Cardiac Regeneration
Time to Revisit Nature

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It is hard to imagine any topic in cardiovascular science that has been more hotly contested for the past 2 decades than cardiovascular regenerative medicine. The flavor of the day with regard to stem cell types fluctuates, yet first-in-human studies of a regenerative approach with definitive results remain elusive. The good news is that many of us continue to toil away in our laboratories and clinical trials in pursuit of this Promethean quest and that most stem cell types used have proven to be safe, if not always effective. We think that past paradigms have not always been organically founded on the basis of prevailing mechanisms found in nature and in our own evolutionary history. Thus, a more rigorous focus on developmental biology and genetics is of paramount importance to identify the best targets. This must be coupled with preclinical testing in large animal models to assess the success of any given gene or cell therapy before clinical trials.

Getting Past Nondividing Cardiomyocytes

Terminal differentiation of the adult mammalian heart without significant regenerative ability has been long-standing dogma in the cardiovascular field, but this concept has only been strengthened by more sophisticated recent studies. Studies from the late 1990/early 2000s,[1] demonstrating that the adult human heart could undergo turnover of cardiomyocytes, evocative as they were, have been superseded with use of more rigorous techniques examining this phenomenon. Through an elegant approach using carbon-14 dating, Bergmann et al[2] have demonstrated that regenerative capacity was not as meaningful as proposed in the earlier findings. Within the measured 0.3% to 1.0% turnover rate reported by this group, most cardiomyocyte renewal takes place in the first decade of life, with older age groups being predisposed to irreversible cardiomyocyte loss in response to cardiac injury. These findings were further corroborated by Mollova et al[3] by examining the hearts of 36 individuals aged 0 to 59 years, with no evidence of cytokinesis found after 20 years of age. Interestingly, these findings also suggest the absence of a significant endogenous stem cell pool in the adult human heart that can enter a regenerative program on injury. Unlike adult mammalian species, other members of metazoan phylogeny have retained the capacity for cardiomyocyte proliferation after injury.[4] Urodele amphibians retain an extraordinary capacity to regenerate damaged anatomic structures through epimorphic regeneration.[4] Urodele regeneration hinges on plasticity of differentiated cells in the area of injury, which reenter the cell cycle, resulting in loss of differentiated characteristics to generate progenitor cells with restricted potentiality.[4]

In the first report of zebrafish heart regeneration, Poss et al[5] reported that regeneration seemed to have resulted from proliferation of cardiomyocytes adjacent to the site of injury. However, in a subset of fish with a temperature-sensitive mutation in *mps1*, a gene encoding a mitotic checkpoint kinase required for cell division in the zebrafish fin, regeneration failed to occur with resulting fibrosis instead. These results imply that a single gene mutation affecting cardiomyocyte mitosis could severely mitigate the regenerative process.

In mammals, regeneration after limited injury (involving ≈20% of the left ventricle) in mice was shown to occur before postnatal day 7.[6] Injury after postnatal day 7 provoked a fibrotic scar. These results support our data spanning >14 years that cyclin A2 (*Ccna2*) is the master regulatory molecule of the cardiomyocyte cell cycle. Progression through the cell cycle is regulated by cyclins complexed with their catalytic subunits known as Cdks (cyclin-dependent kinases). Ccna2 complexed with Cdk2 is essential for the G1/S transition and Ccna2/Cdk1 promotes entry into mitosis.[7] *Ccna2* is absolutely essential for normal embryonic development to occur. A targeted deletion of *Ccna2* in the mouse exhibited embryonic lethality at embryonic day 5.5.[8] *Ccna2* is the only cyclin demonstrated to be transcriptionally silenced when cardiomyocytes exit the cell cycle across mammalian species.[9–13] Our studies revealed a critical role for *Ccna2* in cardiomyocyte mitosis and we have shown that the delivery or activation of *Ccna2* elicits cardiomyocyte proliferation across species in mouse, rat, and porcine models of myocardial infarction and significantly increases post–myocardial infarction cardiac contractile function.[9–12] In fact, in our mouse model of transgenic expression of *Ccna2* in cardiomyocytes, hearts underwent significant regeneration post-myocardial infarction even after large anterior infarcts that encompassed 50% of the left ventricle, which is a much larger area of injury reported in earlier studies of axolotl or zebrafish cardiac injury, as well as the area injured in studies of neonatal mice. In our experiments with the porcine infarct model, the strategy of utilizing vector-mediated delivery of *Ccna2* succeeded in increasing the numbers of cardiomyocytes in the peri-infarct zone by 55%.[12] To our knowledge, this is the first demonstration of de novo cardiomyogenesis from existing cardiomyocytes in a large animal that so closely mimics human cardiac structure and physiology. To mechanistically address cytokinesis of cardiomyocytes in the porcine heart, we devised a research tool comprised of mCherry-labeled α-actinin that allowed dynamic labeling of sarcomeres so that sarcomere dynamics could be followed during live imaging.

