Further Developments In the Electrophysiology of Induced Stem Cell-Derived Human Cardiomyocytes

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Abstract:

Production of human cardiac cells by genetic reprogramming offers exciting possibilities for tissue repair, artificial organs and the testing of potential new drugs. The electrophysiological behavior of the artificial cells is important and must be understood for all of these applications. This review summarizes the findings of a large number of experimental papers that have appeared in the last ten years. Recurring themes in this review are the use of tissue culture factors to "program" the specialized differentiation of cardiac cells, the use of adenoviruses to add specific genes to the cardiomyocyte transcriptome, and the ability of transplanted cells to cause favorable and lasting "paracrine effects" even when the transplanted cells do not permanently survive within the host tissue.

Electrophysiological effects can include up-regulation of ion channels that hyperpolarize the membrane, which reduces beat frequency, or up-regulation of depolarizing ion channels, which increases beat frequency. Evidence that transplanted myocytes can sometimes align and couple with the host myocytes, and thereby augment contractile force is discussed. Most of the studies reviewed here involve animal research, although promising clinical studies have already been reported.

Keywords: Stem Cells; potassium channels; arrhythmias; induced cardiomyocytes.

1. Introduction.

Creation of induced pluripotent stem cells that could differentiate into mature human cells, such as cardiomyocytes, has been an attractive area of research for the past ten years, and offers promise with respect to new therapies for heart disease. This avenue of research first became apparent in 2006 when Takahashi and Yamanaka showed that mouse or human adult fibroblasts could revert to pluripotent stem cells when exposed to four factors, Oct3/4, Sox2, c-Myc, and Klf4 under embryonic stem cell culture conditions. This work was a partial basis for the award of the Nobel Prize in Medicine in 2012. This research opened up a new potential area of "personalized medicine" since the stem cells of a specific patient could be further used to produce cultured cells that were characteristic of a specific organ such as the heart, or a particular type of cell within the heart, such as ventricular myocytes.

However, the most obvious commercial application would be the use of human stem-cell derived cardiomyocytes in safety testing of pharmaceutical products.

Pharmaceutical products not intended for use in heart disease can have unexpected effects on cardiac ion channels that can promote the development of lethal cardiac arrhythmias that produce sudden death in an unpredictable fashion.

Many compounds that seem promising as therapeutic agents have been derailed by arrhythmogenic effects seen in mice, pigs or dogs or by effects on ion channels that are seen in experiments with single cardiac myocytes derived from these species. The best known examples of this phenomenon are drugs that increase the duration of the cardiac action potential. Prolongation of the action potential can lead to a potentially lethal ventricular tachyarrhythmia known as torsades des pointes.

The use of human induced cardiomyocytes from any person rather than animal myocytes is a potential advantage. When large numbers of these cells are produced, "high throughput" assays are feasible. These cells can be cryopreserved so that a single batch of induced cardiomyocytes can be used for a large series of measurements (26). Unfortunately, early efforts to determine the electrophysiological characteristics of stem cell derived cardiac myocytes showed that these cells lacked mature features. Specifically, they showed immature electrophysiological properties including shifted sodium channel activation (leading to a slowed upstroke) and the presence of spontaneous beating.

1.1. Induced stem cell derived cardiomyocytes exhibit spontaneous activity due to low levels of I_{K1}.

I_{K1} is a distinctive potassium current because it exhibits "inward rectification." While the basic function of I_{K1} is to produce an inside negative resting potential during diastole (phase IV), the I_{K1} channels close during the action potential, which gives the membrane a relatively high resistance during the plateau phase. This high resistance minimizes the outward flow of potassium ions (in exchange for sodium and calcium). The I_{K1} channels reopen during repolarization, which contributes to further repolarization. Note that there are additional potassium channels (primarily the slow voltage-gated potassium currents, called I_{Kr} and I_{Ks}) which open during the plateau (phase 2) and are the most important currents leading to time-dependent ventricular repolarization.

