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Cardiac Regeneration: New Hope for an Old Dream

Florian Weinberger[1,2] and Thomas Eschenhagen[1,2]

[1]Institute for Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany; email: f.weinberger@uke.de, t.eschenhagen@uke.de

[2]German Center for Cardiovascular Research (DZHK), Partner Site Hamburg/Kiel/Lübeck, 20246 Hamburg, Germany

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Abstract

The regenerative capacity of the heart has long fascinated scientists. In contrast to other organs such as liver, skin, and skeletal muscle, the heart possesses only a minimal regenerative capacity. It lacks a progenitor cell population, and cardiomyocytes exit the cell cycle shortly after birth and do not re-enter after injury. Thus, any loss of cardiomyocytes is essentially irreversible and can lead to or exaggerate heart failure, which represents a major public health problem. New therapeutic options are urgently needed, but regenerative therapies have remained an unfulfilled promise in cardiovascular medicine until today. Yet, through a clearer comprehension of signaling pathways that regulate the cardiomyocyte cell cycle and advances in stem cell technology, strategies have evolved that demonstrate the potential to generate new myocytes and thereby fulfill an essential central criterion for heart repair.

1. INTRODUCTION

In 1886, Goldenberg published a detailed report on atrophy and hypertrophy of the heart, in which he concluded that hypertrophy results from hypertrophic cardiomyocytes rather than hyperplasia. But he also mentioned that rare events of hyperplasia occur (1). Since then, innumerable studies have evaluated the heart's response to injury. These studies were driven by scientific curiosity but also inspired by a clinical need, because cardiovascular diseases have long been a major medical burden. Almost 150 years later, it seems that Goldenberg's conclusions, solely based on histological analysis, were surprisingly accurate.

The human heart contains 2–4 $\times$ 10^9 cardiomyocytes (2) that make up most of the heart's volume, but they comprise only 20–30% of the heart's cells. In combination with the nonmyocytes, they supply the body with blood, thereby with oxygen and nutrients, for 72 years on average. Heart diseases are common, and heart failure with reduced ejection fraction is the sequela of basically all heart diseases, affecting ~26 million people worldwide. Coronary artery disease, favored by risk factors such as hyperlipidemia, hypertension, age, male sex, and clonal hematopoiesis, is the most frequent cause of heart failure. Myocardial infarction leads to an acute loss of cardiomyocytes (~0.5–1 $\times$ 10^9 when 20% of the myocardium is affected). Interventional therapy has become the standard care for patients with myocardial infarction. As a result, the number of fatal myocardial infarctions has declined, but nonfatal events cause a loss of cardiomyocytes that eventually leads to a decline in left ventricular function and contributes to the increase in heart failure prevalence (3). Current pharmacotherapy targets neurohumoral activation, a hallmark of heart failure, and aims to salvage myocardium. Combination drug therapy is highly effective and has substantially improved life expectancy for heart failure patients. Nevertheless, mortality, particularly in end-stage heart failure, remains high (4). Regenerative therapies are conceptually different, as they aim to rebuild myocardium rather than salvage tissue. Even though they have been discussed for years and a first wave of clinical studies has been conducted, regenerative therapies have remained an unfulfilled dream in cardiovascular medicine until today.

2. CARDIAC REGENERATION: WHAT DO WE AIM FOR?

Tissue regeneration is difficult to define. Brockes & Kumar (5) begin their work "Comparative Aspects of Animal Regeneration" with a paragraph that comes close to a definition when they write that "it involves the recognition of tissue loss or injury followed by mechanisms that reconstruct or restore the relevant structure" (p. 526). Yet, in a later paragraph they state "regenerative phenomena represent a continuum in relation to mechanisms, and exact definitions can be difficult to justify" (p. 527). Therefore, a brief discussion might help to clarify what we understand as cardiac regeneration. One aspect is, in our opinion, central for true heart regeneration: formation of new myocardium. Resolution of fibrosis and the formation of new blood vessels are additional, crucial aspects. Regeneration does not only involve morphological restoration. New myocardium has to integrate functionally, including electrical and mechanical coupling to the host tissue. Remuscularization cannot be achieved with any current pharmacological, interventional, and surgical strategies and is discussed most extensively in this review.

A central question is whether a direct correlation between cardiomyocyte number and left ventricular function exists. At first sight, this seems like a straightforward hypothesis: The lower the number of cardiomyocytes, the worse the heart function. Most evidence that cardiomyocyte number correlates with function derives from acute myocardial infarction. Even though the correlation is not pronounced in the acute phase, infarct size eventually shows a strong correlation with ejection fraction in animal models as well as in patients (6–8) (**Figure 1**). There is also some evidence

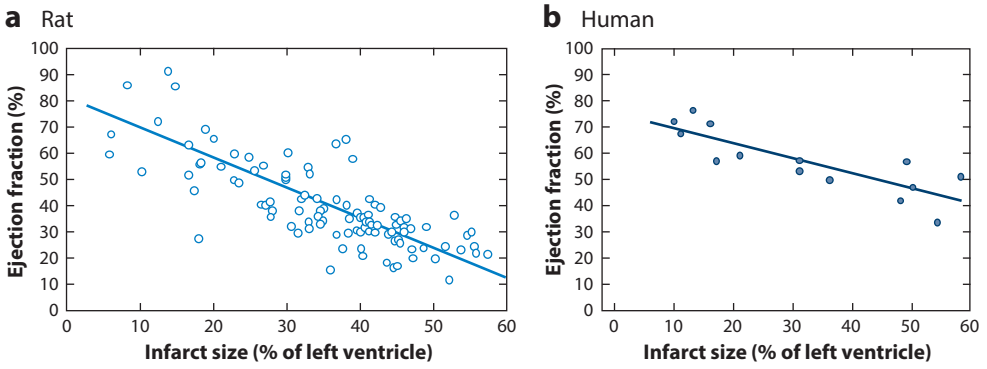

Figure 1

Correlation between infarct size and left ventricular function. (*a*) Data from Pfeffer & Braunwald (6) correlating infarct size and ejection fraction in a rat myocardial infarct model 30 days after infarction. (*b*) Data from Burns et al. (8) correlating the ejection fraction in patients one year after infarction and infarct size measured at the time of hospital discharge.

from studies that analyzed cardiomyocyte apoptosis during heart failure. Assuming that a greater number of apoptotic cardiomyocytes will result in a lower total cardiomyocyte number, one would expect a negative correlation between cardiomyocyte number and ejection fraction. Although the exact numbers vary widely [and studies that rely on DNA end labeling by deoxynucleotidyl transferase (TUNEL) most likely overestimate the true number (9)], myocardial samples from patients with end-stage heart failure consistently showed more apoptotic cardiomyocytes (10, 11) than healthy human myocardium [~0.25% (10) and 5–35% (11) versus 0.001% in control samples (10)], providing some evidence that such a continuous low-level loss of cardiomyocytes perpetuates heart failure. The hypothesis is further strengthened by a mouse model that inducibly expressed activated, apoptosis-inducing caspase 8. As with many complex transgenic models, some cells expressed caspase 8 even without induction. This resulted in a low number of apoptotic cardiomyocytes even without induction (23 per 10^5 nuclei, 0.023%). Though this value is lower than those reported for human end-stage heart failure (see above), the mice exhibited a lethal cardiomyopathy within a few weeks (12). Combined, these findings support the very intuitive hypothesis that remuscularization will improve left ventricular function. There is one more lesson that can be learned from the animal models: It will most likely turn out to be a numbers game; in other words, to achieve a clinically meaningful improvement in left ventricular function, a substantial part of the scar has to be remuscularized, as demonstrated by a simple calculation. In a rat model, large myocardial infarcts affecting ~50% of the left ventricle resulted in an ejection fraction of ~23% compared to a normal mean of ~80%. Remuscularization of ~10% of the scar (a value that is in the upper range of what has been reported so far) would reduce the scar area to 45% of the left ventricle, which correlated with an ejection fraction of ~30% (an absolute increase by 7%) (6). Left ventricular function negatively correlates with mortality in heart failure patients, indicating that an increase in ejection fraction of 5–10% can be meaningful. Yet, it is far beyond complete recovery. There is a great variability in left ventricular function in humans after myocardial infarction, which complicates a direct comparison to the clinical scenario (and other factors like fibrosis and cardiomyocyte hypertrophy also affect heart function), but the correlation is similar (**Figure 1**), highlighting the challenge of regenerative strategies.

3. CARDIAC GROWTH AND PHYSIOLOGICAL RESPONSE TO INJURY

The origin of cardiomyocytes, pre- and postnatally, has been studied intensively for over a century (13). Before discussing these studies, we briefly summarize what we have learned, as a clearer picture has finally emerged (14): (*a*) Growing evidence supports a model that the final cardiomyocyte number is established early after birth; (*b*) the neonatal heart is able to regenerate during a brief postnatal period; (*c*) cardiomyogenesis in the adult heart is rare; and (*d*) physiological and pathophysiological stress appear to increase the number of newly formed myocytes, but even under these circumstances the number of newly formed cardiomyocytes is low.

3.1. The Quest for a Cardiac Stem or Progenitor Cell Population

A well-described dichotomy regarding the origin of regenerative cells exists (15). Regeneration can derive from either the differentiation of progenitor cells or already differentiated cells. Digit tip regeneration represents an example in which lineage-restricted progenitor cell populations regenerate an organ (16). In contrast, regeneration of hepatocytes occurs from differentiated Sox9-positive hepatocytes (17). In the heart, myocyte and nonmyocyte lineages separate early during development (18). Inspired by embryonic heart development and the adult hematopoietic system, researchers started to search for a postnatal cardiac progenitor cell population. A bone marrow transplantation study in *mdx* mice was the first to report a contribution of hematopoietic cells to the myocardium (19), but it was a study in which c-Kit$^+$ bone marrow cells were injected into the injured myocardium that fueled this field because it reported a remuscularization of ~70% of the scar after myocardial infarction (20). This was followed by two other studies: one that also reported that c-Kit$^+$ marked a resident stem cell population in the heart (21) and the other that reported a high percentage of cardiomyocyte chimerism (~10%) after transplantation of female hearts in male donors (22), which paved the way for innumerable studies on cardiac progenitor cells and laid the foundation for clinical studies. Yet early on, studies that applied more rigorous experimental conditions questioned the cardiogenic potential of bone marrow cells (23, 24) and the degree of chimerism after sex-mismatched transplantation (25). It took a long time until the critical view became universally accepted (14), not at least because animal studies consistently reported beneficial effects of bone marrow (and other) cell infusions/injections into the injured heart. However, it seems very likely that such beneficial effects result from paracrine effects and/or the immune response to the injected cells and not from the formation of new myocytes (26).