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After isolation from adult porcine ventricular tissue, cardiomyocytes were cultured, transfected with adenovirus containing *Ccna2* versus null adenovirus, and the actinin-mCherry. We noted that ≈3% of cultured adult porcine cardiomyocytes treated with *Ccna2* adenovirus could be observed undergoing complete cytokinesis, resulting in intact daughter cells with preserved sarcomeres, compared with negligible numbers in cardiomyocytes treated with null adenovirus (see their online supplemental movie[12]). The numbers observed in vitro are not necessarily reflective of the increase in numbers of cardiomyocytes noted in vivo after gene therapy with *Ccna2* and may be attributed to the technical difficulties of coaxing adult porcine cardiomyocytes into culture and maintaining them for the period of time required for such studies. However, this is the only way to verifiably and visually prove that cytokinesis was induced, as this phenomenon is thus far exceedingly difficult to visualize in vivo.

This is an area of technical difficulty that we find in studies that examine proliferation of adult cardiomyocytes: inadequacies in assessing definitive cytokinesis. Furthermore, it is equally imperative to follow the survival and fate of daughter cardiomyocytes. In vitro cytokinesis assessment by dynamically labeling sarcomeres and visualization of cytokinesis using time-lapse fluorescence microscopy is vital to substantiate the incidence of cytokinesis in adult mammalian cardiomyocytes. Relying entirely on cell cycle/proliferation markers (Aurora B kinase or Ki-67) and to an extent the M-phase marker phospho-histone (H3P) may misrepresent actual cytokinesis if not confirmed by visualization of cell division in real time. Our ongoing studies explore the mechanistic basis of cell division in adult cardiomyocytes, more recently in adult human cardiomyocytes (work in progress). More recently, Mohamed et al[14] also demonstrated a role for Cdk1 in combination with 3 other cell cycle regulators in murine cardiomyocyte proliferation. It is interesting to note this group did not report postnatal silencing of *Ccna2* described by others and ourselves in multiple animal models and humans,[9–13] especially because the Ccna2/Cdk1 complex is critical for mitotic entry. Nonetheless, results of all of these studies allude to a potentially powerful strategy to finally achieve the end-goal of human cardiac regeneration, with *Ccna2* gene delivery already having resulted in successful preclinical results of robust cardiac regeneration in the porcine model ready for clinical testing.

Cell Therapy for Cardiac Regeneration: Let's Not Miss a Beat!

One potential strategy that has been the subject of tremendous focus for regeneration apart from coaxing the division of preexisting myocytes involves the use of stem/progenitor cells to generate de novo cardiomyocytes. Animal studies have shown the potential of cell therapy in improving heart function after myocardial infarction; however, the biological or clinical significance of various approaches have been under constant debate and marred with controversy. It has become increasingly apparent that the adult mammalian heart does not harbor endogenous stem cells of any physiological relevance that can regenerate injured myocardium. The interest in cardiovascular stem cell therapy became greatly heightened when it was demonstrated that bone marrow–derived cKit$^+$ cells were capable of generating new cardiomyocytes in vivo.[15] This study by Orlic et al[15] gained tremendous momentum in the stem cell field; however, these data remain at odds with other investigators who contested these observations as they failed to observe any significant cardiomyocyte differentiation from cKit$^+$ cells.[16] Thus, it is likely that functional improvements were mediated through a paracrine mechanism rather than transdifferentiation into working myocardium. In such cases, transplanted stem cells may function as a reservoir for various soluble factors like exosomes and other growth factors that may induce reparative processes in myocardium mainly through reduction of apoptosis and augmenting angiogenesis. Despite investigations with a wide variety of cell types as candidates to attain this goal, the results of stem cell transplantation remain ambiguous.

In the pursuit for an alternative cell source for cardiac repair, we have examined various stem/progenitor cells, including cardiac-derived cKit$^+$ cells, cardiac side population cells, bone marrow side population cells, and cardiac Sca-1$^+$ cells for their ability to differentiate into cardiomyocytes in vitro using a neonatal murine cardiomyocyte feeder layer as a niche to support cardiogenesis. However, no spontaneously beating cardiomyocytes were generated from these cells in culture. We think that manifestation of spontaneous beating in culture is an important criterion that defines the cardiac potential of any stem/progenitor cell type. Interestingly, this integral characteristic feature has been neglected from various studies assessing stem cell-mediated cardiac differentiation. This phenomenon is distinct and cannot be associated with induced beating achieved using a directed differentiation protocol with compounds such as 5-azacytidine, ascorbic acid, and others. This is further complicated by lack of homing of most cell types studied, resulting in few cells finding their way into the heart. Intramyocardial injection has been utilized as an alternative, albeit rather invasive strategy, to circumvent this issue. Retention and survival of the donor cells is the basic challenge faced in cell therapy and a lack of electromechanical coupling may further complicate this scenario. Methods to increase survival and persistence of donor cells should be another key area of study that can enhance current strategies. Nonetheless, preclinical and clinical studies do not argue against further development of cell therapy as a safe option. It would, however, be of great clinical significance to find an alternative primitive cell type other than embryonic stem cells that is cardiogenic.

