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Mini-Review

Cardiac regeneration: Past, present, and future

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ABSTRACT

Cardiac regenerative medicine is evolving towards more organic approaches that are increasingly aligned with our evolutionary biology. This is a much-needed direction given the tumultuous path this field has seen over the last two decades. Limited regenerative potential of the adult mammalian heart is indisputably a major factor contributing to the extensive morbidity and mortality of cardiovascular disease worldwide. Many studies are underway globally in the pursuit of this 'holy grail' of cardiovascular medicine, i.e., strategies to actually repopulate lost cardiomyocytes after myocardial infarction or in the setting of heart failure. A multitude of stem cell types have been tested for cardiac repair with clinical trials in this arena falling short of bona fide regeneration, yet more clinical testing of presumed multipotent stem cells is likely to continue. Growth factors, reprogramming and exosomes are also being examined; yet pre-clinical studies have only been reported for growth factor therapy. Cell cycle regulation of cardiomyocyte proliferation is an area our laboratory was amongst the first to report and this is receiving much greater attention recently in light of marginal results noted in past clinical stem cell trials. Gene and cell-based approaches should carefully leverage underpinnings from developmental pathways, which then progress to preclinical studies in large animal models that mimic human cardiac anatomy and physiology prior to the transition to clinical trials.

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INTRODUCTION

As an undoubtedly remarkable organ that sustains life, the adult heart is vulnerable to injuries resulting in a functional decline that can ultimately trigger cardiovascular disease (CVD). Myocardial infarction (MI)/heart attack in adults, results in the formation of a permanent scar that reduces the pumping efficiency of the heart leading to chronic heart failure. CVDs thus far are the leading cause of morbidity and mortality worldwide taking an estimated 17.9 million lives each year. Unlike the embryonic or neonatal heart, adult mammalian heart has limited capacity to renew the heart muscle cells (cardiomyocytes) that succumb to an injury, signifying an unmet need for regenerative approaches that can restore cardiac function in such conditions.

Tissue regeneration is a dynamic process involving diverse cell fate decisions that are comparable to the events in embryonic development. The ability to regenerate lost tissues varies greatly among species. Complexity in tissue organization and the regenerative capacity follow an inverse evolutionary pattern ranging from metazoans to mammals. Clearly, amphibians and teleost fish have intrinsic ability to regenerate lost tissues by epimorphic regeneration [1, 2]. The body of work involving zebrafish (*Danio rerio*) as an injury model revealed a remarkable ability to regenerate the heart without any evidence of scar formation [3-5]. These studies certainly captivated the interest of the scientific community in tracing factors that can lead to strategies aimed at heart regeneration.

Further, such studies provided a springboard to explore the dynamics of proliferation right from pre-natal - neonatal - adult life. Dissecting the developmental pathways and checkpoints offers a window to explore what regulates cell division and regeneration. This ultimately will result in techniques leading to genetic, cell based and cell-free approaches to treat heart disease. The need of the hour is to critically examine the scientific rigor and feasibility in identifying such strategies. Here we discuss the relevant studies that shaped current cardiovascular research, the ongoing vital findings in this field and the potential caveats that need to be taken care of to accomplish a clinically viable cardiac therapy.

