

Perspective

Cardiomyocyte Proliferation

A Platform for Mammalian Cardiac Repair

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ABSTRACT

Permanent loss of heart muscle cells, cardiomyocytes, is a major mechanism resulting in ventricular dysfunction and heart failure. Potential solutions to this problem could be either to stimulate the heart to generate new cells by inducing existing cardiomyocytes to divide or to activate or deliver stem cells/progenitor cells to multiply and subsequently differentiate into cardiomyocytes. Utilizing *in vitro* and *in vivo* approaches, p38 MAP kinase has recently been identified as a key negative regulator of cardiomyocyte proliferation. This work provides strong evidence that adult mammalian cardiomyocytes can divide. This review discusses the potential of the induction of mammalian cardiomyocyte proliferation as a therapeutic strategy for myocardial repair.

Conventional wisdom says mammalian hearts cannot regenerate. Although recent experiments have cast some doubt on this dogma,^{1,2} from a practical perspective, mammalian hearts respond to injury by scarring. Considerable progress in the treatment of congestive heart failure patients has reduced early mortality from myocardial infarction. However, because of irreversible loss of cardiomyocytes and progressive deterioration of cardiac function, patients will have a diminished quality of life and inevitable progression to overt heart failure. The loss of cardiomyocytes triggers pathological remodeling, a process resulting in structural changes within the healthy myocardium adjacent to as well as remote from the infarcted area. Myocardial remodeling is characterized by progressive changes in ventricular size, shape, and function which leads to further loss of cardiomyocytes (necrosis and apoptosis) and increase in interstitial fibrosis.³⁻⁵ In the developed world, the prognosis for patients with symptomatic heart failure is worse than that associated with most cancers- about 30% mortality rate within 1 year.^{6,7} One of the reasons for this poor outlook is the fact that conventional treatment regimen fail to correct the problem of cardiomyocyte loss. Given the high morbidity and mortality rate associated with heart failure, new approaches to address the pathophysiologic deficits resulting from the loss of cardiomyocytes could have major impact. Two strategies have been employed in recent studies: one is regeneration through stem cells and the other is induction of cardiomyocyte proliferation.

HOW DO WE DEFINE REGENERATION?

"Regeneration" has been used as a broad term to describe many phenomena. In this review, we use the term "regeneration" to describe processes that restore organ function instead of recreating the original morphology of the organ. During liver regeneration, for example, hepatocytes multiply to restore liver function, but proliferation is not limited at the original anatomical site where the injury occurred.^{8,9}

In comparing the underlying mechanisms of different regeneration processes, it seems that regeneration is highly organ-specific and is related to the developmental processes during organ formation. For example, during skeletal muscle development in mammals, early proliferation and terminal differentiation are mutually exclusive.¹⁰ Thus, it makes sense that skeletal muscle regeneration is based on stem cells.¹¹ Another example is red blood cells that lose their nuclei during terminal differentiation, and thus have to be replenished from progenitor cells. On the other hand, liver progenitor cells can differentiate into functional hepatocytes that still retain the ability to proliferate. Thus, liver regenerates by stem cells or hepatocyte proliferation.^{8,9} Mammalian cardiomyocytes display two developmentally programmed forms of growth. *In utero*, ventricular mass is augmented by proliferation of differentiated cardiomyocytes. *In postnatal* life, ventricular cardiomyocytes exit the cell cycle and respond to an increased hemodynamic burden with an adaptive

increase in cardiac mass through increase in cell size.¹²⁻¹⁴ The fact that liver regenerates through hepatocyte proliferation supports and encourages the search for ways to repair the mammalian heart by promoting cardiomyocyte proliferation as an alternative approach to stem cell therapy.

HOW DO NEWT AND ZEBRAFISH HEARTS REGENERATE?

Two model organisms have been used to study heart regeneration, newts and zebrafish. Newts are capable of regenerating 50% of the heart ventricle after mechanical excision.^{15,16} Zebrafish can fully restore lost myocardium with little or no scarring following 20% ventricular resection.¹⁷ Interestingly, in both cases the regenerative response is accomplished through cardiomyocyte proliferation. In vivo experiments in newts have demonstrated a proliferative response in atrial and ventricular cardiomyocytes adjacent to the injured site. Amputation of the apical portion of the newt ventricle, mincing, and placing it back onto the amputation gap was followed by cardiomyocyte proliferation with a peak thymidine incorporation index of 24.6% after 16 days.¹⁸ In zebrafish, amputation of the apical portion of the ventricle was followed by proliferation that peaked at 32% BrdU incorporation (labeling day 7 to 14), with most cycling cardiomyocytes localized to compact muscle at the lateral edges of the wound. While mitoses were rarely seen in the uninjured heart, 3–10 cardiomyocyte mitoses per wound area were observed at day 14 post injury. To date, there has been no indication that cardiac regeneration in these organisms is based on stem cells. However, this possibility cannot be excluded.¹⁷

Interestingly, it has been reported in mammals that adaptive growth after heart injury through increase of cardiomyocyte size is associated with reactivation of cell cycle machinery as well as re-expression of a subset of fetal genes.¹⁹⁻²⁵ These observations suggest that cardiomyocytes initiate an abortive attempt to undergo cell division by dedifferentiation but the process is somehow hindered by negative regulators. This observation suggests that despite of the terminally differentiated status of adult mammalian cardiomyocytes, they are still able to dedifferentiate and proliferate.

