Cardiomyocyte proliferation in cardiac development and regeneration: a guide to methodologies and interpretations

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1 Experimental Renal and Cardiovascular Research, Institute of Pathology, Department of Nephropathology, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany; and 2 Department of Cardiology, Icahn School of Medicine at Mount Sinai Hospital, New York, New York

Leone M, Magadum A, Engel FB. Cardiomyocyte proliferation in cardiac development and regeneration: a guide to methodologies and interpretations. Am J Physiol Heart Circ Physiol 309: H1237–H1250, 2015. First published September 4, 2015; doi:10.1152/ajpheart.00559.2015.—The newt and the zebrafish have the ability to regenerate many of their tissues and organs including the heart. Thus, a major goal in experimental medicine is to elucidate the molecular mechanisms underlying the regenerative capacity of these species. A wide variety of experiments have demonstrated that naturally occurring heart regeneration relies on cardiomyocyte proliferation. Thus, major efforts have been invested to induce proliferation of mammalian cardiomyocytes in order to improve cardiac function after injury or to protect the heart from further functional deterioration. In this review, we describe and analyze methods currently used to evaluate cardiomyocyte proliferation. In addition, we summarize the literature on naturally occurring heart regeneration. Our analysis highlights that newt and zebrafish heart regeneration relies on factors that are also utilized in cardiomyocyte proliferation during mammalian fetal development. Most of these factors have, however, failed to induce adult mammalian cardiomyocyte proliferation. Finally, our analysis of mammalian neonatal heart regeneration indicates experiments that could resolve conflicting results in the literature, such as binucleation assays and clonal analysis. Collectively, cardiac regeneration based on cardiomyocyte proliferation is a promising approach for improving adult human cardiac function after injury, but it is important to elucidate the mechanisms arresting mammalian cardiomyocyte proliferation after birth and to utilize better assays to determine formation of new muscle mass.

cardiac regeneration; cardiomyocyte proliferation; newt; zebrafish

THE MAMMALIAN HEART FORMS from the first and second heart field. The heart field precursors migrate and fuse at the midline of the embryo to form the primary linear heart tube (81). The heart tube then elongates, which occurs primarily via addition of precursor-derived cardiomyocytes to both poles. Subsequent “ballooning” of the chambers and growth of the heart are achieved by proliferation of contracting cardiomyocytes (109). After birth, mammalian cardiomyocytes exit the cell cycle and stop proliferating (Fig. 1) (107, 115).

Cardiovascular diseases are among the leading causes of death worldwide, presenting a major socioeconomic burden whose incidence is expected to increase further (39). Upon injury the adult mammalian heart does not regenerate, neither by activating stem cells nor by induction of cardiomyocyte proliferation (129). Instead, the heart compensates for the loss of cardiomyocytes by cardiomyocyte hypertrophy (increase in cell size). In contrast to reversible physiological hypertrophy during pregnancy or endurance training, this compensatory hypertrophy results in concentric or dilative remodeling (Fig. 1). It is accompanied by inflammation, fibrosis, increased wall stiffness, cardiomyocyte slippage, and continuous apoptotic loss of cardiomyocytes, resulting in reduced heart function (47, 57, 76).

Currently, there are no effective therapies available to reverse cardiac damage and remodeling, and, for a large group of patients, further deterioration cannot be efficiently prevented. Consequently, there is an urgent need for the development of novel therapies. In contrast to adult mammals, newborn mice, newts, and zebrafish regenerate their hearts after experimentally induced injury by the induction of cardiomyocyte proliferation (14, 38, 63). Thus, extensive efforts have been invested to determine whether it is possible to induce adult mammalian cardiomyocyte proliferation. A large body of evidence has accumulated in recent years suggesting that in the future it might be possible to prevent functional deterioration after

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injury and even to reverse cardiac damage by inducing cardiomyocyte proliferation.

**Assays to Determine Cardiomyocyte Proliferation**

Cardiomyocyte cell cycle activity does not necessarily equate with proliferation as it can also reflect pathological hypertrophy, polyploidization, or polynucleation (115, 129). Therefore, it is important to be careful with the interpretation of data from cell cycle assays (Table 1). Misinterpretation of cell cycle assays might provide an explanation for the controversies in the field of cardiac regeneration, as to the extent of cardiomyocyte proliferation. Therefore, we will discuss here their advantages and disadvantages.

**Nucleotide analog incorporation.** A commonly used approach in determining the extent of cardiomyocyte proliferation is the use of a nucleotide analog-incorporation assay (BrdU, EdU, tritiated thymidine). Nucleotide analogs will be incorporated during DNA synthesis, and thus cells will be labeled during S phase. A major advantage of this assay is that it permits the ability to perform long-term labeling, as well as pulse-chase experiments. Long-term labeling is especially helpful for the detection of cell cycle entry when the time course of cell cycle activation is unclear and, furthermore, helps to determine the total number of cycling cardiomyocytes. However, DNA synthesis occurs not only during semi-conservative DNA replication during S phase but also during DNA repair. Moreover, semi-conservative DNA replication is merely an indicator of S-phase cell cycle progression (115, 129). Thus, a disadvantage of this assay is that it does not predict whether a cell will divide or undergo G2/M arrest, polyploidization, or polynucleation. Pulse-chase experiments permit the identification of colony formation and thus, by deduction, cell division, as the label will be reduced by 50% per each cell division. Yet, to our knowledge, this approach has so far not been utilized in the field of cardiac regeneration.

**Colorimetric assays.** Several colorimetric proliferation assays are available. These assays, such as MTT, Alamar Blue, or ViaLight assays, are based on a color change due to the cleavage of tetrazolium salts, reduction of resazurin, or the detection of cellular ATP. An increase in the color change is interpreted as an increase in cell number. However, these assays work on the assumption that mitochondrial activity or the amount of cellular ATP per cell is constant. In this regard, these assays have limitations when applied to cardiomyocytes, as mitochondrial activity is increased during hypertrophy or elevated contractility (100). Moreover, as these assays are not cell-type specific, it cannot be excluded that an increase in the colorimetric signal results from an increase in proliferation of

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>DNA Synthesis</th>
<th>DNA Repair</th>
<th>Polyploidization</th>
<th>Mitosis</th>
<th>Endomitosis</th>
<th>Bi-/Polynucleation</th>
<th>Cytokinesis</th>
<th>Proliferation</th>
<th>Hypertrophy</th>
<th>Cardiac specific</th>
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<tr>
<td>Nucleotide incorporation</td>
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<td>Aurora B/Anillin</td>
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<td>Sarcomere disassembly</td>
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FACS, fluorescence-activated cell sorting.
nonmyocytes, which are present, albeit to a lesser degree, in primary cardiomyocyte cultures.