Lessons learned from the evolutionary loss of cardiac regeneration

The ability to replace lost tissues of any origin is significant in lower vertebrates. Urodeles and teleosts, can regenerate the heart through adulthood, in contrast with the higher order mammalian species. In advanced mammals, skeletal muscle, skin, intestine and liver show a capability to renew to an extent, but comparable ability is missing in the heart [6, 7]. Zebrafish as a model is amenable to genetic and mechanical manipulations and has thus become a benchmark to understand heart regeneration. Adult zebrafish can completely regenerate ~20% of surgically removed ventricular myocardium with no evidence of fibrotic scar formation [3]. However, as we move along the evolutionary tree, this regenerative ability is diminished considerably as heart regeneration capability is lost beyond developmental stages. In mammals, the embryonic heart has the ability to fully regenerate, whereas neonatal heart does proliferate, yet fails to completely regenerate. Rodent studies have confirmed the lack of cardiomyocyte cell cycle activity past post-natal day 7 (P7) [8, 9]. In neonatal mice, the potential to regenerate the injured heart after apical resection was detected [10]. However, some studies failed to endorse this notion as they observed a limited proliferation in neonatal mouse hearts post injury [11]. The adult mammalian heart has traditionally been viewed as a terminally differentiated post-mitotic organ with no capacity to proliferate whatsoever. Bergmann *et al.* have demonstrated that cardiomyocyte renewal does occur, but is mostly limited to the early stages of adult life [12]. They conducted retrospective ^{14}C-dating measurements in the myocardial cells including cardiomyocytes to study the extent of post-natal DNA synthesis. This model predicated a rate of ~1% of renewal for cardiomyocytes per year at the age of 25 and only 0.45% at the age of 75. Further studies by Mollova *et al*. supported these results demonstrating a lack of cardiomyocyte cell division markers found in cardiomyocytes obtained from patients past 20 years of age [13]. These studies were crucial as they unequivocally confirm the presence of turnover in adult mammalian heart, albeit very limited. Understanding what restricts this regenerative capacity during the course of evolution of heart is the central pursuit of this field.

Gupta *et al*. recognized that in zebrafish, cardiomyocytes adjacent to the injury zones were proliferative and hence contributed to the renewal process. The involvement of Gata4+ cardiomyocytes in heart regeneration was observed [14] suggesting the proliferation of pre-existing myocytes in adults. Recently the role of Tbx20 in the induction of dedifferentiation and endocardial expansion in zebrafish heart regeneration has been studied [15]. Prior to these, studies also demonstrated the effective electrical coupling and functional integration of newly formed cardiomyocytes in zebrafish myocardium [16, 17]. Interestingly, in a temperature-sensitive mutant fish that lacked the mitotic checkpoint kinase *mps1*, a fibrotic scar was formed instead of new cardiomyocytes [4]. These observations from lower vertebrate species provide key information that speaks to the role of cell cycle regulation in inducing the division of existing cardiomyocytes.

Developmental regulation of cardiomyogenesis and the role of cell cycle

The view from zebrafish studies suggests that the addition of cardiac precursors and the proliferation of pre-existing cardiomyocytes would play a major role in embryonic heart chamber development. Studies assessing embryonic development among different species have provided information on the factors that are involved in induction of cardiomyogenesis. Cell-cycle regulation is a key mechanism in tissue proliferation and maintenance that involves cyclins (Cyclin A, B, D, E) and

cyclin-dependent kinases, which in turn regulate them (Figure 1). In adult mammals, cardiomyocyte division that establishes the fully formed myocardium is mostly completed in the prenatal stage. What follows injury in the neonatal stage is a compensatory mechanism involving an increase in cardiomyocyte volume leading to a condition termed cardiac hypertrophy. Hypertrophy would indicate that cardiomyocytes simply increase their DNA content without actually undergoing cell division and thus result in multinucleation and polyploidization, ending the ability to divide further. Studies strongly suggested that the regenerative ability is confined to the mononucleated diploid cardiomyocytes and polyploidy act as a barrier that prevents cytokinesis [18, 19]. At this point, the exit of cardiomyocytes from the cell cycle and the initiation of an alternative compensatory mechanism have created significant interest to study the key factors that control this phenomenon. The presence of these cell cycle variants also marks the switching of the heart's metabolic profile into oxidative phosphorylation (OxPHOS) and the silencing of cell cycle proteins including cyclins and cyclin-dependent kinases (CDKs). The mechanistic pathways that influence cyclins are key to their functional relevance. P38-MAPK signaling has been shown to modulate cyclin A and cyclin B expression during mitosis of cardiomyocytes [20]. Gene delivery methods need to be critically fine-tuned as previous studies aimed at delivering miR199 were found to be detrimental and caused arrhythmia leading to mortality in porcine studies [21]. The mode of delivery and the role of the particular cell cycle regulator are thus crucial for positive outcomes. Recently Hippo/Yap pathways have been identified to play a role in cardiomyocyte proliferation [22, 23]. Inhibiting other signaling components including lncRNA CAREL have reportedly resulted in an increase in cardiomyocyte proliferation [24].

