Heart regeneration and repair after myocardial infarction – translational opportunities for novel therapeutics

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Abstract

Current therapies for heart failure after myocardial infarction (MI) are limited and non-curative. Although regenerative approaches are receiving significant attention, clinical efforts involving transplantation of presumed stem and progenitor cells have largely failed. Recent studies of endogenous heart regeneration in model organisms such as the zebrafish and neonatal mouse are yielding novel mechanistic insights into the roles of cardiomyocyte proliferation, resident stem cell niches, neovascularisation, the immune system and the extracellular matrix. These findings have revealed novel pathways which might be therapeutically targeted to stimulate repair following MI, and provided lessons to guide future efforts toward heart regeneration through cellular reprogramming or cardiomyocyte transplantation.

Introduction

Heart failure (HF) is the consequence of cardiomyocyte death or dysfunction, most commonly caused by myocardial infarction (MI), hypertension, valve disease, infiltration, infection, chemotherapy or genetic cardiomyopathy.[1,2] HF is a global disease challenge which affects an estimated 38 million people worldwide.[3] Despite strides forward in the management of acute MI, HF remains common and the incidence may be increasing due to improved early survival with primary percutaneous coronary intervention.[4,5] Healthcare costs associated with HF exceed $30 billion annually in the USA alone and are projected to increase to almost $70 billion by 2030.[6] HF is a leading cause of hospitalization, adverse quality of life, and death, and a new diagnosis carries a worse prognosis than many cancers, with a survival rate of only 50% at 5 years.

Current therapies to prevent or delay progression of HF are limited. Conventional pharmacotherapy targets the maladaptive counter-regulatory mechanisms activated by left ventricular dysfunction (Box 1). This approach has yielded blockbuster agents over the last 20 years, including inhibitors of the renin-angiotensin system, the mineralocorticoid receptor, the sympathetic nervous system, and most recently, the natriuretic system (Figure 1).[7,8] While these drugs reduce mortality, they fail to address the underlying loss of cardiomyocytes and vasculature and are intrinsically non-curative.

Regeneration of the heart by reconstitution of the cardiomyocyte substrate is a tantalising and potentially entirely disruptive approach to HF. Regeneration is seen widely across the animal kingdom, and can occur in humans, for example after liver injury. To date, clinical efforts towards cardiac regeneration have focused on cell-based therapies, including bone marrow-derived cells, mesenchymal stem cells and presumed cardiac progenitor cells (Table 1).[9,10] While these studies have met safety endpoints, the effect on cardiac function has been small or negligible, which has prompted a search for novel approaches. Insights from endogenous heart regeneration in animal models such as the zebrafish and neonatal mouse are now yielding new understanding of innate mechanisms for complex organ repair. This includes the origins of new cardiomyocytes after injury, control of cardiomyocyte proliferation in

development and ageing, and the roles of developmental stem cell niches such as the epicardium.[11,12]

In this Review, we will describe recent insights into the biology of heart regeneration gained in preclinical animal models and extrapolate these to a next generation of regenerative strategies for HF, including methods for augmentation of intrinsic repair, cell reprogramming and extrinsic cardiomyocyte replacement. Key steps needed to translate informed biology to novel therapeutic approaches and compounds, adapt existing clinical trial design and enhance interactions between scientists, clinicians and the pharmaceutical industry will be outlined.

Discovery of heart regeneration [H1]

Organ regeneration has long been recognized. In 1686, lizard tail regeneration was demonstrated to the Paris Academy of Sciences, and the first scientific reports of regeneration in *Hydra* were published by Abraham Trembley in the mid-18th century.

Regeneration of the injured heart was first recognised in amphibians and has now been described in a number of teleost fish and amphibians.[13] The two-chambered heart of the zebrafish (*Danio rerio*) regenerates after damage caused by surgical resection of the cardiac apex, cryoinjury, cardiomyocyte ablation, or hypoxia-reoxygenation.[14-17] Urodele amphibians, including the axolotl and newt, are also capable of complete heart regeneration. In both fish and amphibians, functional cardiomyocytes re-populate the injury site and transient scar is resolved over a variable period of 60-180 days depending on the injury mechanism.[13,18,19]

Although previously thought to be restricted to fish and amphibians, Porrello *et al* reported heart regeneration in the neonatal mouse in 2011.[20,21] After resection of the cardiac apex or surgical coronary artery ligation (to induce MI), regeneration occurred over ~ 21 days leaving only very minor residual scarring at the site of the ligature. Interestingly, this capacity for regeneration was restricted to a temporally privileged window of the first seven days after birth.

The revelation that regeneration can occur in the neonatal mouse has ignited the field and suggests that regenerative repair is not a unique program lost to mammals in evolution. Whilst regeneration in the postnatal setting may involve a repurposing of the ongoing growth response, it remains highly valuable as a means to identify trophic pathways to promote regeneration in the adult. Furthermore, several intriguing (albeit low level) studies support the concept of a temporally privileged period of regenerative repair in humans. These include a case report of a newborn infant with a large anterior myocardial infarction, which healed by regeneration with full functional recovery.[22] Other case reports of functional regeneration after cardiac injury in infants, and a lack of scarring in children after cardiac surgery for congenital heart disease, support the concept of age-dependent regeneration in the human heart.[23,24]

Mechanisms of heart regeneration [H1]

Regeneration is a finely orchestrated process which has parallels to organ formation during embryonic development, requiring control of cell division, differentiation, migration, integration and maturation.[25] Compared to development, regeneration from injury is complicated by the need to clear damaged or dead tissue, regulate inflammation, suppress overactive fibrosis, and reconstitute and integrate only a subsection of cardiomyocytes, extracellular matrix, blood vessel and lymphatic systems.[26] Studies of heart regeneration in key genetic model organisms, the zebrafish and the mouse, have heralded a number of insights into the underlying biological mechanisms of these processes.

Cardiomyocyte regeneration [H2]

Replacement of cardiomyocytes to restore structural and functional integrity is the *sine qua non* of heart regeneration. Identifying the source(s) of new cardiomyocytes and mechanisms controlling cardiomyocyte proliferation is critical to understanding the mechanisms of regeneration and to direct therapeutic strategies for humans.

Sources of cardiomyocytes in endogenous regeneration [H3]

In principle, new cardiomyocytes might be derived from the existing cardiomyocyte pool (either directly from mature cardiomyocytes or an intermediate cell type) or alternatively from a progenitor cell population, either resident in the heart or located remotely. Seminal studies in the zebrafish and mouse have addressed this question using genetic lineage tracing, employing the tamoxifen-inducible Cre-LoxP system to irreversibly label cardiomyocytes with a fluorescent reporter protein prior to injury.[27] Once the label is activated, all cardiomyocytes and their progeny express the fluorescent reporter, meaning that if new cardiomyocytes are labelled they have originated from the pre-existing pool. If not, it can be assumed that they have been derived from a non-cardiomyocyte progenitor. Two studies using this approach in the zebrafish have shown that almost all (~95%) new cardiomyocytes after injury are labelled, suggesting that cells in the pre-existing cardiomyocyte pool are capable of re-entering the cell cycle, dividing and migrating in order to effect regeneration.[28,29] In the mouse, new cardiomyocytes formed during ageing and neonatal heart regeneration are also derived from

the pre-existing cardiomyocyte pool.[21,30] In the infarcted non-regenerating adult mouse heart, some data support a minor contribution of a non-cardiomyocyte progenitor population (e.g. c-kit+ cells) to the development of new cardiomyocytes, but this remains controversial.[31,32]

The concept of regeneration driven primarily by existing cardiomyocytes is a paradigm shift. Many of the first generation cell-therapy trials (Table 1) were inspired by the concept of bone-marrow derived progenitors, which had been reported to differentiate into cardiomyocytes, but this has been progressively discredited.[33-35] The benefits of bone marrow mononuclear cells and mesenchymal stem cells seen in some clinical studies (Table 1) are increasingly ascribed to paracrine effects. Understanding whether human mature cardiomyocytes can proliferate, and the mechanisms by which this is controlled, is now a major research focus.