In a second wave of research, a variety of cardiac resident stem/progenitor cells have been described, despite apparent conceptual problems. (*a*) Any myocardial injury leads to a decline in left ventricular function. Spontaneous recovery is rare and not necessarily complete (e.g., in myocarditis, postpartum cardiomyopathy). (*b*) Myocardial tumors are extremely rare. (*c*) Even highly regenerative organs such as the intestine do not harbor several different (but rather one) progenitor cell populations that eventually form the same single cell type. (*d*) Lower vertebrates that possess the ability to regenerate the heart do so by cardiomyocyte proliferation (27). Nevertheless, several resident cardiac progenitor cell populations have been described. Besides the aforementioned c-Kit$^+$ cells, these were defined by stem cell antigen 1 (Sca-1) (28), the transcription factor Islet-1 (29), the ability to form outgrow cell clusters (30), or the ability to extrude Hoechst 33342, a fluorescent DNA dye (side population) (31). The strongest evidence for the existence of a progenitor cell population evolved from a lineage tracing study in which cardiomyocytes were labeled with green fluorescent protein (GFP). Labeling efficiency was ~80%. While the percentage of GFP$^+$ cardiomyocytes remained stable in normal life (arguing against a role of noncardiomyocytes), it declined to ~65% after myocardial injury, indicating the formation of new myocytes from a GFP$^-$ progenitor cell pool (32). Retrospectively, it is more

likely that GFP$^+$ cells were more susceptible to myocardial injury and therefore preferentially died after myocardial infarction. We believe this to be the case because several recent lineage tracing studies have provided increasingly more evidence against the existence of a progenitor cell population in the heart and found only a very limited cardiogenic potential for c-Kit$^+$ (17, 33), Sca-1$^+$ (34), and Islet-1$^+$ (35) cells in the adult heart. Finally, a comprehensive study by Li et al. (36) followed a strategy that did not lineage trace a specific cell population (with the inherent risk of labeling other cells at low level) but used a dual reporter system to mark cardiomyocytes with a specific fluorochrome and all nonmyocytes with a second one. The authors did not find a single cardiomyocyte derived from a nonmyocyte in this study. In conclusion, these studies provide compelling evidence that neither the adult heart nor the rest of the body harbors a cardiomyocyte progenitor cell population. There is some evidence that pharmacological intervention with THYMOSIN BETA 4 prior to myocardial infarction can activate epicardial cells to differentiate to cardiomyocytes postinjury (37). Yet, when applied after myocardial injury, THYMOSIN BETA 4 treatment did not induce the formation of new myocytes, and further studies are needed to better understand the cardiogenic potential of epicardial cells after injury. Reasons why the field needed so long to solve this issue are complex and include methodological challenges, but we also strongly believe that exaggerated competition and scientific fraud played a role (38).

3.2. Cardiomyocytes as the Source for New Myocytes

Returning to the initially mentioned dichotomy, a second option remains: cardiomyocytes as a source for new myocytes. At birth the human heart weighs about 20 g, doubles in weight within one year, reaches 120 g at the age of 10 years, and weighs about 300 g in young adults (39). This increase in weight by a factor of 15 is paralleled by a similar increase in cardiomyocyte cell volume (2). Hence, postnatal heart growth could be completely explained by cardiomyocyte hypertrophy, but of note, even slight variations in volume determination would leave room for ongoing hyperplasia to contribute to the overall increase in heart weight. Mollova et al. (40) reported a ~3.5-fold increase in cardiomyocyte number within the first two decades in humans. In contrast, stereology studies and retrospective birth dating by ^{14}C labeling (^{14}C increased in the atmosphere from the mid-1950s due to nuclear bomb tests and decreased after the test ban in 1963) provided evidence that the final cardiomyocyte number is established early after birth and postnatal cardiomyogenesis is rare (2), ~0.8% in young adults. It decreases to ~0.3% in the elderly, indicating that only ~40–50% of all cardiomyocytes are exchanged during life. Studies on human samples have inherent limitations but are well in line with animal data. Heart weight in mice is about ~7 mg at birth, increases to ~50–60 mg around day 21 (the time point of weaning), and reaches ~150 mg in adulthood. In parallel, cardiomyocyte volume also increases threefold from day 21 (~8,000 μm^3) to ~25,000 μm^3 in the adult heart (41, 42).

In congruence with the human example, these numbers alone can explain the postnatal heart growth, assuming that there is no substantial cardiomyocyte death. Cardiomyocyte death has not been studied as extensively as cardiomyocyte proliferation, but the existing data provide some evidence that the number of cardiomyocytes undergoing programmed cell death declines during embryonic development and in the early postnatal period (43) and is very low after postnatal day 20 (44). Studies that (*a*) evaluated cell cycle markers, (*b*) performed lineage tracing, and (*c*) conducted clonal analysis further corroborated that cardiomyogenesis after birth is rare. Early studies analyzed DNA synthesis as a marker for proliferation by radioactive labeling with tritiated thymidine in mice and rats. Cardiomyocyte DNA synthesis declined during the late phase of embryonic development in mice, followed by a small temporary increase within the first week. Eventually, there was no detectable degree of DNA synthesis in adult cardiomyocytes. The small increase

after birth paralleled an increase in binucleation, reaching ~80% in adult mice (45). More recent studies labeled DNA with the nonradioactive nucleosides BrdU, EdU, or ^{15}N-thymidine combined with stainings for other cell cycle markers (Ki67, histone H3 phosphorylation, Aurora B kinase, and Anillin) and lineage tracing models. With the exception of one study that reported a burst in cardiomyocyte proliferation in the second postnatal week (46), they reported (very) low indices of cell cycle activity in the healthy neonatal, adolescent (42, 47), and adult heart (48). These results agree with studies that applied clonal analysis (49, 50) and collectively provide undeniable evidence that postnatal cardiomyocyte proliferation is rare under uninjured, physiological conditions. There is some evidence that not all cardiomyocytes are equal and proliferation is not randomly distributed. Based on the hypothesis that progenitor cells, e.g., in the bone marrow, localize in a hypoxic microenvironment, Kimura et al. (51) used a lineage tracing approach in which the oxygen–dependent degradation domain of Hif-1α was fused to an inducible Cre-recombinase (CreERT2). This strategy labeled rare cardiomyocytes in the adult heart (~0.005%) that were either hypoxic or in which Hif-1α was stabilized via nonhypoxic manners. During a one-month follow-up period the number of labeled cardiomyocytes increased (sometimes in clusters), and the authors calculated an annual cardiomyocyte formation rate of 0.6–1%, which could account for all newly formed cardiomyocytes.

3.3. Does Injury Induce Cardiomyocyte Proliferation?

The first experimental studies to answer this question were carried out by Zielonko in 1874 (13). He performed transverse aortic constriction (TAC) surgery in frogs and rabbits but also directly injured the heart with a needle and eventually concluded that he was not able to answer the question with certainty whether new myocytes are formed. Transgenic approaches now allow evaluation of the plasticity of the heart during embryonic development. Heart-specific deletion of the holocytochrome c synthase (*Hccs*), an X chromosomal-localized gene crucial for the mitochondrial respiratory chain, led to embryonic lethality in males. In female animals, in which one X chromosome is randomly silenced, one would assume that 50% of all cardiomyocytes are affected, but during development diseased cardiomyocytes contributed progressively less over time and at birth comprised only 10% of the total cardiomyocyte number, resulting in a mild hypoplastic phenotype (52, 53). The pronounced plasticity of the embryonic heart was also demonstrated by genetic ablation with a diphtheria toxin model. Ablation of up to 60% of cardiac progenitors or cardiomyocytes was compensated without morphological and functional restriction, demonstrating substantial regenerative potential during embryonic development (54).

In 1956, Robledo (55) described mitotic figures in cardiomyocytes of neonatal rat hearts after injury, and the work of Rumyantsev (56) substantiated this injury-induced increase in cardiomyocyte proliferation in neonates. Porrello et al. (57) eventually demonstrated a profound regenerative response after apical resection in the neonatal heart in mice during the first seven days and provided evidence that the newly formed cardiomyocytes derive from the proliferation of pre-existing cardiomyocytes. A brief regenerative window, lasting only two days, has been also described in pigs, during which myocardial injury increased cardiomyocyte proliferation and resulted in only minimal scarring (58). Anecdotal data suggest that a brief regenerative window might exist in humans as well (59). However, after this brief regenerative period, the heart possesses only a minimal regenerative capacity. This conclusion is based on (*a*) consistent clinical observations that left ventricular function declines after myocardial injury and (*b*) findings from postmortem human samples after myocardial infarction that report an increase in cardiomyocyte cell cycle activity resulting in polyploidy but not in cytokinesis (60); and (*c*) it is substantiated by results from animal studies. Tritiated thymidine injections (to visualize DNA synthesis) in a transgenic mouse model specifically labeling cardiomyocyte nuclei yielded 1/180,000 cells (0.00055%) in the healthy heart,

but also only 3/36,000 screened cardiomyocytes (0.0028% = 5 times higher) after injury (61). Very limited numbers of (or even no) proliferating cardiomyocytes were also reported in studies that used transgenic models to visualize cell cycle activity (62), monitored DNA synthesis by the incorporation of ^{15}N-thymidine by multi-isotope imaging mass spectrometry (48), or used transgenic labeling of cycling cells followed by single-cell RNA sequencing to determine cell identity (63). It is important to consider that all biological assays have some background noise. Even the most rigorous assays probably reach their sensitivity threshold when events occur in the magnitude of 1% per year, and the interpretation therefore requires caution (for more discussion, see review in 64).

3.4. Why Do Cardiomyocytes Exit the Cell Cycle but Not Re-Enter It After Injury in the Adult?

Several studies have addressed this issue, indicating that there is not a single factor but rather a compendium of factors that drive cardiomyocytes to exit the cell cycle. The increase in oxygen tension after birth (caused by the establishment of the pulmonary circulation and the closure of the foramen ovale and ductus arteriosus) was shown to increase reactive oxygen species (ROS) production and DNA damage. Scavenging ROS prolonged the regenerative window (65). There is some evidence that polyploidy and multinucleation impede cardiomyocyte proliferation (66). The switch to fatty acid oxidation (67) and changes in the extracellular matrix composition have been linked to cardiomyocyte cell cycle exit (68). Moreover, a recent study revealed a correlation between the loss of postnatal regenerative potential and the development of endothermy (the ability to maintain constant body temperature) and demonstrated that this correlates with a postnatal increase in thyroid hormone signaling (69).