**Histone H3 phosphorylation.** Histones undergo a wide variety of posttranslational modifications, some of which are specific to mitosis (118). The most common histone modification used to identify mitotic cells is the phosphorylation of histone H3 at serine 10 (Ser-10) (H3P). Phosphorylation at Ser-10 begins in G2 phase and dephosphorylation is completed “immediately prior to detectable chromosome decondensation in telophase” (Fig. 2) (24, 44). Thus, the often-used term “mitotic index” for the percentage of H3P-positive cells is misleading, as part of the H3P-positive cells are in G2 phase. In addition, as with detection of S-phase activity, mitosis does not predict cell division as it can lead to polyploidization via endomitosis (78) or binucleation due to failure of cytokinesis (34). Furthermore, unlike nucleotide analog incorporation assays, H3P staining cannot be combined with a cardiac-specific nuclear marker (e.g., Nkx2.5), as prometaphase is coupled with nuclear envelope breakdown. Therefore, to determine if an H3P-positive cell is a cardiomyocyte, H3P staining is generally coupled with staining of the sarcomeric apparatus, which loses in cardiomyocytes its organized structure upon entry into mitosis. In contrast, during endomitosis the sarcomeric apparatus stays intact.

**Ki67.** Ki67 has been shown to be required for cell proliferation, but its function is poorly understood. Ki67 is expressed in all cell cycle phases of proliferating cells but is not expressed in non-cycling cells. Thus, Ki67 has been considered a reliable marker for evaluating cellular proliferation (18, 30). Furthermore, based on its intranuclear staining pattern it is even possible to determine in what cell cycle phase a cell resides, a feature that has not been utilized yet in cardiomyocytes. However, there is evidence that Ki67 is not a reliable marker for determining if a cardiomyocyte can proliferate. For instance, Meckert and coworkers (78) showed that Ki67-positive cardiomyocytes undergo endomitosis. Moreover, it has been shown that cell cycle genes as well as Ki67 are re-expressed in hypertrophy (28, 129). Thus, as with nucleotide analog incorporation and H3P labeling for mitosis, Ki67 does not predict whether a cycling cell will divide, undergo polyploidization, or will binucleate.

**Aurora B and Anillin.** In 2005 we introduced Aurora B as a marker of cytokinesis in cardiomyocytes (33). Aurora B is expressed in G2 phase, phosphorylates histone H3 at Ser-10,
and associates with centromeric heterochromatin early in mitosis (Fig. 2). In contrast to H3P as a marker, Aurora B can be used to analyze cytokinesis as it transfers first from the kinetochore during metaphase to the central spindle during anaphase, ultimately contributing to midbody formation during telophase (24, 121). Unfortunately, we realized later that Aurora B alone is also not a reliable marker for cell division, as its staining pattern in dividing cells is indistinguishable from the pattern in binucleating cells (34). However, Aurora B staining combined with a sarcomere staining allowed us to visualize cytokinesis failure during binucleation based on asymmetric constriction of the cleavage furrow. Subsequently, we introduced Anillin as an additional marker to evaluate whether a cardiomyocyte will binucleate or divide. Like Ki67, Anillin is absent in G0 phase. In proliferating cells it accumulates in the nucleus during G1, S, and G2 phase, then localizes to the cell cortex at the onset of mitosis, and finally locates around the midbody in cytokinesis (36, 111). Aurora B and Anillin costaining identifies binucleating cells based on asymmetric constriction of the cleavage furrow and failure of Anillin to focus at the cortex in anaphase and to concentrate around the midbody during cytokinesis (34). Collectively, the sole presence of Aurora B or Anillin is not proof of cell division. However, the cellular localization of Anillin will indicate if a cell will divide or binucleate.

Fluorescence-activated cell sorting. A method that has rarely been utilized to characterize cell cycle progression in cardiomyocytes is fluorescence-activated cell sorting (FACS). Measuring cell proliferation via FACS is based on labeling isolated cardiomyocytes with a cardiomyocyte-specific marker and a fluorescent DNA dye, which permits determination of the DNA content based on fluorescence intensity. Using this approach, one can determine what fraction of the cardiomyocytes is for example in S phase. Time series experiments help to determine whether DNA synthesis results in binucleation (increase in the G2/M phase peak), polyploidization/polytenization (DNA content ≥ 4n), or cell division (no change in the G1, S, and G2/M distribution) (34). In addition, one can perform a BrdU pulse-chase experiment (diluting the BrdU label due to cell division) or a BrdU-Hoechst assay to prove that cell division occurs (29). The principle of a label being diluted because of cell division (such as BrdU or membrane labeling) even enables investigators to determine how often a cell has divided, as each cell division results in a 50% decrease of the label (71).

Sarcomere disassembly/dedifferentiation. Previously, it has been suggested that newt and zebrafish cardiomyocytes differentiate before undergoing proliferation based on the observation that their sarcomeric apparatus disassembles (52). Yet Ahuja and coworkers (3) have shown in detail that fetal cardiomyocytes disassemble their sarcomeric apparatus in a highly ordered fashion during mitosis and reassemble it during cell division. This suggests that sarcomere disassembly is not a sign of dedifferentiation but is simply a necessary process to allow cardiomyocyte proliferation. That adult mammalian cardiomyocytes are also able to restructure or disassemble their sarcomeric apparatus has been long known. For example, adult cardiomyocytes with disassembled/disorganized/restructured sarcomeric apparatus has been observed in long-term in vitro cultures (92, 110), hypocontractile hibernating myocardium (72, 106), and Tako-Tsubo cardiomyopathy (83). Importantly, we have demonstrated that this complex disassembly-reassembly process occurs not only in neonatal and adult cardiomyocytes after induction of cell division but also during binucleation (33, 34). Thus, sarcomere disassembly, often misleadingly called “dedifferentiation,” is not an unambiguous marker of cardiomyocyte proliferation. However, it can be utilized to exclude proliferation as sometimes H3P-positive cardiomyocytes are reported with an intact sarcomeric apparatus, indicative of endomitosis (32, 115).