Cardiomyocyte proliferation in development, ageing and injury [H3]

Cardiomyocytes in mice and humans proliferate during heart development *in utero* and early postnatal life. After birth, most cardiomyocytes exit the cell cycle, a variable subset (~25% in humans) undergoing a further cycle of nuclear division without cell separation (cytokinesis), resulting in binucleation.[36] In humans, the adult heart has thus traditionally been considered incapable of further cell division, with growth being achieved by hypertrophy. In fact, increasing evidence suggests that cardiomyocytes do renew in the human heart, albeit at a low level. Relying on integration of carbon-14 into DNA during Cold War nuclear testing, Bergmann *et al* estimated that cardiomyocyte self-renewal occurs at a rate of 1% per year for adults aged 25 years, decreasing to 0.45% by 75 years.[37] Histological analysis of the human heart has identified phosphorylated histone H3, a marker of mitosis, in adults up to the age of approximately 20 years.[38] However, despite the apparent capacity for renewal, MI fails to activate effective proliferation. In the adult mouse, using co-registration of cardiomyocyte fluorescent labelling and [^{15}N]thymidine labelling of DNA replication, Senyo *et al* reported that only 3% of infarct zone cardiomyocytes initiate DNA replication and nuclear division. Furthermore, whilst these cells became binucleated, almost none underwent cytokinesis to form a new daughter cell.[30]

Control of cardiomyocyte proliferation [H3]

Environmental cues in the post-natal environment have been implicated in proliferative arrest of mammalian cardiomyocytes. After birth, the heart transitions from the relatively hypoxic intrauterine environment to normoxia, which is associated with a shift from glycolysis to oxidative phosphorylation, increased mitochondrial content and activity, production of reactive oxygen species and cardiomyocyte cell cycle arrest.[39] Scavenging of reactive oxygen species prolongs the neonatal regenerative window, whereas hyperoxia shortens it. During regeneration, re-activation of cell cycle activity is associated with hypoxic activation of Hif-1α.[40] Other factors have been implicated in the loss of proliferative capacity postnatally, including upregulation of p38 MAPK and Meis1.[41 42] Meis1, a member of the three amino acid loop extension transcription factor family, promotes cell cycle arrest via activation of the cyclin-dependent kinase inhibitors p15, p16 and p21. Inhibition of Meis1 extends the regenerative window in neonates and is capable of reactivating the cell cycle in adults.

Many of the emerging ligands and signalling pathways which govern cardiomyocyte proliferation in regeneration are familiar from developmental biology (Figure 2). Neuregulin1 (NRG1), an agonist for the ErbB2 and ErbB4 receptor tyrosine kinases of the epidermal growth factor receptor family, is a key mitogen during heart development.[43,44] It is reactivated in perivascular cells during zebrafish heart regeneration and overexpression enhances cardiomyocyte proliferation even in the uninjured heart.[45] The transcription factor Hand2 is critical to cardiomyocyte development from the second heart field, with zebrafish mutants having a reduction in cardiomyocytes and mice showing abnormalities in the right ventricle and outflow tract region.[46,47] Hand2 is upregulated in the injured zebrafish ventricle, and overexpression is sufficient to drive cardiomyocyte proliferation.[48] Similarly, Gata4, a zinc finger transcription factor known to regulate cardiomyocyte differentiation, migration, hypertrophy and survival, is required for neonatal mouse heart regeneration, acting via FGF16 to stimulate proliferation.[49] The Hippo pathway, comprising series of proteins which regulate the transcription factor YAP and its co-activator TAZ, is an evolutionarily conserved regulator of cell proliferation, growth, viability and organ size.[50] Forced expression of YAP in development leads to hyperproliferation and cardiac enlargement, and in mouse

regeneration, YAP promotes proliferation acting via insulin-like growth factor and Wnt signalling pathways.[51,52] Finally, as in development, regeneration requires chromatin remodelling by Brg1, which controls proliferation through pro-proliferative Bmp10 and inhibition of p57*kip2*.[53] Inhibition of Brg, or other SWI/SNF components such as baf60c and baf180, leads to blunted proliferation and failed regeneration.[54]

Despite these insights, whether a mature mammalian cardiomyocyte can re-enter cell cycle and progress through to cytokinesis is unclear. In the zebrafish, cardiomyocytes are mononuclear and relatively “primitive”, and this appears to be a critical factor determining their ability to divide. Proliferation of the mononuclear cardiomyocyte population can be induced by NRG1 treatment after MI in mice, and based on nuclearity, the adult human heart might be more ‘regenerative’ than the mouse since it contains a higher proportion of mononuclear cardiomyocytes.[55] In addition, distinct subsets of cardiomyocytes may have differing abilities to proliferate: in zebrafish, the outermost ‘cortical’ zone proliferates early and rapidly in regeneration.[56] Cardiomyocyte proliferation requires cellular ‘de-differentiation’, defined by increased intercellular separation and loss of sarcomeric and Z-disc structure.[28] Dedifferentiation has been observed in the mammalian heart, and may be triggered by oncostatin M, a macrophage-derived cytokine related to IL6, but is poorly characterised at a molecular level.[57]

Research priorities in the mammalian heart include identifying and tracking the key cardiomyocyte sub-population(s) capable of division in the adult, based on ploidy or other discriminating features. Characterising the markers and transcriptional pathways of cardiomyocytes undergoing dedifferentiation, replication, migration and maturation would be valuable and is achievable with single cell sequencing approaches.[58] The precise mechanisms which direct cardiomyocytes to undergo cell division as opposed to polyploidization (which seems to suppress further cell cycle-entry) also remain poorly understood.[59] In parallel, understanding the hierarchy, relative importance and overlap of extrinsic cues would help prioritise therapeutic targets. For example, alongside the soluble ligands discussed above, the autonomic nervous system is emerging as a regulator of

cardiomyocyte proliferation and may be potentially ripe for adjunctive targeting.[60,61]

Neovascularisation: the role of the epicardium [H2]

Whilst PPCI is effective at restoring coronary blood flow in the major epicardial vessels following MI, failure to perfuse the microcirculation (microvascular occlusion, MVO) is common and is associated with poor wound healing, ventricular remodelling, heart failure and reduced overall survival.[62] MVO is multifactorial and occurs due to endothelial cell death, inflammation, and physical plugging by thrombotic or plaque debris. Restoration of effective myocardial perfusion by regeneration or repair of the coronary microcirculation, comprising vascular endothelium, smooth muscle, fibroblast and pericytes, will be essential to achieve heart regeneration.

Coronary formation during development [H3]

Coronary formation during development is a blueprint for formation of new vessels. Coronary endothelial cells arise primarily from the sinus venosus, with an additional contribution from the inner lining of the heart, the endocardium, which generates coronary endothelium for the interventricular septum.[63-65] The program of coronary vascular formation is directed by the epicardium, also known as the visceral pericardium, which acts as both a source of trophic factors and progenitor cells.[66] Formed at embryonic day 9.75 in the mouse (human Carnegie stage 11), the epicardium is an epithelial sheet which envelops the growing heart.[67] Epicardial-derived cells (EPDCs) invade the underlying myocardium and undergo epithelial-to-mesenchymal transition (EMT), giving rise to pericytes, smooth muscle and adventitial and interstitial fibroblasts.[63,68 69] The epicardium also begins a signalling *pas de deux* with the myocardium, secreting growth factors supporting vasculogenesis and mitogens which support cardiomyocyte proliferation.[66] Physical or genetic ablation (through deletion of epicardial genes such as *Wt1*) leads to defects in coronary vessel formation and impaired cardiomyocyte proliferation.[70,71]

Coronary revascularization following injury [H3]

The epicardium is quiescent in the adult heart, but is reactivated and expands in response to injury.[72,73] Reactivation is associated with expression of an embryonic gene profile and recapitulation of its developmental functions, supporting repair and neovascularisation. Ablation of tcf21 positive cells in the epicardium after ventricular resection in the zebrafish led to a reduction in cardiomyocyte proliferation, delayed neovascularisation and incomplete regeneration at 30 days.[74] In adult mice, priming of the epicardium prior to injury with the small peptide thymosin β4 (Tβ4), or treatment with epicardial cell conditioned media, improves neovascularisation and functional outcomes after MI.[75-77] Similarly, stimulation of the epicardium with Tβ4 in the neonatal mouse can extend the temporal window for regeneration.[78]

The precise mechanisms of coronary revascularisation following injury are not well defined. There is evidence to support both local proliferation of endothelial cells and a contribution from a remote stem cell source, the endothelial progenitor cell, a controversy which is discussed in detail elsewhere.[79] In the neonatal mouse heart, neovascularisation is achieved primarily by formation of large collateral arteries which bypass the ligation site. These derive from pre-existing arteries through a process of arteriogenesis, rather than arterialization of the pre-existing capillary network.[80] Clues from non-cardiac injury models (e.g. zebrafish fin and retina) suggest that neovascularisation during regeneration is dependent on classical angiogenic signalling mechanisms involving VEGFR, HIF-1, and Cxcl12. [81-83]

