Towards regenerating the mammalian heart: challenges in evaluating experimentally induced adult mammalian cardiomyocyte proliferation

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Submitted 1 September 2015; accepted in final form 23 February 2016

Zebrowski DC, Becker R, Engel FB. Towards regenerating the mammalian heart: challenges in evaluating experimentally induced adult mammalian cardiomyocyte proliferation. Am J Physiol Heart Circ Physiol 310: H1045–H1054, 2016. First published February 26, 2016; doi:10.1152/ajpheart.00697.2015.—In recent years, there has been a dramatic increase in research aimed at regenerating the mammalian heart by promoting endogenous cardiomyocyte proliferation. Despite many encouraging successes, it remains unclear if we are any closer to achieving levels of mammalian cardiomyocyte proliferation for regeneration as seen during zebrafish regeneration. Furthermore, current cardiac regenerative approaches do not clarify whether the induced cardiomyocyte proliferation is an epiphenomena or responsible for the observed improvement in cardiac function. Moreover, due to the lack of standardized protocols to determine cardiomyocyte proliferation in vivo, it remains unclear if one mammalian regenerative factor is more effective than another. Here, we discuss current methods to identify and evaluate factors for the induction of cardiomyocyte proliferation and challenges therein. Addressing challenges in evaluating adult cardiomyocyte proliferation will assist in determining 1) which regenerative factors should be pursued in large animal studies; 2) if a particular level of cell cycle regulation presents a better therapeutic target than another (e.g., mitogenic receptors vs. cyclins); and 3) which combinatorial approaches offer the greatest likelihood of success. As more and more regenerative studies come to pass, progress will require a system that not only can evaluate efficacy in an objective manner but can also consolidate observations in a meaningful way.

heart; cardiomyocyte; cell cycle; proliferation; regeneration; zebrafish; mammal; rat; mouse; human

ISCHEMIC HEART DISEASE IS the leading cause of death in the industrialized world. Cardiac ischemia results from reduced flow of oxygen and metabolites to the muscle cells of the heart (cardiomyocytes). Should ischemia be severe or prolonged, cardiomyocytes die, reducing heart function. As the adult mammalian heart lacks any significant ability to regenerate, which is partially due to the inability of adult mammalian cardiomyocytes to proliferate, a significant loss of cardiomyocytes leads ultimately to heart failure and death. Currently, the only cure for patients with heart failure is heart transplantation.

Since the middle of the 19th century, scientists studying the regenerative capacity of the adult mammalian heart have observed cardiomyocyte cell cycle activity upon injury (61). These studies fostered the idea that the mammalian heart can be regenerated by enhancing cardiomyocyte proliferation. In recent decades, the discovery that newt and zebrafish can regenerate their hearts by cardiomyocyte proliferation (45) intensified the attempts to induce adult mammalian cardiomyocyte proliferation for the purposes of heart regeneration. With this concerted effort, the heart regenerative field is increasingly identifying novel mechanisms and pathways to induce adult cardiomyocytes to enter and progress through the cell cycle to varying degrees. Nevertheless, robust induction of adult cardiomyocyte proliferation remains elusive and several key questions remain unanswered:

1) How efficient is the induction of adult cardiomyocyte proliferation in vivo?
2) Which of the factors described to induce cardiomyocyte proliferation is the most efficient?
3) Is the observed improved cardiac function due to enhanced cardiomyocyte proliferation?

Here we give an introduction into the field of cardiomyocyte proliferation during development and regeneration. Furthermore, we discuss the issue of standardized protocols for eval-
The Cardiomyocyte Cell Cycle During Development and Regeneration

A brief history of mammalian cardiomyocyte cell cycle activity during heart development. Fetal mammalian heart growth occurs by cardiomyocyte proliferation. As gestation progresses, cardiomyocyte cell cycle activity decreases precipitously with nearly all cardiomyocytes entering a quiescent G0 state by birth (69). Shortly after birth, the number of cardiomyocytes increases by ~68% from postnatal day (P) 0 to P3 in rat and by ~40% from P2 to P5 in mouse, suggesting that cardiomyocytes in newborn hearts maintain some ability to proliferate (4, 46). In support of this in vivo observation, in vitro experiments show that cardiomyocytes isolated from P1 rats undergo a 1.6-fold increase in number over 6 days in response to 10% fetal calf serum (40). From approximately P4 to P14, in both rat and mouse in vivo, ~90% of cardiomyocytes undergo a terminal cell cycle resulting in binucleation via cytokinesis failure (46, 69). Completion of this “binucleation-phase” represents an end to cardiomyocyte cell cycle activity during normal heart development (77). After the “binucleation-phase,” cardiomyocytes remain withdrawn from the cell cycle with continued heart growth occurring by cardiomyocyte hypertrophy (i.e., cell enlargement).