To bridge this gap, our laboratory sought to identify potential cell types that may contribute to the reparative process by studying fetal–maternal stem cell transfer during pregnancy.[17] This study illustrated that experimental myocardial injury, induced in a pregnant mouse, triggers flux of fetal cells from placenta via the maternal circulation into the injured heart, where they undergo differentiation into diverse cardiac cell fates. Isolation of the fetal derived placental cells from the maternal hearts was possible via tagging with green fluorescent protein, and we demonstrated spontaneous beating of these cells in vitro. Transfer of fetal cells into maternal circulation, known as fetal-microchimerism, is an evolutionarily conserved phenomenon across eutheria but a functional significance of this phenomenon had not previously been defined. We think that exploiting this intrinsic potential of fetal placental cells toward regenerative medicine is a clinical imperative because it may directly provide a wealth of information regarding the types of stem cells that are naturally poised toward cardiomyogenesis. One subpopulation of placenta-derived cells, which we found to be unique and highly prevalent within the isolated fetal cells

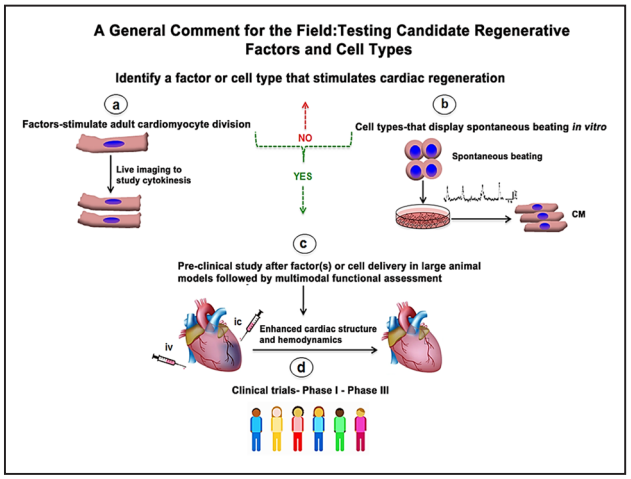

Figure. Candidate regenerative factors and cell types. Isolation of putative cardiac progenitors that are thought to adopt the cardiac fate should undergo ex vivo testing to document contractile activity using live imaging. Proliferative factors should be rigorously tested for ability to induce cytokinesis. Hemodynamic studies to support a true cardiac regenerative process should demonstrate sustained improvements over time. CM indicates cardiomyocytes; ic, intracardiac; and iv, intravenous.

from maternal myocardium, expressed caudal-related homeobox 2 (*Cdx2*). This unexpected observation may represent a paradigmatic shift, as *Cdx2* has previously been associated only with trophoblast stem cell regulation and placenta formation. Because placental stem/progenitors are more primitive than adult tissue-specific stem cells and are closer to embryonic stem cells in their developmental hierarchy (yet do not seem to possess tumorigenic effects like embryonic stem cells), we are studying Cdx2 cells isolated from mouse and human placentas to develop an alternative strategy for allogeneic stem cell therapy for cardiac regeneration. The notion of preset fate choices in embryonic lineages is thus being challenged by such findings by ourselves and others. Hence, more rigorous study of underlying biological pathways in development is crucial for the identification of cardiogenic stem cell types that should then be tested in preclinical large animal models before rushing to clinical trials.

We thus think that parallel comprehensive studies are still critical for therapeutic interventions regarding cardiomyocyte proliferation and cell therapy unless comparative efficiency of either strategy greatly outweighs the other in clinical settings. Much has been learned over the past 2 decades of an intense global focus on cardiac regeneration, and our conclusions regarding the approaches most likely to succeed rest on the premise that lessons learned from nature itself and further focus on developmental biology will best be applied to the clinic (Figure). This should advance us well beyond results obtained by a multitude of earnest clinicians injecting every imaginable cell type and factor into the heart without rigorous analyses ex vivo and testing across species from small to large animals.

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Disclosures

H.W. Chaudhry is founder and equity holder of VentriNova, Inc and listed inventor on multiple patents regarding cyclin A2–mediated cardiac repair and caudal-related homeobox 2 cells for cardiac repair. The other author reports no conflicts.

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