Neovascularization is supported by the epicardium via a number of secreted factors, including retinoic acid, fibroblast growth factors, VEGF-A and SDF1.[84,85] Blockade of Fgf signalling in zebrafish leads to a failure of EMT and neovascularisation (discussed further below), in turn leading to failed regeneration.[73] In the mouse, reactivation of epicardial EMT following MI contributes several cell lineages to support repair. By inducible labelling of the *Wt1* population, EPDCs have been shown to contribute to fibroblasts, myofibroblasts, smooth muscle cells and adipocytes.[77,86] Whether the reactivated epicardium can contribute other cell types, specifically cardiomyocytes or endothelial cells, is debated. When pre-primed with exogenous Tβ4, the epicardium can generate extremely limited numbers of cardiomyocytes,

but this does not occur without priming and is not seen in zebrafish regeneration.[75,87-89] Finally, an emerging mechanism relates to epicardial-immune cell cross-talk. The epicardium is required for seeding of the heart with tissue-resident macrophages during embryonic development, and macrophages colocalise with the epicardium following injury.[90] Recently, the epicardium has been shown to mediate an immunosuppressive response to MI via modulation of cytokines which promote regulatory T-cell recruitment to the heart.[91] In the mouse, genetic knock-out of epicardial YAP/TAZ led to persistent inflammation, widespread fibrosis, heart failure and death following MI.[91]

The epicardium is increasingly recognised to be a highly heterogeneous cell population containing both mesenchymal and haematopoietic cells within specialised clusters.[92] Dissecting apart their respective roles, and separating the paracrine and progenitor cell components of the epicardial response, will require improved markers for different subpopulations. Recently at least three new subpopulations have been reported in zebrafish, including a role for novel genes such as caveolin 1.[93] Understanding how the epicardial response is modulated in the setting of regeneration in comparison to scar formation, for example to repress fibroblast formation, is a key research requirement in order to optimally harness its therapeutic effects.

Inflammation and immune system activation [H2]

MI leads to extensive cell death and is a potent activator of inflammation.[94] In mice and humans, inflammation is linked to repair through a biphasic immune response: an early pro-inflammatory phase characterised by release of cytokines and recruitment of neutrophils and monocytes, followed by a reparative phase with resolution of inflammation, activation of myofibroblasts and deposition of collagen-based scar.[95,96] This process is patterned and under tight spatiotemporal regulation. Macrophages, for example, have diverse functions through the course of injury including pro-inflammatory cytokine production, phagocytosis of necrotic cell debris, pro-angiogenic signalling, activation of fibroblasts and remodelling of the ECM.[97,98] Improved cell surface markers and transcriptional profiling are helping to define the

identity and function of specific monocyte-macrophage, neutrophil and T cell subsets in the injured heart.[99-101]

Inflammation is not a barrier to tissue regeneration and in fact may provide the initial pro-regenerative cues. In the zebrafish, for example, brain injury activates acute inflammation which is sufficient to drive neural proliferation via leukotriene C4.[102] The cellular immune response to injury is has also been directly implicated in healing by regeneration.[103] In regenerating muscle, infiltrating cells including macrophages, eosinophils and T-regulatory cells influence activation of satellite cells and fibroadipogenic progenitors to specify production of new myofibres.[104,105 106] Macrophages also commit endothelial progenitors to capillary formation, suppressing an alternative EMT pathway.[107] After liver injury, macrophage secretion of Wnt3a controls lineage differentiation of hepatic progenitor cells to produce hepatocytes.[108] Similarly, macrophage production of Wnt7b in the injured kidney is required for regeneration.[109]

Inflammation and the immune response has also been linked to heart regeneration. Activation of inflammation stimulates cardiomyocyte proliferation in the neonatal heart, and blockade of IL6 or cardiomyocyte STAT3, its downstream effector, blocks heart regeneration after apical resection.[110] In the neonatal MI model, macrophages are actively recruited to the neonatal heart and treatment with clodronate liposomes to ablate macrophages blocks regeneration through inhibition of angiogenesis.[111] Divergent roles for distinct macrophage subsets are emerging, notably between tissue-resident macrophages, seeded to visceral organs during embryonic development, in comparison to those which derive from circulating monocytes, from bone marrow or splenic reservoirs, during acute inflammation. Using a cardiomyocyte-ablation model, Lavine *et al* found that depletion of resident macrophages in the neonatal heart led to reduced cardiomyocyte and endothelial cell proliferation, interstitial fibrosis and chamber dilatation.[112] In contrast, inhibition of CCR2+ macrophages, derived from monocytes, preserved embryonic subsets and improved myocardial repair.[112]

Multiple injury models suggest that inflammatory signalling is required as a trigger to induce

cell proliferation or differentiation of progenitors to restore the lost tissue substrate. The precise mechanisms behind the divergent outcome of inflammation in regeneration and scar forming models, however, remain to be fully elucidated. The capacity for tissue regeneration appears to be inversely correlated with evolutionary complexity of the immune system, which has led to the suggestion that a component of the mammalian immune response might be a barrier to regeneration.[113] Dissecting apart pro-regenerative signals from those which drive fibrosis or scar deposition, or the mechanisms by which the same signals drive divergent healing, will require detailed comparisons of immune-cell signalling in regenerative and scar-forming models. In addition, further characterisation of the interplay between immune cell subsets, fibroblasts, endothelial cells and cardiomyocytes is required. Potential therapeutic approaches to harness the regenerative potential of inflammation are discussed below.

Role of the extracellular matrix [H2]

The extracellular matrix (ECM) consists of an organised and dynamic meshwork of proteins.[114] Previously thought of as an inert structural scaffold, the ECM is now recognised to have a number of biological effects and influences cell proliferation, migration, lineage specification, intercellular signalling and growth factor presentation.[115] Control of the extracellular matrix is a critical component of regeneration. In the newt limb, upregulation of ECM-remodelling matrix metalloproteinases (MMPs) occurs within hours, and inhibition of MMPs blocks regeneration.[116] This is associated with early deposition of a primitive ECM consisting of hyaluronic acid, fibronectin and tenascin-C, and downregulation of collagen.[117] Production and remodelling of ECM components also occurs in scar-based healing, but this 'scarring' ECM is compositionally distinct and appears to be directed by immune-cell control of fibroblasts to produce collagen.[118]

In the heart, differences in ECM structure may be an important aspect underlying the interspecies capacity for regeneration. While fish have a non-compacted, spongy myocardium designed to function at low arterial blood pressure, the 4-chambered adult mammalian heart is highly compacted in a rigid matrix.[119] In the neonatal mouse, stiffening and maturation of the ECM is correlated with cardiomyocyte cell cycle arrest and *in vitro*, modulation of ECM compliance directly influences the ability of cardiomyocytes to undergo proliferation and cytokinesis.[120] Interestingly, if the failing human heart is off-loaded by implantation of a left ventricular assist device, cell cycle re-entry has been observed – an effect which might be mediated by permissive ECM changes.[121]

The ECM also has direct biological effects on cardiomyocytes. The decellularised zebrafish ECM can induce cardiomyocyte proliferation and cardioprotection in the mouse heart, an effect which is mediated via the ErbB2 receptor.[122] Functional ECM components are emerging from candidate approaches and unbiased screens. Fibronectin, derived from the epicardium after injury, stimulates zebrafish heart regeneration.[123,124] Periostin, a matricellular protein, promotes cell cycle activation in mononucleated cardiomyocytes, but also activates fibroblasts.[125] Small and large animal studies of periostin have shown improved healing after

MI, but at the expense of increased fibrosis.[126,127] Hyaluronic acid and its receptor hyaluronan-mediated motility receptor are required for EMT and zebrafish regeneration.[128]

The roles of the ECM during regeneration and scar formation are still emerging, and thus much of the work is descriptive and early stage. The compositional differences in the ECM during regeneration and scar formation, and the biological activity of these proteins on downstream cell types, are not yet fully defined. Transcriptional profiling of fibroblasts, the major source of ECM components, and mass spectrometry of the ECM in regeneration versus scar formation, would begin to address these questions. It remains unclear to what extent ECM components can drive regeneration *in vivo* in an otherwise non-regenerative environment, and whether the ECM represents a standalone therapeutic target in endogenous regeneration. Harnessing the biological effects of the ECM is also of value to cell therapy strategies, where survival, localisation, and engraftment of cells may be augmented using patches or bioscaffolds with engineered matrix properties.[129]

Scar formation and degradation [H2]

