

Features of cardiomyocyte proliferation and its potential for cardiac regeneration

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Received: March 9, 2008; Accepted: July 16, 2008

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Abstract

The human heart does not regenerate. Instead, following injury, human hearts scar. The loss of contractile tissue contributes significantly to morbidity and mortality. In contrast to humans, zebrafish and newts faithfully regenerate their hearts. Interestingly, regeneration is in both cases based on cardiomyocyte proliferation. In addition, mammalian cardiomyocytes proliferate during foetal development. Their proliferation reaches its maximum around chamber formation, stops shortly after birth, and subsequent heart growth is mostly achieved by an increase in cardiomyocyte size (hypertrophy). The underlying mechanisms that regulate cell cycle arrest and the switch from proliferation to hypertrophy are unclear. In this review, we highlight features of dividing cardiomyocytes, summarize the attempts to induce mammalian cardiomyocyte proliferation, critically discuss methods commonly used for its detection, and explore the potential and problems of inducing cardiomyocyte proliferation to improve function in diseased hearts.

Keywords: regeneration • heart • proliferation • cardiomyocytes

Introduction

Ischaemic heart disease is among the leading causes of death worldwide and thus cardiac regeneration has caught an ever-increasing interest among scientists [1]. The ability of the heart to regenerate has been studied since the mid-nineteenth century and the consistent conclusion has been that the heart has little or no regenerative capacity [2, 3]. Recently, however, an increasing number of studies have reported induction of cardiomyocyte proliferation and cardiac regeneration. Nevertheless, it is still disputed to what extent cardiomyocyte proliferation can be induced and whether induction of proliferation can be utilized therapeutically.

Mammalian cardiomyocytes *in vivo* lose their ability to proliferate and exit the cell cycle during the first weeks after birth [3, 4]. Therefore, the adult mammalian heart is considered incapable of

regeneration after ischaemic or other forms of injury. Natural compensatory processes of the injured heart are limited to hypertrophy of the remaining cardiomyocytes and replacement of necrotic regions with fibrotic scar tissue. Cardiac scarring and loss of contractile tissue can cause arrhythmias, dilation, heart failure and other complications, contributing significantly to morbidity and mortality [1, 5]. Recent progress in conventional treatment has reduced early mortality from myocardial infarction. However, these treatment regimens fail to correct the primary cause of impaired heart function: the loss of cardiomyocytes. Therefore, two strategies have been employed in recent years: cell replacement through cell therapy and induction of endogenous cardiomyocyte proliferation.

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Multiple cell types have been tested for repopulation of the injured myocardium to improve heart function. At first, it had been demonstrated that transplanted foetal and neonatal cardiomyocytes could functionally integrate and enhance recipient cardiac function [6]. Therefore, scientists enhanced their efforts to identify a cell type that can be obtained in large quantities and can be differentiated into cardiomyocytes. Skeletal myoblasts seemed a good alternative, since they are autologous, easy to obtain and they have a high proliferative potential. It has been demonstrated that they incorporate in the myocardium and improve myocardial function upon transplantation after myocardial infarction. However, they do not convert into cardiomyocytes and do not establish connections with the host cardiomyocytes [7]. On the other hand, clinical studies in patients with chronic myocardial disease have consistently reported modest improvements in ventricular function and clinical status [8]. A plethora of different types of stem cells have been tested. Several studies have reported improved cardiac function after transplantation of bone marrow-derived stem cells, *i.e.* mesenchymal stem cells (MSC) and haematopoietic stem cells (HSC), in animal models of myocardial injury [9, 10] as well as in clinical trials [11, 12]. However, the improvement is rather minor and, as recently reported, might be only transient [13, 14].

Many stem cell types can be forced *in vitro* to differentiate into cardiomyocytes [15]. However, various experiments using several stem/progenitor cell types indicate that there is little or no differentiation of the engrafted cells into cardiomyocytes *in vivo* and that their survival rate is low, with the exception of embryonic stem cells [16–18]. Therefore, the mechanism of stem cell therapy is still controversial. The main current opinion is that stem cells as well as skeletal myoblasts secrete cytokines and/or growth factors that cause the functional improvement by stimulating neovascularization, cardiomyocyte proliferation and/or preventing apoptosis [15, 19–23]. Recently, it has been reported that the mammalian heart contains resident cardiac stem cells (CSC). The possibilities of expanding autologous CSCs *ex vivo* or stimulating the regeneration capacity of these cells *in vivo* are exciting options for therapeutic regeneration [24].

Cardiac regeneration through cardiomyocyte proliferation is appealing because mammalian heart growth during foetal development as well as newt heart regeneration is mediated by cardiomyocyte proliferation [3, 23]. The mechanism of cardiac regeneration in zebrafish remains unclear but it appears at least to be partially based on cardiomyocyte proliferation [25]. Cell proliferation is an orderly sequence of events in which cells duplicate their contents and then divide. This cycle of duplication and cell division is known as the cell cycle (Fig. 1). The cell cycle consists of four distinct phases: G1 (gap 1) phase, S (synthesis) phase, G2 (gap 2) phase (collectively known as interphase) and M (mitotic) phase. In S phase, the DNA is replicated. During gap phases, the cell grows and progression to the next cell cycle stage is controlled by a variety of intracellular and extracellular signals. Cells that have temporarily or reversibly stopped proliferating enter a state of quiescence called G0. M phase is composed of two tightly coupled processes: nuclear division (karyokinesis)

in which the chromosomes are segregated, and cell division (cytokinesis), in which the cytoplasm divides forming two distinct daughter cells. Progression through the different phases is highly regulated [4, 26].

