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Towards Comprehensive Cardiac Repair and Regeneration after Myocardial Infarction: Aspects to Consider and Proteins to Deliver

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Abstract

Ischemic heart disease is a leading cause of death worldwide. After the onset of myocardial infarction, many pathological changes take place and progress the disease towards heart failure. Pathologies such as ischemia, inflammation, cardiomyocyte death, ventricular remodeling and dilation, and interstitial fibrosis, develop and involve the signaling of many proteins. Proteins can play important roles in limiting or countering pathological changes after infarction. However, they typically have short half-lives in vivo in their free form and can benefit from the advantages offered by controlled release systems to overcome their challenges. The controlled delivery of an optimal combination of proteins per their physiologic spatiotemporal cues to the infarcted myocardium holds great potential to repair and regenerate the heart. The effectiveness of therapeutic interventions depends on the elucidation of the molecular mechanisms of the cargo proteins and the spatiotemporal control of their release. It is likely that multiple proteins will provide a more comprehensive and functional recovery of the heart in a controlled release strategy.

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Keywords

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1. Introduction

Cardiovascular disease can be very costly and burdensome to society economically, socially, and psychologically. Myocardial infarction (MI), commonly known as heart attack, is a major cardiovascular disease that is responsible for significant morbidity and mortality, causing an estimated 7.3 million deaths per year worldwide [1]. According to the American Heart Association, 720,000 Americans experience new and recurrent heart attacks each year. Approximately, 15% of those experiencing an MI in a given year die because of it. In 2010, the direct and indirect cost of heart disease was approximately $205 billion in the United States [2].

Heart transplantation is the most effective treatment for chronic heart failure (CHF) patients. However, this option is very limited due to the lack of heart donors, highly invasive and complex surgical procedures, and significant cost. Reperfusion methods of the blocked coronary artery through percutaneous coronary intervention (PCI), coronary bypass surgery, and anti-thrombotic therapy are considered the standard of care for MI patients. In addition, angiotensin-converting enzyme (ACE) inhibitors and β-blockers are commonly used in the clinic to prevent adverse cardiac remodeling. Although these treatment methods lead to significant reductions in restenosis and improve lifestyles and long-term survival, the incidence of MI and heart-related mortality have not significantly changed [3, 4]. The conventional medical treatments have reached their practical limits and are not able to regenerate the damaged cardiac tissue and restore heart function. Also, not all patients are eligible for these kinds of interventions. Therefore, the development of alternative MI treatment therapies is paramount.

MI occurs as a result of an occlusion in one of the two main coronary arteries branching into the heart walls. The occlusion is usually due to coronary atherosclerosis and thrombosis that lead to heart muscle damage and likely progression to heart failure (Fig. 1). As a result of the ischemia, many changes occur at the molecular, cellular, and tissue levels of the myocardium. Hypoxia, death of cardiomyocytes, inflammation, ventricular dilation and adverse remodeling, tissue necrosis, interstitial fibrosis, and contractile dysfunction are some of the main features that may present themselves during progression from MI to CHF [5, 6].

In this review, we give overviews on these different pathological aspects of MI and the therapeutic interventions that have been explored to counter them in the last 15 years. We focus on proteins as potential therapies to repair and regenerate damaged cardiac tissue. Gene and cell-based therapies are thoroughly reviewed elsewhere [3, 7-11]. Additionally, we focus on the complexity of tissue regeneration and repair processes and reasons for more comprehensive therapies. Finally, we discuss the importance of using controlled release systems to overcome the limitations of protein therapy. A schematic to explain the process of developing an effective MI protein-based therapy is provided in Figure 2.