Regeneration and scar formation lie at opposite ends of the spectrum of repair. From an evolutionary perspective, it is unclear whether regenerative capacity was lost accidentally, as a neutral trait, or whether it was selected against. While it seems intuitive that regeneration would confer a survival and reproductive advantage, costs associated with energy, time or interim function (e.g. electrical stability) may have made it advantageous to heal by rapid scar formation[130,131]

Following acute MI, scar is rapidly laid down in the mammalian heart by activated myofibroblasts. In the short term this scar is critical to providing mechanical strength and prevention of ventricular rupture. Fibroblasts are originally derived from the epicardium and endocardium during embryonic development through EMT.[132] Using a periostin inducible Cre line for lineage tracing, Kanisicak *et al* showed that activated myofibroblasts derive from tissue-resident (tcf21+) fibroblasts, producing large quantities of extracellular matrix components such as collagen, and deactivating following resolution of injury.[133] In the

reparative phase, fibroblast activation following MI is multifactorial but intricately linked to macrophage cytokine regulation, for example by TGFβ and CTGF. Interestingly, TGFβ has been identified as a key pro-regenerative cytokine in the axolotl – but the mechanisms by which fibroblast activation are suppressed are not clear.[134]

The relationship between scar deposition and regeneration is complex. It has been proposed that these two events are diametrically opposed and compete in order to achieve organ repair, with collagen deposition directly inhibitory to regeneration.[135] However, knock-down of scar formation by astrocytes following spinal cord injury is not sufficient to induce axonal regrowth.[136] Furthermore, in the cryoinjury model in the zebrafish heart, even despite extensive scar deposition during the first 3 weeks following injury, regeneration still occurs, with progressive scar removal and replacement with cardiomyocytes over time.[19] The finding that scar is not necessarily a barrier to regeneration has enormous therapeutic implications, and suggests that the regenerative program does not necessarily need to be established in the early injury phase.

Strategies for therapeutic regeneration [H1]

Efforts towards heart regeneration encompass a broad spectrum of approaches including cell therapy, biomaterials, tissue engineering, reprogramming, and modulation of endogenous repair (Figure 3a). This section will focus primarily on therapeutic strategies which exploit insights from developmental biology, including specification or programming of the cardiomyocyte lineage, and endogenous regeneration.

It should be noted that distinct approaches will be required for patients post-MI and patients with chronic HF. MI provides a dynamic environment of repair in which endogenous pathways can be modulated towards regeneration. In contrast, in the setting of stable HF, strategies to directly provide new cardiomyocytes should be the focus. Current and future strategies for production of new cardiomyocytes and targeting of endogenous repair are discussed in turn (Figure 3a, b).

Cardiomyocyte replacement [H2]

Activation of cardiomyocyte proliferation [H3]

Initial attempts to reactivate cardiomyocyte proliferation were inspired by insights into cell cycle regulation by the cyclin and cyclin-dependent kinase (Cdk) system.[36] Cyclin-Cdk complexes modulate members of the retinoblastoma gene family (Rb, p107, p130), which in turn lead to release of E2F transcription factors which activate genes for DNA synthesis. Overexpression of cyclin B1-CDC2[137] or knockdown of the cyclin-dependent kinase inhibitors p21, p27 and p57 by RNA interference, are effective at inducing DNA replication *in vitro*.[138] *In vivo,* overexpression of cyclin A2, D1 or D2 stimulates DNA synthesis, and cyclin D2 overexpression leads to improved repair following MI.[139,140] Other approaches have included knockout of tumour suppressor genes Rb (Rb1) and p130, and direct targeting of the E2F transcription factor family.[141] Combinatorial reprogramming of cardiomyocytes informed by microarray approaches to define the proliferative state have proved to be more effective at achieving improved repair.[142] In general, however, these approaches have triggered relatively modest DNA replication, very limited cytokinesis and consequently little new cardiomyocyte mass.

Therapeutic targeting of upstream signalling pathways regulating proliferation may reduce the risk of teratogenicity which is inherent in directly targeting the cell cycle. For example, exogenous injection of NRG1 activates proliferation of mononucleated cardiomyocytes via the ErbB4 tyrosine kinase receptor and PI3K, leading to improved repair following experimental MI.[55] Similarly, delivery of FGF1 with blockade of the p38 MAPK pathway, a key mediator of cardiomyocyte differentiation, promotes myocardial repair following injury.[143] A recent porcine MI study showed that percutaneous intramyocardial injection of microparticles loaded with NRG1 and FGF1 is effective at inducing improvement in LV function following MI, with reduced remodelling and improved angiogenesis (Table 2).[144] However, the growth response is exquisitely regulated: in the zebrafish, Notch activation is required for cardiomyocyte proliferation, but hyperactivation of Notch inhibited rather than promoted heart regeneration.[145]A phase I study of recombinant Neuregulin 1β3 (cimaglermin alfa) in patients with chronic heart failure has recently shown early promise, with evidence for safety and preliminary findings suggesting an improvement in LV function at 90 days (Table 3).[146]

Therapeutic cardiomyocyte proliferation can also be induced by micro-RNA targeting. Screening of a whole genome miRNA library, identified 40 miRNAs which increased both DNA synthesis and cytokinesis *in vitro,* two of which, ~~has-~~miR-590 and ~~has-~~miR-199a, stimulated cardiac regeneration in adult mice after experimental MI.[147] Similarly, overexpression of the miR302-367 family is sufficient to improve regeneration following adult MI, acting via repression of the Hippo pathway.[148] In contrast, the miR-15 family is upregulated post-natally, correlating with the shutdown of cardiomyocyte proliferative capacity. Delivery of anti-miR15 led to increased proliferation in both the cardiomyocyte and non-myocyte compartments after MI at day 21, resulting in significant improvement in functional outcome.[21]

A major outstanding challenge in the field is that reactivation of the cardiomyocyte cell cycle is frequently not followed by completion of cytokinesis to generate new daughter cells. Identifying therapeutic factors, miRNAs or small compounds which can drive bona fide cytokinesis would benefit greatly from improved readouts of completed cell division. Efforts

towards this include the anilin-GFP model which provides a potential mechanism for discriminating cytokinesis from endoreduplication.[149] Despite these recent advances, accurately quantifying dividing cardiomyocytes within the adult mammalian heart is extremely difficult and would benefit greatly from further research focus.

De novo cardiomyocytes by cellular (re)programming [H3]

Production of *de novo* cardiomyocytes by directed differentiation of embryonic stem (ES) cells, or reprogramming of differentiated non-myocyte cells (e.g. fibroblasts) to a cardiomyocyte fate has revolutionised therapeutic approaches to regeneration.[26,150] Interestingly, reprogramming by transdifferentiation is a strategy deployed in endogenous regeneration, first recognised in the newt lens over 100 years ago.[151] The zebrafish utilises transdifferentiation of alpha to beta cells to regenerate its islets, and in the heart can reprogram atrial to ventricular cardiomyocytes after injury.[152,153] Reprogramming of hepatocytes to biliary endothelial cells has been described in murine liver regeneration.[154]

Large numbers of human cardiomyocytes can be produced by differentiation of ES cells. Chong *et al* demonstrated that transplanted hESC-CMs (at a dose of 1×10^9 per heart) survive and contribute new myocardium to the macaque heart after direct injection two weeks following MI.[155] Although a powerful proof of concept, evidence of functional improvement was lacking and ventricular arrhythmias were recorded in all animals, suggesting that significant hurdles with electrical integration remain before human trials could safely be undertaken (Table 2). Furthermore, ethical concerns exist about use of embryonic tissue, and as an allogeneic product, recipients would require lifelong immunosuppression to prevent rejection of the cells.

Reprogramming of fibroblasts to cardiomyocytes offers potential advantages: a non-immunogenic cell product, derived from the patient's own cells, and no requirement for the destruction of embryos. Inspired by the revolutionary description of induced pluripotency by Yamanaka, reprogramming approaches were initially undertaken *ex vivo*, relying on an intermediate induced pluripotent stem (iPS) cell stage.[156] Transplanting iPS-derived

cardiomyocytes in a non-human primate model, Shiba *et al* showed improvement in function following cell transplant, but also a significant ventricular arrhythmia rate (Table 2).[157] However, production of an autologous cell product is expensive, subject to variability, and has restricted commercial opportunity. In addition to biological hurdles, the logistical and financial barriers to clinical use of autologous cell therapies are extremely challenging. Current cell numbers in the macaque trials have been of the order of 10^8-10^9 cells, with at least an order of magnitude higher required for humans. The infrastructure and running costs of good manufacturing production at this scale, ideally embedded within or close to clinical cardiac centres, appear prohibitive.