The question of why cardiomyocytes, in contrast to other cell types of the body, lose their regenerative potential postnatally is unanswered. A common hypothesis is that the need for continuous work precludes cell division, during which sarcomers have to be (partially) disassembled. During embryonic development, single myocytes proliferate while remaining connected to their neighboring cells in a beating heart, but the higher afterload during postnatal circulation might preclude this process. A regenerative response after myocardial injury, e.g., myocardial infarction, would require the generation of a huge number of new cardiomyocytes. Therefore, a large number of preexisting cardiomyocytes would have to (partially) disassemble their sarcomeres, which might further reduce left ventricular function. In addition, newly formed cardiomyocytes have to mature and structurally and physiologically integrate during a critical time given the high arrhythmogenic risk in the early postinfarct period. Scar formation is a rapid alternative process to cellular regeneration that acutely stabilizes the heart (as demonstrated by ventricular rupture in mouse models in which scar formation is inhibited) and therefore might have provided evolutionary advantages.

4. STRATEGIES TO INDUCE REMUSCULARIZATION

We currently see three strategies to remuscularize the injured heart: stimulation of cardiomyocyte proliferation, transdifferentiation of nonmyocytes to cardiomyocytes, and transplantation of pluripotent stem cell–derived cardiomyocytes (**Figure 2**).

4.1. Cardiomyocyte Proliferation to Remuscularize the Injured Heart

Field pioneered the study of cardiomyocyte proliferation when he overexpressed the SV40 T antigen under the atrial natriuretic promoter and discovered that the transgenic mice developed

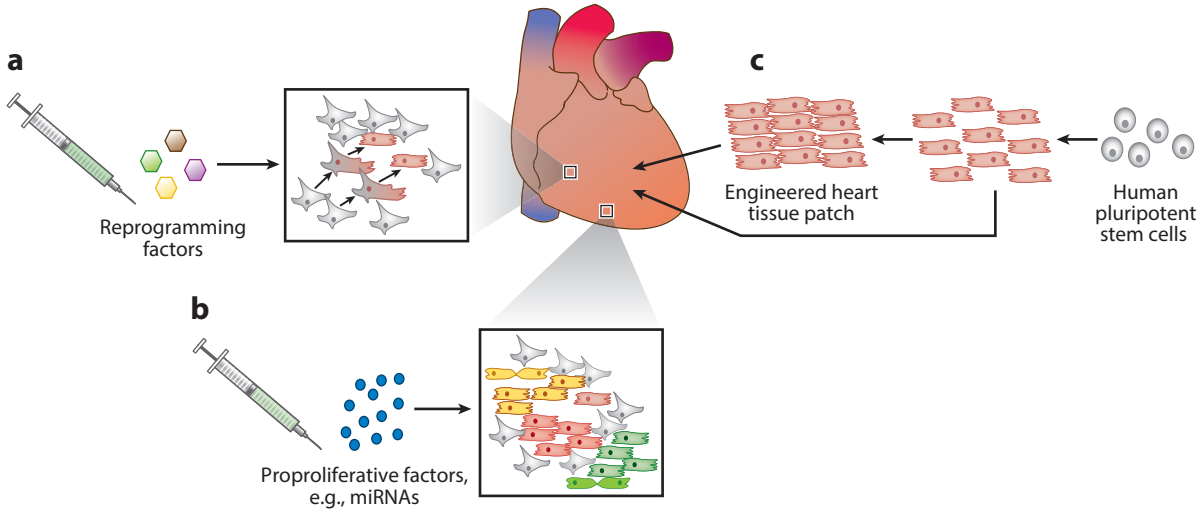

Figure 2

Therapeutic strategies to remuscularize the injured heart. Three strategies have demonstrated the potential to remuscularize the injured heart: (*a*) Reprogramming of nonmyocytes (e.g., fibroblasts) to cardiomyocytes, (*b*) stimulation of cardiomyocyte proliferation (e.g. with miRNAs), and (*c*) transplantation of stem cell–derived cardiomyocytes (either as single-cell injection or as engineered cardiac patch).

hyperplasia of the right atrium (70). Twenty years later, a translational study that therapeutically applied miRNAs to stimulate cardiomyocyte proliferation in a late preclinical study encountered a similar problem and thereby (almost) closes a circle (71). During this period, we have learned that stimulation of cardiomyocyte proliferation can be achieved with several strategies. Conceptually, stimulation of cardiomyocyte proliferation is attractive because it is, per se, autologous and therefore circumvents the immunological problems of cell transplantation. Most studies did not analyze integration and coupling of newly formed myocytes in detail, but gross analysis showed structural integration of the newly formed cardiomyocytes. In addition, stimulation of cardiomyocyte proliferation consistently inhibited the fibrotic response after injury, resulting in smaller scars.

4.1.1. Cyclins: first attempts to stimulate cardiomyocyte proliferation. Cyclins and cyclin-dependent kinases jointly regulate cell cycle progression. Studies showed that after birth, cyclin A and B gene expression decreases over time, whereas gene expression of cyclin D and E is unaltered (72). Field's group targeted D cyclins to induce cardiomyocyte proliferation. They overexpressed the three members of the cyclin D family [cyclin D1, D2, and D3 (Ccnd1/2/3)], which together with the cyclin-dependent kinase 4 regulate G1/S-phase transition. Overexpression of Ccnd1/2/3 increased DNA synthesis in the healthy heart (and altered the degree of multinucleation), but there was a striking difference after injury when Ccnd1 and Ccnd3 localized to the cytoplasm and only Ccnd2 remained in the nucleus, resulting in increased DNA synthesis and eventually profound generation of new myocardium (73, 74). One aspect is particularly remarkable about this study. The new myocardium enveloped the scar within 180 days, thereby partially replacing scar tissue (74), indicating that the generation of new myocytes takes time and resolution of fibrosis is even slower. This stands in contrast to many other studies in which stimulation of cardiomyocyte proliferation also resulted in smaller scars, but in which the new myocardium appeared to take the place of the scar tissue rather than overgrowing it (e.g., 75). Other cyclins have also been targeted

to stimulate cardiomyocyte proliferation. Cardiomyocyte-specific overexpression of cyclin A2, which is usually not expressed in the adult heart, resulted in hyperplastic hearts and eventually a reduced ejection fraction (76), providing a good example that an increase in cardiomyocyte number can be detrimental in a healthy organ. Yet, the increase in cardiomyocyte proliferation improved left ventricular function after injury (77). This strategy has been moved forward, and a gene therapy study in pigs demonstrated increased cell cycle activity and improvement of left ventricular function (78). Follow-up studies using this strategy are awaited.

4.1.2. Hippo signaling: the major hub for cardiomyocyte proliferation. In the past decade, the Hippo pathway has been identified as the major hub for the regulation of cardiomyocyte proliferation. This pathway regulates heart growth during development. Prenatal stimulation or inhibition results in hypo- or hyperplastic hearts, respectively. YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) are the effectors of Hippo signaling. They are transcriptional coactivators that interact with transcription factors, mainly from the targeting the transcriptional enhanced associate domain (TEAD) transcription factor family. Activation of Hippo signaling results in Yap and Taz phosphorylation, which prevents their translocation into the nucleus and promotes degradation in the cytoplasm. Phosphorylation occurs via a kinase cascade in which Mst1 and Mst2 (mammalian STE20-like protein kinase 1/2) in combination with Salv1 (scaffold protein salvador homolog 1) phosphorylate (and thereby activate) Lats1/2 (large tumor suppressor homolog 1/2), which then phosphorylate Yap and Taz. In short, Hippo signaling impedes cardiomyocyte proliferation. As a consequence, inhibition of the Hippo pathway stimulates cardiomyocyte proliferation. This strategy has successfully been used to drive cardiomyocyte proliferation and improve left ventricular function in several small animal myocardial injury models and recently in the first large animal model. Cardiomyocyte-specific deletion of Salv resulted in decreased Yap phosphorylation (i.e., activation), increased cardiomyocyte proliferation, and cardiomegaly (79). Similarly, expression of a constitutively active Yap mutant increased cardiomyocyte proliferation, either during development or in early postnatal hearts (80, 81), whereas cardiomyocyte-specific deletion of Yap (i.e., inactivation) reduced cardiomyocyte proliferation and caused myocardial hypoplasia (80, 81). From a translational perspective, the most remarkable finding is that the Hippo pathway can be effectively targeted for cardiac regeneration. Constitutively active Yap, as well as cardiomyocyte-specific Salv knockout, stimulated cardiomyocyte proliferation after myocardial injury and improved left ventricular function (82–84). Similarly, gene therapy with an adeno-associated virus (AAV) expressing a short hairpin (sh)RNA against Salv three weeks after myocardial infarction, a time point at which a scar was already established, also stimulated cardiomyocyte proliferation. Although left ventricular function improved more slowly than by genetic knockout, it eventually showed the same effect size (84).

Two further aspects regarding the Hippo pathway seem of particular interest. First, the Hippo pathway is connected to other pathways involved in the regulation of cell cycle, e.g., Wnt signaling, which might offer additional therapeutic targets. Growing evidence also suggests a link between the extracellular matrix, mechanical signaling, and the Hippo pathway. There is initial evidence that Yap is restrained to the intercalated discs. Disruption of intercalated discs, by knocking out alpha-catenins (αE- and αT-catenin double knockout), increased nuclear Yap (i.e., activation), indicating that it was released from the intercalated discs and translocated to the nucleus. Knockout of alpha-catenins also resulted in enhanced cardiomyocyte proliferation in the neonatal heart (in which intercalated discs are not fully matured). Inducible deletion of alpha-catenin in the adult heart did not stimulate cardiomyocyte proliferation under physiological conditions, but after myocardial infarction, suggesting that Yap is not only restrained by alpha-catenins in the mature intercalated discs, but that myocardial injury is necessary to release these additional restrains (85).

This indicates that Yap is sequestered by alpha-catenins in mature intercalated discs and upon release translocates to the nucleus and stimulates cardiomyocyte proliferation. Morikawa et al. (86) showed that phosphorylated (i.e., inactivated) Yap is not only bound to intercalated discs that join cardiomyocytes end-to-end, but also to the dystrophin-glycoprotein complex along the lateral cell membrane. This binding sequesters Yap, prohibits translocation to the nucleus and thereby inhibits cardiomyocyte proliferation. Bassat et al. (68) demonstrated that these changes are not only coincidental but causal. They described agrin, an extracellular matrix component that decreases in abundance postnatally, as a regulator of cardiomyocyte proliferation. Interestingly, this protein can be used as a pharmacological agent to release Yap from the dystrophin-glycoprotein complex (by extracellular binding to α-dystroglycan), stimulate cardiomyocyte proliferation, and improve heart function (68).