Although these cell cycle dynamics have been known for over 20 yr, to date, very little is known as to how or why 1) fetal cardiomyocytes exit the cell cycle just before birth, 2) neonatal cardiomyocytes reenter the cell cycle shortly after birth, 3) cardiomyocytes binucleate, and 4) neonatal cardiomyocyte binucleation is intimately linked to cell cycle withdrawal and permanent exit from a proliferative state. It has recently been described in P15 mice that a brief, but intense, proliferative burst of predominantly binuclear cardiomyocytes occurs, increasing cardiomyocyte numbers by ~40% (53). However, additional studies by a number of independent laboratories have since been unable to detect a preadolescent P15 cardiomyocyte proliferative burst (4, 34, 71). The inability to reproducibly identify cardiomyocyte proliferation in P15 mice could potentially be due to subtle differences in mouse strain and housing conditions. However, it appears that a cardiomyocyte proliferative burst is not required for normal heart development.

Adult cardiomyocytes are postmitotic but not permanently withdrawn from the cell cycle. Adult cardiomyocytes are often described as being “permanently withdrawn” from the cell cycle (58). Indeed, there is overwhelming support for permanent cell cycle withdrawal during postnatal heart development (where the heart grows by physiological hypertrophy) (67, 77). However, in response to pathological stimuli (e.g., pressure overload, sympathetic overdrive, infarct), adult cardiomyocytes reexpress factors associated with G1 cell cycle entry (e.g., c-Jun, D-type cyclins, and phosphorylated retinoblastoma protein pRb) (6, 15, 16, 24, 33, 36, 87). This observation has led to the speculation that postnatal pathological cardiac growth (hypertrophy) is a cell cycle-dependent process (85). In this sense, while the vast majority of adult cardiomyocytes are permanently postmitotic, they may not necessarily be permanently withdrawn from the cell cycle. This presents the hypothesis that, to some degree, adult mammalian cardiomyocytes maintain the initial mechanics required to respond to an external mitogenic stimulus (and thus, in a proliferative context, are not completely inert). There is some precedence for such a hypothesis. For instance, isolated adult cardiomyocytes can progress through the cell cycle with the combination of FGF1 and p38 inhibitor (p38i) but neither alone (24). Interestingly, in vivo, after infarct, only p38i is required for cardiomyocyte cell cycle progression (24). Thus injury itself seemingly provides the initial mitogenic signals, and cardiomyocytes merely require downstream signals (e.g., via inhibition of p38) to progress further through the cell cycle.

Nevertheless, adult mammalian cardiomyocytes rarely, if ever, naturally progress beyond late G1 in response to pathological hypertrophic stimuli (65, 68). Although certain factors have been shown to promote adult cardiomyocytes to progress through the cell cycle to varying degrees, manipulations of these factors generally only affect a small fraction of adult cardiomyocytes. Why this is remains largely unknown. One mechanism blocking cell cycle progression in cardiomyocytes appears to be the epigenetic silencing of cell cycle genes via the retinoblastoma protein family (65). In addition, it has been shown that cell cycle arrest is mediated by alterations of centrosome integrity in cardiomyocytes (86). Several other reasons underlying the strict postmitotic state of cardiomyocytes, such as binucleation, alterations of the extracellular matrix, or DNA damage, have been speculated upon but currently lack substantial experimental evidence and/or mechanism (45). Thus the mechanisms underlying a strict postmitotic state have yet to be fully elucidated.