Cell count experiments. Cell count experiments are used as a direct measurement of cardiomyocyte proliferation. Yet, often investigators measure only the total cell number, not distinguishing between cardiomyocytes and nonmyocytes. In contrast, we have for example shown that the total number of neonatal cardiomyocyte increases over time after FGF1/p38 inhibitor stimulation based on total cell number corrected for the percentage of cardiomyocytes as analyzed by FACS (33). In addition, in some studies the cell number after stimulation is compared with control stimulation. A higher cell number can also be explained by a cell-protective effect, as it is known that neonatal as well as adult cardiomyocytes undergo apoptosis in culture. In addition, cell numbers are often determined by isolating cardiomyocytes from tissue or trypsinization from culture dishes. Here, it should be considered that alterations in extracellular matrix composition, cell-to-cell adhesion, and cell size can significantly affect the cell count due to inefficient enzymatic digestion or altered efficiency in cell harvest by centrifugation.

Clonal analysis. Clonal analysis from single cells unambiguously determines if a stimulus induces proliferation. In vitro, clonal analysis has not yet been reported for cardiomyocytes. However, we have introduced the monitoring of cardiomyocytes over time, demonstrating an increase in cardiomyocyte density after FGF1/p38 inhibitor stimulation (33). Recently, Lin and coworkers (65) performed clonal analyses in vivo. The authors utilized the Brainbow technology (68) to induce, in a small number of randomly distributed cardiomyocytes, expression of a fluorescence protein (to obtain individual labeled cardiomyocytes). In addition, they utilized a cardiomyocyte-specific doxycycline-inducible system to induce Yes-associated protein expression in cardiomyocytes (65). If induction of proliferation were successful, one would expect the formation of clones from the individual labeled cardiomyocytes (Fig. 3). It should be noted, however, that the Brainbow system for clonal analysis requires that labeled individual cells with the same color not be adjacent to one another. Given that noninduced/control hearts in the study by Lin and coworkers occasionally exhibited clones of two adjacent cardiomyocytes of the same color, this assay is thus useful only if a significant number of clones of at least four labeled cardiomyocytes are observed. A more direct proof of cardiomyocyte division has been introduced by Ali and coworkers (4), who have utilized the “mosaic analysis with double markers” mouse model (132), which can distinguish between parental and daughter cells. In this system parental cells are GFP- and RFP-double positive. After cell division daughter cells will be RFP or GFP positive, while binucleated cells will be GFP- and RFP-double positive (Fig. 3). As this system is dependent on Cre recombination, proliferation can be analyzed specifically in cardiomyocytes. While this system has been used to determine naturally occurring cardiomyocyte proliferation (4), it has not yet been uti-
lized to test the efficiency of factors to induce cardiomyocyte proliferation.

Cardiomyocyte specificity. A major issue, especially in vivo, is to determine whether the signal of a cell cycle marker indeed belongs to a cardiomyocyte. This is especially critical as often there is an increase of fibroblast proliferation and infiltration of inflammatory cells upon injury. As has been previously shown and discussed, cardiomyocyte-specific cytoplasmic markers are prone to misinterpretations; also the presentation of Z-stacks cannot solve this issue (32, 108). Thus, the detection of Ki67, BrdU, or cell cycle regulators such as cyclins should be combined with staining for a cardiomyocyte-specific nuclear marker such as GATA4 or MEF2. Besides a nuclear marker, cardiomyocyte-specific cytoplasmic membrane markers such...
as caveolin 3 can be utilized to identify unambiguously a cycling cardiomyocyte (32).

The analysis of commonly used methods to determine cardiomyocyte proliferation shows that while many of these techniques can detect cell cycle activity, distinguishing between proliferation, polynucleation, or polyploidization is more difficult and in some cases impossible. Not acknowledging this issue often results in misinterpretation of results and is likely the reason for the contradictions, and even controversies, in the field of cardiac regeneration.

**Naturally Occurring Cardiac Regeneration**

Pondering regeneration is as old as civilization. First written reports by Empedocles and Aristotle describing the observation that certain animals can regenerate their appendages can be found as early as 400 BC. In modern times the first known publication regarding regeneration was by René Antoine Ferchault de Réaumur in 1712, where he described limb regeneration in crustaceans (crayfish, crabs, and lobsters) (41, 99). In 1740, Abraham Trembley perturbed, analyzed, and dissected the process of regeneration in hydra, which is considered to be the birth of “experimental biology” (60). In contrast to common belief, regeneration is a widely distributed event with regenerative species in almost every phylum (101).

**Urodele heart regeneration.** Urodeles (salamanders and newts) are considered to be the champions of regeneration. They can regenerate a variety of structures such as limbs, tails, jaws, eyes, and the heart (16, 114). Urodele heart regeneration was mentioned in the literature as early as 1974 (9, 88). After Oberpriller and Oberpriller (87) reported the appearance of mitotic adult newt cardiomyocytes in *Dieniictus viridescens* 16 days after apical resection, they studied the response of the adult newt ventricle to injury in *Notophthalmus viridescens* (88). The authors resected around 12.5% of the ventricle. At the site of injury a blood clot occluded the ventricle and macrophages accumulated at 10 days within and around it. To determine if the injury induced cardiomyocyte proliferation the authors injected tritiated thymidine 3 h before tissue collection to determine the frequency of cycling cardiomyocytes. The maximal thymidine-incorporation index (9–10%) and mitotic index (0.9%) were observed at 20 days postinjury. Becker and coworkers (9) provided additional support for the capacity of urodeles to regenerate their hearts. They demonstrated that *Triturus viridescens* at the adult aquatic stage survives the resection of 30–50% for at least 2 wk with a mortality rate of 10% (several hundred resections were performed). Normal circulatory dynamics were re-established after 5 h based on microscopic analysis of skin capillaries in the tail fin using transmitted light. These data suggested that, upon injury, the adult newt heart might be able to regenerate its heart by cardiomyocyte proliferation. However, the authors observed the formation of scar tissue at 30 days postinjury, indicating a limited regenerative response (88).