Second, microRNAs can be used pharmacologically to stimulate cardiomyocyte proliferation and repair the injured heart. This finding, per se, is remarkable, but interestingly, recent studies demonstrated that the miRNA effect is mediated via Hippo signaling, further strengthening its role as the main regulator for cardiomyocyte proliferation. The Giacca group (75, 87) performed a high-throughput screening and discovered ~200 miRNAs that increased cardiomyocyte proliferation. Expression of miRNA-199 and miRNA-590 stimulated cardiomyocyte proliferation in the healthy neonatal rat hearts but also after myocardial infarction in mice, resulting in smaller scars and better left ventricular function (75, 87). This proproliferative effect was abolished by Yap knockdown, demonstrating a link between Hippo signaling and these miRNAs. The miRNA-mediated proproliferative effect involved inhibition of the expression of Yap-degrading proteins but also altered cytoskeleton regulation (88). Tian et al. (89) described a similar function for miRNA302. Knockout resulted in myocardial hypoplasia, whereas overexpression stimulated cardiomyocyte proliferation via downregulation of Mst and Lats (thereby preventing Yap phosphorylation). A transient therapy with a miRNA-302 mimic after myocardial infarction decreased infarct size and improved function in mice. However, constitutive (prolonged) miRNA-302 overexpression after myocardial injury decreased left ventricular function despite smaller scars, possibly as a result of an immature cardiomyocyte phenotype (89). This suggests that cardiomyocyte proliferation always happens at the expense of lower contractile function and highlights a caveat for clinical application. The conclusion is further strengthened by a recent study in which sustained inhibition of Hippo signaling induced cardiomyocyte dedifferentiation and had deleterious effects in a chronic pressure overload model (90).

Giacca's group (71) recently moved the miRNA-199 strategy toward clinical application. Direct intramyocardial AAV6 injection encoding for miRNA-199a at the time of injury stimulated cardiomyocyte cell cycle activity after myocardial infarction in pigs, reduced scar size, and improved left ventricular function (assessed by cardiac MRI) after 28 days. This effect was sustained in a small group of animals, but the majority died of sudden cardiac death due to ventricular arrhythmias that occurred 7–8 weeks after treatment. Cardiac histopathology revealed highly proliferative cell clusters infiltrating the myocardium that expressed markers for rhabdo- and leimyosarcomas and were microRNA-199a negative (71). The study thus impressively shows the potential of proproliferative therapies but also that any such strategy needs to incorporate robust means for temporal regulation of cardiomyocyte proliferation before clinical translation.

4.2. Direct Reprogramming

In 1898, Thomas Morgan (who later received the Nobel Prize in physiology for his work on *Drosophila* genetics) described a form of regeneration in worms in which "relative proportions of the planarian are attained by a remodeling of the old tissue" (morphallaxis) (91, p. 385), involving

transdifferentiation from one cellular identity to another. A striking example is the regeneration of the newt eye. After surgical removal of the lens, pigmented epithelial cells of the iris transdifferentiate and form a new lens. During this process they transiently dedifferentiate, but remain committed to a lens fate as exemplified by the fact that they generate lens tissue even if transplanted into other regions of the newt (92).

After myocardial injury, fibroblasts become activated, proliferate, secrete abundant extracellular matrix proteins, and eventually together with the extracellular matrix form the scar tissue. Therefore, transdifferentiation of fibroblasts to cardiomyocytes could offer solutions for two problems: de novo cardiomyogenesis and resolution of scar tissue. Researchers showed in 1987 that forced expression of MyoD transdifferentiates nonmyocytes to striated skeletal muscle myocytes (93). Yet, in contrast to skeletal muscle, a master regulator for cardiomyocytes does not exist. Bruneau's (94) group showed transdifferentiation of noncardiac mesoderm to cardiomyocytes by the forced expression of two transcription factors (Tbx5 and Gata4) and a chromatin-remodeling complex (Baf60c). Based on this result, Ieda et al. (95) demonstrated that (neonatal) murine cardiac and skin fibroblasts can be transdifferentiated to cardiomyocytes (induced cardiomyocytes) by viral overexpression of the three transcription factors Gata4, Mef2c, and Tbx5 (GMT) in vitro.

During the transdifferentiation process, cell identity bypasses a pluripotent or mesodermal progenitor state and converts from fibroblasts (via the intermediate steps referred to as induced fibroblast and preinduced cardiomyocyte) (96) to induced cardiomyocytes, which raises interesting questions about cardiomyocyte identity. Ieda et al. (95) used a GFP reporter system under the alpha-myosin heavy chain reporter to identify induced cardiomyocytes. These cells morphologically resembled neonatal cardiomyocytes. Most expressed sarcomeric alpha-actinin, only 30% expressed cardiac troponin T, a subset showed spontaneous Ca^{2+} oscillations, and, as the most direct evidence of (immature) cardiomyocyte identity, they displayed spontaneous contractions after 4–5 weeks in culture.

The in vivo application of GMT or similar GMHT (Gata4, Mef2c, Hand2, Tbx5), though inefficient, induced a morphologically and electrophysiologically more complete transdifferentiation. Viral delivery of GMT or GMHT resulted in the formation of cardiomyocytes from nonmyocytes [lineage traced by periostin (97), fibroblast-specific protein 1-Cre (97, 98), or capsulin/epicardin-MerCreMer (98)] in mouse myocardial infarct models. The in vivo–induced cardiomyocytes were rod shaped (assessed after cell isolation), and N-cadherin expression was localized to the longitudinal termini of the cells in many cases, whereas connexin, although abundantly expressed, showed a more dispersed expression. Action potentials closely resembled adult mouse ventricular action potentials. Qian et al. (97) reported that four weeks after injury, ~35% of the borderzone cardiomyocytes were derived from transdifferentiation events after GMT treatment, whereas Song et al. (98) reported ~6% new borderzone myocytes three weeks after GMHT delivery and calculated that a minimum of 10,000 new cardiomyocytes were generated. Induction of transdifferentiation with either GMT or GMHT resulted in a sustained improvement of left ventricular function. Similar results were described after viral delivery of four miRNAs in the injured mouse heart (87). After the initial description, research has focused on maximizing the efficiency of the process, e.g., by optimizing the stoichiometry of Gata4, Mef2c, and Tbx5 (99) or inhibition of TGF-β signaling (100). The discovery is fascinating, but major hurdles remain in regard to human heart regeneration. (*a*) Human cells proved to be more resistant than murine cells. For example, reprogramming required more factors [GATA4, HAND2, TBX5, MYOCD, miR-1, and miR-133 (101); GATA4, MEF2c, TBX5, and MYOCD plus inhibitors of WNT and TGF-β signaling (102); GATA4, MEF2c, TBX5, MESP1, MYOCD, and miR-133 (103); or nine small molecular substances (104)], was less efficient, and led to less well-developed cardiomyocytes with no (103) or only weak contractility (101). (*b*) Adult (human) fibroblasts were more difficult to reprogram than

fetal cells, a factor that might particularly affect the elderly population suffering from heart failure, as age has been shown to affect fibroblast composition, activation state, and reprogramming efficiency (105). (*c*) The in vivo–generated cardiomyocytes are not well characterized yet. Proper integration and maturation will be important aspects determining their arrhythmogenic risk.

4.3. Cardiomyocyte Transplantation

It is difficult to imagine a more straightforward approach than cardiomyocyte transplantation. Myocardial infarction kills cardiomyocytes, so replenishment with new myocytes should normalize the situation. Currently, this strategy is being evaluated in late preclinical trials; a first clinical trial was initiated, and more clinical studies are underway. Nevertheless, it took time since the Oberpriller lab (106) performed the first cardiomyocyte transplantation studies in newts in 1978, in which they amputated the heart's apex, minced the myocardium, and retransplanted the tissue. Fifteen years later, embryonic mouse cardiomyocytes were transplanted in syngeneic mouse hearts and structurally integrated (107). Since then the field has progressed significantly and step by step cleared most hurdles toward clinical trials. The main bottleneck for clinical studies has for long been the lack of human cardiomyocytes. As discussed above, cardiac progenitor cells, bone marrow cells, and other mesenchymal stroma cells do not possess quantitatively relevant cardiogenic potential. In this respect, pluripotent stem cells opened the door, as they offer the opportunity to generate human cardiomyocytes in very large numbers (108–110). Fundamental work on cardiomyocyte transplantation has been performed with neonatal rat and mouse cardiomyocytes. These studies (even though not all results are directly translatable to stem cell–derived myocytes) have taught us some lessons: (*a*) Transplanted cardiomyocytes engraft and are able to survive long term. (*b*) Yet, engraftment rates are low (111), and the majority of transplanted cells does not survive transplantation and/or is washed out immediately (112). (*c*) Cardiomyocyte maturation is critical. Adult rat cardiomyocytes did not survive transplantation, whereas immature cells did (113), which probably reflects their remarkable resistance against hypoxia (114). (*d*) Two cell delivery strategies have been developed: direct intramyocardial injection and the transplantation of engineered tissue constructs (113, 115–117).

4.3.1. Stem cell–derived cardiomyocyte transplantation. Initial differentiation protocols produced only a small number of human pluripotent stem cell–derived cardiomyocytes with low purity. Heterogeneous cell populations, containing small numbers of cardiomyocytes, were used for transplantation. These studies demonstrated (somewhat unexpectedly) that while stem cell–derived cardiomyocytes survived transplantation, nonmyocytes did not (118, 119). They also revealed that stem cell–derived cardiomyocytes, which are immature, proliferate after transplantation (118, 119) and that the transplanted myocytes are able to electrically couple to the host myocardium when transplanted in healthy ventricular myocardium in species with a human-like electrophysiology (e.g., pigs) (120).

