REVIEW

doi:10.1038/nature10147

Heart regeneration

Michael A. Laflamme[1] & Charles E. Murry[1,2,3]

Heart failure plagues industrialized nations, killing more people than any other disease. It usually results from a deficiency of specialized cardiac muscle cells known as cardiomyocytes, and a robust therapy to regenerate lost myocardium could help millions of patients every year. Heart regeneration is well documented in amphibia and fish and in developing mammals. After birth, however, human heart regeneration becomes limited to very slow cardiomyocyte replacement. Several experimental strategies to remuscularize the injured heart using adult stem cells and pluripotent stem cells, cellular reprogramming and tissue engineering are in progress. Although many challenges remain, these interventions may eventually lead to better approaches to treat or prevent heart failure.

Heart regeneration has been intensely investigated, and extremely controversial, for more than 150 years[1]. In pursuit of this subject, the heart has been stabbed, snipped, contused, cauterized, coagulated, frozen, injected with toxins, infected and infarcted, in species ranging from marine invertebrates to horses[2,3]. Why has this proven to be such a difficult challenge? The heart is one of the least regenerative organs in the body, so if there is a regenerative response, it is small in comparison to that seen in many other tissues, such as liver, skeletal muscle, lung, gut, bladder, bone or skin. For most investigators, the question is about whether there is no regeneration, which is intrinsically difficult to prove, or whether it occurs but at very low rates, which is not easy to detect but possible using highly sensitive approaches.

This is more than an academic argument. Heart failure is a burgeoning public health problem, and some predict that it will reach epidemic proportions as our population ages. Cardiomyocyte deficiency underlies most causes of heart failure. The human left ventricle has 2–4 billion cardiomyocytes, and a myocardial infarction can wipe out 25% of these in a few hours[4]. Disorders of cardiac overload such as hypertension or valvular heart disease kill cardiomyocytes slowly over many years[5], and ageing is associated with the loss of ~1 g of myocardium (about 20 million cardiomyocytes) per year in the absence of specific heart disease[6]. If the human heart has even a small innate regenerative response, it may be possible to exploit this therapeutically to enhance the heart's function. This fundamental motivation has kept investigators pursuing rare events for more than a century.

Over the past 15 years, researchers have taken a more interventional approach to the injured heart, creating the field of cardiac repair. The ultimate goal of cardiac repair is to regenerate the myocardium after injury to prevent or treat heart failure. This interdisciplinary field draws from advances in areas such as stem cells, developmental biology and biomaterials in an attempt to create new myocardium that is electrically and mechanically integrated into the heart. Cardiac repair has moved rapidly from studies in experimental animals to clinical trials involving thousands of patients. In this Review, we summarize the evidence for heart regeneration in animal models and humans. We discuss the status of research using adult stem cells and pluripotent stem cells for cardiac repair in experimental animals, and explore the promises and problems of cellular reprogramming and tissue engineering. Clinical trials will be covered only briefly, owing to space limitations, so we refer interested readers to recent reviews on this topic[7,8].

Heart regeneration in amphibia and fish

Unlike humans, many amphibia and fish readily regenerate limbs, appendages and internal organs after injury. There is a long history of research on amphibian heart regeneration[9]; more recently, the zebrafish has proven to be a particularly useful model, given its substantial regenerative capacity and amenability to genetic manipulation[10]. The zebrafish heart fully regenerates after the surgical amputation of the cardiac apex — an injury that corresponds to a loss of approximately 20% of the total ventricular mass[10]. In the low-pressure zebrafish heart, this large wound is effectively sealed by an initial fibrin clot, which is gradually replaced by *de novo* regenerated heart tissue rather than by scar tissue[10–13].

Not surprisingly, this regenerative response involves a substantial amount of cardiomyocyte proliferation. Even at baseline levels, zebrafish cardiomyocytes show a much higher degree of cell-cycle activity than equivalent cells from their mammalian counterparts. A recent study showed that approximately 3% of cardiomyocytes in the compact myocardium of uninjured adult zebrafish hearts incorporate the thymidine analogue bromodeoxyuridine (BrdU) during a seven-day pulse-labelling experiment. Two weeks after amputation of the cardiac apex, the fraction of BrdU-positive cardiomyocytes had increased by tenfold, and this parameter remained as high as 20% as late as one month after injury[10].

Initial experiments suggested that undifferentiated progenitor cells were the principal source of regenerating cardiomyocytes in zebrafish[11], but two recent genetic fate-mapping studies unambiguously demonstrated that pre-existing committed cardiomyocytes are instead the main source[12,13] (Box 1). The two groups independently generated transgenic zebrafish in which the cardiomyocyte-specific *cmlc2* (also known as *myl7*) promoter drives the expression of tamoxifen-inducible Cre recombinase. These animals were crossed with a reporter line, in which Cre-mediated excision of a *loxP*-flanked stop sequence induces constitutive expression of green fluorescent protein (GFP). In the offspring of this cross, all pre-existing cardiomyocytes and their progeny can be induced to express GFP by tamoxifen treatment. If the regenerated myocardium were derived from undifferentiated progenitor cells, the new ventricular apex should be GFP^-. Instead, both groups found that the vast majority of the newly regenerated cardiomyocytes were GFP^+ (refs 12, 13). Thus, heart regeneration in zebrafish is principally mediated by the proliferation of pre-existing cardiomyocytes, rather than the generation of new cardiomyocytes from stem cells.

[1]Department of Pathology, Center for Cardiovascular Biology, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, Washington 98109, USA. [2]Deparment of Bioengineering, Center for Cardiovascular Biology, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, Washington 98109, USA. [3]Department of Medicine/Cardiology, Center for Cardiovascular Biology, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, Washington 98109, USA.

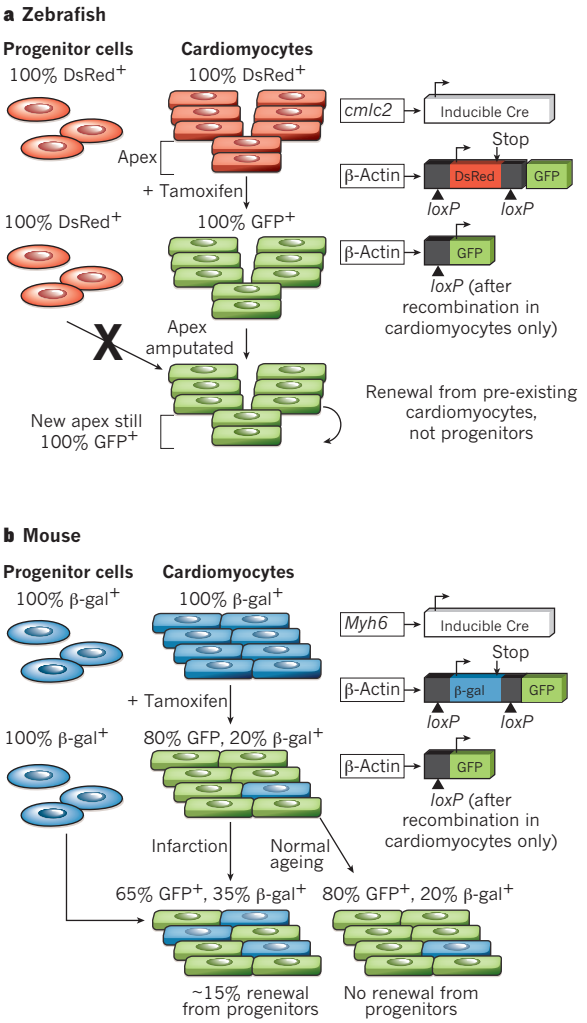
BOX 1

Genetic fate mapping in heart regeneration

Genetic fate mapping has proven to be an invaluable tool for dissecting the mechanisms of endogenous cardiac repair in model organisms, and several laboratories have used an elegant strategy based on the conditional Cre-*loxP* system, which allows both temporal and cell-type-specific control of reporter expression.

After amputation, the apex of the zebrafish heart can fully regenerate. To determine the source of the newly proliferating cardiomyocytes that underlies this regeneration, a zebrafish strain carrying two transgenes was created (see Figure, **a**). In one transgene, the cardiomyocyte-specific *cmlc2* promoter drives the expression of tamoxifen-inducible Cre recombinase. In the second transgene, the constitutive β-actin promoter initially drives expression of the red fluorescent DsRed protein. Cre recombinase induces the excision of *loxP*-flanked stop sequences, causing a permanent switch from constitutive DsRed to constitutive green fluorescent protein (GFP) expression[12]. Thus, when the transgenic zebrafish was pulsed with tamoxifen, all of its cardiomyocytes and their descendants expressed GFP. By contrast, cardiomyogenic progenitor cells should remain DsRed$^+$GFP$^-$, because the cardiomyocyte-specific *cmlc2* promoter would not be active in these undifferentiated cells. If progenitor cells later contributed to cardiomyocyte renewal after injury, one would expect those cardiomyocytes to also be DsRed$^+$GFP$^-$. Instead, after amputation of the apex, the new apical myocardium was 100% GFP$^+$, indicating that heart regeneration in the zebrafish results from the expansion of pre-existing cardiomyocytes, not from the recruitment of cardiomyogenic precursors. Another independent group reached the same conclusions using a similar experimental design[13].