2. Pathological aspects of MI and corresponding therapeutic interventions

Over the last 15 years, many experimental studies provided evidence of the adult heart's limited potential to regenerate and repair, motivating many tests of new therapies [7, 9, 12, 13]. Many of these attempted to overcome the limitations imposed by the endogenous biological system in order to achieve healing rather than scarring of the heart after MI. The full elucidation of the mechanisms of MI pathologies and their role in causing heart failure can help design more effective therapies. Therapeutic strategies, therefore, should take into account the different aspects of pathologies MI causes, and find solutions for the most critical or the complete set of impairments using more comprehensive well-designed approaches in order to restore normal function to the myocardium (Fig. 2). In this section, we describe various pathological changes after MI and proteins that may reverse or counter these changes (Table 1).

2.1. Heart ischemia

2.1.1. Ischemic damage and importance of proper vasculature—The heart vasculature ensures the metabolic and structural homeostases of the heart. Proper perfusion provided by blood vessels is crucial for the growth and survival of cardiomyocytes [14]. For instance, improved activation of hypoxia response pathways by stabilizing hypoxia inducible factor (HIF)1-α in endothelial cells (ECs) leads to increased cardiomyocyte survival, improved LV systolic function, and reduced scar size [15]. The deficiency of laminin-α4, an abundant extracellular matrix (ECM) protein in the basement membrane of myocardial blood vessels, leads to myocyte hypoxia and necrosis, and ultimately to heart failure [16]. Moreover, the interaction between ECs and cardiomyocytes offers increased protection for the myocytes through nitric oxide (NO)-dependent mechanisms and regulate myocyte contractility after an ischemic insult [17, 18]. Inadequate perfusion of the heart muscle can contribute to an irreversible myocardial hibernation and decrease of contractile function [14]. Hence, therapeutic angiogenesis that aims to form new blood vessels from pre-existing ones might contribute to the repair of the infarcted myocardium [19].

2.1.2. Angiogenesis mechanisms and proangiogenic therapies—The hypoxic conditions activate angiogenic growth factors (GFs) that cause pericytes to detach from bloods vessels, allowing the loosening of cell-cell junctions and the migration, proliferation, and differentiation of ECs. Differentiated endothelial tip and stalk cells elongate the sprouting neovessels and form vessel lumens. As two neovessels fuse, blood perfusion can be initiated. Maturation and stabilization follow through the recruitment of pericytes to wrap around the growing neovessels [21]. Angiogenesis is a complex tightly regulated process that requires the cooperation of different cells, GFs, ECM, and signaling molecules (Fig. 3). Spatial and temporal cues are important to ensure an adequate angiogenic outcome.

Vascular endothelial GF (VEGF) and basic fibroblast GF (FGF-2) are key initiators of angiogenesis. VEGF is an endothelial-specific factor that stimulates the proliferation, migration, and survival of ECs. It upregulates in response to hypoxia by HIF1-α signaling more than any other angiogenic factor [21, 22]. Additionally, VEGF induces the production of NO, a critical vasodilator, and promotes vascular permeability [23]. VEGF-mediated

angiogenesis demonstrated therapeutic benefit in many ischemic heart disease animal models [24, 25]. FGF-2 induces proliferation and migration of ECs and smooth muscle cells (SMCs), induces ECs to physically organize into tube-like structures, and triggers angiogenesis [19, 22, 24]. Additionally, FGF-2 promotes survival of ECs, SMCs, and cardiomyocytes [26]. FGF-2 upregulates the expression of VEGF and vice versa [27, 28]. Moreover, FGF-2 increases the expression of other proangiogenic molecules such as hepatocyte GF (HGF), monocyte chemoattractant protein-1 (MCP-1), and platelet-derived GF receptor (PDGFR) on vascular SMCs [29, 30]. Administration of FGF-2 at the infarcted myocardium improves revascularization and cardiac contractility and reduces infarct size [3, 20, 24]. Other proteins that also improve angiogenesis include FGF-1, HGF, granulocyte colony-stimulating factor (G-CSF), sonic hedgehog (Shh), erythropoietin (EPO), and stromal cell-derived factor 1-alpha (SDF-1α) (Table 1) [20, 22, 24, 31]. For example, HGF induces proliferation and migration of ECs, acts in synergy with VEGF, and improves heart function after MI through angiogenesis [32-34]. SDF1-α might contribute to angiogenesis not by direct actions on ECs, but by recruiting endothelial progenitor cells (EPCs) and inducing other angiogenic factors such as VEGF [35, 36]. Some single GF applications, namely VEGF and FGF-2, reached clinical trials, but showed only modest to little benefit in inducing proper revascularization and treating MI patients [24, 37, 38]. Possible reasons behind the limited therapeutic benefit seen in patients include rapid diffusion, large doses, and short half-lives of bolus injections of GFs and the minor attention paid to the spatiotemporal and physiologic presence of different GFs during angiogenesis.