Even though direct cell injection is a very intuitive strategy, it has been troubled by low engraftment rate (121). Further advances in the differentiation protocols allowed the generation of cardiomyocytes in much greater numbers (laboratory scale 10^8–10^9) and of higher purity (routinely around 80–95%), but simple upscaling only partially overcame this problem. Even with modern differentiation protocols, cardiomyocyte differentiation is difficult, error prone, and expensive. Moreover, pluripotent stem cells possess a tumorigenic risk, and large cell numbers increase the risk of remnant pluripotent stem cells. Murry's group (122) developed a strategy combining heat shock and a pharmacological prosurvival cocktail to improve engraftment. This

strategy resulted in substantial larger grafts when transplanting 10×10^6 human embryonic stem cell (hESC)–derived cardiomyocytes in a rat infarct model. Left ventricular function remained stable in the intervention group, whereas the control groups showed a decline in left ventricular function. Follow-up studies demonstrated that the transplanted cells can electrically couple to the host myocardium when injected into the scar (123), but they also revealed that transplantation in a chronically injured heart is more difficult than in the subacute setting (124). Fukuda's group (121) developed a method to generate cardiomyocyte aggregates (spheroids, 150–200 μm in diameter; ~1,000 cardiomyocytes per spheroid) to improve cell retention. Final graft size is a net result of cells that survive transplantation and cells that proliferate after transplantation. Stimulation of cardiomyocyte proliferation, as a way to increase graft size after transplantation, has been achieved by cyclin D2 overexpression, resulting in larger grafts and better left ventricular function (125). As genetic modifications are unfavorable for translation, pharmacological interventions to stimulate cardiomyocyte proliferation could be a practically more applicable strategy to improve graft size. A second approach is the combination of several cell types to promote engraftment; e.g., cardiomyocyte and epicardial cell cotransplantation stimulated cardiomyocyte proliferation, increased angiogenesis, and resulted in larger grafts (126).

Within the last 6 years, the cell injection approach has been advanced to studies in nonhuman primates (127–130) and pigs (131, 132). It is difficult to directly compare these studies because they used such different cell numbers or different cell preparations. With the exception of one study in which nonhuman primate stem cell–derived cardiomyocytes were transplanted (allogeneic) (128), all other large animal studies were xenogeneic (human cells in animal models). Different immunosuppressive regiments were applied and animals were followed over different periods of time. There are, however, a few lessons that can be learned. Substantial remuscularization was achieved only when large numbers of cells were transplanted (127–129, 132). Nonhuman primates (body weight 3–13 kg) received 4×10^8–1×10^9 cardiomyocytes or 1×10^7 cardiac progenitor cells. Pigs (body weight 10–30 kg) received 6×10^6 cells of a mixed population (2×10^6 cardiomyocytes, 2×10^6 endothelial cells, and 2×10^6 smooth muscle cells, all from human-induced pluripotent stem cells, hiPSCs) or 1×10^9 cardiomyocytes. Substantial remuscularization was seen in the four studies that used the largest cell numbers. In one nonhuman primate study, 1×10^9 cardiomyocytes were injected in ~40 g hearts and remuscularized ~40% of the infarct (which affected ~4–10% of the left ventricle). A second study applied ~7.5×10^8 cells that remuscularized ~10% of larger infarcts (~20% of the left ventricle), and a third study used 4×10^8 cells (infarct size was 10% of the left ventricle, and the graft area was ~15% of the scar). The same number of cells (1×10^9) similarly remuscularized substantially larger hearts in a pig model (heart weight ~150 g, infarct size ~25%, and graft size ~15% of the scar) (132). This comparison does not reveal a dose-response curve and indicates that the results probably cannot be simply extrapolated to the human heart one to one. It highlights the challenges when aiming to remuscularize a human heart (300 g). Assuming that ~3×10^9 cardiomyocytes contribute ~75% to the heart mass, 1×10^9 human cardiomyocytes weigh ~75 g and thereby more than a nonhuman primate heart. Even when taking the much smaller size of immature stem cell–derived cardiomyocytes (~20-fold less than adult) into account, the ratio between the input cell mass and target organ remains a substantial challenge for any upscaling strategies aimed at clinical application.

Pluripotent stem cell–derived cardiomyocytes are immature and, compared to the rodent cardiomyocyte transplantation studies, should engraft better in the host myocardium than more mature cells. Yet, cardiac progenitor cells did not survive long term (140 days), and even four weeks after transplantation engraftment was low in a large nonhuman primate study (130). There is also some evidence that cardiomyocyte maturation (at least) to a certain degree promotes engraftment

(133). A major limitation is the lack of long-term follow-up data. Early studies transplanting human cardiomyocytes in mice showed that, despite long-term graft survival, improvement in left ventricular function was temporary (119). The longest follow-up period in the large animal studies that demonstrated remuscularization was 12 weeks (128), during which left ventricular function continued to improve. Of note, even though cardiomyocyte transplantation resulted in large grafts in pigs, it did not improve left ventricular function (132). In contrast, two studies in primates and pigs that saw only modest (or no) cardiomyocyte engraftment reported an increase in left ventricular function (130, 131). It is difficult to reconcile these findings. One general problem in large animal studies is that they are limited to small animal numbers for economic and logistic reasons, making robustly powered efficacy studies difficult (if not impossible). For comparison, the first study with ACE inhibitors in severely sick patients (heart failure class III–IV) was done in 253 patients and showed a number-needed-to-treat (NNT) of six (134). Modern heart failure studies are done with 3,000 or more patients and result in NNTs of 20–30. The interpretation of these results therefore requires caution. Taking all small and large animal studies together, there is evidence that cardiomyocyte transplantation improves function after injury. However, the benefit seems to not purely derive from remuscularization (and thereby active contribution of the new myocardium) but also from paracrine effects, e.g., reduced apoptosis of host cardiomyocytes and increased angiogenesis.

Electromechanical stability is more critical than predicted from small animal models. Small animal models provided no evidence for arrhythmias after transplantation. In contrast, cardiomyocyte transplantation induced ventricular tachycardia in all large animal models with persistent remuscularization (127–129, 132). Even though they were transient (2–4 weeks after transplantation), they represent a significant hurdle for a first clinical trial. Interventional electrophysiological studies revealed that they were caused by focal automaticity (129, 132), indicating that the immature cardiomyocytes initiate ectopic pacemaking. Nonhuman primates (heart rate between 120–170 beats per minute) were clinically mainly unaffected, but one pig (heart rate ~100 beats per minute) died due to ventricular fibrillation. Remarkably, in one study arrhythmias occurred in the presence of amiodarone therapy (127). Humans have an even lower heart rate and therefore might be even more susceptible to these life-threatening arrhythmias after cardiomyocyte transplantation.

4.3.2. Cardiac patch transplantation. Tissue engineering may solve the issue of low engraftment rates after cell injection. Yet, it can also be used to efficiently deliver drugs. Epicardial patches loaded with follistatin-like 1 (Fstl1, which is secreted by epicardial cells) induced cardiomyocyte proliferation (135). This strategy improved left ventricular function in mice and pig models and is currently being developed for clinical application. Several engineering techniques exist to deliver cardiomyocytes as a prefabricated construct (reviewed in 136), with cell sheets (137, 138) and hydrogel-based constructs (139–141) being the most advanced techniques. Transplantation of cell sheets and hydrogel-based constructs resulted in partial remuscularization of the injured heart in small animal models. In a robustly powered guinea pig study, transplantation of two hiPSC-derived tissue constructs (5×10^6 cardiomyocytes per construct) remuscularized about 12% of the scar area and improved left ventricular function (compared to two control groups that either received cell-free constructs or constructs that contained only noncontractile endothelial cells) (140). The study provided indirect evidence that application of cells in a prefabricated contracting patch improves engraftment, because the number of cells required for the same or better graft size and functional benefit was ~10-fold lower than in comparable cell injection studies (8×10^7–1×10^8). The grafts were vascularized by recipient-derived blood vessels (even

when the tissue constructs contained stem cell–derived endothelial cells) (140). The results of one large animal study are published, and several more are currently ongoing. Transplantation of large fibrin-based cardiac patches ($4 \times 2 \times 0.125$ cm with 4×10^6 cardiomyocytes, 2×10^6 endothelial cells, and 2×10^6 smooth muscle cells, all hiPSC-derived) improved ventricular function after transplantation in a pig myocardial infarct model, even though remuscularization was sparse. The latter might result from the low input number of cardiomyocytes, but also from a rather mild immunosuppression (no drug to inhibit T cell costimulation, e.g., abatacept, was used) (142).

The main caveat of the cardiac patch strategy so far is electrical coupling (140, 141, 143). Epicardial transplantation of tissue constructs consistently leads to a scar tissue cap that separates the new myocardium from the host tissue. Even though electrical coupling can progress via nonmyocytes (e.g., fibroblasts or macrophages), this structural barrier very likely impedes electrical integration. There was evidence of electrical coupling in a subset of animals in the guinea pig study (2/7 animals), but two studies in rats found no evidence of electrical coupling (141, 142, 144), suggesting that electrical coupling is the major hurdle for a tissue engineering approach. So far, there are no data on electrical coupling from large animal models with more human-like physiology.

Transplantation of cells in a patch has already been applied in a small clinical trial. Fibrin patches containing a very low number ($5–10 \times 10^6$) of hESC-derived cardiovascular progenitor cells were epicardially transplanted in six patients, not with the aim to remuscularize the heart, but rather to evaluate safety and immune responses in an allogeneic setting with immunosuppression limited to the first 4 weeks after transplantation (144). A similar regimen was applied in a study with stacked cell sheets containing a much higher number of hiPSC-derived cardiomyocytes that was recently initiated in Japan and apparently also does not aim at sustained remuscularization. Details of the study have not been published.

4.3.3. Immune rejection of transplanted cells. Probably the biggest current hurdle for the clinical application of stem cell–derived cells/patches is the unresolved question of efficient and safe immunosuppression. The concept of long-term remuscularization implies that the newly formed myocardium actively contributes to heart function. Unfortunately, the right conditions are difficult to test in current animal models. Xenogeneic studies (the current standard in the absence of robust hESC or hiPSC lines from guinea pigs, rabbits, or pigs) require a harsh immunosuppressive regiment that cannot be extrapolated to clinical use. In an allogeneic setting, HLA matching was necessary to allow cell survival under clinically tolerable medication (128), but HLA matching by itself was not sufficient, and iPSC-derived neurons were rejected without immunosuppression in nonhuman primates (145). Long-term immunosuppression causes severe problems (146). Immunosuppressed patients are at risk of serious infections with clinical symptoms that are often nonspecific and therefore difficult to diagnose. Kidney failure is a common problem of calcineurin inhibitors, and tumors (e.g., nonmelanoma skin cancer) occur more often than in the general population. Immunosuppression could be circumvented by an autologous approach (147) or the use of hypoimmunogenic cell lines. An autologous strategy has serious logistic and economic hurdles and faces regulatory difficulties because genetic aberrations can occur during reprogramming and stringent quality control mechanisms are necessary (147). Moreover, recent evidence suggests the formation of neoantigens (148), questioning this strategy from a biological perspective. Hypoimmunogenic approaches are an attractive alternative. Different genetic modifications have been described to generate hypoimmunogenic pluripotent stem cell lines (149, 150). Each of these strategies contains multiple genetic modifications to escape a T cell answer and simultaneously to not provoke a natural killer (NK) cell response. These strategies circumvent a T cell response by knocking out MHC molecules, which according to the "missing self-hypothesis"

makes them susceptible for NK cells and requires additional genetic modifications. So far, none of these strategies has been applied in humans. Hence, the long-term effects are unknown at present.