Zebrafish can regenerate their heart via cardiomyocyte proliferation. Unlike cardiomyocytes of adult mammals, those of the zebrafish can proliferate throughout adulthood (80). Furthermore, the adult zebrafish heart can fully regenerate in response to a variety of injuries, including apex-resection, cryoinjury, and genetic ablation of cardiomyocytes (1, 28, 30, 31, 35, 59, 63, 64, 78, 79, 83). Lastly, zebrafish heart regeneration occurs primarily by proliferation of endogenous cardiomyocytes, as there is little evidence to suggest progenitor or stem cells contribute to cardiac regeneration (38). Thus work in zebrafish provides proof-of-principle that not only can the vertebrate heart undergo regeneration but indicates that cardiomyocyte proliferation is a requisite feature of this process.

What makes an adult zebrafish cardiomyocyte different from a mammalian cardiomyocyte with regards to proliferative potential is poorly understood. One divergence between cardiomyocytes of these two species is the activity of p38 stress kinase after injury. p38 is active in both adult zebrafish and mammalian cardiomyocytes (39). However, unlike in their mammalian counterparts, p38 is inactive during regeneration and cardiomyocyte cell cycle progression in zebrafish cardiomyocytes. Interestingly, inhibition of p38 in mammalian cardiomyocytes promotes injury-induced cardiomyocyte cell

1 In the absence of a strong mitogenic stimulus, P2-isolated neonatal cardiomyocytes quickly lose proliferative potential (14, 74, 86).

2 Numerous studies over the past decade indicate that a small number of cardiomyocytes undergo turn-over in the aging heart (3, 8).

3 To date, no laboratory has been able to induce adult zebrafish cardiomyocytes to proliferate in vitro.
cycle progression (25). Thus, with regards to proliferative potential, p38 activity represents one difference between adult mammalian and zebrafish cardiomyocytes. What stimulates and maintains p38 activity in adult mammalian cardiomyocytes during injury remains unclear. One possible explanation is the developmentally regulated disassembly of centrosomes in cardiomyocytes of mice and rats, which does not occur in zebrafish or newt (86), as it has previously been shown that loss of centrosome integrity induces p38-mediated cell cycle arrest (50).

Towards regenerating the mammalian heart via cardiomyocyte proliferation

Observations showing that cardiac regeneration occurs in zebrafish, and requires endogenous cardiomyocyte proliferation, have led to a significant increase in the number of studies over the past decade aimed at inducing adult mammalian cardiomyocyte proliferation. A number of reviews have highlighted the successes in experimental methods in this regard, providing optimism that one day we may be able to regenerate the human heart (2, 72, 75, 84, 85). This was recently further supported by the observation that overexpression of constitutively active ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation (22). Classical approaches to initially identify the proliferative potential of a factor include 1) neonatal (P0–P3) cardiomyocyte in vitro screens; 2) transgene overexpression; and, most recently, 3) neonatal cardiac regeneration. Here we discuss limitations of these approaches.

Isolated neonatal cardiomyocytes as a screen for regenerative factors. P0–P3 isolated neonatal rat cardiomyocytes are generally used to screen potential proliferative factors for adult cardiomyocytes. However, as stated earlier, under normal developmental conditions, neonatal cardiomyocytes maintain the ability to enter the cell cycle. Thus it is of little surprise that a number of factors can promote, or increase, cell cycle activity of isolated neonatal cardiomyocytes. However, most factors that promote neonatal cardiomyocyte cell cycle entry appear to have a significantly reduced effect on adult cardiomyocytes (11, 25, 27), an observation that underlines neonates as a poor mitosis-test for regenerative factors in adult cardiomyocytes. What stimulates and maintains p38 activity in adult mammalian cardiomyocytes during injury remains unclear. One possible explanation is the developmentally regulated disassembly of centrosomes in cardiomyocytes of mice and rats, which does not occur in zebrafish or newt (86), as it has previously been shown that loss of centrosome integrity induces p38-mediated cell cycle arrest (50).