Early studies of human induced pluripotent stem cells by Jonsson et al. (17) showed that all of the ion currents present in human adult cardiac myocytes are present in the induced pluripotent stem cells, but that the level of expression of $I_{K1,}$ is much lower than in native ventricular myocytes. Jonsson et al (17) found that two out of five cells studied had no I_{K1} at all. The remaining three had a current that was about one fourth of the normal value (-2.7$\pm$ 0.3 pA/pF vs. -12+1 pA/pF). Similar results were obtained by Ma et al (20) who also measured the principal ionic currents in the stem cells, but noted spontaneous beating in human pluripotent stem-cell derived cardiomyocytes. Ma et al noted that the human stem cell derived cardiomyocytes had an unexpectedly high level of the inward pacemaker current, I_f (also called HCN) but that the level of I_{K1} was very low. The observations of these two groups have been confirmed by physiological studies described below.

1.2. Restitution of the normal resting potential by electronic I_{K1} enhancement.

The first attempt to produce normal electrophysiological behavior in human induced cardiomyocytes was published by Bett et al. in 2013 (4). This was accompanied by an editorial, which discussed the significance of the work and future directions (7). Commercially available human i-PSC (induced pluripotent stem cell)-derived cardiac myocytes of the ventricular type were selected for study on the basis of morphology. In the first part of the paper, spontaneous beating due to early and late afterdepolarizations (EAD-like and DAD-like behavior) was recorded. Repetitive beating of quiescent cells could be induced by stimulation with current

pulses. The principal focus of the study was on i-PSC derived ventricular cells that exhibit regular spontaneous beating. This spontaneous beating is similar to the pacemaker potential of the sinus node or A-V node of adult birds and mammals. As in the work of Jonsson et al (17) and Ma et al. (20) Bett and colleagues attribute the spontaneous beating to an abnormally low level of expression of the long-lasting plateau potassium current I_{K1}. When I_{K1} is low, the progressive turn off of I_K during diastole causes the membrane to depolarize until it reaches threshold for a new upstroke. In order to prove this, Bett et al undertook to "upregulate" I_{K1} to normal levels using an electronic circuit to provide a time-variant artificial I_{K1} that is injected into the cells through a micropipette electrode in the "cell attached" mode. The instantaneous voltage recorded by the micropipette was fed into the input of a variable voltage-to current-converter that was programmed to simulate the behavior of the I_{K1} in normal ventricular myocytes. The current voltage relationship of the artificial I_{K1} is given in the paper by an empirical equation, which makes the experiment reproducible by others. The "artificial I_{K1}" made the cell as a whole behave as if the number of I_{K1} channels present in the membrane was higher than it actually was. While the authors propose that induced pluripotent stem cells with an artificially induced I_{K1} might be used for drug development, they acknowledge that a biological modification of the stem cells to cause biological expression of I_{K1} might be more practical. This was discussed in the accompanying editorial in *Heart Rhythm* (7).

1.3. Restitution of the normal resting potential in stem cell derived cardiomyocytes by biologic I_{K1} enhancement.

Two recent studies have shown that mature electrophysiological properties (i.e. absence of spontaneous beating and stable resting potentials) can be induced in human stem-cell derived human ventricular myocytes by viral expression (19,25). The earlier of these studies, by Lieu et al used human embryonic stem cells derived from cardiogenic embryoid bodies (19). These cells were assessed by patch clamp and showed spontaneous beating in the absence of intervention. An adenovirus was used to mediate expression of I_{K1}, which produced silencing of spontaneous activity. The adenovirus silenced cells had stable resting potentials, and the I_{K1} current could be demonstrated by voltage clamp. The more recent study by Vaidyanathan et al. (25) involved commercially available induced pluripotent stem cell-derived cardiomyocytes (iCells) which were also made to express I_{K1} by adenovirus

expression. The resulting cells had a stable resting potential which remained constant at rapid pacing rates.

While it turned out to be possible to produce human induced pluripotent stem cells with stable resting potentials, quantitative concerns about the resting potential are still raised. In one study, Du et al. (8) used voltage sensitive dyes to record action potentials from populations of induced pluripotent stem cells for the purpose of identifying chamber specific action potential morphologies. These studies were criticized because optical dye recordings do not disclose the resting potential, which is necessary for identification of cell type based on action potential shape (13). Two other studies where APD was measured in ventricular-like cells derived from human embryonic stem cells (15,27) gave resting potential values that were relatively low (e.g. -60 mV) and lead to correspondingly low action potential upstroke velocities (see Hartman et al. ref. 14).