The discovery that delivery of three cardiac developmental transcription factors, Gata4, Mef2c and Tbx5 could drive direct reprogramming of fibroblasts into cardiomyocytes, without an intermediate cell stage, has opened the door to the concept of reprogramming *in vivo*.[158,159] A number of additional factors which improve the efficiency of reprogramming have now been identified, most notably Hand2.[160] This approach requires no cell product, obviating the need for complex manufacturing. Proof of concept for *in vivo* reprogramming has now been demonstrated in mice, with transdifferentiated cells expressing sarcomeric proteins, forming gap junctions, and driving sustained improvement in ventricular function.[161-164] Substantial challenges remain, including achieving selectivity of targeting to cardiomyocytes, reprogramming human cells which have stable epigenetic modifications, and achieving maturation of structure and function in reprogrammed cells.

Neovascularisation & lymphangiogenesis [H2]

Strategies aimed at neovascularisation in MI have been somewhat frustrated in recent years by the failed promise of vascular endothelial growth factor (VEGF-A). Despite a number of animal studies showing efficacy from the recombinant protein or gene therapy with VEGF-A, the double blind EUROINJECT-ONE and NORTHERN clinical trials failed to show benefit.[165,166] Attempting to reactivate more comprehensive developmental programs of coronary vessel formation, including targeting retained adult cell types that previously contributed to the developing coronaries, including the coronary sinus (sinus venosus-

derived), endocardium and epicardium is a more attractive strategy for invoking neovascularization post-MI.

The epicardium in particular has emerged as a viable target and the development of *in vitro* systems for culture of human epicardium from human pluripotent stem cells will facilitate both biological understanding of the heterogeneity of the epicardium and small molecule screening for activating compounds.[167] Recently, epicardial FSTL1 was identified as a key anti-apoptotic and proliferative factor promoting myocardial regeneration after injury. Application of a patch containing recombinant human FSTL1 improved long-term cardiac function in both rodent and swine models of myocardial infarction (Table 2).[168] Furthermore, Zangi *et al* showed that intracardiac injection of a modified RNA (modRNA) encoding VEGF-A led to enhanced epicardial progenitor activation and improved functional outcome after MI.[169] Functionally, VEGF-A modRNA promoted differentiation of EPDCs towards an endothelial (and in small numbers cardiomyocyte) cell fate.[169]

Clinical translation would benefit greatly from the ability to image the epicardium *in vivo*, and emerging technologies using PET, SPECT and molecular imaging to demonstrate angiogenesis will guide future trials.[170] The endocardium continues to provide further endothelium for vessel formation for a short period during postnatal growth and might be reactivated in adulthood to enhance neovascularisation.[171] Interestingly, experimental MI in mice has recently been shown to activate endothelial remodelling on the endocardial surface, leading to outgrowth of pre-existing coronary vessels and *de novo* arteriogenesis.[172]

Stimulation of new lymphatic vessel formation, lymphangiogenesis, is another emerging strategy to augment repair. The cardiac lymphatics remain poorly understood but are important for transport of interstitial fluid and trafficking of immune cells.[173] Following MI, endogenous repair mechanisms activate lymphangiogenesis in mice and humans, a response which is required for clearance of oedema and resolution of inflammation.[174] In rodents, augmentation of lymphangiogenesis by stimulation of VEGF-C signalling, the principal cytokine mediator of lymphatic formation during development, improves healing,

reduces fibrosis and preserves myocardial function.[175,176] The precise mechanisms underlying this are poorly understood but may relate to clearance of oedema and resolution of inflammation.

Immunomodulation [H2]

Components of functional pathways, which can loosely be termed “inflammation”, are intricately linked to healing following MI. A number of unsuccessful clinical trials of immunosuppressive agents (e.g. methylprednisolone, immunoglobulin, pexelizumab, anakinra; see Table 3) suggest that blunt inhibition of inflammation is not effective.[177] Successful immunomodulation is likely to require both more nuance stratification of patients based on known activation (or inhibition) of specific pathways, coupled with identification and successful *in vivo* targeting of specific immune cell subsets, or pathways, to bring about beneficial repair. For example, inhibition of the CCR2+ monocyte population mobilised after MI using nanoparticle-delivered anti-CCR2 siRNA led to reduced injury and cardiac remodelling in mice.[178] Transplantation of specific immune cell populations is a strategy being tested in other diseases: autologous macrophages are being trialled for regression of liver fibrosis[179], and infusion of T-regulatory cells has shown efficacy in reduction of inflammation. Furthermore, understanding the differences of the immune response in the setting of scar formation compared to regeneration will shed light on precise pathways which can be modulated to enhance regeneration without compromising repair. In the chronic HF patient, immunomodulation is unlikely to be sufficient to induce regeneration. However, immunosuppression or induction of tolerance will be required to prevent rejection of allogeneic cardiomyocyte cell therapies, or vectors used to deliver a reprogramming cocktail.

Fibrosis inhibition [H2]

Inhibition of pro-fibrotic signalling in the setting of chronic HF has been suggested to account for some of the existing benefits of beta-blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers and statins. A number of novel anti-fibrotic compounds are emerging and have recently been reviewed in the context of cardiovascular disease.[180] Inhibition of scar formation after MI may have a complementary role to stimulation of endogenous repair or cell therapy, but without effective replacement of cardiomyocytes, remains critical to prevent cardiac rupture. Targeting late ‘reactive’ fibrosis in the uninjured myocardium, however, may reduce ventricular remodelling and progression to heart failure.[181]

For example, in addition to its effects on cardiomyocyte proliferation, NRG1 administered between 7-35 days following MI reduced fibrosis and remodelling in swine through inhibition of myofibroblast transdifferentiation.and TGFβ signalling.[182] Inhibition of chymase, which stimulates fibroblast activation after injury, reduced fibrotic area and improved survival following MI in small animal models.[183,184] In rats, blockade of platelet derived growth factor (PDGF) receptor signalling with imatinib selectively inhibited fibrosis in the non-infarcted myocardium.[185] Other prospective anti-fibrotic strategies in early stage studies include inhibitors of Wnt and histone deacetylases, and modulation of relaxin signalling (Figure 3b).[180] Prior to clinical trials in humans, an improved understanding of the heterogeneity of human post-MI healing is required, to allow targeting of anti-fibrotic therapies to groups at highest risk. For example, the use of advanced imaging and novel biomarkers of inflammation and fibrosis in the early phase of MI may identify outlier patient subpopulations who could then be targeted with specific therapies.

Translational challenges and considerations [H1]

The last decade of clinical cell therapy trials have provided important lessons for the design and translation of future regenerative therapies.[186,187] Issues related to the use of animal models, drug discovery approaches, patient selection, and clinical trial design will be discussed in turn.

Limitations of animal models [H2]

Animal models have unquestionable value as a route to novel biological insights, and there is extensive species conservation in regenerative signalling pathways such as Notch, TGFβ, and JAK/STAT.[188] From a translational perspective, however, there is a pressing need to improve the filtering of therapeutic targets and compounds prior to human studies. Many recent clinical trials of compounds targeting cardiac repair have shown limited efficacy (Table 3) and this has led to questions regarding the validity of animal models for assessing novel therapeutic strategies.

For regenerative therapies, a key problem inherent to existing animal studies is the use of young, healthy, homogeneous populations which lack the comorbidities or drug confounders typically associated with human patients. Age is inversely correlated with capacity for repair, with older mice demonstrating impaired wound healing after MI.[189] In young animals, the 'bar' to improved repair is low, leading to false positive results and subsequent failed large animal or human studies. Development of highly *non-regenerative* animal models, such as aged rodents or pigs, might help reduce false positive studies prior to human trials.

Drug discovery [H2]

Focused drug discovery approaches to cardiac regeneration are complicated by the breadth of therapeutic strategies which persist in the academic arena, ranging from cell replacement, to reprogramming, to stimulation of endogenous regeneration (Table 2; Figure 3a). To address this, a move towards use of phenotypic screens, *in vivo* discovery and combination approaches will be required, with underlying biological insights used for target deconvolution. For example, screening for cardiomyocyte cell cycle reactivation *in vivo* using the FUCCI

fluorescent reporter in zebrafish has been used to identify compounds targeting the Hedgehog, insulin-like growth factor and TGFβ pathways which effectively stimulate cell cycle re-entry.[190] Furthermore, the 'FunSel' screening approach, whereby a cDNA library of the mouse secretome is targeted to cardiomyocytes *in vivo* using an adenoviral vector, has been applied to identify cardioprotective factors.[191] Targeted sequencing to identify factors which are enriched after MI revealed that cardiomyocytes transfected with ghrelin improved cell survival after injury.