CAN ADULT MAMMALIAN CARDIOMYOCYTES PROLIFERATE?

In order to determine cell proliferation, scientists have used several different techniques and markers. Most studies assess cell proliferation rates based on the incorporation of a nucleotide analog, like BrdU. However, this is not a definitive proof of proliferation as DNA repair and polyploidization can also cause BrdU incorporation. Mitotic figures are used as well. However, it is a known fact that cardiomyocytes can undergo karyokinesis without cytokinesis resulting in poly-nucleated cells. Thus, it is important to prove induction of proliferation by demonstrating cytokinesis and cell division. The best evidence may be provided through live cell imaging and an increase in cell number.

As early as 1888, rare mitotic figures were reported in adult cardiomyocytes inside muscle fibers bordering necrotic myocardium.²⁶ However, until today, direct proof of actual cell division has not been reported. In the 1970's several investigators demonstrated by live cell imaging that neonatal cardiomyocytes divide in vitro, albeit rarely. Many attempts have been undertaken to increase the proliferation rate of neonatal and adult cardiomyocytes in culture. DNA synthesis can be induced in vitro by growth factors, viral oncoproteins, and cell cycle activators.^{27,28} However, none of these studies was able to document the induction of cytokinesis in adult cardiomyocytes.

Considerable effort has been invested to accomplish mammalian cardiomyocyte proliferation in vivo. Transgenic overexpression of oncogenes/cell cycle promoters has led to cell cycle activation in adult cardiomyocytes but still failed to prove cell division.^{27,29-33} Recently, a major step forward was made by demonstrating that targeted expression of Cyclin D2 in cardiomyocytes leads to DNA synthesis in vivo and infarct regression. Although this study presents several indirect proofs for cardiomyocyte proliferation (increase in DNA synthesis, mitotic figures, and cell number based on mathematical calculations), the clear evidence that adult cardiomyocytes proliferate is still lacking. Furthermore, gene expression in transgenic approaches began in fetal development when cardiomyocytes still proliferate which raises the possibility that cardiomyocyte differentiation was altered by the transgene. Lastly, experiments to confirm the effect of these genes on proliferation of adult cardiomyocytes have not been successful. For example, de novo expression of c-myc in adult cardiomyocytes in vitro or in vivo (inducible system) failed to promote cardiomyocyte cytokinesis.^{28,34}

Several possibilities may explain the observed limited proliferation potential of adult cardiomyocytes. Mature cardiomyocytes contain highly ordered structures, called sarcomeres, which contain contractile proteins required for force generation. This structure is incompatible with cytokinesis. Secondly, adult cardiomyocytes must continuously beat to sustain cardiac output, and it was not clear how they could simultaneously divide and contract. Thirdly, cardiomyocytes are often bi-nucleated or polyploid.

We have recently identified p38 MAP kinase as a major negative regulator of cardiomyocyte proliferation during development in vivo. p38 activity is inversely correlated with cardiac growth during development. Activation of p38 in vivo by MKK3bE reduces fetal cardiomyocyte proliferation whereas cardiac-specific *p38 α* knockout mice show an increase in neonatal cardiomyocyte mitoses. Importantly, we have shown that mono- and bi-nucleated adult mammalian cardiomyocytes can divide in vitro.³⁵ Simultaneous stimulation with growth factors and inhibition of p38 MAP kinase resulted in transient dedifferentiation by eliminating sarcomeric structures and completion of cytokinesis.³⁵ Thus, our data provide convincing evidence that fully matured mammalian cardiomyocytes can divide in vitro.

In addition, our proliferation data for adult cardiomyocytes resemble data previously described for fetal and neonatal mammalian cardiomyocytes as well as adult newt cardiomyocytes. Of note, the transient dedifferentiation of the sarcomeric structure during cytokinesis supports the notion of normal cell division resulting in two functional cardiomyocytes. This phenomenon was previously demonstrated in fetal cardiomyocytes as early as 1909.²⁶ In 1972 investigators showed that neonatal cardiomyocytes transiently dedifferentiate, stop beating, undergo cytokinesis, redifferentiate and begin beating again.³⁶ The same has been observed in adult newt cardiomyocytes.^{18,37} However, our study has demonstrated neither adult cardiomyocyte proliferation in vivo nor its relevance after myocardial injury.

HOW IS CARDIOMYOCYTE PROLIFERATION REGULATED?