In this review, we highlight the features of natural dividing adult newt and foetal mammalian cardiomyocytes. We describe techniques necessary to identify cardiomyocytes and to determine proliferation. Finally, we critically analyse the recent literature about induction of mammalian cardiomyocyte proliferation and cardiac regeneration.

Newt and zebrafish heart regeneration

Urodele amphibians are generally regarded as the champions of regeneration among vertebrates, as first reported by Spallanzani in 1768. An adult newt is able to regenerate its tail and limbs, upper and lower jaws, ocular tissue such as the retina and lens, and even substantial sections of the heart [27–30].

The cardiac muscle of amphibians is capable of regenerating up to 50% of the ventricle after mechanical excision. This process involves dedifferentiation of the remaining cardiac muscle cells and proliferation of both cardiomyocytes and connective tissue cells [30–32]. To increase the proliferative response, the apical portion of the newt ventricle was amputated, minced and placed onto the amputation gap. This procedure was followed by a period of proliferation that peaked at 16 days after amputation [28]. These data suggest that in urodele amphibians most of the mature cardiomyocytes retain the ability to proliferate.

To further understand the process of newt cardiac regeneration, ventricular cardiomyocytes were placed into cell culture. DNA synthetic and mitotic activity in these cultures was studied using 24-hr exposures to tritiated thymidine and time lapse videotaping. Nuclear labelling was first seen after 6 days (7.5%), increasing to 30.5% after 10 days and subsequently declined. The majority of cardiomyocytes (75%) were found to replicate DNA and 76% of those entered mitosis. Mitotic indices near the peak time were 0.48% (8 days), 1.7% (10 days) and 1.55% (12 days). A total of 81% of the observed mitotic cardiomyocytes underwent cytokinesis. Taken together, approximately one-third of the initial cardiomyocyte population progressed through mitosis and entered successive cell divisions [32–34]. Interestingly, also multinucleated cells were found to replicate DNA and to complete mitosis and cytokinesis. These results show that the formation of multinucleated cardiomyocytes does not preclude further proliferation.

Heart regeneration in zebrafish is poorly understood. Initially, it has been reported that it occurs through robust proliferation of cardiomyocytes after amputation of the apex. The time course of cardiac regeneration as well as the number of cardiomyocytes undergoing DNA synthesis and mitosis highly resembled cardiac regeneration in newt [35]. Unfortunately, zebrafish cardiomyocyte proliferation has so far not been achieved *in vitro*. Recent data

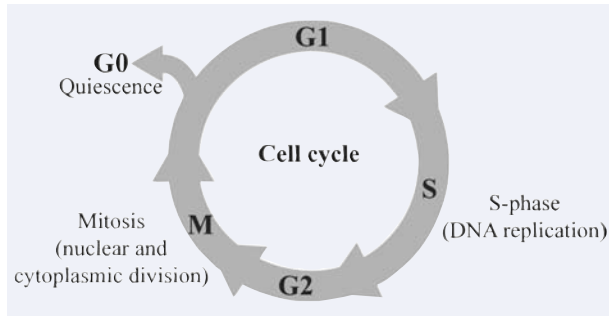


Fig. 1 The cell cycle consists of four distinct phases: G1 phase, S phase, G2 phase and M phase.

suggest that stem/progenitor cells might also be involved in this process [36].

In conclusion, these data strongly suggest that naturally occurring heart regeneration in lower vertebrates is based on cardiomyocyte proliferation.

Features of dividing cardiomyocytes

Shortly after birth, mammalian cardiomyocytes stop proliferating. Continuous heart growth is based on increase in cell size, *i.e.* hypertrophic growth [37]. This transition is characterized by maturation of the contractile apparatus, a cytoplasmic structure that is thought to preclude cytokinesis [3]. Historically, this conclusion was derived from the evidence that during histogenesis of skeletal muscle in vertebrates, only myoblast and/or satellite cells are able to synthesize DNA and to divide mitotically. Cell fusion and the onset of contractile protein synthesis are accompanied by a stable repression of genes controlling the multiplication of the muscle cell. Thus, differentiation and proliferation are mutually exclusive in skeletal muscle [38, 39].

In contrast to skeletal muscle, differentiation of cardiomyocytes proceeds gradually during the course of embryonic and postnatal development. It happens in a step-by-step increase in size, number, and structural complexity of myofibrils, mitochondria and sarcoplasmic reticulum, along with the formation of intercalated disks, desmosomes and tight junctions. At the same time, the relative amount of non-sarcomeric filaments, microtubules, free ribosomes, Golgi and rough endoplasmic reticulum elements diminish visibly, especially during the postnatal stages of cardiac myogenesis [3]. Interestingly, as they differentiate, foetal beating cardiomyocytes still undergo cytokinesis [40, 41]. In chick the mitotic index even increases from 1.5% at 30 hrs after incubation to a peak of 3.2% by 4 days when myofibrils accumulate [42–44]. This demonstrates that differentiation and proliferation are compatible in cardiomyocytes.