Methods to Evaluate Adult Cardiomyocyte Proliferation

Cell cycle activity is not proliferation. Adult cardiomyocytes are naturally resistant to proliferation. To date, by and large, the efficacy of a particular approach to induce mammalian cardiomyocyte proliferation is evaluated by its ability to in-
crease the rate of cardiomyocyte cell cycle activity (and/or progression) relative to controls. Typical readouts in this context include DNA-synthetic activity [e.g., BrdU or 5-ethynyl-2’-deoxyuridine (EdU) incorporation], Ki67 expression, Chromosome Passenger Complex (CPC) protein (e.g., Aurora B) midbody localization, and/or H3P-positive nuclei or condensed chromosomes. However, none of these approaches can predict whether a cardiomyocyte will divide or multinucleate (26, 45, 75). This is evident through numerous studies that show while cell cycle entry can be achieved, attaining proliferation is much more difficult. For instance, cardiomyocyte-specific inducible overexpression of cyclin D1 resulted in \(~40\)% of adult cardiomyocytes to undergo DNA-synthetic activity in vivo (73). Similarly, overexpression of FN14 and subsequent stimulation with TWEAK in isolated adult cardiomyocytes results in \(~35\)% DNA-synthetic activity (after 6 days of BrdU labeling) (55). However, cardiomyocytes do not appear to enter mitosis in response to either of these manipulations. Furthermore, Ki67, which is expressed from G1 to mitosis, and is often used as a marker of proliferation, is expressed even when a cell has undergone G1/S cell cycle arrest (Fig. 1). Taken together, this emphasizes that 1) multiple hurdles must be overcome in adult cardiomyocytes before proliferation (i.e., division) can be achieved and that 2) the described readouts do not allow correct measurement of a factor’s efficacy to promote proliferation.

Interpreting Aurora B staining patterns. Evaluation of cell cycle entry and progression requires not only the analysis of markers, but their critical interpretation to mitigate potential false positives. For instance, Naqvi et al. (53) reported “Nuclear localization of Aurora B in most of the mitotic cardiomyocytes indicated that these cells were in prophase.” Similar analysis by an independent laboratory confirmed this observation (54). However, images provided by the authors in both publications are not representative of Aurora B-specific staining patterns. Aurora B expression appears from prophase (i.e., before nuclear envelope breakdown) through metaphase as punctate signals, consistent with its centromere localization, verified in numerous studies using both antibody-based immunofluorescence staining in fixed cells and overexpression of
Aurora B-GFP constructs in live cell imaging (19, 41, 52). To determine if anti-Aurora B antibodies can result in nonspecific staining, we conducted staining with two different Aurora B antibodies (designated AB1 and AB2). Staining cryosections of P14 mouse heart ventricles with AB1 resulted in a positive nuclear signal in a large number of both cardiomyocytes and nonmyocytes (Fig. 2A). As there is little cell cycle activity in cardiomyocytes in P14 hearts (4, 34, 71) and as the staining pattern was uncharacteristic for Aurora B, this suggested a nonspecific nuclear staining by AB1. To further support this conclusion, cryosections of E15 rat heart ventricles, known to contain proliferating cardiomyocytes, were stained with AB1 and AB2 resulting in a positive nuclear signal in most cells for AB1 and fewer cells for AB2. Unlike AB1, which showed no specific nuclear staining pattern, the staining pattern of AB2 in nuclei was punctate and thus characteristic of prophase to metaphase Aurora B localization (19, 41, 52) (Fig. 2B). This further supported that AB1 is nonspecific. To verify the specificity of AB2, co-staining with anti-H3P antibodies was performed, which shows that AB2 stains specifically cells in mitosis (Fig. 2C). Thus, when interpreting experimental readouts, one should be sure that the antibody’s signal matches the protein’s biology.

**Ranking the Efficacy of Regenerative factors**

An increasing body of work indicates that the experimental induction of cardiomyocyte cell cycle entry can be achieved at a number of different levels (i.e., receptors, second messengers, and transcription factors). Some regenerative factors are efficient at inducing significant numbers of cardiomyocytes to enter the cell cycle but do not induce proliferation (e.g., cyclin D1; Ref. 70). In contrast, other factors induce relatively low numbers of cardiomyocytes to enter the cycle but are capable of inducing cardiomyocyte proliferation (e.g., YAP1; Ref. 48). This underlines the importance to precisely interpret the data in regards to cardiomyocyte proliferation. Moreover, this issue highlights the methodical differences in the field of cardiac regeneration, like distinct time points of analysis after injury or different labeling periods (see next section), making it difficult to compare a factor’s potential to induce proliferation between individual studies. Thus it remains unclear if one factor, or one level of regulation, is better than another.