Unfortunately, most of the studies in small and large animal models failed to convincingly demonstrate events of cytokinesis in 'real time' causing ambiguity in such studies. What we have seen and successfully demonstrated in our studies is the discovery and crucial role of cell cycle regulator cyclin A2 (CCNA2) in cytokinesis of adult mammalian hearts [8, 9, 25-27]. Importantly, CCNA2 is silenced in the post-natal heart coinciding with the absence of cell cycle activity. We have established that exogenous delivery of CCNA2 can induce the proliferation of cardiomyocytes in small and large animal models leading to significant improvement in cardiac function post injury. As a preclinical gene therapy approach, adeno-Ccna2 delivery in a porcine myocardial infarction model achieved ~55% increase in the formation of new cardiomyocytes in the peri-infarct zone with enhanced contractile function of the heart (Left ventricular ejection fraction-LVEF) [27]. Moreover, this was safely achieved as we had no mortality of the experimental pigs whereas deaths were observed in the control group. This level of safety has not been observed in any other study of regenerative therapy in large animals that we are aware of while repairing the largest infarct area induced in large animal studies of regenerative therapy. Furthermore, live cell imaging of isolated adult porcine cardiomyocytes carrying adeno-Ccna2 showed significantly higher (~15 fold higher) cytokinesis (live imaging of the cells in culture) in culture compared to the control cardiomyocytes carrying adeno-null vector [27]. Since mitosis of cardiomyocyte may not necessarily reflect cytokinesis, it is critical to observe cytokinesis in real time to confirm the success of studies describing adult mammalian heart division. This key observation further supports our ongoing translational work in adult human cardiomyocytes paving the way for future clinical trials using adeno-Ccna2 as a gene therapy approach for cardiac repair.

Cell-based approaches for cardiac repair: Preclinical and clinical studies

Regenerative medicine is in a constant quest for pluripotent/multipotent cell types that can give rise to all germ layers and regenerate tissues after an injury. It is imperative to address how cell therapy approaches have tuned the field of cardiac regeneration. Stem cells in general possess diverse functional facets based on the tissues in which they reside. Adult tissue specific stem cells are widely used in bone marrow transplantation studies and in other regenerative approaches. As such, the initial stage of cardiac regenerative medicine