Naturally occurring proliferation of cardiomyocytes from newt and developing chick, rat and mouse myocardium have been

studied with several techniques including high laser confocal microscopy and live cell imaging [3, 31, 40–46]. All these studies observed in essence the same changes in cardiomyocytes during the course of cell division: cardiomyocytes in prophase do not differ in their appearance from non-dividing cardiomyocytes except for the condensation of the chromosomes. The myofibrils remain unchanged up to the late prophase whereas the nucleolus persists frequently up to mid-prophase. Disintegration of the nuclear membrane occurs in late prophase. During prometaphase the majority of Z-disks of cardiomyocyte myofibrils undergo degradation. After the metaphase plate has formed almost all the myofibrils are seen subdivided into isolated sarcomeres and myofilament bundles as a result of the progressive Z-disk degradation. This is followed by a progressive isolation and scattering of the myofilament bundles and of the whole sarcomeres during the subsequent phases of mitosis. Consistent with this observation contractions become weaker during metaphase and cease during anaphase.

Molecular analyses have shown that myofibrillar breakdown occurs in a biphasic manner. Z-disk and thin-filament associated proteins appear in a diffuse localization pattern before M-band and thick-filament-associated proteins [40]. Complete myofibril disassembly is only seen in cardiomyocytes in telophase. Importantly, no myocardial cell has been observed in these studies that underwent mitosis without these disruptive changes of the myofibril striation patterns [40, 41, 44].

Cleavage furrow formation begins in late telophase and continues until complete separation of both daughter cells. Next, both nuclei increase in size. When each daughter nucleus attains around half of its volume of the interkinetic nucleus the first signs of gradual restoration of contrast rich z-bands interconnecting the previously isolated sarcomeres are observed. The re-assembled myofibrils are first disorganized, crossing one another. Throughout the subsequent growth and maturation, myofibrils become ultimately oriented parallel to the long axis of the cell [41].

In 1977, it was concluded, from the observation of mitotic figures in injured adult hearts, that 'a limited percentage' of adult mammalian cardiomyocytes can 'pass through all phases of the mitotic cycle' and that this was associated with the transient disassembly of the contractile apparatus (Fig. 2) [3].

Interestingly, dividing cardiomyocytes *in vitro* and in whole mount preparations retain close contact with their non-dividing neighbours throughout all phases of mitosis. Persistence of desmosome-like structures prevents the mitotic cell from a completely rounding off and from the loss of its association with the neighbouring cells [40, 44]. This evidence from static pictures has also been observed in live cell imaging studies depicting actively contracting rat cardiomyocytes in a state of division. The authors noted that the myocardial cell, in contrast to non-myocytes, did not round up prior to division [46].

Taken together, a plethora of characteristic features of dividing cardiomyocytes has been collected over the last decades. These data provide a framework for careful analysis of recent data reporting the induction of proliferation of cardiomyocytes and cardiac regeneration.

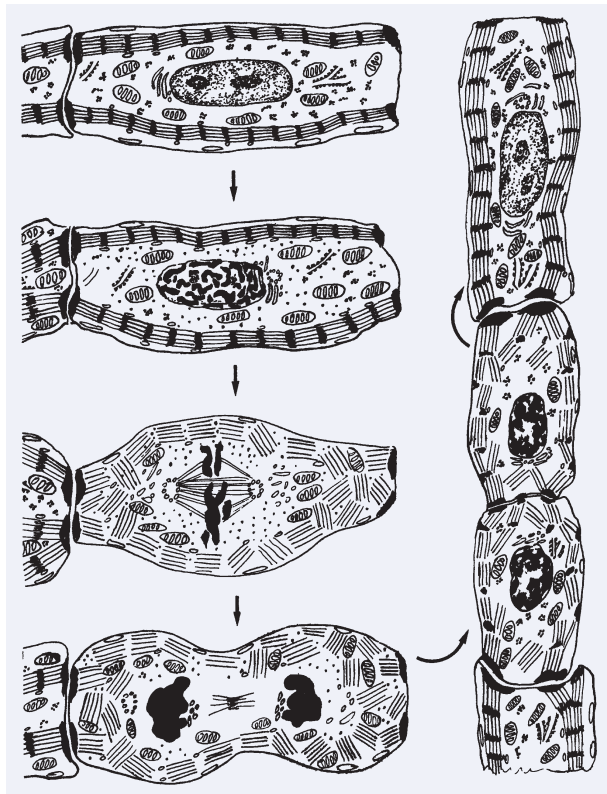


Fig. 2 Suggested scheme of myofibril changes throughout mitosis and cell division of adult mammalian cardiomyocytes *in vivo*. The myofibrils remain unchanged up to late prophase. After the metaphase plate has formed almost all Z-disks are degraded and the myofibrils are subdivided into isolated sarcomeres and myofilament bundles. Complete myofibril disassembly is only seen in telophase. During cytokinesis, the first signs of gradual restoration of Z-disks interconnecting the previously isolated sarcomeres are observed. These early myofibrils are often in disorder, crossing one another. Throughout the subsequent growth and maturation myofibrils become rearranged and oriented parallel to the long axis of the cell. This Figure was published in Romyantsev, P.P. (1977). Interrelations of the proliferation and differentiation processes during cardiac myogenesis and regeneration. *Int Rev Cytol* 51, 186–273. Copyright Elsevier (1977).