Despite the fact that we have made considerable progress in inducing cardiomyocyte proliferation, cell cycle control in cardiomyocytes remains enigmatic. Why do cardiomyocytes lose the ability to proliferate? What are the mechanisms that regulate the essentially irreversible cell cycle withdrawal of cardiomyocytes? Why does the heart respond to injury by hypertrophy but not by proliferation?

Why do primary cardiac tumors almost never occur?^{38,39} Is the lack of cardiomyocyte proliferation due to heart function?

In general, there is an inverse relationship between proliferation and differentiation,⁴⁰ and molecules that promote differentiation may also repress cell cycle reentry. However, the mechanism that regulates cell cycle exit of cardiomyocytes is unknown. This might be the reason why most studies trying to induce cardiomyocyte proliferation have focused on inducers of proliferation, namely growth factors, cell cycle genes, and oncogenes.²⁷⁻³³ However, these attempts were not successful. Based on the observation that cardiac tumors occur very rarely, this is not a surprising result. It appears that cardiomyocytes have acquired several mechanisms that prevent proliferation. In contrast to previous studies, we have established that both negative and positive regulatory pathways need to be targeted in order to promote cardiomyocyte proliferation. First, we have identified p38 as a key negative regulator of cardiomyocyte proliferation. However, inhibition of p38 was not sufficient to result in cardiomyocyte proliferation. Second, we screened several growth factors and confirmed FGF1 as a positive regulator of cardiomyocyte proliferation. Stimulation of cardiomyocytes with FGF1 resulted in DNA synthesis but not proliferation. However, simultaneous inhibition of p38 and stimulation with FGF1 initiated cardiomyocyte proliferation.³⁵ Our study suggests that in addition to provide factors promoting cell cycle progression, it is important to neutralize mechanisms responsible for the cell cycle blockade. This finding reveals the importance to identify the mechanisms that regulate differentiation and cell cycle exit of cardiomyocytes.

IS CARDIOMYOCYTE PROLIFERATION AN ALTERNATIVE TO STEM CELLS?

Recent studies have reported improved cardiac function after transplantation of various stem cell populations in animal models of myocardial injury.⁴¹⁻⁴⁴ Clinical trials have also been performed with encouraging results for some types of stem cells. However these studies are controversial because it is unclear why stem cell therapies result in an improvement of cardiac function. So far, none of these studies has unambiguously demonstrated that the utilized stem cells give rise to new cardiomyocytes. It rather seems that stem cells secrete survival factors that rescue damaged heart muscle cells from undergoing apoptosis and/or promote an innate regeneration program that might include proliferation of existing cardiomyocytes.

Recent studies by others and us suggest that cardiac regeneration through cardiomyocyte proliferation has potential to be an alternative approach to stem cell therapy. First, naturally occurring heart regeneration in newt and zebrafish is based on cardiomyocyte proliferation. Second, mammalian heart growth during fetal development is mediated by cardiomyocyte proliferation.²⁶ Third, the heart reactivates the cell cycle machinery after damage and reexpresses a subset of fetal genes suggesting that adult cardiomyocytes are not fixed in a terminally differentiated state. Fourth, adult mammalian cardiomyocytes can divide in vitro. Fifth, targeted expression of Cyclin D2 leads to cardiomyocyte DNA synthesis in vivo and infarct regression.

FUTURE DIRECTIONS

Rumyantsev speculated in 1977 that a "limited percentage of adult mammalian ventricular myocytes may overcome the most rigid restraints regarding the resumption of DNA synthesis and pass through all phases of the mitotic cycle".²⁶ Since then we have collected considerable evidence that cardiomyocyte proliferation can be

a platform for myocardial repair. However, we have to overcome several major hurdles and many questions still remain: Does cell division of adult mammalian cardiomyocytes in vitro results in two functional and fully differentiated cardiomyocytes? Is it possible to induce cardiomyocyte proliferation in vivo through pharmacological means? Are there other positive and negative regulatory pathways involved in cardiomyocyte proliferation? What is the mechanism underlying cell cycle regulation in adult cardiomyocytes? Why does the mammalian heart respond to injury by hypertrophy and not proliferation? Can cardiomyocyte proliferation be used to rebuild the heart after acute injury?

In summary, both the use of stem cells and the induction of proliferation in surviving cardiomyocytes provide new promising approaches to repair heart muscle after damage. The research of cardiac regeneration is a fast moving field and is of tremendous clinical importance. However, moving too fast into clinical trials without a profound knowledge of the underlying mechanisms bears tremendous risk from unforeseen medical complications. To realize the goal of regenerative medicine, it is imperative to work as multi-disciplinary teams including basic scientists, biotechnologist, bioengineers and physicians. If we are patient and persistent in our pursuit of the underlying mechanisms of improved heart function in animal models, we might be able to apply our findings to humans without serious medical complications. In that case cell-based repair of the heart will save and improve the lives of millions of people.

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