2.1.3. Importance of temporal cues in therapeutic angiogenesis—The involvement of many signals, GFs, ECM components, and different cell types in the process of angiogenesis suggests that relying on a single factor might not be enough (Fig. 3). It has been shown that VEGF or FGF-2 alone can lead to the formation of aberrant and leaky vessels that might regress quickly [39, 40]. Angiopoietin (Ang)-2 destabilizes blood vessels by weakening the interactions between ECs and pericytes [41]. Platelet-derived GF (PDGF) and Ang-1 are involved in stabilizing neovessels. PDGF triggers the recruitment of SMCs that cover the newly formed vessels, thus improving their functionality and reducing the possibility of regression and leakiness [42]. VEGF was shown to be a negative regulator of PDGF that inhibits its signaling and recruitment of pericytes. VEGF activates its receptor VEGFR-2, which complexes with PDGFR-β to block its signal transduction [43]. In contrast to Ang-2, Ang-1 strengthens the interactions between ECs and pericytes [41]. Approaches that sequentially delivered early angiogenic factors such as FGF-2, VEGF, and Ang-2 followed by late factors such as PDGF and Ang-1 demonstrate a more robust angiogenesis process and mature neovasculature than single factors [44-48]. Therefore, therapies that aim to form mature vasculature in ischemic tissues should take into account the proper time to administer GFs and limit any potential antagonism between different GFs (Fig. 3).

2.1.4. Role of vasodilation—Nitric Oxide (NO) is a potent vasodilator that helps regulate blood vessel tone and cardiac function [49]. It has been shown that reduced endothelial NO after MI contributes to pathophysiology and heart failure [50]. It is involved in the tissue response to ischemia and improves angiogenesis through HIF1-α and VEGF-mediated mechanisms [19]. Loss of function models targeting nitric oxide synthase (NOS)

enzymes can lead to pathological consequences for vascular function [52, 53]. Mice lacking NOS show reduced left ventricle (LV) function and increased adverse remodeling after MI [54, 55]. It has been reported that specific NOS1 overexpression in cardiomyocytes reduces infarct size and oxidative stress and improves cardiac function after infarction [56]. NOS3 can help recruit EPCs, induce neovascularization, and limit LV remodeling and dysfunction after infarction [49, 57]. Improving the bioavailability of endothelial NO after MI using statin treatment leads to increased angiogenesis and EPC mobilization and reduces fibrosis and cardiac dysfunction [58].

Relaxin is another molecule with potent vasodilation properties and can affect cardiac remodeling [59]. It has also been shown to have anti-inflammatory, anti-fibrotic, and angiogenic effects, all considered beneficial to teat MI patients. Relaxin exerts its effects on the cardiovascular system by binding to relaxin family peptide receptor 1 (RXFP1) and triggering intracellular signaling pathways that induce cyclic adenosine monophosphate (cAMP) production, NO signaling, tyrosine kinases, and others. Clinical trials of relaxin suggest it has important cardioprotective roles and can relieve symptoms of acute heart failure [59].