2. Plans For Use of Induced Pluripotent Stem Cells in Drug Discovery.

The status of cardiac safety testing in the pharmaceutical industry has recently been reviewed by Fermini, et al. (11). The Comprehensive In Vitro Proarrhythmia Assay (CiPA) is a public-private initiative designed to update existing cardiac safety studies. This initiative is mainly focused on reducing the cost of testing pharmaceutical products for their capacity to produce action potential prolongation and torsades de pointes arrhythmias. Previous research of this type has already classified 28 commonly used drugs as either high risk, intermediate risk or very low risk. Compounds that prolong the QT interval have typically been identified by whole animal and single cell electrophysiological studies that focus on a specific potassium channel (I_{Kr}) that is involved in ventricular repolarization. These would continue under CiPA, but would be expanded to multiple ion channels with in silico modelling to determine the net effect of a prospective drug on action potential duration. It is thought that the expanded ionic current data and in silico modelling could obviate the need for whole animal studies in which super-therapeutic levels of the drug are administered to see if the animal develops torsade de pointes. CiPA would expand the testing protocols to include additional studies in stem-cell derived cardiomyocytes. An obvious advantage of induced stem cell-derived human cardiomyocytes is that research in human cells is considered more relevant to human disease, and avoids the need to sacrifice experimental animals. Furthermore, stem-cell derived

cardiomyocytes can be cultured for long periods of time, during which chronic exposure of the cells to a test compound is possible. The CiPA initiative is being modified so that studies in induced pluripotent stem cells will be added to the existing drug safety testing procedures. It is possible that the use of induced stem-cell derived cardiomyocytes would largely replace animal electrophysiology, although this is an area of active debate and rapid technological progress.

3. Stem Cell Treatments to Regenerate Myocardium.

The ability of physicians to harvest and purify any sort of stem cell that might replace damaged myocardium is of obvious interest in terms of possible therapy. Myocardial scarring predisposes to unfavorable left ventricular remodeling, heart failure and sudden death. The ability to assess the degree of scarring and its exact location has been facilitated by development of contrast enhanced MRI. In addition, the old notion that ventricular cardiac myocytes terminally differentiate and cannot replicate has been challenged by Bergmann, et al, (3) who published evidence in 2009 that a low level of cardiomyocyte renewal (about 1% per year) can be documented by careful measurement of radioactive ^{14}C decay resulting from exposure of humans to atmospheric nuclear tests. These results support the existence of a small population of cardiac stem cells in normal, healthy human hearts. However, at the time of this publication, there is substantial evidence that the regenerative potential of human myocardial stem cells is greatly increased in diseased hearts.

In 2003, Beltrami et al (2) reported that adult cardiac stem cells can support myocardial regeneration in the setting of coronary artery disease. They found that adult cardiac stem cells are self-renewing, clonogenic and can support regeneration when injected into ischemic hearts. In 2004, Messina et. al. (22) developed a method to isolate and expand adult cardiac stem cells from human and mouse hearts. Human cells were derived from myocardial biopsy specimens and could be expanded by inclusion of certain growth factors in the medium along with thrombin. Expansion caused the formation of cardiospheres, which could be shown to be clonogenic, and not simply the result of cell aggregation. When transplanted into an infarcted mouse heart, these cells could differentiate into myocytes and vascular cells, and the myocytes exhibited contractile activity.

In 2010, Chiementi et al (6), working in the same laboratory as Messina et al (22) reported that the effects of transplanted cardiospheres in infarcted

mouse hearts may largely have been due to paracrine effects rather than persistence of the transplanted cells in the tissue. While differentiation of the transplanted cells was still believed to play a role, paracrine effects could be of equal or even greater importance to the overall effect.