4.4. Where Things Stand Today

One hundred and fifty years after Goldenberg's surprisingly accurate description of the heart's minimal regenerative capacity, cardiac regeneration remains an unfulfilled medical need. Yet, three strategies to remuscularize the injured heart have emerged. These are based on rigorous work from many groups spanning several decades. All three strategies demonstrated the potential to fulfill the central requirement of remuscularization and showed promising results in preclinical models. Yet, a few words of caution are necessary. Our knowledge mainly stems from animal models with (sub)acute anterior myocardial infarction/injury. However, human patients with advanced coronary artery disease generally exhibit much more diverse disease characteristics. They often suffer from multivessel disease and experience repeated ischemic events, which most likely will impede every regenerative approach. Pharmacologists aim to understand a drug's mode of action, and even though the integrated effect of many drugs remains incompletely understood, the primary drug target is generally undisputed. This brings us back to the beginning of this article, where we demonstrated that remuscularization will, most likely, be a numbers game. Many studies [including our own work (140)] report increases in left ventricular function that are larger than one would expect from the degree of remuscularization or the percentage of cycling cardiomyocytes. While part of this discrepancy may be explained by technical limitations in analyzing left ventricular function or threshold effects, another likely reason encompasses additional, yet unknown mechanisms that need to be scrutinized and interpreted with an open mind. Pharmacologists teach introductory classes explaining that therapeutics without side effects most likely also do not have effects. Assuming that regenerative therapeutics can have substantial effects, more work is still required to evaluate the risk-benefit ratio. Eventually, clinical trials will tell whether our hypothesis turns out to be true and heart failure patients can benefit from regenerative therapeutics.

DISCLOSURE STATEMENT

Florian Weinberger filed a patent for the generation of human-scale engineered heart patches. Thomas Eschenhagen is co-founder of EHT Technologies GmbH, a university spin-off providing equipment for the generation and analysis of EHTs. No other affiliations, memberships, funding, or financial holdings are declared that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED

1. Goldenberg B. 1886. Ueber Atrophie und Hypertrophie der Muskelfasern des Herzens. *Arch. Pathol. Anat. Physiol. Klin. Med.* 103(1):88–130
2. Bergmann O, Zdunek S, Felker A, Salehpour M, Alkass K, et al. 2015. Dynamics of cell generation and turnover in the human heart. *Cell* 161(7):1566–75

3. Khera R, Pandey A, Ayers CR, Agusala V, Pruitt SL, et al. 2017. Contemporary epidemiology of heart failure in fee-for-service Medicare beneficiaries across healthcare settings. *Circ. Heart Fail.* 10(11):e004402
4. Crespo-Leiro MG, Anker SD, Maggioni AP, Coats AJ, Filippatos G, et al. 2016. European Society of Cardiology Heart Failure Long-Term Registry (ESC-HF-LT): 1-year follow-up outcomes and differences across regions. *Eur. J. Heart Fail.* 18(6):613–25
5. Brockes JP, Kumar A. 2008. Comparative aspects of animal regeneration. *Annu. Rev. Cell Dev. Biol.* 24:525–49
6. Pfeffer MA, Braunwald E. 1990. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation* 81(4):1161–72
7. Chareonthaitawee P, Christian TF, Hirose K, Gibbons RJ, Rumberger JA. 1995. Relation of initial infarct size to extent of left ventricular remodeling in the year after acute myocardial infarction. *J. Am. Coll. Cardiol.* 25(3):567–73
8. Burns RJ, Gibbons RJ, Yi Q, Roberts RS, Miller TD, et al. 2002. The relationships of left ventricular ejection fraction, end-systolic volume index and infarct size to six-month mortality after hospital discharge following myocardial infarction treated by thrombolysis. *J. Am. Coll. Cardiol.* 39(1):30–36
9. Kanoh M, Takemura G, Misao J, Hayakawa Y, Aoyama T, et al. 1999. Significance of myocytes with positive DNA in situ nick end-labeling (TUNEL) in hearts with dilated cardiomyopathy. *Circulation* 99(21):2757–64
10. Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, et al. 1997. Apoptosis in the failing human heart. *N. Engl. J. Med.* 336(16):1131–41
11. Narula J, Haider N, Virmani R, DiSalvo TG, Kolodgie FD, et al. 1996. Apoptosis in myocytes in end-stage heart failure. *N. Engl. J. Med.* 335(16):1182–89
12. Wencker D, Chandra M, Nguyen K, Miao W, Garantziotis S, et al. 2003. A mechanistic role for cardiac myocyte apoptosis in heart failure. *J. Clin. Investig.* 111(10):1497–504
13. Zielonko J. 1874. Pathologisch-anatomische und experimentelle Studien über Hypertrophie des Herzens. *Arch. Pathol. Anat. Physiol. Klin. Med.* 62(1):29–57
14. Eschenhagen T, Bolli R, Braun T, Field LJ, Fleischmann BK, et al. 2017. Cardiomyocyte regeneration: a consensus statement. *Circulation* 136(7):680–86
15. Brockes JP, Kumar A. 2008. Comparative aspects of animal regeneration. *Annu. Rev. Cell Dev. Biol.* 24:525–49
16. Rinkevich Y, Lindau P, Ueno H, Longaker MT, Weissman IL. 2011. Germ-layer and lineage-restricted stem/progenitors regenerate the mouse digit tip. *Nature* 476(7361):409–13
17. He L, Li Y, Li Y, Pu W, Huang X, et al. 2017. Enhancing the precision of genetic lineage tracing using dual recombinases. *Nat. Med.* 23(12):1488–98
18. Li Y, Lv Z, He L, Huang X, Zhang S, et al. 2019. Genetic tracing identifies early segregation of the cardiomyocyte and nonmyocyte lineages. *Circ. Res.* 125(3):343–55
19. Bittner RE, Schöfer C, Weipoltshammer K, Ivanova S, Streubel B, et al. 1999. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic *mdx* mice. *Anat. Embryol.* 199(5):391–96
20. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, et al. 2001. Bone marrow cells regenerate infarcted myocardium. *Nature* 410(6829):701–5
21. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, et al. 2003. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 114(6):763–76
22. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, et al. 2002. Chimerism of the transplanted heart. *N. Engl. J. Med.* 346(1):5–15
23. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, et al. 2004. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 428:13–15
24. Nygren JM, Jovinge S, Breitbach M, Säwén P, Röll W, et al. 2004. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nat. Med.* 10(5):494–501
25. Müller P, Pfeiffer P, Koglin J, Schäfers H-J, Seeland U, et al. 2002. Cardiomyocytes of noncardiac origin in myocardial biopsies of human transplanted hearts. *Circulation* 106(1):31–35

26. Vagnozzi RJ, Maillet M, Sargent MA, Khalil H, Johansen AKZ, et al. 2020. An acute immune response underlies the benefit of cardiac stem cell therapy. *Nature* 577(7790):405–9
27. Uygur A, Lee RT. 2016. Mechanisms of cardiac regeneration. *Dev. Cell* 36(4):362–74
28. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, et al. 2003. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *PNAS* 100(21):12313–18
29. Genead R, Danielsson C, Andersson AB, Corbascio M, Franco-Cereceda A, et al. 2010. Islet-1 cells are cardiac progenitors present during the entire lifespan: from the embryonic stage to adulthood. *Stem Cells Dev*. 19(10):1601–15
30. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, et al. 2004. Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ. Res.* 95(9):911–21
31. Hierlihy AM, Seale P, Lobe CG, Rudnicki MA, Megeney LA. 2002. The post-natal heart contains a myocardial stem cell population. *FEBS Lett*. 530(1–3):239–43
32. Hsieh PCH, Segers VFM, Davis ME, MacGillivray C, Gannon J, et al. 2007. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat. Med.* 13(8):970–74
33. van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, et al. 2014. c-kit^{+} cells minimally contribute cardiomyocytes to the heart. *Nature* 509(7500):337–41
34. Neidig LE, Weinberger F, Palpant NJ, Mignone J, Martinson AM, et al. 2018. Evidence for minimal cardiogenic potential of stem cell antigen 1-positive cells in the adult mouse heart. *Circulation* 138(25):2960–62
35. Weinberger F, Mehrkens D, Friedrich FW, Stubbendorff M, Hua X, et al. 2012. Localization of islet-1-positive cells in the healthy and infarcted adult murine heart. *Circ. Res.* 110(10):1303–10
36. Li Y, He L, Huang X, Bhaloo SI, Zhao H, et al. 2018. Genetic lineage tracing of nonmyocyte population by dual recombinases. *Circulation* 138(8):793–805
37. Smart N, Bollini S, Dubé KN, Vieira JM, Zhou B, et al. 2011. De novo cardiomyocytes from within the activated adult heart after injury. *Nature* 474(7353):640–44
38. Chien KR, Frisén J, Fritsche-Danielson R, Melton DA, Murry CE, Weissman IL. 2019. Regenerating the field of cardiovascular cell therapy. *Nat. Biotechnol.* 37(3):232–37
39. Shankle WR, Landing BH, Gregg J. 1983. Normal organ weights of infants and children: graphs of values by age, with confidence intervals. *Pediatr. Pathol.* 1(4):399–408
40. Mollova M, Bersell K, Walsh S, Savla J, Das LT, et al. 2013. Cardiomyocyte proliferation contributes to heart growth in young humans. *PNAS* 110(4):1446–51
41. Bensley JG, De Matteo R, Harding R, Black MJ. 2016. Three-dimensional direct measurement of cardiomyocyte volume, nuclearity, and ploidy in thick histological sections. *Sci. Rep.* 6:23756
42. Alkass K, Panula J, Westman M, Wu T-D, Guerquin-Kern J-L, Bergmann O. 2015. No evidence for cardiomyocyte number expansion in preadolescent mice. *Cell* 163(4):1026–36
43. Martínez-Lagunas K, Yamaguchi Y, Becker C, Geisen C, DeRuiter MC, et al. 2020. In vivo detection of programmed cell death during mouse heart development. *Cell Death Differ*. 27(4):1398–414
44. Fernandez E, Siddiquee Z, Shohet RV. 2001. Apoptosis and proliferation in the neonatal murine heart. *Dev. Dyn.* 221(3):302–10
45. Soonpaa MH, Kim KK, Pajak L, Franklin M, Field LJ. 1996. Cardiomyocyte DNA synthesis and binucleation during murine development. *Am. J. Physiol.* 271(5 Part 2):H2183–89
46. Naqvi N, Li M, Calvert JW, Tejada T, Lambert JP, et al. 2014. A proliferative burst during preadolescence establishes the final cardiomyocyte number. *Cell* 157(4):795–807
47. Hirai M, Cattaneo P, Chen J, Evans SM. 2016. Revisiting preadolescent cardiomyocyte proliferation in mice. *Circ. Res.* 118(6):916–19
48. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, et al. 2013. Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* 493(7432):433–36
49. Ali SR, Hippenmeyer S, Saadat LV, Luo L, Weissman IL, Ardehali R. 2014. Existing cardiomyocytes generate cardiomyocytes at a low rate after birth in mice. *PNAS* 111(24):8850–55
50. Lin Z, von Gise A, Zhou P, Gu F, et al. 2014 Cardiac-specific YAP activation improves cardiac function and survival in an experimental murine MI model. *Circ. Res.* 115(3):354–63