2.2. Inflammatory response

2.2.1. Effects of inflammation after MI—Inflammatory cells such as neutrophils and monocytes rush into the ischemic heart in the early stages after MI triggering a strong inflammatory response. The therapeutic targeting of the inflammatory response after MI has been met with controversy mainly because the presence of inflammatory cells acts as a double-edged sword. Inflammatory cells can promote beneficial effects such as inducing angiogenesis by monocytes secreting proangiogenic factors and phagocytosis of dead cells and their cellular debris. However, they can also have detrimental effects on cell survival and cause tissue damage, infarct expansion, and LV dilation [60, 61]. Neutrophils produce large amount of reactive oxygen species (ROS) and elastase which cause cell apoptosis and elastin degradation [61, 62]. In addition, they can reduce the proangiogenic effects of progenitor cells and bolster ischemic conditions [63, 64]. A limited presence of neutrophils is necessary to initiate the inflammatory response. Macrophages are also strong regulators of post-infarction events such as angiogenesis and scar formation. Macrophage activation can lead to two major distinct phenotypes: M1 and M2. M1 macrophages promote further inflammation and ECM degradation, while M2 macrophages contribute to anti-inflammation, angiogenesis, and ECM reconstruction [65]. Therefore, specific reduction in the levels of specific inflammatory mediators might show a therapeutic benefit after MI.

2.2.2. Implicated proteins and anti-inflammation therapy—Proinflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-1 levels are elevated in the infarct zone and activate matrix metalloproteinases (MMPs), which degrade the ECM(Table 1) [61]. For example, leukocyte-derived MMP-9 deletion protects the ischemic heart from LV dilation and cardiac rupture, but also disrupts angiogenesis [66]. LV dilation and inflammatory response are significantly attenuated in IL-1 and MCP-1 knockout mouse models, but not infarct size [67]. TNF-α upregulates in heart failure, promotes invasion of inflammatory cells to the ischemic myocardium, induces MMP

production, triggers cell apoptosis, and exacerbates adverse LV remodeling [68, 69]. Transforming GF (TGF)-β can deactivate macrophages, downregulate pro-inflammatory cytokines, and promote ECM preservation [67]. Tissue inhibitor of MMPS (TIMP)-3 inhibits TNF-α-converting enzyme (TACE), the enzyme activator of TNF-α [70]. Other studies suggest a cytoprotective role of TNF-α in preventing myocyte apoptosis after MI [71]. Ultimately, clinical trials using TNF inhibitors were unsuccessful [37]. This might suggest pleiotropic actions of some cytokines such as TNF-α or that the failed outcome might be as a result of toxic effects due to high doses used. IL-10 is an anti-inflammatory cytokine that inhibits the production of pro-inflammatory cytokines. IL-10 can induce TIMP-1 production by mononuclear cells which may help in reducing ECM degradation by MMPs [60]. Treating the infarcted heart with IL-10 can improve ejection fraction and angiogenesis, and reduce infarct size, fibrosis, and cardiomyocyte death [72, 73]. In contrast, a knockout study reported that IL-10 does not have a critical role in LV remodeling [74]. Decreasing the neutrophil invasion after ischemia through the inhibition of CCAAT/enhancer binding protein (C/EBP) pathway results in less fibrosis and improves cardiac function [75].

It seems logical that quenching the inflammatory response completely will not yield the desired functional benefits for the injured heart, because clearing dead cells from the infarct area helps reduce tissue necrosis and damage. It appears imperative that a tightly regulated inflammatory response, both temporally and spatially, should be available for a limited time after MI. Therefore, the goal of anti-inflammation therapy should not be a complete suppression of the post-infarction inflammatory response, but rather to properly modulate it in order to reduce the potentially dangerous consequences of uncontrolled activity. Because the MI inflammatory response activates both detrimental and protective signaling pathways, it is crucial for therapeutic strategies to respond to the pathophysiologic complexity of the infarct environment and optimize dosage and spatiotemporal profiles of applied agents in order to achieve a successful outcome.