In 2011, Bolli et al. (5) described the use of cardiac stem cells in patients with ischemic cardiomyopathy. Compared to control patients, those who received autologous stem cells showed a highly significant improvement in ejection fraction, which was even more significant at 12 months. Bolli et al. (5) point out that the beneficial effects they observed do not necessarily mean that the cardiac stem cells differentiated into adult ventricular myocytes which directly augmented myocardial contraction. Bolli et al. (5) point out that paracrine actions of the cardiac stem cells, including inhibition of apoptosis, inhibition of fibrosis, or improved contractile function of resident cardiac myocytes, could potentially have explained their results.

In 2012, Makkar et al (21) published results from patients with recent myocardial infarction who had autologous cells grown from endomyocardial biopsy specimens infused into the infarct related artery 1.5 to 3 months after the infarct. Magnetic resonance imaging at 6 months showed that compared to controls the treated patients had a highly significant reduction in scar mass, significant increases in viable heart mass, regional contractility and systolic wall thickening. However, no increase in left ventricular ejection fraction was demonstrable at six months.

It is important to note that in the above referenced studies, teratomas have not been observed. The concern about teratomas is greatest when pluripotent stem cells derived from a fetus are used. A human fatality occurred in a trial involving the use of embryo-derived pluripotent stem cells in the brain. It is believed that teratomas can be avoided by the use of highly purified cells that are almost entirely cardiac myocytes, with avoidance of immature cell forms.

4. Neonatal Cardiac Sheet Transplantation Into Injured Adult Rat Hearts.

Effective transplantation of neonatal rat cardiac myocytes into superficially injured adult rat hearts has been accomplished by Furata, et al. (12). To do this they used the technique of fabricating pulsatile cardiac tissue grafts by growing neonatal cardiomyocytes in layers on a polymer-coated dish as described by Shimizu, et al. (24). The matrix-free cardiac cell sheets can be stacked up to form up to 100 micron thick 3D cardiac

tissue sandwiches (24). In the experiments of Furata et al. (12), the surface of the left ventricle was injured with a heated probe. Then, the sheet graft was transplanted onto the injured region and the chest was closed. As explained in an accompanying editorial by Eschenhagen, et al. (9), the key objective of Furata et al. (12) was to determine whether the implanted "tissue sandwiches" electrically integrate with the host myocardium and participate in synchronous contraction. To demonstrate electrical integration, the recipient rat hearts were removed seven days after transplantation and stained with the fluorescent voltage-sensitive potentiometric dye di-4-ANEPPS. The stained hearts were imaged with a CCD camera, which permitted acquisition of an isochronal map of action potential propagation. Recordings from the engrafted hearts were compared against a control group that had not been engrafted. There were no arrhythmias (spontaneous or pacing-induced) in either group. Bidirectional smooth AP propagation between the host heart and the grafted cell sheets (CS) was observed. This indicated functional integration, presumably mediated by gap junctions, between the cardiac cell graft and the host heart.

Similar studies using human stem-cell derived cardiac cell sheets have recently been conducted by Eschenhagen's group and are in press (10).

5. Cell Therapy and Gene Therapy for Biological Pacing.

Besides the absence of teratomas, the clinical use of cardiac stem cells in heart failure or infarction does not involve the creation of subsidiary ventricular pacemakers or an idioventricular rhythm. This clinical reality is consistent with the absence of arrhythmias or automaticity in the engrafted rat myocyte sheets described above. Nevertheless, the idea that either gene therapy or cell therapy could be used to produce an ectopic "biological pacemaker" in the heart has recently been proposed (16, 18, 23). Adenovirus vectors have been used to cause expression of Tbx 18 in isolated rat neonatal myocytes. The transformed neonatal myocytes developed spontaneous beating and the action potential acquired characteristics of adult SA node pacemaker cells. Rosen (23) suggests that this pacemaker is due to "overexpression" of the hyperpolarization-induced pacemaker current, HCN. Along with the electrical transformation, Tbx 18 produced morphological changes in the myocytes which included degradation of the contractile proteins and a change in cell shape from rod shaped to spindle shaped (18). Tbx18 converts ventricular myocytes to replicas of sinus node