An analogous genetic fate-mapping approach was used to investigate the mechanisms of cardiac regeneration in mammalian hearts[22]. Here, a double-transgenic mouse was generated in which the cardiomyocyte-specific *Myh6* promoter drives tamoxifen-inducible Cre recombinase, and Cre-mediated excision of *loxP*-flanked stop sequences induces a switch from constitutive β-galactosidase (β-gal) to constitutive GFP expression (see Figure, **b**). After tamoxifen treatment, ~80% of the cardiomyocytes in the transgenic animal became GFP$^+$, and 20% remained β-gal$^+$GFP$^-$. As in the analogous zebrafish experiment, any progenitor cells should remain GFP$^-$ after the tamoxifen pulse. During normal ageing for up to 1 year after tamoxifen treatment, the ratio of GFP$^+$ to β-gal$^+$ cardiomyocytes remained fixed at 80:20, indicating no significant cardiomyocyte renewal by unlabelled progenitor cells. However, after infarction, the ratio of GFP$^+$ to β-gal$^+$ shifted to ~65:35 in the peri-infarct zone, indicating that newly differentiated cardiomyocytes (β-gal$^+$GFP$^-$ because they had not undergone Cre-mediated recombination) had been recruited from the progenitor pool. Hence, in mice, the small amount of regeneration that occurs after injury involves the cardiac induction of progenitor cells.



Limited regeneration in rodent hearts

Although they lack the remarkable regenerative capacity of the zebrafish heart, postnatal mammalian hearts also undergo some degree of cardiomyocyte renewal during normal ageing and disease. Despite all the recent attention by the field, this is not a new concept. Extremely low but detectable levels of cardiomyocyte cell-cycle activity have been reported in rodent studies dating back to the 1960s[3,14]. Capturing the rare dividing cardiomyocytes present in mammalian hearts is technically challenging, but recent work has taken advantage of the greater specificity and throughput afforded by transgenic mouse models. For example, transgenic mice were created in which the cardiomyocyte-specific α-myosin heavy chain (*Myh6*, also known as α-*MHC*) promoter drives nuclear-localized expression of β-galactosidase[15]. This convenient read-out allowed researchers to screen more than 10,000 cardiomyocyte nuclei in histological sections for the incorporation of radiolabelled thymidine, and they found labelling indices of 0.0006% for adult ventricular cardiomyocytes in intact hearts and 0.0083% for cardiomyocytes in the border zone of injured hearts[14,15].

Although such proliferative indices are small, they raise the possibility that such phenomena could be augmented therapeutically. Proof of concept for this approach has come from transgenic mice with cardiomyocyte-restricted overexpression of the cell-cycle activator cyclin D2, because these animals show reduced scar tissue and improved mechanical function after myocardial infarction[16]. Other efforts to enhance the proliferation of adult cardiomyocytes — by manipulating oncogenes or cell-cycle regulators — have proven less consistent in improving outcomes after infarction (see ref. 17 for a comprehensive review). Pharmacological enhancement of cardiomyocyte cell-cycle activity would be more practical clinically than gene therapy, and the signalling molecules periostin[18], fibroblast growth factor-1 (ref. 19) and neuregulin 1 (NRG1)[20] have all been reported to act as mitogens for adult ventricular cardiomyocytes and to exert beneficial effects on

cardiac structure and function after infarction. (It should be noted that a more recent study has called into question the effects of periostin on cell-cycle activity or cardiac repair[21].) A recent study of the mitogenic effects of NRG1 showed that simple systemic injection of this growth factor into adult mice enhanced infarct scar shrinkage and improved mechanical function[20]. The effects of NRG1 on the cell cycle were dependent on the expression of its tyrosine kinase receptor, ERBB4, by cardiomyocytes and seem to stimulate mononucleated, but not binucleated, cardiomyocytes to divide. Although this intriguing result awaits independent confirmation, it suggests a straightforward approach to enhancing ventricular repair through the administration of recombinant growth factors.

Most of the above studies focused on the proliferation of existing cardiomyocytes, and were not designed to detect cardiomyocytes formed from progenitor cells. To determine whether such progenitor cells contribute to cardiomyocyte renewal, researchers have performed an elegant genetic fate-mapping experiment in transgenic mice[22], akin to those previously described in the zebrafish model, in which cardiomyocytes were indelibly labelled after a tamoxifen pulse (Box 1). This system allowed the authors to distinguish between cardiomyocyte renewal from pre-existing (and therefore fluorescently labelled) cardiomyocytes and cardiomyocyte renewal from unlabelled progenitor cells. Interestingly, they found no significant contribution by such progenitor cells during normal ageing, up to one year after tamoxifen treatment. However, they observed a reduction in the fraction of labelled cardiomyocytes after infarction, indicating dilution by unlabelled progenitor cells. When combined with the findings that the rate of cardiomyocyte proliferation is very low in both normal and injured rodent hearts, these data indicate that the limited endogenous reparative mechanisms in the adult mammalian heart operate differently from those in zebrafish, and depend more on replenishment by cardiomyogenic progenitor cells than on replacement by cardiomyocyte proliferation.

A recent report suggests that these differences between mammalian and fish hearts do not necessarily apply earlier in development[23]. Borrowing approaches from the zebrafish model, the authors resected the left ventricular apex of one-day-old neonatal mice and observed a brisk regenerative response reminiscent of that in the adult zebrafish. By three weeks after injury, the defect had been replaced by normal myocardial tissue, which showed normal contractile function by eight weeks. Genetic fate-mapping studies indicated that this regeneration was mediated by the proliferation of pre-existing cardiomyocytes, again as in the zebrafish. Notably, this regenerative capacity was not observed in seven-day-old mice, suggesting that its loss may coincide with cardiomyocyte binucleation and reduced cell-cycle activity. Nonetheless, in addition to representing a surgical tour de force, this study indicates that zebrafish-like regenerative mechanisms are latent in mammalian hearts. It also provides a genetically tractable model for dissecting the blocks to these mechanisms in the mammalian adult.

The evidence for human heart regeneration

Before addressing whether new cardiomyocytes are generated in the human heart after injury, it is instructive to review a few points about normal cardiac growth and adaptation to workloads (Box 2). In brief, most human cardiomyocyte nuclei are polyploid by the onset of puberty[24]. In response to pathological workloads, such as hypertension, valvular disease and post-infarction overload, human cardiomyocytes commonly reinitiate DNA synthesis without nuclear division[24,25]. This increases cardiomyocyte nuclear ploidy further, reaching levels as high as 64*n* (in which *n* represents the haploid set). Unlike rodent cardiomyocytes[26], most human cardiomyocytes seem to remain mononucleated throughout life[27]. Thus, DNA synthesis is common in the adult human heart, but this cannot be equated to cardiomyocyte proliferation without accounting for the process of polyploidization.

Historically, regenerative responses have been detected by either the macroscopic regrowth of the tissue or the microscopic presence of mitosis. Macroscopic regeneration of the human heart clearly does not occur. Mitosis occupies only ~2% of the cell cycle, making it hard to quantify meaningfully. Experiments have confirmed this, with some investigators reporting no mitosis after injury, and others reporting rare (and potentially abnormal) mitotic figures around the injured site[28–30]. One factor contributing to these discrepancies has been the inherent difficulty in recognizing cardiomyocyte nuclei in conventional histological sections[14]. Many of the published images are persuasive for cardiomyocyte mitosis and provide important evidence that this can occur in humans. However, extrapolation to organ turnover rates from such low numbers is perilous.