To demonstrate that the newt heart regenerates from cardiomyocyte proliferation several assays have been performed. Mitotic cardiomyocytes in newts were initially identified by electron microscopy based on condensed mitotic chromatins, “fibrils, which appeared to be myofibrils approximately the length of a sarcomere” in the periphery of the cell, a basal lamina, and glycogen granules (87). In addition, these mitotic cells are characterized by the absence of thin filaments and Z bands (87). These features have also been observed in dividing fetal mammalian cardiomyocytes, indicating that the adult newt heart might indeed regenerate its heart by cardiomyocyte proliferation. However, this “transient disassembly of the sarcomeric apparatus” occurs also when cells fail to undergo cell division after mitosis resulting in binucleated cardiomyocytes (34). Thus, it was important to determine whether the number of binucleated cardiomyocytes increases upon injury. Oberpriller and coworkers (89) reported that the vast majority (>98%) of adult newt cardiomyocytes are mononucleated. Importantly, they have demonstrated that the majority of newt cardiomyocytes (~85%) that underwent DNA synthesis 45 days after injury (apex resection and reinduction after mincing) were still mononucleated and diploid. These data strongly indicated that the vast majority of adult newt cardiomyocytes that enter the cell cycle complete cell division.

To prove that adult newt cardiomyocytes are indeed able to complete cell division and to proliferate, Matz and coworkers (77) performed life cell imaging experiments (day 8–19 in culture). These experiments demonstrated that 80% of mononucleated cardiomyocytes produced mononucleated daughter cells, while the remaining failed to complete cytokinesis, resulting in binucleated cells. In addition, 32% of binucleated cardiomyocytes produced binucleated daughter cells. Bettencourt-Dias and coworkers (12) confirmed the proliferative competence of adult newt cardiomyocytes in vitro. They observed that approximately one-third of the cells completed cell division and part of those entered successive cell divisions. These data suggest that the newt heart contains a subpopulation of cardiomyocytes that retain a higher proliferative potential, while the remaining newt cardiomyocytes behave similarly to their mammalian counterparts.

To determine whether new myocardium is formed during newt regeneration, Piatkowski and coworkers (91) introduced “scanning electron microscopy analysis in combination with stereological estimation.” Injury induced by “repeated orthogonal squeezing with fine forceps” was followed by cardiac regeneration associated with a significant increase in the volume fraction of trabeculae. In contrast to initial studies, this and other studies have demonstrated, by investigating later time points, that cardiac newt regeneration results in a morphologically fully regenerated heart with no signs of scarring (91, 124).

To provide evidence that newts can functionally regenerate their hearts, a standardized ventricular resection model was established, whereby “a more lateral region of the ventricle, as opposed to the apex,” (5–10%), was resected (124). Echocardiography was utilized for the analysis of heart function and revealed that heart function was reduced at day 7 postinjury from around 38-34% fractional shortening. Full function was reported at 23 days and 60 days postinjury (~40% fractional shortening) (124). However, it remains unclear whether the functional improvement is indeed due to newly formed heart tissue. Considering that a rather small amount of muscle tissue was resected, the rather small drop in function might also be due to the initial formation of the blood clot and scar at day 7, impairing wall motion. In the future it would be important to repeat such a study in a model with more severe damage to the heart. This is especially important as recent studies in neonatal
mice suggest that the regenerative response of the heart depends on the size of injury (19, 26).

Collectively, the available data demonstrate that adult newt cardiomyocytes can proliferate in vitro and indicate that newts can repair their hearts after several different kinds of injury by cardiomyocyte proliferation (Table 2). To further characterize newt heart regeneration, echocardiographic studies should be performed comparing the functional recovery in injury models of varying degrees of damage. Finally, it remains unclear how adult proliferating newt cardiomyocytes differ from nonproliferating newt or mammalian cardiomyocytes.

**Zebrafish heart regeneration.** Zebrafish are known to regenerate a variety of organs, and, in contrast to urodeles, zebrafish are amenable to molecular analysis and genetic manipulation. In 2002, Poss and coworkers (97) tested whether zebrafish can regenerate their hearts. Similar to the experiments with urodeles, Poss and coworkers amputated around 20% of the apex. On the basis of histological data, nucleotide analog (BrdU)-incorporation assays, and an analysis of H3P for mitosis, the authors concluded that the zebrafish can regenerate its heart by cardiomyocyte proliferation after mechanical injury. Subsequently, similar data were obtained for other injury models including cryoinjury (21) and genetic ablation (25, 119). That zebrafish heart regeneration is based on cardiomyocyte proliferation was supported by the observation that zebrafish carrying a mutation in a cell cycle gene failed to regenerate, forming a scar (97). However, this mutation was not cell-type specific and might have affected heart regeneration by inhibiting proliferation of another cell type. Thus, it remained unclear if regeneration occurred by proliferation of remaining endogenous cardiomyocytes or by stem cells (or some other cardiomyocyte progenitor cell) differentiating into cardiomyocytes. In addition, in contrast to newt cardiomyocytes (12, 77), no protocol exists to induce proliferation of isolated adult zebrafish cardiomyocytes (64, 102) in vitro.

Thus, the source of newly formed cardiomyocytes remained controversial for several years until Jopling and coworkers (52) as well as Kikuchi and coworkers (54) introduced the tamoxifen (4-OHT)-inducible Cre/lox system to the zebrafish model to conduct lineage-tracing experiments. These lineage-tracing approaches demonstrated that stem/progenitor cells are not significantly involved in zebrafish cardiac regeneration but indicated that regenerated heart muscle in zebrafish is derived from proliferation of differentiated cardiomyocytes. Jopling and coworkers (52) observed a global proliferative response of cardiomyocytes to mechanical injury, which was supported by the finding that an inhibitor of the cell cycle kinase Plk1 blocked cardiac regeneration. In contrast, Kikuchi and coworkers (54) identified a cardiomyocyte subpopulation (GATA4-positive) throughout the subepicardial ventricular layer that contributed to heart regeneration. The importance of this subpopulation for cardiac regeneration has been proven by inducible, tissue-specific overexpression of a dominant-negative Gata4 cassette (g4DN), which blocked regeneration and caused severe scarring. The data indicate that proliferation of trabecular myocytes was not affected while cortical cardiomyocyte proliferation was markedly reduced (43).