Once novel screening systems are established, partnerships with pharma should be established early in order to access screening libraries and improve the process of lead generation and optimization. Many paradigms which are effective *in vitro* or in pre-clinical models may of course fail to translate into the clinic. Assessment of compounds across multiple assays, or screening to assess the cumulative benefit of targeting of multiple parallel pathways for a given endpoint (for example, cardiomyocyte proliferation) may result in improved clinical efficacy downstream.[192,193]

Delivery systems for regenerative therapies [H2]

Targeting the heart is achievable through a combination of local delivery, biomaterial adjuncts and biological selectivity. For delivery, advances in catheter technology have made transendocardial injection, subepicardial access and intracoronary injection available by percutaneous, minimally-invasive approach.[194] Surgical approaches by median sternotomy may be reasonable for proof of principle but are not realistic for the frail, comorbid HF population at large. Alongside delivery, major advances have been made in the field of biomaterials and bioscaffolds.[129] Pre-seeded scaffolds, patches or injectable hydrogels have been shown to improve retention and survival of transplanted cells.[195] For non-cell based approaches, delivery of factors within hydrogels or coated on microparticles can prevent rapid clearance or degradation.[196] Local delivery and retention must be complemented by biological targeting (e.g. targeted liposomes, exosomes, or viral vectors) or biological selectivity for cardiomyocyte pathways. The risks of off-target effects are greatest for pro-proliferative or reprogramming approaches, which may disrupt remote cell function or be tumorigenic.[197]

Patient selection [H2]

Targeting therapies to selected patient groups will be key to the success of future regenerative therapy trials. Two distinct groups with separate biological and logistical challenges are patients with acute MI and patients with chronic HF.

The acute MI population is attractive for regenerative therapies which modulate or harness existing repair pathways, for example factors to promote cardiomyocyte proliferation or angiogenesis, epicardial targeting, immunomodulation or inhibition of fibrosis. The gain from such therapies might be expected to be greatest in young patients without comorbidities, in whom the barrier to endogenous regeneration is lowest. However, identifying precisely which MI patients to target is difficult as accurate predictors of future HF are lacking. Although age, diabetes, coronary physiology or imaging parameters (e.g. oedema, haemorrhage or microvascular obstruction) are predictors of final infarct size in cohorts at large, it remains difficult to accurately predict future HF events for a given patient. Practically, this means it may be difficult to justify the use of the highest-risk interventions (e.g. cell reprogramming) in patients.

Better identification of patients at risk of HF is required: emerging possibilities for enhanced characterisation derive from novel, mechanistically relevant biomarkers, including exosome characterisation, miRNA profiling and cellular transcriptomics.[94] The goal is to develop diagnostics that provide quantifiable data on therapeutically relevant targets, which can be used to recruit patients to specific therapies. As noted in the cancer field, it seems likely that optimal characterisation will require approaches that combine imaging and panels of biomarkers for mechanistic staging.

In patients with chronic HF, the disease is established and in those with severely impaired left ventricular function and NYHA III-IV symptoms, the prognosis is extremely poor. As such, regenerative strategies which carry higher risk may be reasonable. Implantable cardioverter defibrillators are already indicated in this patient group to protect from arrhythmia and

therefore cardiomyocyte cell therapy, reprogramming or cell patches, which may be arrhythmogenic, should be targeted at this cohort. The subgroup of patients on LVAD therapy as a bridge to heart transplant should also be recruited, as cardiac tissue may become available (at heart transplantation) to examine biological endpoints such as cell engraftment.

Clinical trial design [H2]

Regenerative therapies require re-evaluation of their clinical trial paradigm.[198] Early trials of cell therapies have been small, heterogeneous, and subject to small study effect, in which subsequent studies fail to reproduce an apparently large effect. Improvements in trial design, particularly at the phase II stage, have the potential to reduce this problem.[199] Wherever possible, phase II clinical studies should be designed to demonstrate a defined biological effect as a surrogate for efficacy. This might include angiogenesis, evidence of engraftment, or cell cycle re-entry, and in turn needs development and validation of novel imaging strategies for regeneration.[200] This key step would allow biological insights to be correlated with clinical outcomes, in order to prioritise some therapies for large-scale, placebo controlled trials. These should ideally be carried out as part of consensus regenerative medicine networks such as the Cardiovascular Cell Therapy Research Network, which has been instrumental in organising large studies to show that BMMNCs in acute MI or ischaemic cardiomyopathy do not improve LV function. Furthermore, there is increasing recognition of reporting issues with cell therapy trials, with over 600 reporting discrepancies identified across 49 trials.[201] Of concern, the number of discrepancies correlated with the reported effect size in the trial. There is a need for standardization of cell characterisation, handling, methodology and reporting practice (akin to PRISMA or CONSORT) for trials.

Conclusions

Novel regenerative therapies inspire great hope amongst patients, scientists, physicians and the media. Although this excitement can be warranted, it can easily lead to exaggeration of actual benefits. Balancing the needs of a desperate patient population with the requirements for scientific rigour is challenging. Guidelines from the International Society for Stem Cell Research specifically warn against the dangers of hype as novel therapies emerge.[202,203]

Progress with regenerative therapies is likely to be incremental and iterative rather than a quantum leap.

While complete regeneration of the infarcted heart is the end goal, marginal gains in cardiomyocyte number, neovascularisation or scar reduction are a realistic first step and would have therapeutic value. The emerging biology of endogenous regeneration and cardiomyocyte biology is exposing a multiplicity of therapeutic targets which might be exploited by conventional small molecule approaches, recombinant factors, microRNAs, reprogramming or cell transplantation.

Improving the commercialisation of regenerative therapies requires closer partnership of scientists, spin-outs, pharmaceutical industry and clinical trialists. There is a role for pharma in early partnership with scientists to test compound libraries in novel *in vitro* or *in vivo* screens. Improved pre-clinical models, patient selection and design of early clinical studies to establish biological efficacy in human subjects will help streamline a next generation of regenerative therapy trials. At the national level, governmental support is required, through facilitation of regulation and funding for incubator/facilitator organisations such as the Cell Therapy Catapult (U.K.) and the Centre for Commercialization of Regenerative Medicine (Canada) and CellCAN in Canada.[230]

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Conflict of interest statement

None to declare

Boxes

Box 1: Development of heart failure: pathophysiological mechanisms

Heart failure following acute MI is a paradigm for failed regeneration. Following coronary artery occlusion, ischaemic death of cardiomyocytes begins within hours. Cell injury and death trigger release of proinflammatory cytokines, infiltration of neutrophils and mobilisation of monocytes from the spleen.[204] Opening of the occluded coronary artery by PPCI improves salvage of the injured myocardium, but in the short term leads to a burst of oxidative stress and further cardiomyocyte death. Even after reperfusion, microvascular obstruction (caused by thrombotic and plaque debris and by endothelial damage) persists in up to 50% of patients.[205] Over subsequent days, inflammation drives further infarct expansion at the border zones. A transition from inflammation to repair is characterised by activation of fibroblasts to myofibroblasts, which deposit collagen matrix leading to scar formation.[206]

Chronic remodelling of the damaged left ventricle subsequently occurs over weeks to months, with ventricular dilatation, scar thinning and activation of interstitial fibrosis.[207,208] Reduced cardiac output triggers activation of neurohormonal systems which act to maintain the circulation.[209] Release of angiotensin II and aldosterone drive sodium and fluid retention, and adrenergic system activation maintains blood pressure through vasoconstriction.[210] These mechanisms are initially compensatory but become maladaptive, driving fluid overload, myocardial hypertrophy, and slow but ongoing cardiomyocyte death, leading to further deterioration in ventricular function.[211,212] Incremental benefits may be gained by optimising early salvage, no-reflow and further inhibition of maladaptive physiology, but entirely novel approaches are required to address the fundamental issue of cardiomyocyte death.

Figure Legends

Figure 1

Heart failure therapy pipeline, to present day. In green: landmark breakthroughs in heart failure therapy, with key trials indicated by *. In purple: progress in regenerative therapies for heart failure. Abbreviations: ACEI – angiotensin converting enzyme inhibitors; ARB – angiotensin receptor blockers; BB – beta-blockers; BMMCs – bone marrow mononuclear cells; CMs – cardiomyocytes; ES – embryonic stem cell; CRT – cardiac resynchronisation therapy; HF – heart failure; ICD – implantable cardioverter defibrillator; iPS – induced pluripotent stem cell; MRA – mineralocorticoid receptor antagonists; MSCs – mesenchymal stem cells.