**An absence of comparative standards.** Although there are few methods by which to accurately measure cardiomyocyte proliferation in vivo, measuring cell cycle activity (e.g., via EdU incorporation) represents a reasonable first-pass qualifier to determine the proliferative potential of a particular factor or manipulation. Yet, currently, it is difficult to evaluate the relative effectiveness of one regenerative factor over another. This is primarily due to an absence in standardized methods to evaluate cardiomyocyte cell cycle activity. For instance:

1) Rarely do mammalian regeneration studies share a common time point for H3P analysis. For instance, Engel and collaborators analyzed H3P 2 wk after myocardial infarction (MI) (24), Lin and collaborators analyzed H3P 6 wk after MI (48), and Cheng and collaborators analyzed H3P 7 wk after MI (18).

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**Fig. 2. Nonspecific Aurora B nuclear staining.** A: cryosection of postnatal day 14 mouse left ventricle immunostained with Nkx2.5 (cardiomyocyte nuclei: 1:200; sc-8697; Santa Cruz Biotechnology), AuroraB (AB1: 1:500; ab2254; Abcam lot no. GR139258-1), and DAPI (nuclei). B: cryosection of embryonic day 15 rat ventricle immunostained with AB1 (1:500; ab2254; Abcam), AB2 (1:500; AIM1; 611083; BD Biosciences lot no. 76738), and DAPI (nuclei). C: cryosection of embryonic day 15 rat ventricle immunostained with H3P (1:500; Millipore), AuroraB (AB2: 1:500), and DAPI. Yellow scale bar = 50 μm. Red scale bars = 10 μm. *AB2-positive nuclei.
2) There is a variation among mammalian regeneration studies regarding DNA labeling by nucleotide analog incorporation. Bersell and collaborators added BrdU to the drinking water 7 days before analysis (11), Kuhn and collaborators performed three BrdU injections over the last 7 days (44), Eulalio and collaborators performed six injections over the last 12 days (27), Woo and collaborators performed weekly BrdU injections (18) or injected every 4 days (81), and Reuter and collaborators delivered BrdU continuously via osmotic pump (60).

In contrast to the mammalian regeneration community (Table 1), the zebrafish regeneration community generally operates with some appreciable level of standardization (Table 2). For example, in many Zebrafish regeneration studies DNA synthetic activity (by BrdU, EdU, or PCNA labeling) is assessed 7 days after injury and only long-term analysis (>10 days postinjury) differs between the studies.

**Comparison of regenerative therapies to natural occurring regeneration.** How is the best way to go about creating a comparative metric? As noted, the adult zebrafish can regenerate its heart and we know the levels of zebrafish cardiomyocyte cell cycle activity during regeneration (Table 2). Thus the frequency of zebrafish cardiomyocyte cell cycle activity might present a reasonable, initial, benchmark by which to evaluate whether a mammalian regenerative factor:

1) can elicit regenerative levels of cell cycle activity seen in zebrafish cardiomyocytes; and

2) is better than another mammalian regenerative factor.

Although such comparison appears useful and rather simple, this approach has several limitations. For instance, the rate of endogenous cardiomyocyte proliferation differs between zebrafish and mammals (3, 8, 10, 80) and it is unknown if 1) the length of the cardiomyocyte cell cycle is the same or different between mammals and zebrafish; 2) differences in the form [apex resection, cryoinjury, left anterior descending artery (LAD) ligation], or in the extent of injury (how long LAD ligation takes place, intermural vs. transmural cryoinjury), may elicit different numbers of cycling cardiomyocytes as well as rate of cell cycle progression; 3) whether the same gene has conserved functions in two different species; 4) how much proliferation is required to regenerate the mammalian heart relative to zebrafish; and 5) additional species-specific factors may influence the rate or frequency of mammalian cardiomyocyte cell cycle progression (i.e., angiogenesis, wound healing processes, inflammation, hypoxia, etc.). Furthermore, the amount of proliferation to achieve zebrafish heart regeneration might present a reasonable, initial, benchmark by which to evaluate whether a mammalian regenerative factor:

1) can elicit regenerative levels of cell cycle activity seen in zebrafish cardiomyocytes; and

2) is better than another mammalian regenerative factor.