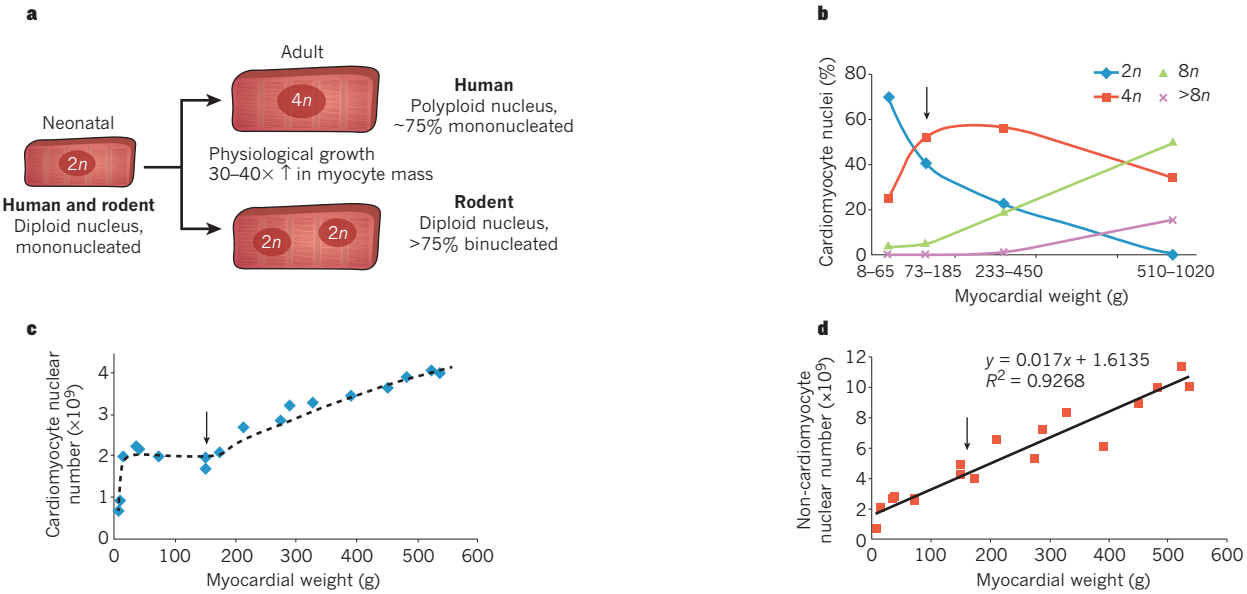
Several investigators have taken the approach of counting the number of cardiomyocytes in the human heart during normal and pathological growth[24,25,31,32], but this method is surprisingly difficult and requires many assumptions. Using a combination of meticulous dissection, histopathology, biochemical measurements of tissue DNA content and fluorescent analysis of individual nuclear DNA content, researchers have shown that from myocardial weights of 50–350 g, the cardiomyocyte nuclear number is steady at ~2 billion. Beyond that, there is a linear increase in nuclear number with increasing heart weight, reaching 4 billion cardiomyocyte nuclei in hypertrophied hearts weighing 700–900 g. The number of non-cardiomyocytes such as fibroblasts and vascular cells increases linearly with heart weight throughout life. If correct, these data indicate that cardiomyocyte renewal occurs during pathological hypertrophy. An important caveat is that the assignment of cardiomyocyte versus non-cardiomyocyte nuclear identity was based on the size and morphology of isolated nuclei. Because we know that postnatal growth and pathological hypertrophy are accompanied by increases in nuclear ploidy (and hence in size), it is possible that diploid cardiomyocyte nuclei in smaller hearts were mistakenly classified as non-cardiomyocyte nuclei. Increases in cardiomyocyte nuclear number in pathological hypertrophy have also been reported[33], using histological sections in which the cardiomyocyte nuclei can be more readily identified.

Two studies have attempted to use more direct means to measure the rates of cardiomyocyte DNA synthesis in human hearts. The first approach, by Bergmann *et al.*[34], was based on the worldwide pulse of ^{14}C that occurred during the atmospheric testing of nuclear weapons in the cold war. The atmospheric ^{14}C became incorporated into plants and entered the human food chain, labelling the DNA of dividing cells. After the Limited Nuclear Test Ban Treaty of 1963, atmospheric ^{14}C levels dropped rapidly. This provided researchers with pulse–chase conditions that can be used to date cells, simply by identifying when atmospheric ^{14}C levels match those of the DNA. As expected, non-cardiomyocytes in the normal human heart were found to be substantially younger than the patient, with ~18% turnover per year and a mean age of only four years. Notably, DNA from isolated cardiomyocyte nuclei (sorted by nuclear troponin staining) was also younger than the patient, although not nearly as young as that from non-cardiomyocytes. As indicated earlier, before one can infer cell division, it is essential to rule out a contribution from polyploidization. To do this, the authors sorted cardiomyocyte nuclei by DNA content, and analysed only the diploid DNA subset. The diploid cardiomyocyte nuclei were also younger than the patient, providing good evidence for cardiomyocyte division. Mathematical modelling suggested that cardiomyocyte renewal was age-dependent, with ~1% of cardiomyocytes being renewed per year at age 20, and 0.4% at age 75. On the basis of these kinetics, ~45% of cardiomyocytes would be predicted to be renewed over a normal human lifespan, whereas 55% would be cells persisting since birth.

In the second approach, by Kajstura *et al.*[35], the rates of cardiac DNA synthesis were obtained by examining post-mortem hearts from patients with cancer that had been treated with the thymidine analogue iododeoxyuridine (IdU). This agent is incorporated into nascent DNA, where it sensitizes cells to radiation therapy. IdU was given as bolus injections or multiweek infusions, and the time between treatment and death ranged from 7 days to 4.3 years. Using immunohistochemistry to

BOX 2

Nuclear dynamics during human heart growth



Fetal hearts in humans and rodents grow through the proliferation of mononucleated cardiomyocytes with diploid nuclei. In the first few days after birth, rodent cardiomyocytes withdraw from the cell cycle. By contrast, human cardiomyocytes seem to proliferate for the first few months after birth, after which replication slows markedly. The cells of both rodents and humans then undergo a period of physiological growth, increasing in size by 30–40-fold. Although most cardiomyocytes cannot grow this much with a single diploid genome, different species have taken varying approaches to solve this problem. Nearly all rodent cardiomyocytes undergo a final round of DNA replication followed by nuclear division without cytokinesis, resulting in a heart with more than 75% binucleated cells with a normal diploid (2*n*) content of DNA in each nucleus (see Figure, **a**)[26]. In humans and other primates, most cardiomyocytes undergo a final round of DNA replication, without nuclear division or cytokinesis, resulting in mononucleated cells[27] with tetraploid (4*n*) or higher DNA content (**a**)[31]. (Estimates of human cardiomyocyte binucleation rates range from 25% in enzymatically dispersed fresh tissue[27] to more than 60% in potassium-hydroxide-digested formaldehyde-fixed tissue[99]. We favour the 25% value, because the harsh potassium hydroxide digestion may selectively eliminate smaller mononucleated cardiomyocytes.) Notably, pacemaker cells of the sinoatrial and atrioventricular nodes remain small and diploid throughout life[100].

As the myocardial mass of the human heart increases, the percentage of diploid cardiomyocyte nuclei decreases steadily. This has been demonstrated by cytofluorometric analysis of human cardiomyocyte nuclear DNA content as a function of myocardial weight, after carefully removing valves, vessels and fat (see Figure, **b**). (Hearts were grouped by weight into four bins for clarity.) Studies of paediatric human hearts indicate that polyploidization occurs in the pre-adolescent growth phase, from 8 to 12 years of age. Tetraploid nuclei (4*n*) are most common in the adult heart. During cardiac hypertrophy, octaploid nuclei (8*n*) become most common, with a substantial number of 16*n* nuclei or those with higher polyploidy. These data demonstrate that human cardiomyocytes have a substantial capacity for DNA replication.

Morphometric analysis has shown that the normal human adult number of 2 billion cardiomyocyte nuclei is reached by about 2 months of age. During physiological hypertrophy, the cardiomyocyte nuclear number remains steady. However, when the heart weight exceeds approximately 450 g (myocardial weight roughly 210 g), there seems to be a linear increase in cardiomyocyte nuclear number with increasing cardiac mass (see Figure, **c**). Because human hearts do not change nuclear number with hypertrophy, this is evidence for the generation of new cardiomyocytes, either from pre-existing cardiomyocytes or from stem cells. Non-cardiomyocyte nuclei increase linearly with increasing myocardial mass (see Figure, **d**), indicating that proliferation of these cells accompanies all phases of cardiac growth. The arrows in **b**–**d** denote the upper limits of normal for human myocardial weight. The data in panels **b**–**d** are derived from ref. 31; the trend line in **c** is hand drawn for illustration purposes only.

detect the IdU signal and identify cardiomyocytes, the researchers found remarkably high rates of cardiomyocyte DNA labelling, ranging from 2.5% to 46%. No IdU staining was found in control hearts from patients without cancer who had not been exposed to the radiosensitizer. Mathematical modelling suggested that cardiomyocytes turn over at a rate of 22% per year, compared with 20% for fibroblasts and 13% for endothelial cells. Furthermore, 83% of the cardiomyocyte nuclei were reported to be diploid, suggesting that this turnover reflects cell division, not increased nuclear ploidy.

It is hard to reconcile these two studies, which differ by nearly 50-fold in their estimates of cardiomyocyte turnover. An important difference seems to be related to the higher rates of cardiomyocyte DNA synthesis activity in IdU-treated patients with cancer. Kajstura *et al.*[35] reported threefold lower DNA synthesis rates (based on immunolabelling of the cell proliferation marker Ki-67) in control hearts from patients without cancer than in hearts from IdU-treated patients with cancer. Neither study adequately rules out a contribution from DNA repair, which can masquerade as DNA replication in these assays. This is of particular concern in patients with cancer receiving radiation treatment plus a radiosensitizer. Kajstura *et al.* suggested that only senescent cardiomyocyte nuclei contain troponin, which could bias the turnover studies of Bergmann and his colleagues[34] towards low proliferation. However, a follow-up paper by Bergmann *et al.*[36] provided evidence that nearly all cardiomyocyte nuclei were identified by troponin

staining. Furthermore, the findings of Kajstura *et al.* contradict two well-accepted principles. First, the findings suggest that more than 80% of cardiomyocyte nuclei are diploid, in contrast to most other reports that suggest they are polyploid. If the nuclei were in fact polyploid, then polyploidization could underlie the authors' high estimates of DNA synthesis. Second, the authors conclude that cardiomyocytes are as proliferative as non-cardiomyocytes, whereas most other investigators find greater orders of magnitude of proliferation in non-cardiomyocytes. Indeed, the cardiomyocyte IdU-incorporation rate (2.5–46%) detected by Kajstura *et al.*[35] approaches the rate reported previously for sarcomas targeted by IdU (50–70%)[37]. The heart does not proliferate like a sarcoma, so these cardiac IdU-incorporation estimates must be too high.