Earlier studies were significantly limited by the lack of longitudinal studies (observing the process of regeneration of an individual heart over time) and functional studies. As it is

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**Table 2. Summary of cardiac regeneration in model systems**

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<thead>
<tr>
<th>Species</th>
<th>Cardiomyocyte Proliferation</th>
<th>Heart Regeneration/Cardiac Tissue Regrowth after:</th>
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<tbody>
<tr>
<td></td>
<td>in vitro</td>
<td>in vivo after Injury</td>
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LAD, left anterior descending coronary artery ligation.
difficult to identify the amputation plane and to identify newly formed cardiomyocytes, it remained unclear if zebrafish can fully restore lost myocardium and whether the observed morphological regeneration is indeed due to cardiomyocyte proliferation. For example, the morphologically repair of a ventricle might be due to cardiomyocyte migration, which has been shown to be essential for zebrafish cardiac regeneration (50). To address this issue, the group of Ken Poss (22) recently introduced so-called “zebraflash” transgenic lines for in vivo bioluminescence imaging. Expression of firefly luciferase under the cardiomyocyte-specific promoter cmci2 allows for direct monitoring of the recovery of heart size. However, this transgenic line cannot distinguish whether recovery is due to either proliferation or hypertrophy. Moreover, these transgenic lines cannot be used to determine functional recovery.

To demonstrate that the zebrafish can fully regenerate heart function after injury, physiological parameters have been analyzed, resulting in contradictory results. Analyses of cardiac conduction postresection in regenerating and scarring hearts initially suggested that zebrafish might not fully recover function. J-point depression was statistically significant 10 days postinjury and gradually normalized at 60 days postresection, independent of scarring or regeneration. In contrast, QTc intervals remained prolonged in both scenarios, indicating delayed electric repolarization (127). However, Kikuchi and coworkers (54) reported normalization of conduction 30 days postresection, which was later confirmed in the cryoinjury model (21). While these studies provided a more physiological insight into cardiac regeneration in zebrafish, recovery of cardiac conduction is a weak indicator of improved cardiac function.

In 2011, Wang and coworkers (119) introduced “swimming endurance” as a read-out to evaluate functional recovery after injury. Zebrafish were placed in a swim tunnel respirometer (MiniSwim-170; Loligo Systems, Tjele, Denmark) and allowed to acclimatize for 20 min at a low flow velocity of 3 cm/s. Swimming speed was then slowly increased to a velocity of ≥20 cm/s, and the endurance of zebrafish was monitored. The authors utilized this system to test the effect of inducible genetic ablation of a high percentage of cardiomyocytes through overexpression of cytotoxic diphtheria toxin A chain. They demonstrated that inducible genetic ablation of >60% of cardiomyocytes in contrast to apex resection results in reduced performance in a swim tunnel. Recovery of this phenotype, considering an initial loss of <60% together with recovery of the density of cardiomyocyte nuclei, provided strong evidence of regeneration from proliferation, even though binucleation had not been excluded (119).

Recently, Gonzalez-Rosa and coworkers (40) introduced the use of 2D echocardiography to directly determine the pump function of the zebrafish heart. In a longitudinal study analyzing individual zebrafish over time the authors observed that global systolic function but not ventricular wall motion was fully recovered following cardiac cryoinjury. This was in agreement with an increased density of cardiomyocyte nuclei in the injured ventricular wall that might affect normal contraction due to ultrastructural changes.

In conclusion, the available data indicate that zebrafish can, to a large extent, regenerate their hearts by means of cardiomyocyte proliferation (Table 2). In addition, it appears that in case of incomplete regeneration zebrafish compensate for impaired wall motion by increased pumping capacity of the remainder of the ventricular wall. However, it remains unclear if lost cardiac tissue is fully replaced by cardiomyocyte proliferation or whether hypertrophy and/or binucleation contribute to this process. In the future, it will be important to demonstrate that adult zebrafish cardiomyocytes can proliferate in vitro and to elucidate how adult zebrafish cardiomyocytes differ from postnatal mammalian cardiomyocytes.

**Newborn mouse heart regeneration.** Fetal and newborn cardiomyocytes have the ability to proliferate (115). Consequently, the question was raised if this ability is enough to allow the fetal, embryonic, or newborn heart to regenerate after injury. In 1975 the first evidence for fetal heart regeneration after mechanical injury in rabbits was reported (74, 75). In 2008, Drenckhahn and coworkers (27) introduced a mouse model that demonstrated that the fetal heart can compensate for the genetic inhibition of proliferation in around 50% of fetal cardiomyocytes. Proliferation was inhibited through inactivation of the X-linked gene encoding holocytochrome c synthase (Hccs). While deletion of Hccs in knockout males resulted in midgestational lethality, heterozygous knockout female mice were not affected, even though around 50% of the cardiomyocytes were Hccs deficient because of the random nature of X-chromosome inactivation. The gradual increase of complex III activity of the mitochondrial respiratory chain, the decrease in Hccs-deficient cardiomyocytes, and a detailed cell cycle analysis showed that the reduced proliferation rate of Hccs-negative cells is compensated for by an increased proliferation rate of the healthy cardiomyocytes. However, hearts of Hccs-deficient female mice at postnatal day 1 were still hypoplastic. Importantly, despite an increased proliferation rate adult Hccs-deficient females exhibit a range of cardiac phenotypes (28). These data suggest that the fetal heart has only a limited capacity for cardiac regeneration.