### Table 1. Overview of cardiomyocyte proliferation assays used in case studies of adult heart regeneration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BrdU/EdU/3H-Thy, %</th>
<th>PCNA, %</th>
<th>Ki67, %</th>
<th>H3P, %</th>
<th>Species</th>
<th>Age/Weight</th>
<th>Refs.</th>
</tr>
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<tbody>
<tr>
<td>p38 Inhibition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>In vitro1</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>12 wk</td>
<td>25</td>
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<tr>
<td>In vitro + FGF1</td>
<td>4.5</td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>12 wk</td>
<td>44</td>
</tr>
<tr>
<td>Injection after MI2</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>8–10 wk</td>
<td>24</td>
</tr>
<tr>
<td>Injection after MI + FGF12</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>8–10 wk</td>
<td>24</td>
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<tr>
<td>Neuregulin 1 (NRG1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mouse</td>
<td>12 wk</td>
<td>60</td>
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<tr>
<td>In vitro3</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>12 wk</td>
<td>11</td>
</tr>
<tr>
<td>In vivo4</td>
<td>m: 14:bi: 3</td>
<td></td>
<td></td>
<td></td>
<td>Mouse</td>
<td>12 wk</td>
<td>22</td>
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<td>After MI5</td>
<td>0.14–0.18</td>
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<td>Mouse</td>
<td>8 wk</td>
<td>60</td>
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<td>In vivo6</td>
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<td></td>
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<td></td>
<td>Mouse</td>
<td>12 wk</td>
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<td>After MI7</td>
<td>0.027</td>
<td></td>
<td></td>
<td></td>
<td>Mouse</td>
<td>12 wk</td>
<td>60</td>
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<td>Periostin</td>
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<tr>
<td>In vitro10</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>300 g</td>
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<tr>
<td>Injection in vivo11</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>300 g</td>
<td>44</td>
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<td>Gelfoam patch after MI11</td>
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<td></td>
<td>Rat</td>
<td>300 g</td>
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<td>miRNA 990</td>
<td>0.1–0.2</td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>300 g</td>
<td>44</td>
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<td>Adenoviral expression after MI12</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td>Mouse</td>
<td>8–12 wk</td>
<td>27</td>
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<td>Adenoviral expression after MI12</td>
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<td></td>
<td></td>
<td>Mouse</td>
<td>8–12 wk</td>
<td>27</td>
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<td>YAP</td>
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<td>Isolated cells from transgenic mice13</td>
<td>0.09:bi: 0.01</td>
<td></td>
<td></td>
<td>0.025</td>
<td>Mouse</td>
<td>4–8 wk</td>
<td>48</td>
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<tr>
<td>Transgenic mice after MI14</td>
<td>0.18–0.3</td>
<td></td>
<td></td>
<td>0.015–0.065</td>
<td>Mouse</td>
<td>8 wk</td>
<td>48</td>
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<tr>
<td>Cyclin A2</td>
<td>0.58–4.4</td>
<td>1–4</td>
<td>1.9–4.6</td>
<td></td>
<td>Rat</td>
<td>250–300 g</td>
<td>81</td>
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<tr>
<td>Transgenic mice after MI16</td>
<td>0.116–0.478</td>
<td></td>
<td></td>
<td>0.016–0.018</td>
<td>Mouse</td>
<td>8 wk</td>
<td>17</td>
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</table>