Cell division *versus* poly-nucleation and endoreduplication

Cardiac injury is followed by down-regulation of cell cycle inhibitors like p21 and p27 and up-regulation of cell cycle perpetuating factors like PCNA and cdk/cyclin complexes in cardiomyocytes. Several studies have concluded from these observations and the occurrence of mitotic figures that fully matured mammalian cardiomyocytes can undergo cell division after injury as a normal, although limited, physiological response to injury [3, 47–53]. However, induction of DNA synthesis or mitosis after

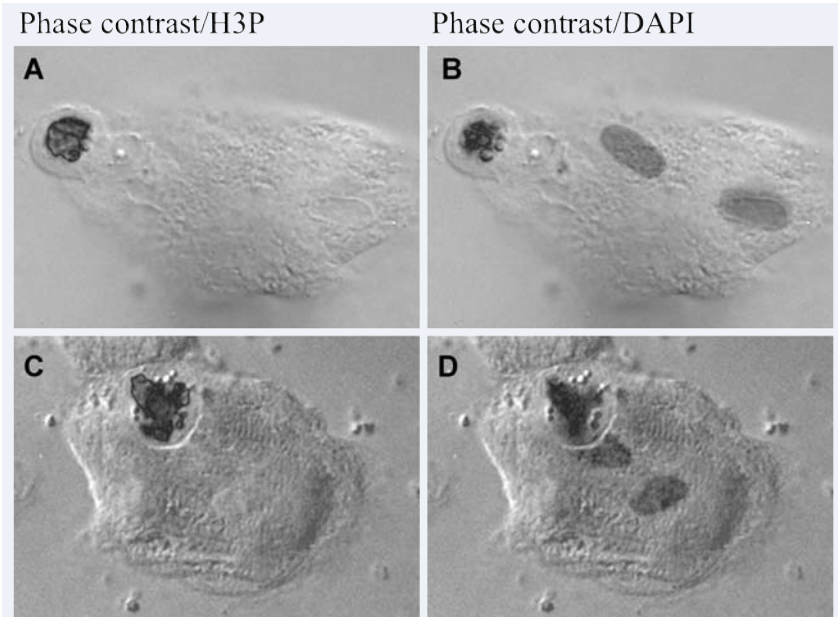
injury could simply result in endoreduplication or poly-nucleation (increased DNA per nuclei or increased nuclei per myocyte) as cell cycle activity during development leads to cell division but also to poly-nucleation [54]. Thus, re-expression of cell cycle genes, the detection of DNA synthesis and mitotic figures are no proof of cardiomyocyte cell division. This phenomenon has been reported for human myocardium [3, 54, 55] and for the myocardium of c-myc transgenic mice [56].

Recently, a study concluded from Ki-67 stainings and mitotic figures that human cardiomyocytes undergo cell division after acute myocardial infarction [49]. However, Ki-67 stainings and mitotic figures cannot be used to distinguish between cell division and poly-nucleation/endoreduplication. Thus, this study has been repeated including additional techniques [55]. The authors reported similar numbers of Ki-67 positive cardiomyocytes in different stages of mitosis. However, they showed that metaphase and late anaphase chromosomes were always located within a preserved nuclear envelope, a clear sign of endoreduplication. In contrast, the nuclear membrane disintegrates during cell division at the end of prophase. This demonstrated that expression of Ki67, usually a marker for proliferating cells [57], and mitotic figures alone are inadequate to distinguish between cell division, endoreduplication and poly-nucleation in cardiomyocytes.

Detection and induction of cardiomyocyte proliferation

Considerable effort has been invested to achieve mammalian cardiomyocyte proliferation to prove that it can be utilized for cardiac regeneration. Various growth factors (*e.g.* FGF1, Neuregulin), small molecules (*e.g.* BIO, SB203580), viral oncoproteins (*e.g.* E1A, SV40 T antigen), an extracellular matrix protein (Periostin) and cell cycle activators (*e.g.* Cyclin A2, Cyclin D2, E2F1, E2F2, c-myc) were studied [4, 58–60]. These studies proved beyond doubt that cell cycle can be re-induced in postnatal cardiomyocytes. To what extent postnatal cardiomyocytes can undergo mitosis, however, is controversial. Mitosis is rare and it is not always simple to determine whether a mitotic nucleus actually belongs to a cardiomyocyte or non-myocyte. As shown in Figure 3, it is not obvious whether the mitotic nuclei are cardiomyocyte nuclei even when using phase contrast microscopy and DNA- as well as phosphorylated Histone H3 (H3P)-staining. This is also a problem *in vivo* as in the injured heart large amounts of proliferating inflammatory cells and (myo)fibroblasts infiltrate the myocardium laying in close proximity to the cardiomyocytes. Moreover, *in vivo* cardiomyocytes display a highly irregular shape and non-cardiomyocytes are often found in the indentations in the cardiomyocytes (Fig. 4). Similar problems have been described in an editorial questioning the occurrence of cardiac chimerism [61] and in a review on stem cell-based cardiac regeneration [62]. Thus, cardiomyocyte-specific cytoplasmic markers are not suitable to distinguish cardiomyocyte from non-myocyte nuclei [63, 64].

Fig. 3 Examples of the problem of interpreting H3P staining in cultured adult cardiomyocytes. (**A, C**) The H3P-positive nuclei (black) appear to be mitotic cardiomyocyte nuclei indicating proliferation. However, DNA stain (DAPI, black, **B, D**) reveals in each cell two H3P-negative nuclei, which argue against proliferation as does the presence of intact myofibrils. Possibly, the H3P-positive nuclei belong to overlaying non-myocytes explaining the membrane separating the chromosomes from the cardiomyocytes. Another explanation for C, D is that the membrane is a nuclear membrane separating an endomitotic cardiomyocyte nucleus.