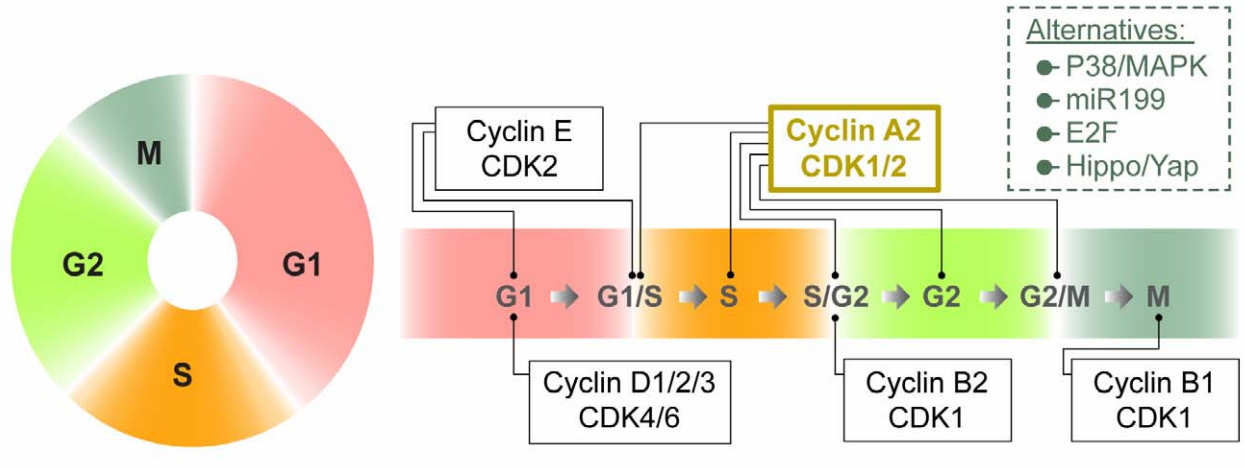

Figure 1. Schematic of cell cycle progression phases G1, S, G2, and M. Solid boxes: Cyclin/CDK regulation of cell cycle progression. Each line indicates the active Cyclin/CDK complex at a specific cell cycle or transition between phases. Notice that Cyclin A2 is critical for various major cell cycle phases and transitions such as G1/S, S, G2/M, and M, making Cyclin A2 the center of interest among all cell cycle regulators. Dotted box: alternative compensatory cell cycle factors.

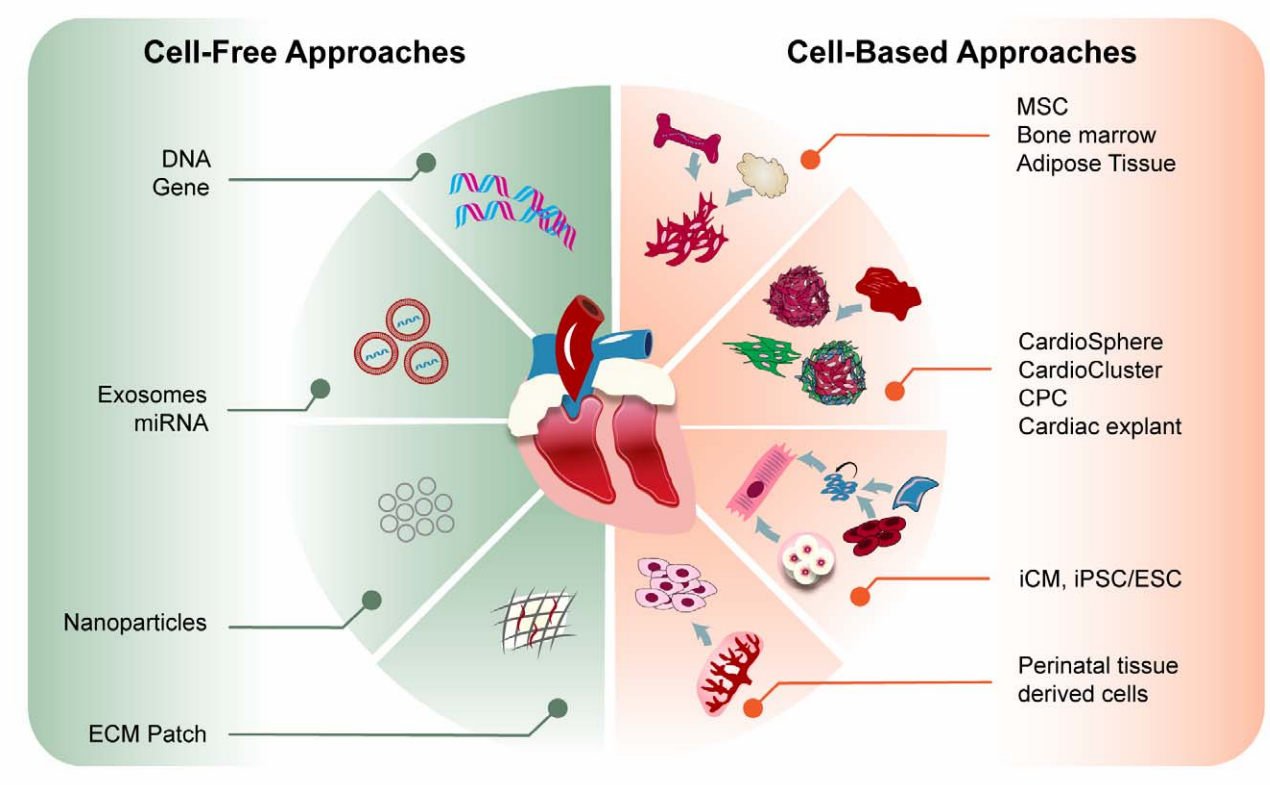

Figure 2. Translational cell-free and cell-based cardiac repair approaches. Left: Cell-free approaches such as DNA, exosome/miRNA, nanoparticle, and ECM patches. Right: Cell-based therapies with various cell sources such as bone marrow or adipose tissue-derived MSCs, cardiac explant-derived progenitor cells, iPSCs, and human placenta-derived stem cells. Some of these cell products require *in vitro* bioengineering (eg. CaridoSphere, CardioCluster, iPSC derived CM) prior to cell transplantation.