Taken together, these human studies provide strong evidence for plasticity in the adult human heart. There is extensive morphometric evidence for DNA synthesis and an increase in cardiomyocyte number in diseased human hearts. Cardiomyocyte division or generation from progenitor cells probably occurs in the human heart, but it seems to be a very slow process. We need better tools to study this process quantitatively, and better ways to model it, if we hope to exploit it therapeutically.

Stem cells and cell therapy

Stem-cell biology is one of the fastest moving areas of biomedical research, and among all of the solid organs, the heart has one of the most active regeneration research programmes. The field can be conceptually organized into work involving endogenous and exogenous cells. The many exogenous cell types can be further divided into pluripotent cells (such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)) and adult cells of more limited potential (such as circulating progenitor cells, resident cardiac progenitor cells and cells native to other tissues). Here we focus on the cells closest to clinical trials and those for which there are the most reliable data.

Cardiac progenitor cells

Several investigators have reported resident populations of cardiac progenitor cells (CPCs) in postnatal hearts. These were identified using a variety of approaches, including studying the expression of surface markers such as c-KIT or SCA-1 (also known as LY6A; note that SCA-1 has no apparent human orthologue) and physiological properties such as the ability to efflux fluorescent dye or form multicellular spheroids (reviewed in refs 38 and 39). Initially, it seemed that there was little overlap among CPCs identified by the different methods, and some scientists suggested that several populations of CPC exist. More recent studies indicate shared markers among once-distinct populations or different stages of maturation in the same line of cells[40,41], so the field may be converging.

CPCs expressing the tyrosine kinase receptor c-KIT are the most extensively studied. In the human adult, c-KIT is expressed by telocytes (formerly known as the interstitial cells of Cajal), the thymic epithelium and mature circulating cells such as haematopoietic stem cells and mast cells. Immature endothelial cells and cardiomyocytes also express c-KIT during development[42]. Small round cells expressing c-KIT have been identified in the perivascular compartment of the adult heart, and their abundance increases in human heart failure[43]. After isolation from rat and human hearts, c-KIT$^+$ cells have been reported to give rise to cardiomyocytes, smooth muscle cells and endothelial cells. Some studies indicate that, when transplanted, c-KIT$^+$ cells induce large-scale regeneration of myocardial infarcts and contribute to the formation of new myocardium and vessels[44], whereas others suggest smaller-scale regeneration[45]. On the basis of these data, a clinical trial is under way, testing the safety and feasibility of autologous c-KIT$^+$ cells as an adjunctive treatment for patients undergoing coronary bypass surgery (ClinicalTrials.gov identifier NCT00474461).

Not all studies with c-KIT$^+$ CPCs gave robustly positive results. In studies with genetic read-outs for lineage tracing and differentiation state, c-KIT$^+$ cells from the adult mouse heart have not been shown to differentiate into cardiomyocytes *in vitro* or after transplantation into infarcted hearts[46]. Another study using transgenic reporter mice found no evidence to suggest that endogenous c-KIT$^+$ cells differentiate into cardiomyocytes, although re-expression of c-KIT in pre-existing cardiomyocytes was identified after injury[42]. Others point out that myocardium, like all solid tissues, contains mast cells. Mast cells are small round cells that reside in clusters in the perivascular space, strongly express c-KIT and increase in number in failing hearts[47]. Studies in humans suggest that 90–100% of all of the cardiac c-KIT$^+$ cells are actually mast cells. However, expansion in culture seems to select for c-KIT$^+$ cells that lack mast-cell markers, indicating that freshly isolated cells and cultured cells are different populations[48].

Another CPC population in clinical trials is cardiosphere-forming cells. These cells are isolated on the basis of their ability to migrate out of cultured cardiac tissue fragments and form spheroids in suspension cultures[49,50]. As one might predict, this yields a mixture of cells, some of which express stem-cell markers such as c-KIT, and others that seem to come from the stromal-vascular compartment. CPCs have been reported to give rise to cardiomyocytes *in vitro* and *in vivo* after transplantation, and to enhance cardiac function after infarction[50]. On the basis of these data, a clinical trial of autologous CPCs has been initiated for patients with recent myocardial infarctions (NCT00893360). The 'stemness' of CPCs has recently been questioned, and it has been suggested that these cells are principally cardiac fibroblasts and that CPC-derived cardiomyocytes are contaminants derived from the original tissue[51].

Thus, although the study of CPCs is an exciting, new area of cardiac research, it is also one of the most controversial. Most of the work has focused on cell culture and transplantation, driven by the clinical need for cardiac repair. We know almost nothing about the endogenous behaviour of CPCs, however. An important question remains about the role of these cells in development, homeostasis, ageing and reaction to injury. The field needs models that permit unambiguous tracing of CPC lineage and phenotype without resorting to transplantation or cell culture (Box 1).

Bone marrow cells

Considerable interest in bone-marrow-derived cells for cardiac repair was prompted by reports of haematopoietic stem cells transdifferentiating into cardiomyocytes[52]. Subsequent studies have shown that haematopoietic stem cells do not form cardiomyocytes but instead become mature blood cells after transplantation[53,54]. Nevertheless, animal studies show improvements in ventricular function when haematopoietic cells are administered after infarction, implicating paracrine signalling as the major mechanism of action.

Work with marrow-derived stromal cells (MSCs) has followed a similar trajectory. MSCs were originally reported to transdifferentiate into cardiomyocytes[55] but are now thought to exert their main actions in a paracrine manner through the release of cytokines[56]. Interestingly, most MSCs die within days or weeks of transplantation into infarcts, yet their beneficial effects can be seen long term, suggesting a critical window of time for the action of MSCs after infarction. MSCs probably operate by many mechanisms, but considerable evidence points towards regulation of the WNT pathway. MSCs secrete antagonists of canonical WNT ligands, such as secreted frizzled related protein 2 (ref. 56). Blocking the production of WNT antagonists limits the beneficial effects of mouse MSCs. A recent report has shown that the administration of MSCs to pig infarcts stimulated endogenous CPCs to contribute to the repair of the infarcts[57]. Further identification of paracrine mediators may allow the development of simpler, cell-free treatments based on proteins or small molecules.

Clinical trials have mostly focused on the delivery of bone marrow mononuclear cells by the coronary circulation. It should be emphasized that >99.9% of bone marrow mononuclear cells are not stem cells, but are committed, although immature, granulocytes or other haematopoietic lineages. These trials indicate that the delivery of bone marrow

derivatives through the coronaries is feasible and safe, but the benefits are modest. MSCs are also in clinical trials (NCT00587990). There are few published results with these cells, but one of the strongest cardiac-repair treatment effects seen so far (a 14% improvement in ejection fraction — the fraction of blood ejected from the left ventricle during one contraction) was reported after the intracoronary administration of large numbers of autologous MSCs[58]. Allogeneic MSCs administered to patients intravenously within ten days of infarction were well tolerated and were associated with decreased arrhythmias and an improvement in some indices of contractile function[59].

Taken together, the best current evidence indicates that bone marrow cells do not work by directly differentiating into new cardiomyocytes. Instead, the cells have been shown to elaborate signals that control the response of cells native to the myocardium, and thereby regulate healing. Although many view this as a novel aspect of stem-cell biology, students of pathology will recognize that this phenomenon fits under a more familiar heading: inflammation. We find it useful to consider the participation of marrow derivatives in cardiac repair as part of the inflammatory response, which is known to regulate angiogenesis, cardiomyocyte survival and left ventricular remodelling after infarction.

Pluripotent stem cells

Many types of adult stem cell are unable to generate large numbers of unambiguous cardiomyocytes. This limitation does not apply to ESCs or their more recently developed 'man-made' counterpart, iPSCs. Because both ESCs and iPSCs can be propagated indefinitely, while still retaining the capacity to differentiate into almost all cell types, they are a potentially inexhaustible supply of human cardiomyocytes. Our current thinking about how cardiomyocytes arise from ESCs is shown in Fig. 1. Human ESC-derived cardiomyocytes express early cardiac transcription factors such as NKX2.5, as well as the expected sarcomeric proteins, ion channels, connexins and calcium-handling proteins (Fig. 2). They show similar functional properties to those reported for cardiomyocytes in the developing heart, and undergo comparable mechanisms of excitation–contraction coupling and neurohormonal signalling[60–63]. Although human ESC-derived cardiomyocytes have been more intensively studied, data indicate that human iPSC-derived cardiomyocytes have a very similar phenotype[64,65]. Importantly, cardiomyocytes from either pluripotent stem-cell type are immature and so lack the expression profile, morphology and function of adult ventricular cardiomyocytes.