Even a 3D reconstruction of cytoplasmic-stained cardiomyocytes appears to be inconclusive [59, 65].

To avoid false-positives, markers that stain the cell membrane of cardiomyocytes, such as Caveolin 3, could be used [66, 67]. Non-myocyte but not cardiomyocyte nuclei will be separated from the cardiomyocyte cytoplasm by cell membrane (Fig. 4) [66]. Another elegant way of detecting cardiomyocyte nuclei is labelling the cardiomyocyte nuclei with markers like Nkx2.5 or utilizing genetic models like the MHC-nLAC reporter mice introduced by Loren Field's group [68, 69]. However, nuclear markers cannot be used to identify mitotic cardiomyocytes as the nuclear membrane disintegrates during prophase. However, they allow distinguishing endoreduplication from karyokinesis.

Some examples of mitotic cardiomyocytes presented in the literature do not match the features of naturally occurring cardiomyocyte proliferation. For instance, mitotic figures within striated cardiomyocytes have been shown after *bcl2* overexpression as well as in miRNA-1-2 deficient mice [64, 70]. This is unusual, as disassembly of the myofibrils has to take place to allow cell division. Another atypical observation has been reported after Cyclin A2 overexpression: cardiomyocytes detach and round up during mitosis [71]. Finally, to define cardiomyocyte cytokinesis it is important to observe the precise localization of cytokinetic markers and not simply evaluate the generalized expression of these markers. Aurora B, for example, associates with centromeric heterochromatin early in mitosis, transfers to the central spindle and finally localizes to the contractile ring and midbody [72]. However, in cardiomyocytes it can localize at the midbody during proliferation as well as binucleation. Furthermore, it has been suggested that contraction of the contractile ring and cleavage furrow ingression is disturbed during binucleation [73]. In a recent study, several

examples of Aurora B-positive cardiomyocytes were presented. Whereas some of the examples indicate cell division others are rather indicative for binucleation as the cardiomyocytes undergo a one-sided furrow ingression [59].

Although it is impossible to confer from BrdU-labelling, H3P- or Aurora B staining that cells proliferate the combination of these results can indicate problems in cell cycle progression. DNA synthesis is detected by incorporation of BrdU during S phase. This method allows detecting all cells that have entered S phase during the labelling period. In contrast, mitosis can only be detected at the moment it occurs, usually utilizing antibodies against H3P. In addition, mitosis and cytokinesis are the shortest phases of the cell cycle. Therefore, even fast proliferating cells like foetal cardiomyocytes *in vivo* or HeLa cells show a relatively low percentage of H3P-positive cells (3.7% or 7%, respectively) [67]. Thus, a fast proliferating cell population will have a mitotic/cytokinetic index of approximately 7% and a BrdU-labelling index of 100%, when labelled for 24 hrs, giving a ratio of 14.29. If there are problems in executing or in progressing through mitosis/cytokinesis this ratio will decrease. In a study describing the effect of Periostin, the authors reported a BrdU-labelling index of 1.1% (3 days BrdU-labelling) and a cytokinesis index of 0.5% resulting in a surprisingly low ratio of 2.2 [59]. Another study describing the effect of GSK-3 inhibitor Bio reports for adult cardiomyocytes a higher mitotic index than for neonatal cardiomyocytes (2.41% versus 2.25%) although the BrdU-labelling index was lower (13.1% versus 47.2%) [60]. There are several possible explanations for those data: (1) The cells accumulate during cytokinesis due to a cell division defect or (2) the cells have been induced to enter the cell cycle and almost synchronously progressed through the cell cycle into cytokinesis.