pacemaker cells both in vivo and in vitro. This can be accomplished in small or large animals, such as the pig, including adult animals. In guinea pig hearts, injection of Tbx18 (with adenovirus) into the apex of the left ventricle produced a wide complex ectopic rhythm when the native sinus node activity was suppressed with methacholine. Kapoor et al (18) note that "Direct conversion of ventricular myocytes to pacemaker cells by Tbx18 obviates the need for passage through a pluripotent state." Rosen (23) speculates that the clinical use of a TBX18-transduced biological pacemaker in patients might be feasible, although the long term stability of these "biological pacemakers" needs to be established through further animal experimentation. TBX1-transduced biological pacemakers produce a stable heart rate in the mid 70's. The ability of electronic implanted pacemakers to deliver "rate responsive pace making" in response to physical activity of the patient cannot not be mimicked by a "biological pacemaker" such as TBX18-transduced cardiac myocytes.

6. Conclusion.

Stem cell derived human cardiomyocytes have been tested and show promise in a number of areas. For certain applications, such as drug testing, their value can be increased by the use of adenoviruses to upregulate potassium channels. Transplantation of stem cells or stem cell-derived cardiac myocytes to augment contractility in heart failure could be considered the "holy grail". The most convincing progress along these lines is still with animal models and involves myocardial cell sheet transplantation into nude rat hearts recovering from focal epicardial ablation. Expansion of cardiac cells derived from human myocardial biopsies followed by intracoronary injection of these cells into the patient is another technique that shows promise. However, benefits found so far may be due to paracrine effects and are not necessarily long lasting. This is an area of rapid progress that needs to be reviewed every few years, with significant new work currently in press.

7. References.

1. Bakker M.L., Boink G.J., Boukens B.J. et al. 2012. T-box transcription factor TBX3 reprogrammes mature cardiac myocytes into pacemaker-like cells. *Cardiovasc. Res.* 94: 439-449. doi: 10.1093/cvr/cvs120

2. Beltrami A.P., Barlucchi, L., Torella D., et al. 2003. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 114: 763-776. http://dx.doi.org/10.1016/S0092-8674(03)00687-1

3. Bergmann O., Bhardwaj R.D., Bernard, S., et al. 2009. Evidence for cardiomyocyte renewal in humans. *Science* 324: 98-102. doi: 10.1126/science.1164680.

4. Bett G.C.L, Kaplan A.D., Agnieszka L., et al. 2013. Electronic "expression" of the inward rectifier in cardiocytes derived from human induced pluripotent stem cells. *Heart Rhythm* 10: 1903-1910. http://dx.doi.org/10.1016/j.hrthm.2013.10.015

5. Bolli R., Chugh A.R., D'Amario D., et al. 2011. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomized phase 1 trial. *The Lancet* 378: 1847-1857. doi: 10.1016/S0140-6736(11)61590-0.

6. Chementi I., Smith R.R., Tao-Sheng Li, et al. 2010. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. *Circ. Res.* 106: 971-980. doi: 10.1161/CIRCRESAHA.109.210682

7. Clusin W.T. 2013. Restoring the resting potential brings maturity to induced stem cell-derived human cardiac myoctyes. *Heart Rhythm* 10: 1911-1912. http://dx.doi.org/10.1016/j.hrthm.2013.10.015

8. Du D.T., Hellen, T.N., Kane C. et al. 2015. Action potential morphology of human induced pluripotent stem cell-derived cardiomycytes do not predict cardiac chamber specificity and is dependent on cell density. *Biophys. J.* 108: 14. doi: 10.1016/j.bpj.2014.11.008.