While fetal cardiomyocytes contribute significantly to heart growth by proliferation, cardiomyocytes of newborn mice have a limited proliferation capacity, and cell cycle activity generally results in binucleation instead of cell division (61). Despite this fact, Porrello and coworkers (93) observed that neonatal mice at postnatal day 1, but not day 7, exhibit a transient cardiac regeneration capacity. In addition, Naqvi and coworkers (82) have recently reported a brief but intense proliferative burst of predominantly binuclear cardiomyocytes at postnatal day 15 in mice. Moreover, they report that mice at postnatal day 15 were able to partially regenerate upon experimental myocardial infarction. Apex resection (15%) at postnatal day 1 was restored within 21 days following a similar process as described for zebrafish (see above) (93). Cardiomyocyte proliferation was assessed by BrdU incorporation assays, phosphorylation of histone H3, aurora B kinase expression, and sarcomere disassembly, indicating an up to sevenfold increase in cell cycle activity. As described above these markers do not allow one to distinguish between proliferation and binucleation (34). However, the observation that ventricular weight and surface area in the absence of hypertrophy were recovered within 21 days supports the conclusion that the rate of cardiomyocyte proliferation is increased upon resection. In addition, cardiac function of resected animals was comparable to sham-operated hearts. Yet it remains unclear whether heart function was affected and whether the normal differentiation process resulting in binucleation was inhibited by the resection or not.

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In a subsequent study, Porrello and coworkers (94) investigated whether the neonatal heart also regenerates after an ischemic insult induced by permanent ligation of the left anterior descending (LAD) coronary artery, a more disease-related injury. A major advantage of this study is the marked decrease in cardiac function 4 days after ligation, which was normalized at day 21. Moreover, the scar that formed within 7 days was resolved at day 21. In addition, in contrast to the Hcxs-deficient female mice, LAD-ligated mice showed no obvious pathology at 9 mo of age, indicating full regeneration. Utilizing the same assays as in their previous studies, the authors concluded that regeneration is due to induction of cardiomyocyte proliferation. In addition, Aurora and coworkers (6) have shown that the regenerative response in the LAD model depends on macrophages.

Recently, it has been suggested that, similar to the study of Drenckhahn and coworkers (28), the regenerative capacity of newborn mouse hearts is limited. Andersen and coworkers (5) published the first study that challenged the apex resection model of regeneration. The authors observed neither a recovery in heart size and weight nor an increased proliferation rate of cardiomyocytes, even though they used the same mouse strain and a similar injury protocol as Porrello and coworkers. However, loss and recovery of heart tissue were assessed differently (heart to body weight vs. ventricular weight and surface). Instead of regeneration, the authors observed fibrosis and cardiac hypertrophy. Interestingly, Jesty and coworkers (51) had already shown that cryoinjury in neonatal mice does not result in scar-free regeneration within 3 mo. In addition, signs of fibrosis and hypertrophy were reported in a diphtheria toxin receptor-based injury model studying the dependence of neonatal regeneration on macrophages (59). Recently, Konfino and coworkers (56) challenged the regeneration upon LAD ligation. While they observed complete regeneration after apical resection, they reported scar formation after LAD ligation in neonatal mice with a lower cardiomyocyte proliferation rate than in the apex resection model.

Collectively, these studies demonstrate that, under certain circumstances, neonatal hearts can recover from injury through cardiomyocyte proliferation (Table 2). It is possible that the observed differences are due to varying injury protocols. Recently, two reports have indicated that the regenerative response depends on the severity of the injury. Durezhershki and coworkers (26) demonstrated that, in contrast to superficial cryoinjuries, transmural cryoinjuries resulted, even after 120 days, in severe scarring and marked reduced function. In addition, Bryant and coworkers (19) reported that the regenerative capacity depends on the size of the resected piece of the apex. In the future, it will be important to determine how robust the cardiac regenerative capacity of newborn mice is and to identify the reasons for the failure of inducing regeneration upon different injury models in several laboratories. Finally, as for zebrafish, it will be important to analyze the effect of the injuries on binucleation.

**What Can We Learn from Naturally Occurring Cardiac Regeneration for Human Cardiac Therapy?**

It is assumed that studying animal models that can naturally regenerate their hearts will result in novel therapies to treat human cardiac disease. In regard to cardiomyocyte proliferation, it is important to understand the molecular mechanisms controlling proliferation and to determine whether the identified molecular mechanisms can be applied to human cardiomyocytes. Critical questions are:

- Are adult cardiomyocytes from model organisms comparable to adult mammalian/human cardiomyocytes?
- Are positive regulators enough to induce adult mammalian/human cardiomyocyte proliferation?
- Urodele and zebrafish heart regeneration. Urodeles have been used in classical experiments to study cardiogenesis as its development is slow and its embryos are large and develop externally (84). However, due to the lack of molecular tools the use of urodeles is limited with regard to the elucidation of molecular mechanisms regulating heart development and thus also heart regeneration. Consequently, so far only microRNA (miR)-128 (123) and the process of RNA editing (125) have been implicated in newt heart regeneration. To harness the advantages of newt heart regeneration, the group of Thomas Braun (15, 69, 70) has made significant progress in pioneering work to elucidate the transcriptome and proteome of newt heart development and regeneration. Even though the zebrafish heart has only two chambers, one ventricle and one atrium, the myocardial wall is similar in structure to that of mammals, being composed of three layers: myocardium, epicardium, and endocardium. In addition, as in mammals, the zebrafish myocardium can be divided into compact and trabecular layers (48). That the zebrafish is a useful model in which to study the human heart and human disease has been demonstrated in a large number of embryonic studies and forward genetic screens (7). However, in contrast to mammalian cardiomyocytes, zebrafish cardiomyocytes are small and mononucleated and retain proliferative potential throughout life (96, 122). Electrophysiological studies revealed that the adult zebrafish myocardium is very similar to that of humans based on acetylcholine-activated K⁺, Na⁺, L-type Ca²⁺, and ßK channels. However, these studies also revealed that adult zebrafish cardiomyocytes display a robust T-type Ca²⁺ current, which, in mammals, is observed only in development or disease. These data further suggest that adult zebrafish cardiomyocytes are less mature than adult mammalian cardiomyocytes (85).