Figure 2

Endogenous mechanisms controlling cardiomyocyte proliferation. Initiation of cardiomyocyte proliferation is regulated by both intrinsic and extrinsic factors. A number of soluble ligands have been identified which promote cardiomyocyte proliferation during development and in models of heart regeneration, including NRG1, FGF1, antagonists of Hippo, Wnts and TWEAK. Other soluble cues implicated in proliferation include OSM1, a macrophage-derived cytokine which directs cardiomyocyte de-differentiation. Downstream transcriptional regulators of cardiomyocyte cell-cycle re-entry include Hand2, Gata4, YAP/TAZ, Hif1α and miRNAs. In the adult mammalian heart, cardiomyocytes exit the cell cycle postnatally and are resistant to cell cycle re-entry, with proliferation inhibited by p38MAPK, Meis1, miR15 and reactive oxygen species. Structural and functional aspects of the extracellular matrix also regulate cardiomyocyte proliferation: periostin and a lack of matrix rigidity promote cardiomyocyte proliferation. Factors which promote cytokinesis following binucleation, to generate new daughter cells, remain poorly defined.

Figure 3

Therapeutic strategies for heart regeneration

a) Strategies for replacement of cardiomyocytes (CMs). From top: (1) Reactivation of cardiomyocyte proliferation is the major mechanism by which endogenous regeneration

occurs in the zebrafish and neonatal mouse, and has been achieved in preclinical models by targeting upstream ligands such as NRG1 and FGF1, and downstream cell cycle pathways mediated by Meis1, YAP/TAZ, p38MAPK, and miRNAs including ~~has-~~miR-590, ~~has-~~miR-199a, and miR302-367. (2) Stimulation of progenitor populations such as the epicardium or cardiac progenitor cells (CPCs) leads to pleiotropic effects to support CM survival and proliferation, but current strategies do not appear to directly lead to new CMs in signficant numbers. (3) *In vivo* reprogramming of fibroblast to produce CMs has been achieved in preclinical models using defined transcription factor cocktails (e.g. GHMT) and microRNAs, and *in vitro* by small molecules. (4). Replacement of CMs by transplantation of exogenous mature CMs, derived from iPS cells or ES cells, have shown proof of concept in large animal studies.

b) Therapeutic targeting of the non-cardiomyocyte compartment for cardiac regeneration. (1) Epicardial activation (top right). During cardiac development and after injury, soluble factors derived from the activated epicardium supports angiogenesis, cardiomyocyte proliferation and survival, including FSTL1, FGF2 and VEGFa. Identifying factors (such as TB4) which activate or direct the epicardium to promote repair, or delivery of specific recombinant factors identified from the epicardium, are unexploited therapeutic strategies. (2) Angiogenesis (red vessels)/lymphangiogenesis (green vessels). Replacement of damaged vasculature will be vital to support new survival of transplanted, reprogrammed or proliferated CMs. Delivery of recombinant VEGFc, a macrophage-derived cytokine which promotes lymphangiogenesis, improves healing and functional outcome following MI in mice. (3) Immunomodulation (bottom right): inhibition of CCL2/CCR2 signalling in monocyte-macrophages by delivery of siRNA nanoparticles reduces monocyte infiltration and infarct size in mice. Signals from the epicardium have recently been identified which recruit regulatory T cells to dampen inflammation following injury. (4) Fibrosis: (left): harnessing endogenous anti-fibrotic pathways mediated by NRG1 or relaxin may be harnessed to halt progression of remodelling and heart failure. Targeting pro-fibrotic pathways with inhibitors of PDGFR, Wnts, chymase or HDAC, have all shown promise in small animal models. Finally, *in vivo* reprogramming of fibroblasts into CMs, using GATA4, Hand2, Mef2c and Tbx5, may allow replacement of CMs, restoration of function and prevent fibrosis. Current therapeutic strategies shown in green,

solid lines; endogenous signalling pathways shown in orange, dotted lines.

Table 1: Landmark studies from the infancy of regeneration: cell therapy trials in acute myocardial infarction & heart failure

Name	Design	Patient group/no.	Cell type/dose/delivery route	Primary endpoint	Outcome(s)	Comment	Ref
Menasche *et al* 2001	Case report	Ischaemic HF undergoing CABG n = 1	Skeletal myoblasts 800 x 10^6 cells Intramyocardial injection during CABG	NA	NA	First-in-man report of skeletal myoblast injection. Improved wall motion and perfusion on PET	213
Strauer *et al* 2002	Non-randomised Open label	Acute MI n = 20	Autologous BM cells 2.8 x 10^7 cells Intracoronary delivery	Not specified	Reduced infarct size in cell therapy arm	First study of BM cells in acute MI	214
Perin *et al* 2003	Non-randomised Open label	Ischaemic HF n = 21	Autologous BM mononuclear cells Mean 25.5 ± 6.3 x 10^6 cells Transendocardial injection	Safety	Improved LV function, reduced reversible perfusion defect	First study of BM cells in HF	215
BOOST 2004	Randomised Non-placebo controlled	Acute MI n = 60	Autologous BM cells 24.6 x 10^8 nucleated cells Intracoronary delivery	Change in LVEF	Improved global LV function	First randomised study of BM cells	216
ASTAMI 2006	Randomised Non-placebo controlled	Acute MI n = 100	Autologous BM mononuclear cells Median 68 x 10^6 cells Intracoronary delivery	Change in LVEF	No change in LVEF or LVEDV or IS at 6 months	Negative trial concurrent with REPAIR-AMI	217
REPAIR-AMI 2006	Randomised Double blind Placebo controlled	Acute MI n = 204	Autologous BM progenitor cells 236 x 10^6 cells Intracoronary delivery	Change in LVEF	Significant improvement in global LV function at 4 months	Largest trial of BMCs. Showed reduction in clinical endpoint of death, recurrent MI & revasc	218
Janssens *et al* 2006	Randomised Double blind Placebo controlled	Acute MI n = 67	Autologous BM stem cells 304 x 10^6 nucleated cells Intracoronary delivery	Change in LVEF	Negative for primary endpoint	Reduction in infarct volume	219
MAGIC 2008	Randomised Double blind Placebo controlled	HF & previous MI undergoing CABG n = 97	Skeletal myoblasts 400 (low dose) – 800 (high dose) x 10^6 cells Surgical injection during CABG	Change in regional and global LV function	Negative for primary efficacy endpoints	High cell dose arm had reduced LV remodelling with decreased LV volumes. Increased arrhythmias in the cell therapy arms.	220
SCIPIO* 2011	Randomised Open label Non-placebo controlled	Ischaemic HF n = 23	Autologous c-kit+ cardiac stem cells 1 x 10^6 cells Intracoronary delivery	Safety	No adverse events reported	Increase in LVEF and decrease in infarct size reported in cell therapy recipients (secondary endpoints)	221
CADUCEUS 2012	Randomised Non-placebo controlled	Acute MI n = 25	Cardiosphere-derived cells 12.5-25 x 10^6 cells Intracoronary delivery	Safety: arrhythmic or unexpected death, MI, tumour formation or MACE	Met safety endpoint	Reduction in scar size/mass in cell therapy arm	222
FOCUS-CCRTN 2012	Randomised Double blind Placebo controlled	Ischaemic HF n = 92	Autologous BM stem cells 100 x 10^6 cells Transendocardial injection	Change in LV end-systolic volume; maximal O2 consumption, reversibility on SPECT	Negative for primary endpoints		223
SWISS-AMI 2013	Randomised Open label Non-placebo controlled	Acute MI n = 200	Autologous BM mononuclear cells 140-160 x 10^6 nucleated cells Intracoronary delivery either early (5-7 days) or late (3-4 weeks) after MI	Change in LVEF	Negative for primary endpoint		224
PROMETHEUS 2014	Non-randomised Non-placebo controlled	Ischaemic HF undergoing CABG n = 6	Mesenchymal stem cells 2 x 10^7 (low dose) - 2 x 10^8 (high dose) cells Intramyocardial injection during CABG	NA	NA	Increased EF, decreased scar mass	225
Menasche *et al*. 2015	Case report	Ischaemic HF undergoing CABG n = 1	Human ES-derived cardiac progenitor cells on a fibrin scaffold Surgical patch implantation	NA	NA	First in man study. New onset contractility observed in the patched region	226
REGENERATE-AMI 2016	Randomised Double blind Placebo controlled	Acute MI n = 100	Autologous BM-derived cells 59.8 x 10^6 cells Intracoronary delivery	Change in LVEF	Negative for primary endpoint	Large, double-blinded study of autologous BM cells which failed to meet primary efficacy endpoint	227