9. Eschenhagen T., Zimmermann W.H., Kleber, A.G. 2006. Electrical coupling of cardiac myocyte cell sheets to the heart. *Circ. Res.* 98: 573-575. DOI:10.1161/01.RES.0000215627.13049.5d

10. Eschenhagen,T. Personal communication. Article in Press.

11. Fermini B., Hancox J.C., Najah A.-G., et al. 2016. A new perspective in the field of cardiac safety testing through the comprehensive in vitro proarrhythmia

assay paradigm. *J. Biomolecular Screening* 2: 1-11. DOI 10.1177/1087057115594589

12. Furata A., Miyoshi S., Itabashi Y., et al. 2006. Pulsatile cardiac tissue grafts using a novel three-dimensional cell sheet manipulation technique functionally integrates with the host heart, in vivo. *Circ. Res.* 98: 705-712. http://dx.doi.org/10.1161/01.RES.0000209515.59115.70

13. Giles W.R., Noble D. 2016. Rigorous phenotyping of cardiac iPSC preparations requires knowledge of their resting potential(s). *Biophys. J.* 110: 278-280. http://dx.doi.org/10.1016/j.bpj.2015.06.070

14. Hartman M.E., Dai, D.-F., Laflamme M.A. 2016. Human pluripotent stem cells: Propects and challenges as a source of cardiomyoctyes for in vitro modeling and cell-based cardiac repair. *Advanced Drug Delivery Rev.* 96: 3-17. doi: 10.1016/j.addr.2015.05.004.

15. He J.Q., Ma, Y., Lee J.A., et al. 2003. Human embryonic stem cells develop into multiple types of cardiac myoctyes: action potential characterization. *Circ. Res.* 93: 32-39. DOI:10.1161/01.RES.0000080317.92718.99

16. Hu Y.-F., Dawkins, J.F., Cho H.C. et al. 2014. Biological pacemaker created by minimally invasive somatic reprogramming in pigs with complete heart block. *Sci Trans Med.* 6:245ra94. DOI: 10.1126/scitranslmed.3008681

17. Jonsson M.K.B., Vos M.A., Mirams G.R., et al. 2012. Application of human stem cell-derived cardiomyocytes in safety pharmacology requires caution beyond hERG. *J. Mol. Cell. Cardiol.* 52: 998-1008. doi:10.1016/j.yjmcc.2012.02.002

18. Kapoor N., Liang W., Marban E., et al. 2013. Direct conversion of quiescent cardiomyocytes to pacemaker cells by expression of Tbx 18. *Nat.Biotechnol.* 31: 54-62. doi:10.1038/nbt.2465

19. Lieu D.K., Fu J.-D., Chiamvimonvat N., et al. 2013. Mechanism-based facilitated maturation of human pluripotent stem cell-derived cardiomyocytes. *Circ. Arrhythm. Electrophysiol.* 6: 191-201. doi:10.1161/CIRCEP.111.97342

20. Ma, J., Guo, L., Fiene, S.L., et al. 2011. High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents. *Am. J. Physiol. Heart Circ. Physiol.* 301: 2006-2017. doi: 10.1152/ajpheart.00694.2011

21. Makkar R.R., Smith, R.R., Cheng, K., et al. 2012. Intracoronary

cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. *The Lancet* 370: 895-904. doi: 10.1016/S0140-6736(12)60195-0.

22. Messina E., De Angelis, L., Frati, et al. 2004. Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ. Res.* 95: 911-921. DOI:10.1161/01.RES.0000147315.71699.51

23. Rosen, M.R. 2014. Gene therapy and biological pacing. *New Eng. J. Med.* 371: 1158-1159. DOI: 10.1056/NEJMcibr1408897

24. Shimizu T., Yamato M., Isoi Y., et al. 2002. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature responsive cell culture surfaces. *Circ. Res. 90: e40.* http://dx.doi.org/10.1161/hh0302.105722

25. Vaidyanathan R., Markandeya Y.S., Kamp T.J. et al. 2015. I_{K1}-enhanced human-induced pluripotent stem cell-derived cardiomyocytes: an improved cardiomyocyte model to investigate inherited arrhythmia syndromes. *Am. J. Physiol.—Heart and Circ. Physiol.* 310: H1611-H1621. doi: 10.1152/ajpheart.00481.2015

26. Xu C., Police S., Hassanipour, M., et al. 2011. Efficient generation and cryopreservation of cardiomyocytes derived from human embryonic stem cells. *Regen. Med. 6: 53-66.* doi: 10.2217/rme.10.91

27. Zhang J., Wilson G.F., Soerens A.G., et al. 2009. Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ. Res.* 104: e30-e41. doi: 10.1161/CIRCRESAHA.108.192237