2.3. Myocardial cell death and strategies to regenerate viable myocardium

A human LV contains up to 4 billion cardiomyocytes. In a few hours, an MI can kill 25% of them [76]. The intense inflammatory reaction, ischemic conditions, adverse remodeling, LV dilation, and infarct expansion put millions of surviving cardiomyocytes at risk of death through apoptosis or necrosis (Fig. 4). The immediate generation of ROS after ischemia, mainly by inflammatory cells, induces apoptosis among myocytes [77]. This massive death of myocytes sets into motion a cascade of events that lead to the replacement of damaged tissue with a scar. Scar tissue reduces the ability of the LV to contract and pump blood efficiently, thus markedly reducing overall cardiac function.

2.3.1. Cell apoptosis mechanisms and anti-apoptotic therapy—Apoptosis is characterized by cell shrinkage, fragmentation of intracellular structures, and phagocytosis into neighbor cells [78]. The balance between pro-apoptotic proteins such as Bax, Bak and Bid, and anti-apoptotic proteins such as Bcl-2 and Bcl-x_L is essential to determine a cell's survival or death after an apoptotic signal (Fig. 4). In the post-MI environment, elevated expression levels of Fas receptor, an apoptosis mediator, were reported [79]. In addition,

increased activation of caspases, the key executioner proteins of cell apoptosis, and increased ratio of Bax to Bcl-2 have been linked to cardiomyocyte apoptosis [80, 81]. The activation of the PI3K/Akt and Ras-Raf-MEK-ERK pathways inhibits apoptosis and imparts cardioprotective effects (Fig. 4) [82-84].

Insulin-like GF (IGF)-1 and HGF can activate the PI3K/Akt pathway, enhance cell survival, and reduce cardiomyocyte apoptosis resulting in improved heart function [85]. G-CSF is another chemokine that prevents apoptosis of myocytes and inhibits the decrease in levels of Bcl-2 and Bcl-x_L forced under oxidative stress conditions [89]. EPO has demonstrated anti-apoptotic activities in many studies [90-92]. In a rat MI model, EPO upregulated Bcl-2 and downregulated Bax, which led to improvements in the heart hemodynamic function [93]. FGF-2, Shh, SDF1-α, Thymosin-β4, PDGF-BB, IL-33, and TIMP-1 can also reduce cardiomyocyte apoptosis and improve overall cardiac function after MI (Table 1) [20, 94-97]. Targeting of specific miRNAs has also been recently investigated to prevent cardiomyocyte death [98, 99]. Additionally, β-blockers demonstrate anti-apoptotic actions that ameliorate ischemic effects [100].

Cardiomyocyte apoptosis is detected during all phases after MI and not only in the infarct zone, but also extends to the viable myocardium in remote noninfarcted region [81, 101]. This suggests that apoptosis could be responsible for a significant amount of myocyte death from the onset of MI injury and throughout the progression to heart failure. It is therefore crucial to design anti-apoptotic therapeutic interventions that counter cell death following MI.

2.3.2. Cardiomyocyte proliferation—The view that adult mammalian cardiomyocytes lose their regenerative capacity shortly after birth has been long-held. The rarity of primary myocardial tumors, the limited recovery after myocardial injury, and the difficulty to stimulate proliferation in mature adult cardiomyocytes, all support the view of the heart as an organ with effectively no regenerative capacity [102]. However, recent findings contested the notion that cardiomyogenesis in adult hearts doesn't occur and proved that new cardiomyocytes can arise to replace old or dead ones even though the turnover rate is very low [12, 13, 103, 104]. The controversy about the origin of new cardiomyocytes persists. Do these new myocytes result from the division of pre-exiting ones or are they a result of differentiation of resident or recruited progenitor cells? It seems there is evidence for both origins and mechanisms, but possibly with different extents of contribution. In this subsection, we focus on factors that promote proliferation of existing cardiomyocytes; and in the next subsection we focus on cardiomyogenic differentiation of progenitor cells.