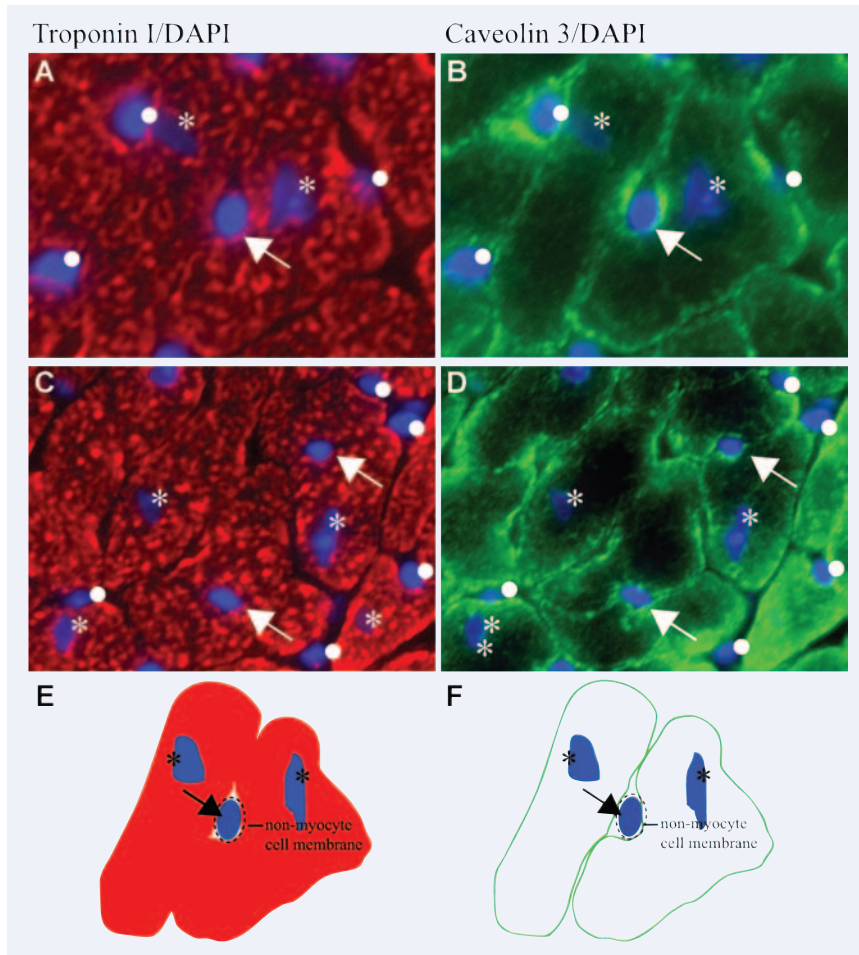


Fig. 4 Examples of the problem of identifying cardiomyocyte nuclei in transversal tissue sections of murine myocardium (A–D, 5 μm -thick). Cardiomyocyte-specific cytoplasmic markers (Troponin I, red) are often used to determine the identity of nuclei (DAPI, blue). Cardiomyocyte nuclei (asterices) are embedded in cardiomyocyte cytoplasm whereas non-myocyte nuclei (dots) are not. A muscle-specific membrane marker (Caveolin 3, green), however, shows that some of the embedded nuclei are separated from the cardiomyocyte cytoplasm by a muscle-membrane (arrows). In addition, these nuclei are not surrounded by a continuous muscle membrane. This indicates that these nuclei belong to non-myocytes located in between two cardiomyocytes. The drawings give a schematic overview of this phenomenon (E and F). In case, that this nucleus would indeed belong to a cardiomyocyte one should see a double muscle-membrane.

Very few studies have combined detection of DNA synthesis, mitosis and cytokinesis and added cell count assays to their analyses. This is important as detection of proteins involved in cytokinesis does not unequivocally prove proliferation. For example, it has been demonstrated that septins, another protein family involved in contractile ring formation, are re-expressed during pathological hypertrophy [74]. Furthermore, it has been shown that cells in the end stage of cytokinesis can fail to undergo cell division and reverse their cytoplasmic constriction resulting in poly-nucleated cells [75]. A molecular marker that has been described to indicate whether a cytokinetic cardiomyocyte will undergo binucleation rather than cell division is Anillin [73]. Anillin was originally identified as an actin-binding protein. It localizes to the cleavage furrow, binds septins, associates with myosin II, and is required for cytokinesis [76].

The first attempts to induce neonatal cardiomyocyte proliferation employed viral proteins two decades ago. Expression of T antigen was associated with mitotic figures and a 3-fold increase in the number of myocardial cells 72 hrs after infection. Within a week, the density of myocardial cells reached confluence. It is noteworthy that in this study not all mitotic cells underwent cytokinesis.

The mitotic index was more than 20-fold increased (4.3% *versus* 0.2%). However, the number of poly-nucleated cells also increased by almost 4-fold (8.6% *versus* 2.3%) [77]. Busk and co-workers used similar approaches to demonstrate that Cyclin D2 overexpression results in an increase in cell number of neonatal cardiomyocytes (1.8-fold) and of 21 days old cardiomyocytes in the presence of serum (2-fold) at 6 days after infection [78].

Recently, it has also been demonstrated that FGF1/p38 inhibitor stimulation induces cardiomyocyte proliferation. This approach increased the mitotic index in neonatal cardiomyocytes after 72 hrs from 0.06% to 3.72%. Cell number increased by 2-fold. Confluence was reached 8 days after stimulation. In addition, cell division was visualized using Aurora B and Anillin staining as well as FACS analysis to monitor binucleation [73]. FGF1/p38 inhibitor stimulation induced also cell division in cultured adult cardiomyocytes demonstrated by transient dedifferentiation combined with cleavage furrow formation and separation of the cytoplasm [67]. A similar strategy was followed to investigate the effect of Periostin on cardiomyocyte proliferation. The authors utilized Aurora B staining to detect cardiomyocyte cytokinesis and reported that stimulation resulted in 0.5% Aurora

B-positive adult cardiomyocytes *in vitro* and 0.1% in healthy myocardium [59].

An interesting technique has recently been introduced that follows a very different approach. Instead of concentrating on markers for the different cell cycle phases this study utilized the so-called flowcytometric BrdU-Hoechst assay [79]. In the absence of BrdU, all cells within G0/1-phase show the same intensity of fluorescence for the DNA staining using both propidium iodide and Hoechst 33258. When BrdU is incorporated into the DNA, it selectively quenches the Hoechst fluorescence but does not interfere with that from PI. Hence, cardiomyocytes that have progressed through S-phase and subsequently divided will have the same PI fluorescence but a reduced Hoechst fluorescence compared with G0/1 cells, which did not proliferate. Using this method the authors demonstrated that expression of E2F1 and E2F2 significantly increased the number of dividing newborn cardiomyocytes [80].