The cardiac potential of ESCs and iPSCs is indisputable, but their unique origin and pluripotency presents a new set of challenges. ESCs are derived from the inner cell mass of preimplantation-stage blastocysts[66], and this contributes to the ethical controversy surrounding their use. Moreover, ESC-based therapies will be allogeneic and require immunosuppression. iPSCs were originally generated by the reprogramming of adult somatic cells such as dermal fibroblasts by the forced expression of up to four stem-cell-related transcription factors[67–69]. As such, their derivation does not involve the destruction of embryos, and they could be used in autologous cell therapies. Nonetheless, first-generation iPSCs were problematic because the reprogramming factors were introduced using integrating viruses, raising concerns about neoplastic transformation. More recently, there have been a variety of refinements to iPSC generation that should reduce or eliminate this risk, including the use of episomal gene delivery, excisable transgenes, cell-permeable recombinant proteins and synthetic messenger RNA (see ref. 70 and references therein). Perhaps most notably, several small molecules have been shown to greatly enhance the efficiency of reprogramming[71], inviting speculation that iPSCs may be generated using such factors alone in the near future. Further work will be required to more precisely define the phenotype and maturation potential of cardiomyocytes derived from iPSCs generated by these methods.

Another concern relating to the clinical application of pluripotent stem cells is their capacity to form teratomas after transplantation[72]. To overcome this, the field needs to develop methods to enrich ESC and iPSC derivatives for cardiomyocytes or other useful cell types

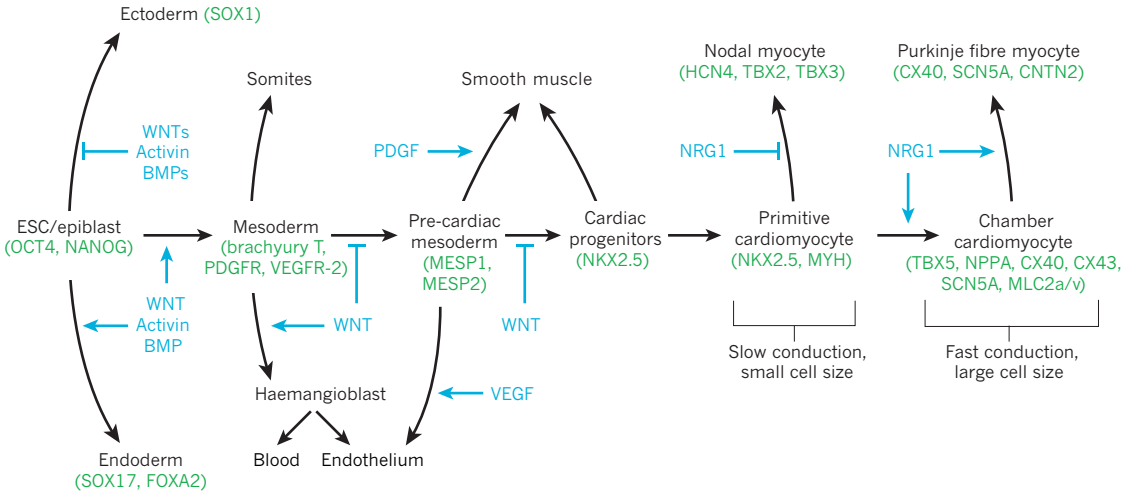


Figure 1 | Cardiovascular lineages during embryonic development and ESC differentiation. Cardiac differentiation from ESCs closely mimics cardiac development in the embryo. In either case, the specification of the cardiovascular lineages involves a transition through a sequence of increasingly restricted progenitor cells, proceeding from a pluripotent state to mesoderm and then to cells committed to cardiovascular fates. Growth factors that regulate fate choices are listed at branch points (blue), and key transcription factors and surface markers for each cell state are listed under the cell types (green). The growth factors are useful for directing the differentiation of ESCs, whereas the markers are useful for purifying cells at defined developmental states. Primitive cardiomyocytes in the embryonic heart tube and nodal or pacemaker cells show slow electrical propagation and a small cell size. By contrast, the eventual specification of working atrial and ventricular cardiomyocytes is accompanied by more rapid conduction, ion-channel remodelling and increased cell size. Although the field has made considerable progress towards determining the early events of cardiogenesis, a better understanding of how pacemaker and chamber-specific cardiac subtypes are formed is required for clinical applications. BMPs, bone morphogenetic proteins; CNTN2, contactin-2; CX, connexin; FOXA2, forkhead box protein A2; HCN4, potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4; MESP, mesoderm posterior protein; MLC2a/v, myosin light chain 2a and/or 2v; MYH, myosin heavy chain; NPPA, natriuretic peptide precursor A; NRG1, neuregulin 1; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; SCN5A, sodium channel protein type 5 subunit α; SOX, SRY-related high-mobility-group box; TBX, T-box transcription factor; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2.

(such as endothelial, smooth muscle and stromal cells). Historically, ESCs have been differentiated by culture in three-dimensional aggregates known as embryoid bodies, in medium containing a high percentage of fetal calf serum. This method is poorly cardiogenic, and differentiated human embryoid bodies are typically composed of less than 1% cardiomyocytes[73]. More recently, our group and others have used insights from developmental biology to devise better controlled approaches in which human ESCs and iPSCs are treated with defined factors, resulting in highly enriched populations of cardiomyocytes[74–76]. A common theme with such methods has been the manipulation of cardioinductive molecules belonging to the transforming growth factor-β superfamily — specifically, activin and the bone morphogenetic proteins (BMPs). Our group has reported a protocol involving the serial application of activin A and BMP4, for example, which reliably yields ~60–80% human ESC-derived cardiomyocytes in large-scale preparations (~10^8–10^9 total cells)[74,77]. Further refinements are possible by manipulating the WNT–β-catenin signalling pathway[78], which mediates biphasic effects on ESC cardiogenesis, promoting mesodermal induction early but inhibiting cardiogenesis late[79].

A complementary approach involves the isolation of mesodermal progenitor cells with a more restricted potential, such as cardiovascular progenitor cells that can differentiate into cardiomyocytes, smooth muscle cells and endothelial cells. Such multipotent progenitor cells have been identified in differentiating ESC cultures on the basis of their expression of transcription factors such as mesoderm posterior protein 1 (MESP1)[80], NKX2.5 (ref. 81) and ISL1 (refs 82 and 83). Arguably more useful for eventual clinical application are progenitor populations that can be sorted on the basis of their expression of a cell-surface marker, such as the cardiovascular progenitor cells marked by expression of vascular endothelial growth factor receptor-2 (VEGFR-2, also known as FLK1 and KDR)[75]. If such cells could be induced to self-renew, they would potentially be very useful for cardiac repair.

Human ESC-derived cardiomyocytes have been shown to engraft in infarcted mouse, rat, guinea pig and pig hearts (Fig. 3), forming islands of nascent, proliferating human myocardium within the scar zone[74,84,85]. This partial remuscularization was accompanied by beneficial effects on regional and global cardiac function[74,84], although some investigators have questioned whether these effects are sustained at later time points[86]. Notably, the mechanism (or mechanisms) underlying the observed improvements in contractile function remains unresolved. In the aforementioned rodent studies, most of the graft tissue was isolated from the host myocardium by means of scar tissue, which may prevent synchronous beating. Furthermore, these human cells, which fire *in vitro* at ~50–150 beats per minute (b.p.m.)[77], may not keep pace with the rapid rate of rats (~400 b.p.m.) and mice (~600 b.p.m.). If they cannot, then the observed salutary effects probably resulted from an indirect, paracrine mechanism, like those described above for adult cells. This also indicates that further beneficial effects on cardiac function may be possible after transplantation to a slower-rated recipient, such as a canine or porcine infarct model.

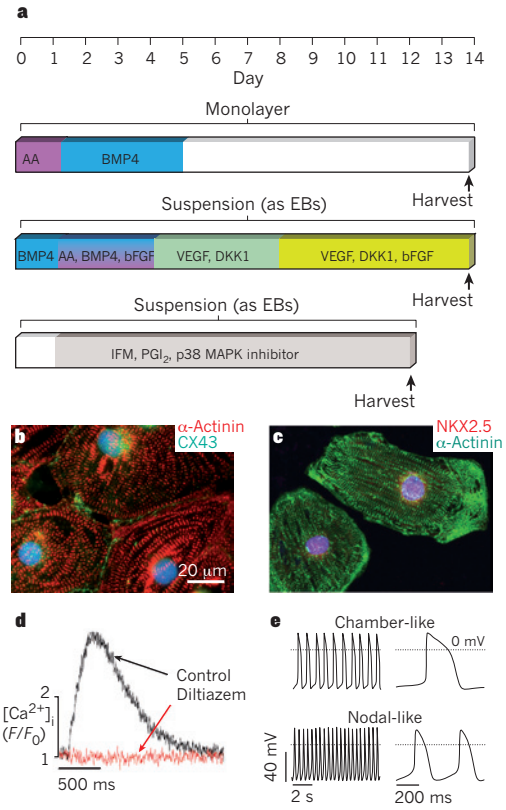


Figure 2 | Guided differentiation and phenotype of cardiomyocytes from pluripotent stem cells. **a**, Selected protocols for the guided differentiation of human ESCs and iPSCs into cardiomyocytes using chemically defined factors. The top timeline shows a protocol from our group in which differentiating cells are serially pulsed with activin A (AA) and BMP4 under monolayer culture conditions[74]. The middle timeline shows a protocol from ref. 75 that involves embryoid body (EB) formation in suspension cultures, and the application of several signalling molecules such as activin A, BMP4, basic fibroblast growth factor (bFGF), dickkopf-related protein 1 (DKK1) and VEGF. The bottom timeline shows a protocol from ref. 76, in which embryoid bodies in suspension are continuously cultured in insulin-free medium (IFM) supplemented with prostaglandin I_2 (PGI_2) and an inhibitor of p38 MAP kinase (MAPK). **b**, Representative human ESC-derived cardiomyocytes, differentiated using the monolayer protocol (top timeline in **a**), immunostained for α-actinin (red) and CX43 (green). Nuclei are shown in blue. **c**, Representative human iPSC-derived cardiomyocytes, differentiated using the monolayer protocol (top timeline in **a**), immunostained for α-actinin (green) and the transcription factor NKX2.5 (red). **d**, Intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) transients in a human ESC-derived cardiomyocyte before (black) or after (red) the application of diltiazem, an L-type Ca^{2+}-channel blocker. The absence of $[Ca^{2+}]$ transients after diltiazem treatment indicates that extracellular Ca^{2+} is required to initiate intracellular Ca^{2+} release, just as in adult cardiomyocytes. F/F_0 denotes the change in fluorescence intensity. **e**, Human ESC-derived cardiomyocytes show the characteristic action-potential properties of either working chamber (top) or nodal (bottom) cardiomyocytes, indicating early subtype specification.