There have been several attempts to induce cell cycle reentry for cardiomyocytes by removing inhibitors such as p27 or triggering activators such as cyclinD1 and E2F2 [108, 109]. Activating the Hippo signaling pathway increases cardiomyocyte proliferation postnatally in mice [110, 111]. Moreover, regulation of the expression of certain miRNAs can affect cardiomyocyte proliferation and heart function [112, 113]. Periostin, an ECM protein, was shown to stimulate a cardiomyocyte subpopulation to reenter the cell cycle and proliferate. Periostin treatment improved cardiac function and angiogenesis, and reduced fibrosis and infarct size after MI in rodents [114]. However, another study reported no

increase in cardiomyocyte proliferation after periostin treatment [115]. Another protein, neuregulin (NRG)-1, has recently shown ability to stimulate survival, differentiation, and proliferation of cardiomyocytes through ErbB2 and ErbB4 [116]. In a cardiomyopathy model, NRG-1 administration improved cardiac function and survival; and when combined with ACE inhibitor therapy, the effects were additive [117]. Another study demonstrated that NRG-1 therapy reduces infarct size and improves cardiac function due to proliferation of a small subpopulation of existing adult mouse cardiomyocytes rather than an increased differentiation of resident or recruited progenitor cells or decreased cardiomyocyte apoptosis [118]. Ongoing human clinical trials suggest promising results of NRG-1 therapy on increasing ejection fraction of heart patients [119]. FGF-1 administration, in conjunction with p38 inhibition, can induce cardiomyocyte proliferation, improve angiogenesis and cardiac function, and reduce scarring and wall thinning [120].

The formation of new cardiomyocytes happens at a low rate even with the highest estimates, and therefore remains inadequate for the full replacement of lost myocardial tissue after infarction. It is thus important for therapies that aim to repair infarcted myocardiums to be designed with a broader focus than just aiming to boost the cardiomyocyte turnover with either mechanism. In addition, protein therapies that focus on cardiomyocyte proliferation need to consider the duration of the signal required to trigger significant cardiomyocyte mitosis. They also need to be localized and selective for myocytes, so as to prevent any potential tumor formation in remote tissues.

2.3.3. Stem/progenitor cell homing and differentiation—The envisioned goal of having stem/progenitor cells in the injury site after MI, whether transplanted or recruited by chemokines, is to differentiate into functional cardiomyocytes to replace the lost ones and improve cardiac performance. Genetic fate mapping provides evidence that some endogenous progenitor cells undergo myogenic differentiation after MI and give rise to new cardiomyocytes [103, 105-107]. Cardiosphere-derived cells (CDCs) have been suggested to express a cardiomyocyte phenotype and electrically couple to surrounding cardiomyocytes [122]. Other studies provided evidence suggesting that most progenitor cells being investigated in cell therapies do not differentiate into cardiomyocytes, but rather might improve heart function via paracrine signaling that activates repair and regeneration pathways [103, 123-125]. Regardless of the mechanisms that progenitor cells undertake in the infarcted region, it seems there is a consensus that they result in benefits at the tissue and functional levels, which explains why many cell therapies have reached the clinical trials stage [7-9].

Many molecules play important roles in the repair of the myocardium by recruiting stem/progenitor cells to the injury site. Mobilizing endogenous progenitors might compensate for the low retention and survival of exogenous transplanted cells. SDF1-α is a powerful chemokine that can mobilize EPCs, hematopoietic stem cells (HSC), mesenchymal stem cells (MSCs), and cardiac stem cells (CSCs) to the infarct zone [94]. Recruitment of one or more kinds of progenitor cells to the heart by SDF1-α promotes beneficial effects after MI, possibly through enhancing angiogenesis and myocyte survival and differentiation [94, 126-128]. G-CSF induces proliferation and mobilization of stem cells to the infarcted myocardium [20, 31]. It exerts beneficial effects on heart function after MI [132, 133].