1) treatment every 3rd day; analysis after 12 days; 2) FGF1 intracardiac after myocardial infarction (MI); p38 inhibitor intraperitoneally after MI (H3P: 2 wk after MI); 3) 9 days of stimulation [bromodeoxyuridine (BrdU): last 3 days]; 4) 9 days of daily NRG1 injections (BrdU: drinking water); 5) daily NRG1 injections starting 7 days after MI, analysis 2 or 15 wk after MI (BrdU: drinking water, last week prior sacrifice); 6) 9 days of daily NRG1 injections (BrdU: osmotic minipump); 7) NRG1 daily injections for 7 days after MI (H3-Thy: injection on day 7); 8) induction of constitutively active (ca) ErbB2 overexpression starts 8–9 days after removal of doxycycline (Dox)-mediated repression (Ki67: 28 days after Dox removal); 9) induction of caErbB2 overexpression simultaneously with MI (Ki67: 3 wk after MI); 10) treatment every 3rd day for 9 days (BrdU: last 3 days); 11) induction of constitutively active (ca) ErbB2 overexpression starts 8-9 days after removal of doxycycline (Dox)-mediated repression (Ki67: 28 days after Dox removal); 9) induction of caErbB2 overexpression simultaneously with MI (Ki67: 3 wk after MI); 10) treatment every 3rd day for 9 days (BrdU: last 3 days); 11) induction of constitutively active (ca) ErbB2 overexpression starts 8-9 days after removal of doxycycline (Dox)-mediated repression (Ki67: 28 days after Dox removal); 9) induction of caErbB2 overexpression simultaneously with MI (Ki67: 3 wk after MI); 10) treatment every 3rd day for 9 days (BrdU: last 3 days);
is likely less than that required for the regeneration of larger hearts, such as the mouse or human heart. Note that if the radius of a spherical injury increases by a factor of 2, the tissue volume that needs to be regenerated increases by a factor of 8, while the wound area increases only by a factor of 4. These unknown variables are valuable areas of research, which, in time, will help to establish a benchmark to assess the efficacy of regenerative therapies.

**Potential benefits of comparative analysis.** Being able to compare the efficacy of one mammalian regenerative factor to another provides knowledge that ultimately will improve how we approach regeneration as a community. For instance, knowing if one factor is better than another may allow us to determine 1) which mammalian regenerative factors should be pursued in large animal studies; 2) if a particular point of cell cycle regulation presents a better therapeutic target than another (e.g., mitogenic receptors vs. cyclins); and 3) which combinatorial approaches offer the greatest likelihood of success. Furthermore, as mammalian regenerative studies become more detailed, we might find that certain factors function particularly well on binucleated cardiomyocytes, others on diploid mononucleated cardiomyocytes, and still others on tetraploid mononucleated cardiomyocytes. Knowing which factors work on which populations will predict their usefulness on human cardiomyocytes (which have different degrees of cell cycle) and/or on which populations will predict their usefulness on human cardiomyocytes (which have different degrees of cell cycle). Furthermore, as mammalian regenerative studies become more detailed, we might find that certain factors function particularly well on binucleated cardiomyocytes, others on diploid mononucleated cardiomyocytes, and still others on tetraploid mononucleated cardiomyocytes. Knowing which factors work on which populations will predict their usefulness on human cardiomyocytes (which have different degrees of cell cycle).

**Assessing regenerative therapies based on functional improvement.** From a clinical point of view, the golden standard to assess regenerative therapies is “improvement in function.” However, if a therapy improves heart function it does not necessarily mean that new heart muscle tissue was generated. Despite the low induction efficiencies of adult mammalian cardiomyocyte proliferation, a variety of approaches have been reported to result in marked improvement of cardiac function after MI. This suggests alternative mechanisms, beyond proliferation, are likely responsible for the observed improvement in cardiac function. Yet, only very few heart regeneration studies have investigated whether the observed functional improvements might be due to additional effects of the used treatment such as inhibition of apoptosis, enhancement of angiogenesis, induction and/or stabilization of hypertrophy, modulation of ventricular wall flexibility or other mechanical parameters, or prevention of remodeling. For example, FGF1/p38i treatment after MI does not have an obvious effect on apoptosis, but it enhances angiogenesis (29, 49, 62). FGF1/p38i treatment after MI does not have an obvious effect on apoptosis, but it enhances angiogenesis (24). Thus, in the future, it will be essential to develop models to demonstrate that an observed improvement in cardiac function is specifically due to cardiomyocyte proliferation. For example, experiments could be performed in animals in which proliferation of cardiomyocytes can be blocked (for example via inhibition of G2/M transition by cardiomyocyte-specific knockout of cyclin B1).