Abbreviations: BM – bone marrow; ES – embryonic stem; CABG – coronary artery bypass grafting; HF – heart failure; LVEF – left ventricular ejection fraction; MACE – major adverse cardiovascular events; NA – not applicable; PET – positron emission tomography, SPECT - single-photon emission computed tomography; * subject to expression of concern

Table 2: Translational pipeline: preclinical large animal regeneration studies in heart failure following myocardial infarction

Drug/therapy	Mechanism	Model/Delivery	Outcome	Comment	Ref
Human ES-derived CMs	Direct CM replacement	HF (2 weeks post-I/R) Surgical intramyocardial injection	Successful transplantation, electromechanical integration and partial maturation of ES-derived CMs No significant change in LVEF	First study to demonstrate regeneration by transplantation of CMs. All transplanted animals experienced ventricular arrhythmias	[155]
Allogeneic iPS-derived CMs	Direct CM replacement	HF (2 weeks post-I/R) Surgical intramyocardial injection	Significant improvement in LVEF (~10%) at 12 weeks	Proof of principle study. All transplanted animals experienced sustained ventricular tachycardia peaking at day 14 post-transplantation	[157]
FGF1 & NRG1-loaded microparticles	Angiogenesis Reversal of fibrosis	HF (4 weeks post-I/R) Catheter-based transendocardial injection	Approximate 8-10% improvement in fractional shortening with both NRG1 & FGF1 microparticles	Reduced ventricular remodelling observed	[144]
MR- 409 (GHRH agonist)	Pleiotropic effects Activation of the GH/IGF-1 axis	HF (2 weeks post-I/R) Subcutaneous injection	Reduced scar size after 4 weeks	Not accompanied by improved cardiac function	[228]
Recombinant FSTL1 in patch	Pleiotropic effects Stimulation of CM proliferation Arteriogenesis	HF (1 week post I/R) Surgical patch implantation	Approximate 10% improvement in LVEF		[168]
IGF1 & HGF within hydrogel	Stimulation of CM proliferation Angiogenesis	HF (4 weeks post-I/R) Catheter-based transendocardial injection	Small improvement in LVEF Reduced scar formation	Overall small functional effects observed; trend towards reduced fibrosis	[229]

Abbreviations: ES – embryonic stem (cell); I/R – ischaemia/reperfusion; LVEF – left ventricular ejection fraction; GHRH – growth hormone releasing hormone; GH – growth hormone; HGF – hepatocyte growth factor; IGF-1 – insulin-like growth factor 1; iPS – induced pluripotent (stem cell); CM – cardiomyocyte

Table 3: Recent clinical trials targeting cardiac injury and repair after acute myocardial infarction

Trial	Drug	Mechanism	Phase/ Patient cohort	Primary Endpoint(s)	Outcome	Ref
Lenihan *et al* 2016	Cimaglermin-α	Recombinant full length neuregulin 1β3	Phase I 40 patients with symptomatic HF and LVEF ≤ 40%. HF aetiology not reported	Safety/tolerability	No severe adverse effects. Improvement in LVEF in the high-dose groups (~7-9%) lasting for study duration (90 days)	[146]
Gao *et al* 2010	Neucardin	Recombinant epidermal growth factor-like domain of neuregulin-1β	Phase II 44 patients with symptomatic HF and LVEF ≤40%. HF aetiology not reported	Change in LVEF, end-systolic volume, or end-diastolic volume at 90 days	Non-significant trend towards improved LVEF and reduced LV remodelling in the neuregulin arms	[230]
LATITUDE-TIMI 60	Losmapimod	p38 MAP kinase inhibitor	Phase III 3503 patients with ACS	Major adverse cardiovascular events at 24 weeks	No significant difference in primary endpoint or HF events	[231]
TIPTOP	Doxycycline	Oral MMP inhibitor	Phase II 110 patients with STEMI	Change in LVEDVI at 6 months	Significant reduction in LVEDVI and infarct size with doxycycline	[232]
VCU-ART2	Anakinra	IL1-receptor antagonist	Phase II 30 patients with STEMI	Change in LVESVI at ~ 3 months	No signficant change in LVESVI, LVEDVI or LVEF. Combined data from VCU-ART2 & VCU-ART suggested a reduction in HF events in the anakinra arm. Larger trial (VCU-ART3) currently recruiting	[233]
VCU-ART	Anakinra	IL1-receptor antagonist	Phase II pilot 10 patients with STEMI	Change in LVESVI at ~ 3 months	Significant improvement in LVESVi on cardiac MRI	[234]
Gullestad *et al* 2013	IVIG	Immunomodulation	Phase II 62 STEMI patients	Change in LVEF at 6 months	No significant difference in change in LVEF or scar size	[235]
REVEAL	Erythropoietin	Pleiotropic effects on injury/repair	Phase II 222 patients with STEMI	Infarct size	No significant difference in infarct size. Increased rate of adverse events with erythropoietin.	[236]
APEX AMI	Pexelizumab	Humanised monoclonal antibody neutralising C5 component of complement	Phase III 5,745 patients with STEMI	All-cause mortality at 30 days	No significant difference in mortality, or in secondary composite endpoint of death, shock or HF at 30 and 90 days	[237]
PREMIER	PG-116800	Oral MMP inhibitor	Phase II 253 patients with STEMI & impaired LV function (LVEF 15-40%)	Change in LVEDVI at 90 days	No significant difference in LVEDVI after 30 or 90 days	[238]

Abbreviations: ACS – acute coronary syndrome; CMs – cardiomyocytes; HF – heart failure; LVEF – left ventricular ejection fraction; iPS – induced pluripotent (stem cell); LVEDVI – left ventricular end-diastolic volume index; LVESVI – left ventricular end-systolic volume index; MI – myocardial infarction; MMP – matrix metalloproteinase; PCI – percutaneous coronary intervention; STEMI – ST-elevation myocardial infarction

Glossary:

Autologous: derived from cells/tissues of the same individual

Allogeneic: derived from genetically different individuals from the same species

Binucleation: division of the nucleus leading to formation of two nuclei within a cell, without division of the cytoplasm

Cytokinesis: division of the cell cytoplasm to complete the cell cycle and create a membrane barrier between two daughter cells

Embryonic stem (ES) cells: pluripotent stem cells that are derived from the inner cell mass of human embryos

Epicardium: the outer layer of the heart, also known as the visceral pericardium

Fibrosis: a pathological process characterised by deposition of interstitial fibrous or scar tissue

Heart failure: a pathological state defined by the inability of the heart to pump blood to support the requirements of the body. Typical symptoms include shortness of breath, fluid retention and fatigue

Induced pluripotent stem (iPS) cells: pluripotent stem cells that are reprogrammed from somatic cells by introducing pluripotency factors

Lymphangiogenesis: the growth of new lymphatic vessels

Ploidy: the number of sets of chromosomes in a cell

Myocardial infarction (heart attack): acute injury to the heart caused by occlusion of the coronary blood supply, usually due to atherosclerotic plaque rupture

Remodelling: a process characterised by a change in size, shape and structure of the ventricle. After MI, pathological remodelling causes the ventricle to enlarge, become spherical in shape, and functionally deteriorate

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Key points

Endogenous regeneration seen in animal models provides a template for optimal repair of the human heart following MI.

In the regenerating heart new cardiomyocytes are produced by proliferation of the existing cardiomyocyte pool. Understanding and targeting the intrinsic mechanisms which regulate cardiomyocyte cell cycle re-entry could enable therapeutic regeneration in the human heart.

Repair is modulated by epicardial activation, neoangiogenesis, the immune response and the extracellular matrix. Biological insights from regenerative models, combined with use of high-throughput phenotypic screens and *in vivo* discovery approaches, are uncovering novel therapeutic targets and compounds to improve repair.

Regenerative strategies emerging from increased understanding of cardiomyocyte lineage specification include transplantation of *in vitro*-produced cardiomyocytes and *in vivo* reprogramming of fibroblasts. Current efforts to improve engraftment, maturation, and targeting will enable a next generation of trials.

Distinct approaches are required for patients in the immediate post-MI period and for those with chronic HF, and high risk strategies should be targeted at the latter group. Clinical trial design should be tailored to incorporate informed biological endpoints alongside functional endpoints.

TOC

Regeneration of the heart by cardiomyocyte reconstitution represents an attractive approach to treat heart failure. Here, Riley and colleagues discuss recent insights into the biology of heart regeneration and highlight emerging therapeutic regenerative strategies for HF. Challenges and considerations in the translation of regenerative therapies into the clinic are discussed. **[Au:OK?]**

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