Whether the underlying mechanisms of adult zebrafish regeneration can be utilized to induce mammalian cardiomyocyte proliferation remains uncertain. To date only a few regulatory mechanisms underlying zebrafish cardiomyocyte proliferation have been identified. Ventricular injury induces raldh2 expression, which is known to result in retinoic acid (RA)-induced fetal cardiomyocyte proliferation. In addition, induced transgenic inhibition of RA signaling blocked cardiac regeneration (53). Besides RA signaling, it has been suggested that IGF2 signaling (49) and Notch signaling (131) are required for zebrafish cardiomyocyte proliferation and cardiac regeneration. In addition, it has been shown that the cardiomyocyte-specific miR-133 is a negative regulator of zebrafish cardiomyocyte proliferation (126). All of these pathways/ regulators have previously been shown to be involved in cardiomyocyte proliferation during mammalian development (62, 67, 105, 112, 116). However, none of these factors has been shown to have a significant effect on adult mammalian cardiomyocyte proliferation. While they might be necessary, to date they have
not been shown to be sufficient to induce proliferation in adult mammalian cardiomyocytes.

In contrast to the above-mentioned factors, Fang and coworkers (35) have reported a Stat3-dependent program as injury-specific regulator of zebrafish cardiomyocyte proliferation. They identified several Jak/Stat3 pathway members by screening translating RNAs in zebrafish cardiomyocytes during heart regeneration. Cardiomyocyte-specific Stat3 inhibition blocked proliferation during regeneration but not development. Retro-orbital introduction of the secreted protein Rhn3 partially rescued Stat3-inhibited cardiomyocyte proliferation (35). In addition, Aguirre and coworkers (2) have recently suggested that a microRNA program (miR-99/100, Let-7a/c, fltb, smarca5) responsible for zebrafish heart regeneration can be utilized to regenerate the mammalian heart. Manipulation of these factors indicated that they regulate zebrafish cardiomyocyte proliferation after cardiac injury. The authors subsequently utilized these factors to induce adult mammalian cardiomyocyte proliferation. They provided in vitro-only data regarding re-expression of proliferating cell nuclear antigen (PCNA), a DNA clamp that acts as a processivity factor for DNA polymerase-δ in eukaryotic cells and is essential for DNA replication. In vivo, they determined expression of GATA4, PCNA, Aurora B kinase, and Anillin as well as histone H3 phosphorylation, and BrdU incorporation. While these data indicate proliferation, they don’t prove cardiomyocyte proliferation (see above). Yet cardiac function (% fractional shortening and ejection fraction) was also significantly improved, and ventricular wall thickness was recovered to around 50% (2). Whether this is due to adequate levels of cardiomyocyte proliferation, activation of a compensatory hypertrophic response, or a combination of both remains unclear.

Newborn mouse heart regeneration. The mouse heart is anatomically highly similar to the human heart (120). However, the difference in size results in different physiological demands and thus in differences to human cardiac physiology. In contrast to the human heart, the mouse heart can beat up to 800 times per minute and exhibits significantly different calcium handling. On a molecular level, the human and mouse hearts differ, for example, in the phosphorylation and isoform expression of a variety of contractile proteins (e.g., myosin heavy chain, titin, troponin) (79, 86). Whether these differences affect cardiomyocyte proliferation is unclear. Yet the human heart might have a greater regeneration potential than those of mice and rats, as >60% of adult human cardiomyocytes remain mononucleated (but mainly polyploid) (80), while >90% of adult mouse and rat cardiomyocyte are bi-/polynucleated (61, 107). In addition, it has been suggested that low proliferation rates of human cardiomyocytes can be observed until the second decade of life (80). Yet a recent paper challenges this view and provides data demonstrating that the final number of human cardiomyocytes is reached at 1 mo of birth and remains constant thereafter with a low turnover rate of around 1% (11). Newborn heart regeneration as well as development studies will help to identify mechanisms required for cardiomyocyte proliferation. Moreover, they might help to elucidate the mechanism underlying the cell cycle arrest. However, it remains unclear whether this knowledge will lead to reversal of cell cycle arrest in adult cardiomyocytes, allowing heart regeneration. Yet several genes have been identified as associated with or required for neonatal heart regeneration with the potential to induce adult mammalian cardiomyocyte proliferation or at least to improve cardiac function (95, 104). For example, Mahmoud and coworkers (73) have shown that the transcription factor Meis1 is downregulated upon myocardial infarction at postnatal day 1, yet only by around 25%. siRNA-mediated knockdown in rat neonatal cardiomyocytes (age is unclear) increased rates of histone H3 phosphorylation. Cardiomyocyte-specific (αMHC) Meis1 knockout mice exhibited increased cell cycle activity (BrdU, H3P, and Aurora B) at low levels (BrdU index of 0.3%) 14 days after birth and contained an increased number of total and mononuclear cardiomyocytes. Analyses of adult Meis1 knockout hearts suggested that cardiomyocyte proliferation markedly decreased with age. Considering that the use of αMHC-Cre results in Meis1 deletion before birth, these data suggest that deletion of Meis1 might prevent or delay cardiomyocyte differentiation. Yet the authors confirmed induction of cell cycle activity in adult cardiomyocytes upon Meis1 deletion utilizing αMHC-MerCreMer mice, which allowed Meis1 deletion in cardiomyocytes upon tamoxifen administration. In fact, the cell number data indicate that Meis1 deletion induced the production of 2 million cardiomyocytes in less than 5 wk by an H3P as well as Aurora B fold increase of less than fourfold. This is an increase of around 40% of the total cardiomyocyte. The heart-to-body weight ratio increased by 20%. In conclusion, these data support the idea that inhibitors control the cell cycle arrest in cardiomyocytes and that this arrest is reversible. This is in accordance with the observation that Meis1 deletion results in upregulation of positive cell cycle regulators and downregulation of other negative regulators (73). Still, it is important that these data are confirmed independently, ideally by clonal assays.

Porrello and coworkers (94) reported that the miR-15 family regulates neonatal heart regeneration. They reported that neonatal hearts of miR-195-overexpressing transgenic mice failed to regenerate after LAD ligation because of reduced cardiomyocyte proliferation. Subsequently, they tested whether inhibition of the miR-15 family would enable adult cardiomyocyte proliferation. To address this question they injected locked nucleic acid-modified anti-miRs postnatally at days 1, 7, and 14 and induced cardiac injury at day 21 by using an LAD ligation/reperfusion model. Even though the authors observed an increase from 1 to around 5 H3P-positive cardiomyocytes per section, the treatment had only a minor effect on cardiac function.