witnessed a surge in the use of skeletal myoblasts and bone marrow-derived mesenchymal stem cells and mononuclear cells, which were demonstrated to be considerably safe as a cell therapy approach in clinical settings [28-30] (Figure 2). These cell types however did not give rise to new cardiomyocytes in injured rodent models but appeared to assist *via* a sequela of paracrine signals that supported angiogenesis and reduced cell death in the injured heart. Investigating the existence of a bonafide 'cardiac resident stem cell' witnessed a flurry of pre-clinical studies of which much focus was directed towards c-Kit cells even leading to the initiation of clinical trials utilizing them. The foundation of these studies, however, was revealed to be dependent on falsified/fraudulent data leading to the retraction of a series of research publications resulting in a temporary pause on some clinical trials, including CONCERT-HF, which tested autologous MSCs and cKit+ cells *via* transendocardial injection [31]. The recent phase II study shows the feasibility of this approach and suggests the possible role of paracrine cellular mechanisms [32]. In the meantime, several elegant studies by then clearly established the limited cardiogenic potential within c-Kit+ cells [33, 34]. A mixed population of cells termed cardiospheres mostly derived from the explant cultures from the endocardial biopsy were also investigated for cardiac regenerative ability [35, 36]. Encompassing a heterogeneous (stromal, hematopoietic and non-hematopoietic cells) population, such cells from small animal models were also studied for regeneration [37, 38]. However, conflicting observations from various groups failed to establish any genuine cardiac differentiation from these cell populations [39, 40]. More recently CardioClusters, a 3D aggregate cell cluster harnessing the possibility of a combinatorial approach, showed enhanced functional recovery following MI in NOD/SCID mice *via* a paracrine mode of action [41]. These studies point out to the importance of key factors to be considered while using a cell-based approach such as effective cell retention and survival for engraftment in injured myocardium. In addition, the odds of immune responses being elicited with adult tissue-specific stem cells need close monitoring as this will adversely affect the overall cell survival and long-term retention. The quest for resident cardiac-specific stem cells that can undergo cardiac differentiation has not been successful thus far.

In view of these limitations, the intrinsic lineage-specific differentiation of pluripotent stem cells appeared promising, as adult stem cells have so far failed to demonstrate remuscularization of the damaged myocardium. Pluripotent stem cells are unique but come with the caveat of unwanted proliferation and thus the incidence of teratoma when compared to much safer cells down the hierarchy that are multipotent. However, the use of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) gained much impetus in both cardiac and other fields of regenerative medicine. ESCs and iPSCs can be reliably propagated *in vitro*, allowing for large-scale production adhering to GMP guidelines, making them a promising target for cell replacement therapies. ESC-derived cardiomyocytes and progenitors have been demonstrated to engraft and differentiate into cardiomyocytes respectively in small and large animal models improving left ventricular ejection fraction (LVEF) after myocardial infarction. Menasche *et al*. have shown the safety endpoint after administration of cardiac progenitor cells derived from hESC in a 1-year follow-up study ESCORT [42]. Although promising, at present the incidence of arrhythmia and tumorigenicity makes it difficult to bank on ESC-derived cell products for clinical trials. More understanding of cellular phenotype and stringent culture conditions are required to establish a safety profile in the case of ESC-derived cell types. A preclinical study using guinea pigs showed that hESC-derived cardiomyocytes were able to electrically couple and protect against arrhythmia [43]. However, multiple studies in large animals and non-human primate hearts that followed have shown an incomplete maturation of cardiomyocytes *in vivo* and higher arrhythmic incidents [44, 45]. This may arise out of the immature fetal-like organization of sarcomeres and lack of T-tubules and differences in the calcium handing inclusive of the changes associated with electrophysiological features of hESC-derived cardiomyocytes. Interestingly a porcine study by Zhu *et al*. challenged any cardiomyogenic potential of ESCs as their results showed an absence of remuscularization of the injured myocardium [46]. They reasoned that the paracrine-mediated effects could be attributed to any modest increase in cardiac function that was observed.