Taken together, many studies have shown that DNA synthesis, mitosis and karyokinesis can be induced in cardiomyocytes. However, whether induction results in cardiomyocyte proliferation is still a matter of interpretation. Therefore, cell cycle control in cardiomyocytes remains enigmatic. It will be important to understand why it is easier to induce neonatal than adult cardiomyocyte proliferation. An obvious reason is the increased maturity of the contractile apparatus in adult cardiomyocytes and thus the need for dedifferentiation, *i.e.* disassembly of the myofibrils. Another reason might be changes in the distribution and composition of adherence junctions, gap junctions and desmosomes (combined intercalated discs) between embryonic and adult cardiomyocytes. Early embryonic cardiomyocytes have these structures distributed circumferentially at the peripheral membrane whereas these components are restricted to the bipolar ends in adult cardiomyocytes. While adherence junctions and desmosomes seem to play mainly a mechanical role, gap junctions maintain the ionic and thus electrical coupling of individual cardiomyocytes [81]. Interestingly, the postnatal gap junction protein connexin 43 but not the embryonic isoform connexin 40 has been demonstrated to be involved in cell cycle control in cardiomyocytes as well as in other cell types [82, 83]. Finally, the recent finding that an extracellular matrix protein can induce cardiomyocyte proliferation highlights the importance of the microenvironment [59]. Previously, it had already been suggested that extracellular matrix proteins play an important role in peripheral nerve regeneration and cancer development [84, 85].

Whether cardiac dedifferentiation can be induced *in vivo* or whether the re-expression of the foetal gene program during hypertrophy or cardiac injury is comparable to the observed cardiac dedifferentiation during natural occurring regeneration remains unclear.

Induction of cardiac regeneration

Studies to date have shown that cyclin D2 [86], cyclin A2 [63], FGF5 [87, 88], VEGF165 [89], Periostin [59] and FGF1/p38

inhibitor [66] treatment promote cardiac regeneration due to induction of cardiomyocyte proliferation. Most of these studies have in addition shown improved heart pump function (ejection fraction or fractional shortening) after myocardial infarction. The question is whether the newly formed cardiomyocytes can directly account for the improved heart function. The mentioned studies demonstrate a mitotic index between 0.2% and 1.2%, which is in the same range as for the regenerating newt and zebrafish heart (<2%). Therefore, cardiomyocyte proliferation could indeed account for cardiac regeneration.

The only study that directly demonstrated that cardiomyocyte proliferation can be utilized to improve heart function has been conducted by the group of Loren Field [90]. Other studies are dependent on estimations by combining quantitative data obtained by histological techniques to suggest formation of new myocardium. For example, Kühn and colleagues calculated that treatment of the infarcted heart with Periostin resulted in the regeneration of 7.2×10^6 cardiomyocytes based on BrdU incorporation, or 5.5×10^6 cardiomyocytes based on quantification of cardiomyocytes with metaphase chromosomes over a period of 12 weeks. The total number of cardiomyocytes in the left ventricle of the rat heart was previously calculated to be 18.5×10^6 [91]. Based on these numbers Periostin treatment resulted in a surprisingly high amount of cardiomyocyte restoration of approximately 30%. Interestingly, both Periostin treatment and Periostin deficiency lead to decreased fibrosis and improved cardiac function after myocardial infarction [92, 93].

As indicated above, improved cardiac function after treatment does not directly indicate that cardiomyocytes have proliferated. For example, growth factors could improve cardiac function by other mechanisms as they display pleiotropic actions [94]. First, there could be a direct effect of the treatment on the remaining cardiomyocytes after injury, such as protection against irreversible injury [95, 96], increased compensatory hypertrophy [87], or alterations in calcium handling that could improve contractility [97]. Second, the genetic or therapeutic manoeuvres could have additional indirect positive effects, *e.g.* on neovascularization promoting cell survival and contractility of surviving cardiomyocytes or on scar formation preventing ventricular rupture.

There are many examples that improvement of the latter parameters alone can have a substantial effect on heart function [98]. It has been shown that induction of vascularization after myocardial infarction by administration of CD34+ cells enabled survival of the remaining cardiomyocytes and led to a sustained improvement in heart function [99]. Fazel and co-workers showed that bone marrow-derived c-kit+ cells can improve heart function after myocardial infarction, which is independent of transdifferentiation of these cells into cardiomyocytes. Instead, c-kit+ cells release cytokines, which improve vascularization and potentiate the formation of myofibroblast-rich scar tissue [21].

Studies where cardiomyocyte proliferation is observed often show additional effects of the treatment. Hearts treated with Periostin after myocardial infarction had a 4-fold increase in capillary and a 1.5-fold increase in arteriolar density in the infarct and in the border zone [59]. FGF1 and FGF1/p38 inhibitor treatment

resulted in a more than 2.5-fold increased capillary density [66]. Intramyocardial injection of a plasmid encoding the 165 isoform of human vascular endothelial growth factor (pVEGF165) in sheep hearts after myocardial infarction increased arteriolar density 6.4-fold and capillary density 2.6-fold and decreased myofibroblast proliferation and fibrosis. In this study, all of these effects may be involved in the observed reduction in infarct size together with cardiomyocyte proliferation [99–101]. Intracoronary injection of an adenoviral construct overexpressing FGF-5 into hibernating myocardium led to significant myocardial hypertrophy with a $\pm 30\%$ increase in LV mass within 2 weeks. The authors concluded that stimulation of hypertrophy together with induction of cell cycle activity in a small number of cardiomyocytes was responsible for the improvement in heart function [87].