Critical Open Questions Regarding Human Heart Regeneration

Is the cell cycle arrest in adult mammalian/human cardiomyocytes actively maintained and reversible? Decades ago it was demonstrated that the expression of a large variety of positive cell cycle factors is downregulated in cardiomyocytes during development, while negative regulators, such as cyclin-dependent kinase inhibitors (CDKIs), are transiently upregulated (113, 115, 129). After birth, the majority of cardiomyocytes become binucleated or polyploid (Fig. 1) (17, 45, 107), the contractile apparatus changes dramatically into a highly ordered structure (66), and cardiomyocytes increase in size almost 20-fold (13, 37). These changes might explain why the proliferative potential and thus the efficiency to induce cardi-
omyocyte proliferation are markedly decreased in adult mammalian cardiomyocytes. Yet the mechanism that induces CDKI-dependent cell cycle arrest of mammalian cardiomyocytes after birth, as well as its purpose, remains elusive. Sdek and coworkers (103) have shown that the regulation of cell cycle genes in cardiomyocytes is mediated by histone methylation. Possible inducers of this epigenetic regulation might be alterations of the extracellular matrix around birth, as well as elevated levels of reactive oxygen species inducing DNA damage (98, 128, 129). Yet there is also evidence that the cell cycle arrest might be initiated even before birth. For example, fetal cardiomyocytes cannot be maintained in a proliferative state in vitro. They exit the cell cycle in vitro in a similar temporal pattern as it occurs in vivo, suggesting that an intrinsic process is activated in cardiomyocytes before birth, limiting the proliferative capacity of cardiomyocytes to a distinct number of cell divisions (20). Moreover, it has recently been shown that mammalian cardiomyocytes, in contrast to newt and zebrafish cardiomyocytes, start to disassemble their centrosomes in rats as early as embryonic day 15, a process that is completed shortly after birth (130). As recent data have demonstrated that the centrosome is a critical signaling hub for the cell cycle regulatory machinery, these data indicate the loss of centrosomes after birth as a possible reason why the hearts of mammals are unable to regenerate after injury. Yet the observations that inhibition of p38 MAP kinase enhances cardiomyocyte proliferation in the absence of a functional centrosome (31, 130) and that newborn mice can regenerate their heart after apex resection through cardiomyocyte proliferation (93), as well as several other studies (reviewed in Refs. 95, 104), provide some optimism for heart regeneration via proliferation of endogenous cardiomyocytes. Does a subpopulation of adult mammalian/human cardiomyocytes with a regenerative potential exist? In recent years evidence has accumulated suggesting that cardiomyocyte proliferation turnover occurs in both mice and humans throughout their lifetimes (10, 108). Yet it is unclear if all cardiomyocytes contribute to this turnover or whether a subpopulation with a higher proliferative capacity exists. The existence of such a subpopulation of mononucleated cardiomyocytes was suggested as early as in 2007 (58) and has recently been substantiated by a fate mapping study identifying a “rare population of hypoxic cardiomyocytes that display characteristics of proliferative neonatal cardiomyocytes, such as smaller size, mononucleation and lower oxidative DNA damage” (55).

Conclusions

A large amount of data has been accumulated that demonstrates that, in newt, zebrafish, and newborn mice, a variety of injuries induce heart regeneration by cardiomyocyte proliferation. In addition, it has been shown that it is possible to efficiently induce neonatal mammalian cardiomyocyte proliferation. In contrast, with regard to adult cardiomyocytes, little evidence exists supporting true proliferation (i.e., successful completion of cytokinesis resulting in two cells). Moreover, much of the data suggestive of adult cardiomyocyte proliferation is mainly based on immunofluorescence analysis, which, depending on the scrutiny and method of analysis, can lead to misinterpretations. Ultimately, much of the data underlying claims of regeneration by cardiomyocyte proliferation has yet to be confirmed independently. A major issue here is that often the limitations of the different proliferation assays are not considered. In addition, there are no studies that follow individual cardiomyocytes to determine if they divide, if the resulting daughter cells are viable and functional, and whether they can continue to proliferate. Moreover, available clonal assays that can unambiguously demonstrate cardiomyocyte proliferation in vivo are rarely utilized. Importantly, the induction efficiency of mammalian cardiomyocytes decreases dramatically with age, which might be at least in part due to epigenetic mechanisms (103) and centrosome disassembly (130).

An important goal in achieving mammalian heart regeneration is to understand naturally occurring heart regeneration. While newt and zebrafish regeneration is well established, it appears that cardiomyocytes of these species do not undergo a similar cell cycle arrest as mammalian cardiomyocytes after birth (130). Thus, it might not be possible to learn from newt and zebrafish regeneration how to reverse the cell cycle arrest in adult mammalian cardiomyocytes. The same might be true for neonatal heart regeneration. In addition, it remains unclear to what extent neonatal mice can regenerate their hearts (19, 26, 28).

Furthermore, one has to consider carefully the mammalian model system in which one studies the effect of candidate factors in improving cardiac function. For example, pigs are commonly used in large-animal studies for translational cardiac studies as they share more similar function and structure to humans than do rodents. However, porcine cardiomyocytes contain 4–16 nuclei (1, 42). Thus, it is unclear whether large-animal models are suitable for studies of cardiac regeneration based on cardiomyocyte proliferation. However, data are accumulating, at least in the adult mouse heart, for the existence of a subpopulation of cardiomyocytes with neonatal characteristics (55). These cells might be responsive to the mechanisms utilized during naturally occurring heart regeneration. Thus, in future study it will be important to determine in which species such subpopulation of cardiomyocytes exist.

Finally, it should be considered that the heart does not consist only of cardiomyocytes. Cardiomyocytes contribute only around 40–60% of the cells in mammalian hearts (8, 23, 46, 90, 117). Thus it appears important to better understand how the current regenerative approaches contribute to the formation of new complex cardiac tissue, how the dynamic of the process is, and whether dividing cardiomyocytes actively contribute to it.

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