iPSCs on the other hand have an advantage that they can be derived from patients' own somatic cells and thus negate any ethical aspects. In addition, the incidence of immune reaction and rejection is lessened to a higher extent compared to the hESC-derived counterparts. Many studies involving small and large animal models have demonstrated the use of iPSC-derived cardiomyocytes for cardiac regeneration after MI [47, 48]. Pre-clinical allogeneic setting studies in non-human primates have shown the functional improvement of injured hearts but the outcomes were complicated with the presence of persistent tachycardia in animals transplanted with iPSC-derived cardiomyocytes [49]. The reasons may be diverse including the differences in action potential or the subsets of cardiomyocytes that are generated from them. Investigations for an alternative to a cell-based approach have also prompted studies utilizing the microvesicles and exosomes [50, 51]; however, the basic mechanism in such cases involves paracrine responses as described.

Searching for cells that have unique immunomodulatory properties and multipotency, we have shown that placenta-derived stem/progenitor cells possess immense potential in tissue regeneration [52, 53]. Perinatal stem cells from the placenta are an easily accessible source that is developmentally closer to embryonic stem cells, yet multipotent and non-tumorigenic [52]. The most studied among these are the mesenchymal stem cells for their angiogenic induction in wound healing [53, 54]. We have demonstrated yet another unique aspect of the placental stem cells in a pregnant mouse model of myocardial infarction. We observed the specific trafficking of fluorescently labeled fetal-derived placental stem cells into injured maternal myocardium where they differentiated into cardiovascular lineages demonstrating for the first time a functional effect of fetal microchimerism [55-57]. Our recent studies proved that Cdx2-derived cells from murine placenta can be isolated and differentiated into spontaneously beating cardiomyocytes and vascular cells [58]. These cells possess an immunologically naïve phenotype and retain all the stem-related proteins of embryonic stem cells but have a unique proteome that supports homing and survival signaling. Furthermore, intravenous administration of placental Cdx2-eGFP cells into male mice subjected to MI, showed specific homing to the injured heart with subsequent cardiovascular differentiation [57]. This further lead to a significant improvement in the contractile function of the heart (as assessed with serial MRIs) and reduced adverse cardiac remodeling signifying a potential role of placental Cdx2 cells in cardiac repair. These studies open the door to investigating the translational role of human CDX2 cells in cardiovascular regeneration, which is our current focus and may further result in other types of organ regeneration due to their multipotency. An overview of current cell-based and cell-free approaches are provided in Figure 2.

CONCLUSIONS

Overall, we are yet to reach a consensus regarding a better therapeutic strategy among cell-based, cell-free or gene therapy approaches. Gene therapy approaches coaxing the division of existing cardiomyocytes in the injured myocardium is a promising strategy. However, as we stated, demonstration of true cytokinetic events is critical in such experiments to ascertain the approach is indeed inducing division in adult mammalian cardiomyocytes. Consequently, for cell-based approaches, generation of spontaneously beating cardiomyocytes *in vitro* and their subsequent differentiation in the injured heart is crucial for remuscularization of the failing heart. The other factors that need refinement include the mode of delivery and maintaining a sustained engraftment of the cells in the injured heart. All other therapeutic strategies rely on paracrine modes of action involving the induction of angiogenesis, pro-survival and pro-regenerative inflammatory responses. Hence, to accomplish the goal of seamless remuscularization of the heart, current and future research should emphasize adequate and comprehensive preclinical studies using small and large animal models. This would unequivocally affirm the promise of any gene-based/cell-based therapeutic in question for cardiac regeneration.

CONFLICT OF INTEREST STATEMENT

Vadakke-Madathil, S. and Wang, B. J. reports no conflict of interest. Chaudhry, H. W. is the 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.

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