Clinical trials using G-CSF have not been as promising possibly due to patient ages or timing of administration, but research on G-CSF therapy continues [31]. In addition, HGF has been reported to be chemotactic on CSCs and to improve cardiac function after MI when applied alongside IGF-1 [134]. Establishing an IGF-1 gradient at the infarct border zone leads to the recruitment of endogenous CSCs and improvement in myocardial regeneration [134]. Moreover, Thymosin-β4 can induce the mobilization of adult epicardial progenitor cells and coronary vasculogenesis and angiogenesis [135]. EPO has also been suggested to mobilize endothelial progenitors [136], with positive effects on cardiac function in heart disease patients [137, 138]. More recently, prostaglandin E2, an endogenous fatty acid derivative, was reported to recruit CSCs and potentially regulate their differentiation to cardiomyocytes after infarction [139]. Other mobilizers of stem cells to the ischemic myocardium include MCP-1, MCP-3, stem cell factor (SCF), VEGF, and nerve GF (NGF) (Table 1) [140]. As for differentiation, IGF-1 is suggested to induce the differentiation of CSCs into myocytes and contribute to the recovery of heart function and structure after infarction [141]. FGF-2 has also been suggested to differentiate resident CSCs into functional cardiomyocytes in vitro [142].

The ideal route for recruited endogenous or transplanted adult progenitor cells in the infarcted myocardium is to differentiate into cells of cardiac lineages, including cardiomyocytes, vascular endothelial, and mural cells, and become properly integrated into the tissue to replace the lost dead cells. However, although these progenitors' ability to differentiate is still controversial, their variable but widely-accepted therapeutic benefit after MI, likely through paracrine activities, is a testimony to their importance in advancing cardiac repair after infarction. The identity of the most efficient progenitor cells needed after MI and a suitable strategy to improve their presence in the infarct zone are still matters of debate and extensive investigation.

2.4. ECM degradation and ventricular remodeling

2.4.1. ECM structure and imbalance after MI—MI results in an adverse remodeling process in the cardiac muscle manifesting clinically by LV dilation and heart pump dysfunction ultimately progressing to heart failure [5, 6]. The remodeling process brings about major alterations in the structure of the ECM. Serving as the base that provides structural stability, contractile force transmission, and correct cardiomyocyte geometry, the ECM composition and orientation are strictly regulated in a healthy myocardium mainly by MMPs and their endogenous inhibitors, the TIMPs (Fig. 5) [143]. The imbalance in the MMP/TIMP ratio contributes to the abnormal remodeling of the ECM post MI.

The appropriate presence of important structural proteins in the ECM, collagen and elastin, allows the optimal transmission of contractile force (Fig. 5). Approximately 85% of the myocardial collagen is type I and 11% is type III. Type I collagen fibrils have very high tensile strength and provide resistance to deformation, while type III collagen fibrils are more distensible and provide resilience. The extent of collagen fibril maturation through crosslinking helps determine ventricular compliance. On the other hand, elastic fibers allow passive recoil in the myocardium after stretching [143, 144].

After an ischemic insult, the profile of collagen changes at different phases of cardiac remodeling and the region within the myocardium. For instance, during the initial phase, collagen is degraded in the infarct region; however in later stages, abnormal collagen synthesis, orientation, and crosslinking in the infarct region and then in remote noninfarct areas lead to fibrosis and further pathological remodeling [144]. Collagen has a long half-life and a slow turnover compared to other proteins [145]; thereby ECM replacement after degradation will also be slow, which places the myocardium at increased vulnerability for adverse remodeling after MI.

2.4.2. Implicated proteins in adverse remodeling—Many MMPs have been implicated in cardiovascular diseases. During cardiac remodeling, MMPs are released by different cells including cardiac fibroblasts, cardiomyocytes, vascular cells, and inflammatory cells [146, 147]. MMPs are usually activated by serine proteases and other MMPs through the cleavage of a propeptide in the amino terminus [148]. The functions of MMPs after infarction are complex and could involve both positive and negative effects, because multiple molecular pathways are involved and can regulate distinct and overlapping processes including angiogenesis, wound healing, ECM homeostasis, proliferation, and apoptosis [149, 150]. MMP activity can be regulated by transcriptional and post-translational factors such as TNF-α, IL-1β, TGF-β, other MMPs, oxidative stress, and mechanical stretch [151]. Additionally, MMP activity can be regulated by TIMPs which prevent MMP access to its substrates by binding to the MMP's catalytic domain; however affinities differ (Fig. 5) [148, 152].