Reprogramming fibroblasts to cardiomyocytes

Fifteen years ago, researchers showed that fibroblasts could be transdifferentiated into skeletal muscle *in vitro* or in the injured heart by overexpressing the gene encoding the myogenic transcription factor, MyoD[87]. Despite an intensive search by several groups, no comparable master gene for cardiac muscle was found, and interest in reprogramming waned. Spurred by the discovery of iPSCs, scientists have returned to this field, using combinations of transcription factors to reactivate core transcriptional networks of desired cell types. In an attempt to induce cardiac differentiation, researchers performed a systematic screen of 14 cardiac transcription factors for their ability to activate a cardiac-specific transgene — the *Myh6* promoter driving yellow fluorescent protein (YFP) expression — in cardiac fibroblasts[88]. The full cocktail activated fluorescence in ~1% of cells. A systematic winnowing yielded three transcription factors (MEF2C, GATA4 and TBX5) that activated the transgene in 20% of fibroblasts. About 4% of the cells expressed endogenous sarcomeric proteins such as cardiac troponin T, and only ~1% showed functional properties such as spontaneous beating. Thus, most of the YFP^+ cells were only partially reprogrammed, although their global gene expression patterns had shifted markedly from fibroblast to cardiomyocyte.

While this manuscript was under review, a different method of reprogramming mouse embryonic fibroblasts to cardiomyocytes was reported[89]. This group used the 'Yamanaka factors' — OCT4 (also known as POU5F1), SOX2, KLF4 and c-MYC — to initiate

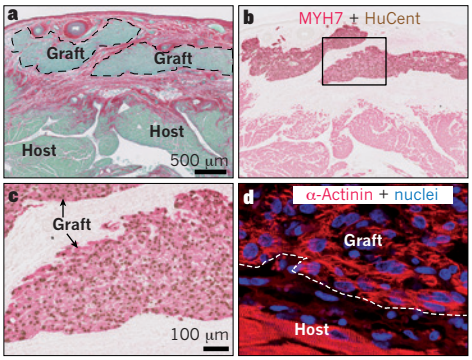


Figure 3 | Grafts of human ESC-derived cardiomyocytes in the cryoinjured guinea-pig heart. Representative photomicrographs demonstrating substantial implants of human myocardium within the scar tissue. **a**, Using picrosirius red stain, the scar appears red, and viable tissue is green. **b**, The human origin of the graft myocardium was confirmed in an adjacent section by combined *in situ* hybridization, with a human-specific pan-centromeric (HuCent; brown) probe, and β-myosin heavy chain (MYH7; red) immunohistochemistry. **c**, Inset from **b** at higher magnification. The nuclear localization of the HuCent signal confirms the human origin of these cells. **d**, Immunostaining for α-actinin (red) highlights the sarcomeric organization of the graft cardiomyocytes. Nuclei have been counterstained with Hoechst 33342 (blue).

reprogramming, but they blocked signalling through the JAK–STAT pathway, which is required for pluripotency in the mouse, and added the cardiogenic factor BMP4. These modifications yielded minimal generation of iPSCs, but instead activated the cardiac progenitor program and, within 2 weeks, generated substantial numbers of beating colonies. By 18 days after induction, approximately 40% of the cells expressed cardiac troponin T. The authors attributed the increased efficiency to the generation of highly proliferative progenitor cells, as opposed to the formation of cardiomyocytes with low proliferative potential. It should also be noted that this study used mouse embryonic fibroblasts, whereas the systematic screen of 14 transcription factors was principally in postnatal mouse cardiac fibroblasts.

Reprogramming the scar-forming fibroblast to a cardiomyocyte is intuitively appealing, particularly if it can be done directly in the infarct. To succeed clinically, we need to know how normal these reprogrammed cardiomyocytes are, and the process will have to be much more efficient and transgene-free. Despite some challenges, this is an exciting avenue of research and could be a game changer.

Tissue engineering

Tissue engineering refers to the growth of three-dimensional tissues *in vitro*, with the aim of building more biologically relevant models for *in vitro* study or tissues for *in vivo* regenerative therapy. Most commonly, this involves the use of porous, biodegradable scaffolds onto which cells are seeded, but other approaches include casting cells into hydrogels or creating scaffold-free tissues composed only of cells and the matrix they secrete. Synthetic materials have big advantages in manufacturability, typically being easy, cheap and reproducible to make. However, synthetics generally have worse biocompatibility, because they cause foreign-body inflammatory reactions and, sometimes, release locally toxic degradation products. Bioreactors are often used in tissue engineering to provide electrical and mechanical conditioning or to deliver nutrients to the tissue by perfusion systems.

A major aim of bioengineering is to improve the host response to biomaterials, in essence, to make materials that can heal[90]. Surprisingly, the chemical composition of a material does not have a major influence on how the body responds to it. Whether materials are organic or metallic, hydrophobic or hydrophilic, or positively or negatively charged, they all cause similar foreign-body reactions. Instead, what the body seems to sense is the surface topography of a material[90]. When surfaces are smooth, there is intense inflammation and scarring, creating a fibrotic capsule around the implant. If a surface is given a more complex topography, for example, by creating pores or grooves, there is less inflammation, scarring diminishes and blood vessels grow into the implant. Systematic variation in the topology can 'tune' this host response. For example, our tissue-engineering group has developed scaffolds with two compartments: cylindrical channels to generate cables of cardiomyocytes, surrounded by a network of smaller interconnected pores for stromal and vascular ingrowth[91]. The pores are optimally sized to maximize vascularization within the implant and minimize fibrosis around it.

The cardiomyocytes used in tissue engineering have been immature cells derived from young animals or stem cells. To take on an adult workload, these cells will need to organize into the cable-like structure of myocardium and increase their size by more than 20-fold compared with the neonatal stage. There is a continuing debate in tissue engineering about whether this maturation should take place before or after transplantation. On the one hand, electrical[92] and mechanical[93] stimulation *in vitro* enhance hypertrophy, alignment and electromechanical function of rat cardiomyocyte constructs. On the other hand, greater cell differentiation is associated with worse survival after transplantation[94], so there is probably a point of diminishing returns. This needs to be explored further experimentally.

One of the big lessons from tissue engineering has come from studies comparing cardiomyocyte-only with mixed-cell constructs. When cardiomyocyte-only constructs are transplanted, the tissue survives poorly. When vascular endothelial cells together with a stromal cell population are included, the endothelial cells form networks resembling a primitive vascular plexus, and the stromal cells form a provisional matrix that enhances mechanical integrity[95,96]. After transplantation, the endothelial network organizes into a definitive vascular network that connects to the host circulation, bringing blood flow into the tissue several days sooner than would otherwise be seen. Indeed, our group and others have demonstrated improved survival of prevascularized human myocardial constructs incorporating vascular and stromal elements compared with constructs containing cardiomyocytes alone[95,97]. This indicates that there is considerable synergy to including vessels and connective tissue elements when engineering tissue.

Tissue engineering has not been as extensively studied as cell transplantation in preclinical disease models, but initial studies are promising. A recent study[93] prepared constructs of engineered rat heart tissue from neonatal cardiomyocytes and conditioned them for several days using a cyclic stretch system. The constructs were sutured to the surface of rat hearts that had been infarcted two weeks previously, and were studied one month after implantation. Compared with infarcted hearts receiving non-contractile constructs, hearts receiving the engineered heart tissue had better contractile function, and interestingly, conduction velocities across the infarct were improved, probably because the grafts had electrically connected to the surrounding viable myocardium. Another group reported that patches generated from cardiosphere-derived CPCs can enhance heart function after infarction[98], and there are hints that tissue engineering also provides a larger graft size compared with cell transplantation.

Perspective

After more than a decade of furious activity, the science of stem cells seems to be catching up with its promise. Clinical-scale preparations of the main cardiac cell types can now be generated, and we are learning the rules for building myocardium and keeping it alive after transplantation. Clinical trials have established techniques for cell delivery, and protocols for establishing feasibility, safety and early-stage efficacy in humans are in place. The first patient trials have demonstrated safety with hints of efficacy. So far, so good.