**Considering Additional Concepts for the Evaluation of Regenerative Studies**

The *subpopulation hypothesis*. Presumably, a factor that drives one cardiomyocyte to progress through the cell cycle (and achieve division) should act equally on another cardiomyocyte. Yet, it appears in a number of studies that only a small fraction of the overall cardiomyocyte population ever achieves S phase and mitosis (or even divide). This has led to a highly touted “subpopulation hypothesis” (i.e., only a small number of adult mammalian cardiomyocytes have retained the ability to proliferate/regenerate). Yet, the cellular and molecular phenotype of this hypothetical subpopulation remains unknown. Interestingly, however, a subpopulation of hypoxic cardiomyocytes has recently been identified as having greater proliferative potential than nonhypoxic cardiomyocytes (43).
Furthermore, also in zebrafish a subpopulation of gata4-positive cardiomyocytes has been described that is required for heart regeneration (32, 42). However, whether regenerative factors are indeed acting on a bona fide proliferative-competent subpopulation remains unknown. Furthermore, it remains to be determined if such a subpopulation is large, or capable, enough to achieve regeneration upon therapeutic mitogenic stimulation.

The aberrant proliferation hypothesis. An alternative explanation for the observations giving rise to the “subpopulation hypothesis” is that the small fraction of adult cardiomyocytes observed to progress through the cell cycle merely reflects “aberrant proliferation.” That being, while most cardiomyocytes are resistant to mitogenic manipulations, a small percentage of cardiomyocytes can “slip” past a G1 block and progress through the cell cycle to varying degrees. In this sense, “slip-cardiomyocytes” do not represent a bona fide regenerative subpopulation but merely reflect a statistical representation of aberrant cell cycle progression. In such a case, the cell cycle-inducing factor would not be restoring a normal proliferative state and thus would provide little insight into the mechanisms that underlie the strict postmitotic state of adult cardiomyocytes.

Identification of “turn-over” cardiomyocytes for regenerative purposes. Although the vast majority of postnatal cardiomyocytes are postmitotic, accumulating evidence indicates that there is a low level of cardiomyocyte turn-over in humans (8, 10) and mice (3). While the origin of these newly formed cardiomyocytes remains unclear in humans, in mice, they appear to be generated from preexisting cardiomyocytes (3). These observations suggest the potential presence of a novel cardiomyocyte subpopulation with bona fide proliferative potential and thus represent a target population for regenerative therapy. Yet, identifying a specific phenotype of these turn-over cardiomyocytes has yet to be achieved. With regards to human studies by Bergmann and Jovinge (9), who first observed cardiomyocyte turn-over, identification and quantification of postnatal human cardiomyocytes were achieved by isolating cardiomyocytes based on localization of centrosomal protein PCM1 at the nuclear envelope, a phenotype specific to cardiomyocytes and no other cardiac cell type. Interestingly, this phenotype also associates with cardiomyocytes that lack the ability to proliferate (86). For instance, PCM1 localizes to the centrosome, and not the nuclear envelope, of fetal mammalian cardiomyocytes, which are well known to proliferate (86). Thus, using PCM1 nuclear envelope localization may not be a suitable marker for identifying cardiomyocytes with turn-over potential (i.e., cardiomyocytes with turn-over potential would be those that lack PCM1 at the nuclear envelope).

Conclusions

In recent years, the field of cardiac regeneration via proliferation of adult cardiomyocytes has seen impressive growth. However, to date, we understand little as to the mechanisms that underlie the strict postmitotic state of cardiomyocytes. A better understanding of cardiomyocyte cell cycle behavior, limitations of regenerative models, and careful interpretation of results will reduce discrepancies in the field. Furthermore, given the speed of discoveries and the number of researchers contributing to this field, it might accelerate regenerative research if standardized methods are introduced to evaluate cell cycle progression and proliferation. A similar situation exists also since a long time in the stem cell-based cardiac regeneration field (7, 51). Developing standardized means permitting comparative analysis offers the potential to greatly enhance not only evaluation, and/or predictability, of the effectiveness of regenerative factors currently identified, but for those to come as well.

ACKNOWLEDGMENTS

We thank the rest of the Engel laboratory for critical reading and suggestions.

GRANTS

This work was supported by grants from the Emerging Fields Initiative (EFI) for Cell Cycle in Disease and Regeneration (CYDER) from the Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU to F. B. Engel) and the Deutsche Forschungsgemeinschaft (IRTG 1566; PROMISE; EN 4539-1 to F. B. Engel).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.C.Z., R.B., and F.B.E. drafted manuscript; D.C.Z., R.B., and F.B.E. edited and revised manuscript; D.C.Z., R.B., and F.B.E. approved final version of manuscript; F.B.E. prepared figures.

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