Taken together, there are studies that demonstrate cardiomyocyte proliferation together with functional improvement after myocardial injury. However, it is unclear how much of the beneficial effects were related to cardiomyocyte proliferation or to secondary effects like vascularization and survival. An important advance would be to perform proliferation inductive therapies as a control on animals in which cardiomyocytes were genetically disabled to undergo proliferation.

Cardiac regeneration needs more than proliferation

Currently, the main goal of cardiac regeneration is the restoration of lost cardiomyocytes. However, it is rather unlikely that providing new cardiomyocytes alone is enough to regenerate the injured heart.

Cardiac fibroblasts, myocytes, endothelial cells and vascular smooth muscle cells are the major cellular constituents of the heart. The relationship of these cellular populations depends on developmental stage and is species-specific. Cardiomyocytes make up 30% (rat) to 56% (mouse) of the total number of cells in the adult ventricle and thus a large part of the myocardium consists of other cell types [102]. It has been previously discussed for other organs that the induction of proliferation of one cell type will be corrected during development, for example, by apoptosis. It appears that cells can sense whether the appropriate number and type of cells as well as extracellular matrix surround them [103, 104]. Therefore, the ratio between cardiomyocytes, supporting cells and extracellular matrix provided by the cells in the myocardium has to be kept in balance. Following myocardial injury cardiomyocytes but also other necessary cells, such as endothelial cells, fibroblasts, smooth muscle cells and purkinje cells die. Many of these cells have, in contrast to cardiomyocytes, high proliferation capacity and are restored to some extent during wound healing without any need of therapeutical intervention. For example, blood vessels re-form after myocardial infarction. However, mostly large arterioles were found in the infarct area, and not capillaries, which usually supply the cardiomyocytes with oxygen and nutrients [105]. Regeneration of the infarct area

through cardiomyocyte proliferation without increasing capillary density is impossible. In the peri-infarct region, insufficient capillary perfusion leads to apoptosis of the hypertrophied but viable cardiomyocytes that remain after infarction [106, 107]. Therefore, induction of cardiomyocyte proliferation in the peri-infarct region might just have a transient effect on heart regeneration if the vasculature and other supporting cells are not restored. This might explain why p38 inhibition that has no effect on capillary density causes only a transient effect in improving heart function despite induction of cardiomyocyte proliferation [66]. Insufficient revascularization might also be the reason why overexpression of *c-myc* [108–110] and *cdk2* [111] resulted only in a transient induction of proliferation as well as smaller cardiomyocytes.

Another challenge is that the contraction of all cardiomyocytes within the myocardium has to be coordinated to guarantee maximal possible cardiac output. Therefore, it has to be taken into account that dividing cardiomyocytes cease to contract during anaphase. Thus, for optimal myocardial regeneration the organization and orientation of the new cardiomyocytes, their proper timely contraction as well as a slow proliferation rate is important. This might be the reason why newt and zebrafish heart regeneration takes several weeks [30, 35].

It remains a challenge to understand precisely how the combination of tissue repair mechanisms with reactivation of embryonic programs in urodele and zebrafish can generate growth, pattern formation, and morphogenesis in an adult animal. Whether this kind of regeneration can be achieved in mammals is difficult to estimate. However, full regeneration might not be necessary. Functional regeneration to improve cardiac output might suffice in most clinical settings to restore a satisfactory functional performance without achievement of complete tissue repair. A good example is the liver, which regenerates very efficiently although the original tissue architecture is not rebuilt completely. After excision of one of the five liver lobes the liver does not restore the original morphology regrowing the excised lobe. In fact, after injury the remaining four lobes grow to compensate for the loss in liver mass [112].

Conclusion

Can cardiomyocytes divide? The answer is yes! Cardiomyocytes proliferate during embryonic development. The correct question regarding regeneration is: Can we induce proliferation of postnatal cardiomyocytes after injury and cause improvement of heart function? As outlined in this review, neonatal mammalian cardiomyocytes are endowed with the full cell cycle program and can be efficiently induced to proliferate. However, so far it has not been tested whether this can be used to improve heart function, for example in patients with Congenital Heart Defect (CHD); the most frequently occurring birth defect and leading cause of birth-defect-related death [113, 114].

Recently, it has also been demonstrated that complete cell division can be induced in adult cardiomyocytes [67]. However, in

contrast to neonatal cardiomyocytes, this induction is not efficient. The main problem hereby appears to be the transition from mitosis to cytokinesis and its completion.

Taken together, the scientific community has gathered a plethora of data that allows the encouraging conclusion that repopulation of the heart by inducing cardiomyocyte proliferation is a realistic future option for cardiac repair. At the same time, we urge to a critical analysis of data regarding this hot topic to avoid misinterpretations and too fast conclusions.

Acknowledgements

We thank T. Braun, F. Ferrazzi, M. Harmsen, B. Jungblut, M. Diehl, R. Ross, Y. Wang and all members of the Engel laboratory for suggestions and critical review of the manuscript. This work was supported by a grant from the Charles H. Hood Foundation, Inc., Boston, MA (Child Health Research Grant to F. B. E.), the Alexander von Humboldt Foundation (Sofja Kovalevskaja Award to F. B. E. and a Research Fellowship to M. J. v. A.) and by the Excellence Cluster Cardiopulmonary System (DFG).

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