That said, many short- and long-term challenges remain. In the near term, it will be important to derive the right subtype of cardiomyocyte, for example, ventricular cardiomyocytes that are free of pacemaker cells for repair of an infarct. The major challenge facing the field of

adult CPCs is to develop protocols with higher yields of definitive cardiomyocytes. Researchers studying pluripotent stem cells need to identify the optimal stage of differentiation and demonstrate that these cells can be used without tumorigenesis. The question of allogeneic versus autologous cells remains open. Although desirable, autologous cells will be more expensive, more variable, and the time needed to expand them precludes their use in any acute setting. Allogeneic cells will provide the only off-the-shelf product, but we need to learn how best to manage the immune response to prevent their rejection. All of these efforts will be advanced by improvements in integration of the graft, including control of vascularization (growth of both arterial conduits and microvasculature), inflammation and scarring.

Further ahead, *in situ* manipulation of cells in the heart may allow us to control their fates, thereby obviating transplantation. For example, it may be possible to control CPCs using small molecules or growth factors to enhance their regenerative abilities. Fibroblasts in infarcts could potentially be reprogrammed directly to cardiomyocytes. Given our increasing ability to control the fates of cells and tissues, the debate over whether the heart is intrinsically terminally differentiated seems anachronistic, for the heart does not exist apart from the person who knows how to manipulate it. It is more useful to ask what we can do to promote cardiac regeneration best, and then do it. ■

1. Carvalho, A. B. & de Carvalho, A. C. Heart regeneration: past, present and future. *World J. Cardiol.* **2,** 107–111 (2010).
2. Rumyantsev, P. P. in *Muscle Regeneration* (ed. Mauro, A.) 335–355 (Raven, 1979).
3. Rumyantsev, P. P. *Growth and Hyperplasia of Cardiac Muscle Cells* (Harwood Academic, 1991).
4. Murry, C. E., Reinecke, H. & Pabon, L. M. Regeneration gaps: observations on stem cells and cardiac repair. *J. Am. Coll. Cardiol.* **47,** 1777–1785 (2006).
5. Whelan, R. S., Kaplinskiy, V. & Kitsis, R. N. Cell death in the pathogenesis of heart disease: mechanisms and significance. *Annu. Rev. Physiol.* **72,** 19–44 (2010).
6. Olivetti, G., Melissari, M., Capasso, J. M. & Anversa, P. Cardiomyopathy of the aging human heart. *Circ. Res.* **68,** 1560–1568 (1991).
7. Laflamme, M. A., Zbinden, S., Epstein, S. E. & Murry, C. E. Cell-based therapy for myocardial ischemia and infarction: pathophysiological mechanisms. *Annu. Rev. Pathol.* **2,** 307–339 (2007).
8. Povsic, T. J. & O'Connor, C. M. Cell therapy for heart failure: the need for a new therapeutic strategy. *Expert Rev. Cardiovasc. Ther.* **8,** 1107–1126 (2010).
9. Oberpriller, J. O. & Oberpriller, J. C. Response of the adult newt ventricle to injury. *J. Exp. Zool.* **187,** 249–253 (1974).
10. Poss, K. D., Wilson, L. G. & Keating, M. T. Heart regeneration in zebrafish. *Science* **298,** 2188–2190 (2002).
11. Lepilina, A. *et al.* A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* **127,** 607–619 (2006).
12. Kikuchi, K. *et al.* Primary contribution to zebrafish heart regeneration by *gata4*$^{+}$ cardiomyocytes. *Nature* **464,** 601–605 (2010).
13. Jopling, C. *et al.* Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464,** 606–609 (2010).
References 12 and 13 demonstrate that heart regeneration in zebrafish is mediated by the proliferation of pre-existing cardiomyocytes, not by the mobilization of undifferentiated precursors.
14. Soonpaa, M. H. & Field, L. J. Survey of studies examining mammalian cardiomyocyte DNA synthesis. *Circ. Res.* **83,** 15–26 (1998).
15. Soonpaa, M. H. & Field, L. J. Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts. *Am. J. Physiol.* **272,** H220–H226 (1997).
16. Hassink, R. J. *et al.* Cardiomyocyte cell cycle activation improves cardiac function after myocardial infarction. *Cardiovasc. Res.* **78,** 18–25 (2008).
17. Ahuja, P., Sdek, P. & MacLellan, W. R. Cardiac myocyte cell cycle control in development, disease, and regeneration. *Physiol. Rev.* **87,** 521–544 (2007).
18. Kuhn, B. *et al.* Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. *Nature Med.* **13,** 962–969 (2007).
19. Engel, F. B., Hsieh, P. C., Lee, R. T. & Keating, M. T. FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. *Proc. Natl Acad. Sci. USA* **103,** 15546–15551 (2006).
20. Bersell, K., Arab, S., Haring, B. & Kuhn, B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* **138,** 257–270 (2009).
This study identifies neuregulin as a mitogen for cardiomyocytes, and shows that systemic NRG1 administration improves function in infarcted mouse hearts.
21. Lorts, A., Schwanekamp, J. A., Elrod, J. W., Sargent, M. A. & Molkentin, J. D. Genetic manipulation of periostin expression in the heart does not affect myocyte content, cell cycle activity, or cardiac repair. *Circ. Res.* **104,** e1–e7 (2009).
22. Hsieh, P. C. *et al.* Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nature Med.* **13,** 970–974 (2007).
These authors use transgenic mice and a genetic fate-mapping strategy to show that progenitor cells contribute to cardiomyocyte renewal after injury.
23. Porrello, E. R. *et al.* Transient regenerative potential of the neonatal mouse heart. *Science* **331,** 1078–1080 (2011).
24. Adler, C. P. Relationship between deoxyribonucleic acid content and nucleoli in human heart muscle cells and estimation of cell number during cardiac growth and hyperfunction. *Recent Adv. Stud. Cardiac Struct. Metab.* **8,** 373–386 (1975).
This study uses careful biochemical and cytophotometric techniques to determine cardiomyocyte number and DNA content in human hearts during normal ageing and disease.
25. Adler, C. P. & Friedburg, H. Myocardial DNA content, ploidy level and cell number in geriatric hearts: post-mortem examinations of human myocardium in old age. *J. Mol. Cell Cardiol.* **18,** 39–53 (1986).
26. Li, F., Wang, X., Capasso, J. M. & Gerdes, A. M. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J. Mol. Cell Cardiol.* **28,** 1737–1746 (1996).
27. Olivetti, G. *et al.* Aging, cardiac hypertrophy and ischemic cardiomyopathy do not affect the proportion of mononucleated and multinucleated myocytes in the human heart. *J. Mol. Cell Cardiol.* **28,** 1463–1477 (1996).
28. MacMahon, H. E. Hyperplasia and regeneration of the myocardium in infants and in children. *Am. J. Pathol.* **13,** 845–854 (1937).
29. Beltrami, A. P. *et al.* Evidence that human cardiac myocytes divide after myocardial infarction. *N. Engl. J. Med.* **344,** 1750–1757 (2001).
30. Rumyantsev, P. P. in *Growth and Hyperplasia of Cardiac Muscle Cells* (ed. Carlson, B. M.) 210–238 (Harwood Academic Publishers, 1991).
31. Adler, C. P. & Costabel, U. Cell number in human heart in atrophy, hypertrophy, and under the influence of cytostatics. *Recent Adv. Stud. Cardiac Struct. Metab.* **6,** 343–355 (1975).
32. Herget, G. W., Neuburger, M., Plagwitz, R. & Adler, C. P. DNA content, ploidy level and number of nuclei in the human heart after myocardial infarction. *Cardiovasc. Res.* **36,** 45–51 (1997).
33. Grajek, S. *et al.* Hypertrophy or hyperplasia in cardiac muscle. *Eur. Heart J.* **14,** 40–47 (1993).
34. Bergmann, O. *et al.* Evidence for cardiomyocyte renewal in humans. *Science* **324,** 98–102 (2009).
In this study, the authors took advantage of the global pulse of ^{14}C during the cold war and estimated a rate of cardiomyocyte renewal in human hearts of up to 1% per year.
35. Kajstura, J. *et al.* Cardiomyogenesis in the adult human heart. *Circ. Res.* **107,** 305–315 (2010).
36. Bergmann, O. *et al.* Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover. *Exp. Cell Res.* **317,** 188–194 (2010).
37. Kinsella, T. J. *et al.* A phase I study of intermittent intravenous bromodeoxyuridine (BUdR) with conventional fractionated irradiation. *Int. J. Radiat. Oncol. Biol. Phys.* **10,** 69–76 (1984).
38. Segers, V. F. & Lee, R. T. Stem-cell therapy for cardiac disease. *Nature* **451,** 937–942 (2008).
39. Passier, R., van Laake, L. W. & Mummery, C. L. Stem-cell-based therapy and lessons from the heart. *Nature* **453,** 322–329 (2008).
40. Smith, R. R. *et al.* Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation* **115,** 896–908 (2007).
41. Pfister, O. *et al.* Role of the ATP-binding cassette transporter Abcg2 in the phenotype and function of cardiac side population cells. *Circ. Res.* **103,** 825–835 (2008).
42. Tallini, Y. N. *et al.* c-kit expression identifies cardiovascular precursors in the neonatal heart. *Proc. Natl Acad. Sci. USA* **106,** 1808–1813 (2009).
43. Kubo, H. *et al.* Increased cardiac myocyte progenitors in failing human hearts. *Circulation* **118,** 649–657 (2008).
44. Bearzi, C. *et al.* Human cardiac stem cells. *Proc. Natl Acad. Sci. USA* **104,** 14068–14073 (2007).
This study describes the isolation of c-KIT^{+} CPCs from human hearts, and reports their differentiation into cardiomyocytes and vascular elements both *in vitro* and after transplantation.
45. Tang, X. L. *et al.* Intracoronary administration of cardiac progenitor cells alleviates left ventricular dysfunction in rats with a 30-day-old infarction. *Circulation* **121,** 293–305 (2010).
46. Zaruba, M. M., Soonpaa, M., Reuter, S. & Field, L. J. Cardiomyogenic potential of c-kit^{+}-expressing cells derived from neonatal and adult mouse hearts. *Circulation* **121,** 1992–2000 (2010).
47. Patella, V. *et al.* Stem cell factor in mast cells and increased mast cell density in idiopathic and ischemic cardiomyopathy. *Circulation* **97,** 971–978 (1998).
48. Sandstedt, J., Jonsson, M., Lindahl, A., Jeppsson, A. & Asp, J. C-kit^{+} CD45^{-} cells found in the adult human heart represent a population of endothelial progenitor cells. *Basic Res. Cardiol.* **105,** 545–556 (2010).
49. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ. Res.* **95,** 911–921 (2004).
50. Chimenti, I. *et al.* Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. *Circ. Res.* **106,** 971–980 (2010).
51. Andersen, D. C., Andersen, P., Schneider, M., Jensen, H. B. & Sheikh, S. P. Murine 'cardiospheres' are not a source of stem cells with cardiomyogenic potential. *Stem Cells* **27,** 1571–1581 (2009).
52. Orlic, D. *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* **410,** 701–705 (2001).