51. Kimura W, Xiao F, Canseco DC, Muralidhar S, Thet S, et al. 2015. Hypoxia fate mapping identifies cycling cardiomyocytes in the adult heart. *Nature* 523(7559):226–30
52. Drenckhahn JD, Schwarz QP, Gray S, Laskowski A, Kiriazis H, et al. 2008. Compensatory growth of healthy cardiac cells in the presence of diseased cells restores tissue homeostasis during heart development. *Dev. Cell* 15(4):521–33
53. Drenckhahn JD, Strasen J, Heinecke K, Langner P, Yin KV, et al. 2015. Impaired myocardial development resulting in neonatal cardiac hypoplasia alters postnatal growth and stress response in the heart. *Cardiovasc. Res.* 106(1):43–54
54. Sturzu AC, Rajarajan K, Passer D, Plonowska K, Riley A, et al. 2015. Fetal mammalian heart generates a robust compensatory response to cell loss. *Circulation* 132(2):109–21
55. Robledo M. 1956. Myocardial regeneration in young rats. *Am. J. Pathol.* 32(6):1215–39
56. Rumyantsev PP. 1991. *Growth and Hyperplasia of Cardiac Muscle Cells*. London/New York: Harwood Acad.
57. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, et al. 2011. Transient regenerative potential of the neonatal mouse heart. *Science* 331(6020):1078–80
58. Ye L, D'Agostino G, Loo SJ, Wang CX, Su LP, et al. 2018. Early regenerative capacity in the porcine heart. *Circulation* 138(24):2798–808
59. Haubner BJ, Schneider J, Schweigmann U, Schuetz T, Dichtl W, et al. 2016. Functional recovery of a human neonatal heart after severe myocardial infarction. *Circ. Res.* 118(2):216–21
60. Meckert PC, Rivello HG, Vigliano C, González P, Favaloro R, Laguens R. 2005. Endomitosis and polyploidization of myocardial cells in the periphery of human acute myocardial infarction. *Cardiovasc. Res.* 67(1):116–23
61. Soonpaa MH, Field LJ. 1997. Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts. *Am. J. Physiol.* 272(1 Part 2):H220–26
62. Hesse M, Raulf A, Pilz G-A, Haberlandt C, Klein AM, et al. 2012. Direct visualization of cell division using high-resolution imaging of M-phase of the cell cycle. *Nat. Commun.* 3:1076
63. Kretzschmar K, Post Y, Bannier-Hélaouët M, Mattiotti A, Drost J, et al. 2018. Profiling proliferative cells and their progeny in damaged murine hearts. *PNAS* 115(52):E12245–54
64. Leone M, Magadum A, Engel FB. 2015. Cardiomyocyte proliferation in cardiac development and regeneration: a guide to methodologies and interpretations. *Am. J. Physiol. Heart Circ. Physiol.* 309(8):H1237–50
65. Puente BN, Kimura W, Muralidhar SA, Moon J, Amatruda JF, et al. 2014. The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell* 157(3):565–79
66. Patterson M, Barske L, Van Handel B, Rau CD, Gan P, et al. 2017. Frequency of mononuclear diploid cardiomyocytes underlies natural variation in heart regeneration. *Nat. Genet.* 49(9):1346–53
67. Mills RJ, Titmarsh DM, Koenig X, Parker BL, Ryall JG, et al. 2017. Functional screening in human cardiac organoids reveals a metabolic mechanism for cardiomyocyte cell cycle arrest. *PNAS* 114(40):E8372–81
68. Bassat E, Mutlak YE, Genzelinakh A, Shadrin IY, Umansky KB, et al. 2017. The extracellular matrix protein agrin promotes heart regeneration in mice. *Nature* 547(7662):179–84
69. Hirose K, Payumo AY, Cutie S, Hoang A, Zhang H, et al. 2019. Evidence for hormonal control of heart regenerative capacity during endothermy acquisition. *Science* 188:eaar2038
70. Field LJ. 1988. Atrial natriuretic factor-SV40 T antigen transgenes produce tumors and cardiac arrhythmias in mice. *Science* 239(4843):1029–33
71. Gabisonia K, Prosdocimo G, Aquaro GD, Carlucci L, Zentilin L, et al. 2019. MicroRNA therapy stimulates uncontrolled cardiac repair after myocardial infarction in pigs. *Nature* 569(7756):418–22
72. Yoshizumi M, Lee WS, Hsieh CM, Tsai JC, Li J, et al. 1995. Disappearance of cyclin A correlates with permanent withdrawal of cardiomyocytes from the cell cycle in human and rat hearts. *J. Clin. Investig.* 95(5):2275–80
73. Pasumarthi KBS, Nakajima H, Nakajima HO, Soonpaa MH, Field LJ. 2005. Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice. *Circ. Res.* 96(1):110–18

74. Hassink RJ, Pasumarthi KB, Nakajima H, Rubart M, Soonpaa MH, et al. 2008. Cardiomyocyte cell cycle activation improves cardiac function after myocardial infarction. *Cardiovasc. Res.* 78(1):18–25
75. Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, et al. 2012. Functional screening identifies miRNAs inducing cardiac regeneration. *Nature* 492(7429):376–81
76. Chaudhry HW, Dashoush NH, Tang H, Zhang L, Wang X, et al. 2004. Cyclin A2 mediates cardiomyocyte mitosis in the postmitotic myocardium. *J. Biol. Chem.* 279(34):35858–66
77. Cheng RK, Asai T, Tang H, Dashoush NH, Kara RJ, et al. 2007. Cyclin A2 induces cardiac regeneration after myocardial infarction and prevents heart failure. *Circ. Res.* 100(12):1741–48
78. Shapiro SD, Ranjan AK, Kawase Y, Cheng RK, Kara RJ, et al. 2014. Cyclin A2 induces cardiac regeneration after myocardial infarction through cytokinesis of adult cardiomyocytes. *Sci. Transl. Med.* 6(224):224ra27
79. Heallen T, Zhang M, Wang J, Bonilla-Claudio M, Klysik E, et al. 2011. Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science* 332(6028):458–61
80. Xin M, Kim Y, Sutherland LB, Qi X, McAnally J, et al. 2011. Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Sci. Signal.* 4(196):ra70
81. von Gise A, Lin Z, Schlegelmilch K, Honor LB, Pan GM, et al. 2012. YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *PNAS* 109(7):2394–99
82. Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, et al. 2013. Hippo pathway effector Yap promotes cardiac regeneration. *PNAS* 110(34):13839–44
83. Monroe TO, Hill MC, Morikawa Y, Leach JP, Heallen T, et al. 2019. YAP partially reprograms chromatin accessibility to directly induce adult cardiogenesis in vivo. *Dev. Cell* 48(6):765–79.e7
84. Leach JP, Heallen T, Zhang M, Rahmani M, Morikawa Y, et al. 2017. Hippo pathway deficiency reverses systolic heart failure after infarction. *Nature* 550(7675):260–64
85. Li J, Gao E, Vite A, Yi R, Gomez L, et al. 2015. Alpha-catenins control cardiomyocyte proliferation by regulating Yap activity. *Circ. Res.* 116(1):70–79
86. Morikawa Y, Heallen T, Leach J, Xiao Y, Martin JF. 2017. Dystrophin-glycoprotein complex sequesters Yap to inhibit cardiomyocyte proliferation. *Nature* 547(7662):227–31
87. Lesizza P, Prosdocimo G, Martinelli V, Sinagra G, Zacchigna S, Giacca M. 2017. Single-dose intracardiac injection of pro-regenerative microRNAs improves cardiac function after myocardial infarction. *Circ. Res.* 120(8):1298–304
88. Torrini C, Cubero RJ, Dirkx E, Braga L, Ali H, et al. 2019. Common regulatory pathways mediate activity of microRNAs inducing cardiomyocyte proliferation. *Cell Rep.* 27(9):2759–2771.e5
89. Tian Y, Liu Y, Wang T, Zhou N, Kong J, et al. 2015. A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. *Sci. Transl. Med.* 7(279):279ra38
90. Ikeda S, Mizushima W, Sciarretta S, Abdellatif M, Zhai P, et al. 2019. Hippo deficiency leads to cardiac dysfunction accompanied by cardiomyocyte dedifferentiation during pressure overload. *Circ. Res.* 124(2):292–305
91. Morgan TH. 1898. Experimental studies of the regeneration of *Planaria maculata*. *Arch. Entwickelungsmech. Org.* 7(2–3):364–97
92. Del Rio-Tsonis K, Tsonis PA. 2003. Eye regeneration at the molecular age. *Dev. Dyn.* 226(2):211–24
93. Davis RL, Weintraub H, Lassar AB. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51(6):987–1000
94. Takeuchi JK, Bruneau BG. 2009. Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature* 459(7247):708–11
95. Ieda M, Fu J-D, Delgado-Olguin P, Vedantham V, Hayashi Y, et al. 2010. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 142(3):375–86
96. Liu Z, Wang L, Welch JD, Ma H, Zhou Y, et al. 2017. Single-cell transcriptomics reconstructs fate conversion from fibroblast to cardiomyocyte. *Nature* 551(7678):100–4
97. Qian L, Huang Y, Spencer CI, Foley A, Vedantham V, et al. 2012. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature* 485(7400):593–98
98. Song K, Nam Y-J, Luo X, Qi X, Tan W, et al. 2012. Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature* 485(7400):599–604