53. Murry, C. E. *et al.* Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* **428,** 664–668 (2004).
54. Balsam, L. B. *et al.* Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* **428,** 668–673 (2004).
55. Toma, C., Pittenger, M. F., Cahill, K. S., Byrne, B. J. & Kessler, P. D. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105,** 93–98 (2002).
56. Mirotsou, M. *et al.* Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc. Natl Acad. Sci. USA* **104,** 1643–1648 (2007).
57. Hatzistergos, K. E. *et al.* Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. *Circ. Res.* **107,** 913–922 (2010).
58. Chen, S. L. *et al.* Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am. J. Cardiol.* **94,** 92–95 (2004).
59. Hare, J. M. *et al.* A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. *J. Am. Coll. Cardiol.* **54,** 2277–2286 (2009).
60. Kehat, I. *et al.* Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J. Clin. Invest.* **108,** 407–414 (2001).
61. Sartiani, L. *et al.* Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: a molecular and electrophysiological approach. *Stem Cells* **25,** 1136–1144 (2007).
62. He, J. Q., Ma, Y., Lee, Y., Thomson, J. A. & Kamp, T. J. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. *Circ. Res.* **93,** 32–39 (2003).
63. Zhu, W. Z., Santana, L. F. & Laflamme, M. A. Local control of excitation-contraction coupling in human embryonic stem cell-derived cardiomyocytes. *PLoS ONE* **4,** e5407 (2009).
64. Zhang, J. *et al.* Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ. Res.* **104,** e30–e41 (2009).
65. Zwi, L. *et al.* Cardiomyocyte differentiation of human induced pluripotent stem cells. *Circulation* **120,** 1513–1523 (2009).
66. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282,** 1145–1147 (1998).
67. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131,** 861–872 (2007).
68. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126,** 663–676 (2006).
69. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318,** 1917–1920 (2007).
70. Warren, L. *et al.* Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* **7,** 618–630 (2010).
71. Shi, Y. *et al.* Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell Stem Cell* **3,** 568–574 (2008).
72. Nussbaum, J. *et al.* Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *FASEB J.* **21,** 1345–1357 (2007).
73. Kehat, I. *et al.* Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J. Clin. Invest.* **108,** 407–414 (2001).
74. Laflamme, M. A. *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature Biotechnol.* **25,** 1015–1024 (2007).
This study describes methods for guiding the differentiation of human ESCs into cardiomyocytes, and shows that transplantation of these cells improves function in a rat infarct model.
75. Yang, L. *et al.* Human cardiovascular progenitor cells develop from a KDR$^+$ embryonic-stem-cell-derived population. *Nature* **453,** 524–528 (2008).
This paper describes the guided differentiation and isolation of multipotent cardiovascular progenitor cells from human ESCs.
76. Xu, X. Q. *et al.* Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. *Differentiation* **76,** 958–970 (2008).
77. Zhu, W. Z. *et al.* Neuregulin/ErbB signaling regulates cardiac subtype specification in differentiating human embryonic stem cells. *Circ. Res.* **107,** 776–786 (2010).
78. Paige, S. L. *et al.* Endogenous Wnt/β-catenin signaling is required for cardiac differentiation in human embryonic stem cells. *PLoS ONE* **5,** e11134 (2010).
79. Ueno, S. *et al.* Biphasic role for Wnt/β-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc. Natl Acad. Sci. USA* **104,** 9685–9690 (2007).
80. Bondue, A. *et al.* Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* **3,** 69–84 (2008).
81. Wu, S. M. *et al.* Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* **127,** 1137–1150 (2006).
82. Moretti, A. *et al.* Multipotent embryonic *Isl1*$^+$ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell* **127,** 1151–1165 (2006).
83. Domian, I. J. *et al.* Generation of functional ventricular heart muscle from mouse ventricular progenitor cells. *Science* **326,** 426–429 (2009).
84. Caspi, O. *et al.* Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. *J. Am. Coll. Cardiol.* **50,** 1884–1893 (2007).
85. Fernandes, S. *et al.* Human embryonic stem cell-derived cardiomyocytes engraft but do not alter cardiac remodeling after chronic infarction in rats. *J. Mol. Cell Cardiol.* **49,** 941–949 (2010).
86. van Laake, L. W., Passier, R., Doevendans, P. A. & Mummery, C. L. Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. *Circ. Res.* **102,** 1008–1010 (2008).
87. Murry, C. E., Kay, M. A., Bartosek, T., Hauschka, S. D. & Schwartz, S. M. Muscle differentiation during repair of myocardial necrosis in rats via gene transfer with MyoD. *J. Clin. Invest.* **98,** 2209–2217 (1996).
88. Ieda, M. *et al.* Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* **142,** 375–386 (2010).
This study describes the direct reprogramming of cardiac fibroblasts to a cardiomyocyte-like state by the forced expression of three cardiac transcription factors.
89. Efe, J. A. *et al.* Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. *Nature Cell Biol.* **13,** 215–222 (2011).
90. Ratner, B. D. & Bryant, S. J. Biomaterials: where we have been and where we are going. *Annu. Rev. Biomed. Eng.* **6,** 41–75 (2004).
91. Madden, L. R. *et al.* Proangiogenic scaffolds as functional templates for cardiac tissue engineering. *Proc. Natl Acad. Sci. USA* **107,** 15211–15216 (2010).
92. Radisic, M. *et al.* Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. *Proc. Natl Acad. Sci. USA* **101,** 18129–18134 (2004).
93. Zimmermann, W. H. *et al.* Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nature Med.* **12,** 452–458 (2006).
This paper describes the formation of engineered heart tissue using neonatal rat cardiomyocytes, and shows that their implantation improves electrical and mechanical function in injured hearts.
94. Reinecke, H., Zhang, M., Bartosek, T. & Murry, C. E. Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. *Circulation* **100,** 193–202 (1999).
95. Stevens, K. R. *et al.* Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. *Proc. Natl Acad. Sci. USA* **106,** 16568–16573 (2009).
96. Caspi, O. *et al.* Tissue engineering of vascularized cardiac muscle from human embryonic stem cells. *Circ. Res.* **100,** 263–272 (2007).
97. Dvir, T. *et al.* Prevascularization of cardiac patch on the omentum improves its therapeutic outcome. *Proc. Natl Acad. Sci. USA* **106,** 14990–14995 (2009).
98. Zakharova, L. *et al.* Transplantation of cardiac progenitor cell sheet onto infarcted heart promotes cardiogenesis and improves function. *Cardiovasc Res.* **87,** 40–49 (2010).
99. Brodsky, V. Y., Chernyaev, A. L. & Vasilyeva, I. A. Variability of the cardiomyocyte ploidy in normal human hearts. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* **61,** 289–294 (1991).
100. Rumyantsev, P. P., Erokhina, I. L., Antipanova, E. M. & Martynova, M. G. DNA and sex chromatin content in nuclei of conductive system and working myocytes of normal and hypertrophied human heart. *Acta Histochem. Suppl.* **39,** 225–237 (1990).

Acknowledgements We thank our many colleagues whose useful discussions and work contributed to the material presented here. This work was supported by grants from the US National Institutes of Health.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence should be addressed to C.E.M. (murry@u.washington.edu).