99. Wang L, Liu Z, Yin C, Asfour H, Chen O, et al. 2015. Stoichiometry of Gata4, Mef2c, and Tbx5 influences the efficiency and quality of induced cardiac myocyte reprogramming. *Circ. Res.* 116(2):237–44
100. Zhao Y, Londono P, Cao Y, Sharpe EJ, Proenza C, et al. 2015. High-efficiency reprogramming of fibroblasts into cardiomyocytes requires suppression of pro-fibrotic signalling. *Nat. Commun.* 6:8243
101. Nam Y-J, Song K, Luo X, Daniel E, Lambeth K, et al. 2013. Reprogramming of human fibroblasts toward a cardiac fate. *PNAS* 110(14):5588–93
102. Mohamed TMA, Stone NR, Berry EC, Radzinsky E, Huang Y, et al. 2017. Chemical enhancement of in vitro and in vivo direct cardiac reprogramming. *Circulation* 135(10):978–95
103. Miyamoto K, Akiyama M, Tamura F, Isomi M, Yamakawa H, et al. 2018. Direct in vivo reprogramming with Sendai virus vectors improves cardiac function after myocardial infarction. *Cell Stem Cell* 22(1):91–103.e5
104. Cao N, Huang Y, Zheng J, Spencer CI, Zhang Y, et al. 2016. Conversion of human fibroblasts into functional cardiomyocytes by small molecules. *Science* 352(6290):1216–20
105. Mahmoudi S, Mancini E, Xu L, Moore A, Jahanbani F, et al. 2019. Heterogeneity in old fibroblasts is linked to variability in reprogramming and wound healing. *Nature* 574(7779):553–58
106. Bader D, Oberpriller JO. 1978. Repair and reorganization of minced cardiac muscle in the adult newt (*Notophthalmus viridescens*). *J. Morphol.* 155(3):349–57
107. Soonpaa MH, Koh GY, Klug MG, Field LJ. 1994. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. *Science* 264(5155):98–101
108. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, et al. 2001. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J. Clin. Investig.* 108(3):407–14
109. Breckwoldt K, Letuffe-Brenière D, Mannhardt I, Schulze T, Ulmer B, et al. 2017. Differentiation of cardiomyocytes and generation of human engineered heart tissue. *Nat. Protoc.* 12(6):1177–97
110. Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, et al. 2011. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 8(2):228–40
111. Müller-Ehmsen J, Whittaker P, Kloner RA, Dow JS, Sakoda T, et al. 2002. Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. *J. Mol. Cell. Cardiol.* 34(2):107–16
112. Dow J, Simkhovich BZ, Kedes L, Kloner RA. 2005. Washout of transplanted cells from the heart: a potential new hurdle for cell transplantation therapy. *Cardiovasc. Res.* 67(2):301–7
113. Reinecke H, Zhang M, Bartosek T, Murry CE. 1999. Survival, integration, and differentiation of cardiomyocyte grafts a study in normal and injured rat hearts—a study in normal and injured rat hearts. *Circulation* 100(1):193–202
114. Pritchett-Corning KR. 2009. Euthanasia of neonatal rats with carbon dioxide. *J. Am. Assoc. Lab. Anim. Sci.* 48(1):23–27
115. Zimmermann W, Didié M, Wasmeier GH, Nixdorff U, Hess A, et al. 2002. Cardiac grafting of engineered heart tissue in syngenic rats. *Circulation* 106(12):151–57
116. Zimmermann W-H, Melnychenko I, Wasmeier G, Didié M, Naito H, et al. 2006. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nat. Med.* 12(4):452–58
117. Sekine H, Shimizu T, Hobo K, Sekiya S, Yang J, et al. 2008. Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. *Circulation* 118(14 Suppl. 1):S145–52
118. Laflamme MA, Gold J, Xu C, Hassanipour M, Rosler E, et al. 2005. Formation of human myocardium in the rat heart from human embryonic stem cells. *Am. J. Pathol.* 167(3):663–71
119. van Laake LW, Passier R, Monshouwer-Kloots J, Verkleij AJ, Lips DJ, et al. 2007. Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. *Stem Cell Res.* 1(1):9–24
120. Kehat I, Khimovich L, Caspi O, Gepstein A, Shofti R, et al. 2004. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat. Biotechnol.* 22(10):1282–89
121. Hattori F, Chen H, Yamashita H, Tohyama S, Satoh Y-S, et al. 2010. Nongenetic method for purifying stem cell-derived cardiomyocytes. *Nat. Methods* 7(1):61–66

122. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, et al. 2007. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat. Biotechnol.* 25(9):1015–24
123. Shiba Y, Fernandes S, Zhu W-Z, Filice D, Muskheli V, et al. 2012. Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. *Nature* 489:322–25
124. Shiba Y, Filice D, Fernandes S, Minami E, Dupras SK, et al. 2014. Electrical integration of human embryonic stem cell-derived cardiomyocytes in a guinea pig chronic infarct model. *J. Cardiovasc. Pharmacol. Ther.* 19(4):368–81
125. Zhu W, Zhao M, Mattapally S, Chen S, Zhang J. 2018. CCND2 overexpression enhances the regenerative potency of human induced pluripotent stem cell-derived cardiomyocytes: remuscularization of injured ventricle. *Circ. Res.* 122(1):88–96
126. Bargehr J, Ong LP, Colzani M, Davaapil H, Hofsteen P, et al. 2019. Epicardial cells derived from human embryonic stem cells augment cardiomyocyte-driven heart regeneration. *Nat. Biotechnol.* 37(8):895–906
127. Chong JJH, Yang X, Don CW, Minami E, Liu Y-W, et al. 2014. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. *Nature* 510(7504):273–77
128. Shiba Y, Gomibuchi T, Seto T, Wada Y, Ichimura H, et al. 2016. Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. *Nature* 538(7625):388–91
129. Liu Y-W, Chen B, Yang X, Fugate JA, Kalucki FA, et al. 2018. Human embryonic stem cell-derived cardiomyocytes restore function in infarcted hearts of non-human primates. *Nat. Biotechnol.* 36(7):597–605
130. Zhu K, Wu Q, Ni C, Zhang P, Zhong Z, et al. 2018. Lack of remuscularization following transplantation of human embryonic stem cell-derived cardiovascular progenitor cells in infarcted nonhuman primates. *Circ. Res.* 122(7):958–69
131. Ye L, Chang Y-H, Xiong Q, Zhang P, Zhang L, et al. 2014. Cardiac repair in a porcine model of acute myocardial infarction with human induced pluripotent stem cell-derived cardiovascular cells. *Cell Stem Cell* 15(6):750–61
132. Romagnuolo R, Masoudpour H, Porta-Sánchez A, Qiang B, Barry J, et al. 2019. Human embryonic stem cell-derived cardiomyocytes regenerate the infarcted pig heart but induce ventricular tachyarrhythmias. *Stem Cell Rep.* 12(5):967–81
133. Funakoshi S, Miki K, Takaki T, Okubo C, Hatani T, et al. 2016. Enhanced engraftment, proliferation and therapeutic potential in heart using optimized human iPSC-derived cardiomyocytes. *Sci. Rep.* 6(1):19111
134. CONSENSUS Trial Study Group. 1987. Effects of enalapril on mortality in severe congestive heart failure. *N. Engl. J. Med.* 316(23):1429–35
135. Wei K, Serpooshan V, Hurtado C, Diez-Cunado M, Zhao M, et al. 2015. Epicardial FSTL1 reconstitution regenerates the adult mammalian heart. *Nature* 525(7570):479–85
136. Weinberger F, Mannhardt I, Eschenhagen T. 2017. Engineering cardiac muscle tissue: a maturating field of research. *Circ. Res.* 120(9):1487–500
137. Masumoto H, Ikuno T, Takeda M, Fukushima H, Marui A, et al. 2014. Human iPS cell-engineered cardiac tissue sheets with cardiomyocytes and vascular cells for cardiac regeneration. *Sci. Rep.* 4:6716
138. Matsuo T, Masumoto H, Tajima S, Ikuno T, Katayama S, et al. 2015. Efficient long-term survival of cell grafts after myocardial infarction with thick viable cardiac tissue entirely from pluripotent stem cells. *Sci. Rep.* 5:16842
139. Riegler J, Tiburcy M, Ebert A, Tzatzalos E, Raaz U, et al. 2015. Human engineered heart muscles engraft and survive long term in a rodent myocardial infarction model. *Circ. Res.* 117(8):720–30
140. Weinberger F, Breckwoldt K, Pecha S, Kelly A, Geertz B, et al. 2016. Cardiac repair in guinea pigs with human engineered heart tissue from induced pluripotent stem cells. *Sci. Transl. Med.* 8(363):363ra148
141. Shadrin IY, Allen BW, Qian Y, Jackman CP, Carlson AL, et al. 2017. Cardiopatch platform enables maturation and scale-up of human pluripotent stem cell-derived engineered heart tissues. *Nat. Commun.* 8(1):1825
142. Gao L, Gregorich ZR, Zhu W, Mattapally S, Oduk Y, et al. 2018. Large cardiac muscle patches engineered from human induced-pluripotent stem cell-derived cardiac cells improve recovery from myocardial infarction in swine. *Circulation* 137(16):1712–30

143. Gerbin KA, Yang X, Murry CE, Coulombe KLK. 2015. Enhanced electrical integration of engineered human myocardium via intramyocardial versus epicardial delivery in infarcted rat hearts. *PLOS ONE* 10(7):e0131446
144. Menasché P, Vanneaux V, Hagège A, Bel A, Cholley B, et al. 2018. Transplantation of human embryonic stem cell-derived cardiovascular progenitors for severe ischemic left ventricular dysfunction. *J. Am. Coll. Cardiol.* 71(4):429–38
145. Aron Badin R, Bugi A, Williams S, Vadori M, Michael M, et al. 2019. MHC matching fails to prevent long-term rejection of iPSC-derived neurons in non-human primates. *Nat. Commun.* 10(1):4357
146. Söderlund C, Rådegran G. 2015. Immunosuppressive therapies after heart transplantation—the balance between under- and over-immunosuppression. *Transplant. Rev.* 29(3):181–89
147. Eschenhagen T, Weinberger F. 2019. Heart repair with myocytes. *Circ. Res.* 124(6):843–45
148. Deuse T, Hu X, Agbor-Enoh S, Koch M, Spitzer MH, et al. 2019. De novo mutations in mitochondrial DNA of iPSCs produce immunogenic neoepitopes in mice and humans. *Nat. Biotechnol.* 37:1137–44
149. Gornalusse GG, Hirata RK, Funk SE, Riolobos L, Lopes VS, et al. 2017. HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells. *Nat. Biotechnol.* 35(8):765–72
150. Deuse T, Hu X, Gravina A, Wang D, Tediashvili G, et al. 2019. Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients. *Nat. Biotechnol.* 37(3):252–58