After MI, a significant increase in MMP activity leads to an imbalance in the MMP/TIMP ratio favoring the degradation of the myocardial ECM over deposition. MMP-2 and MMP-9 have been implicated in early ECM degradation [153] and in advancing contractile dysfunction by degrading cardiac proteins such as myosin heavy chain, myosin light chain-1, troponin I, and α-actinin [143]. MMP-7 null mice show improved survival after MI [154]. Fibrosis and LV dilation are reduced when MMP-9 was deleted [155]. Knocking out MMP-2 or MMP-9 in mice protects them from cardiac rupture post infarction [66, 156]. However, healing and angiogenesis are impaired in the long term indicating different effects temporally and spatially [66].

2.4.3. Therapeutic interventions to alter ECM remodeling—The inhibition of NF-κB by IκB leads to a reduction in MMP-2 and MMP-9 expression, thereby reducing LV dilation after MI [157]. TIMP-4 null mice show increased LV dysfunction, fibrosis, hypertrophy, and MMP activity [158]. Deficiencies of TIMP-1 or TIMP-3 in mice can lead to increased LV remodeling, dilation, and dysfunction after MI [159-162]. Cell-based TIMP-3 gene delivery improves cardiac function [163]. TIMP-1 or TIMP-3-based therapiesare able to improve ejection fraction and reduce MMP-2 activity and apoptosis in the ischemic myocardium of rats [97]. Greater functional improvement and preservation of elastic fibers are observed in TIMP-3-treated group, possibly because TIMP-3 is ECM-bound, giving it a greater advantage in protecting the myocardial ECM [164]. ACE inhibitors, β-blockers, and statins have also been suggested as anti-remodeling agents that reduce MMP activity and ECM degradation after MI [166-168].

MMP/TIMP-based therapies need to be applied soon after MI because excessive ECM degradation can accelerate adverse remodeling and result in wall thinning and cardiac rupture. On the other hand, some therapies might also need to focus on preventing excessive ECM deposition which could promote fibrosis that spreads in the later stages after MI and extends from infarct to noninfarct zones leading to LV stiffness and contractile dysfunction [169]. ECM homeostasis is urgently needed to be restored in the post-MI environment, with therapies preventing ECM degradation favored early on in infarct region, while therapies preventing excessive ECM deposition may be beneficial at the later stages in noninfarct regions. While a few-fold increase in collagen above normal levels in the myocardium can cause ventricular stiffness and moderate malfunction [170], only a slight collagen level decrease below normal can lead to detrimental effects including dilation, rupture, and adverse remodeling [66, 152, 171]. So, determining which MMPs and/or MMP functions to inhibit, the optimal timing of intervention, the optimal dose of therapeutic agents, and the myocardium regions to be treated are all essential parameters to achieve a successful cardiac repair and ECM homeostasis.

2.5. Fibrosis

2.5.1. Role of cardiac fibroblasts and myofibroblasts—Cardiac fibroblasts make up 70% of the cells within the myocardium while only occupying a quarter of the tissue volume. They synthesize ECM components, regulate their turnover and maintain homeostasis through MMPs and TIMPs, and help transport mechanical and chemical signals [172]. After MI injury, many fibroblasts differentiate into their activated form, myofibroblasts, under the actions of mechanical stress and different chemical stimuli such as TGF-β [143, 173]. Myofibroblasts express α-smooth muscle actin (α-SMA) and are not normally found in healthy adult hearts. They are attracted to the infarct region and participate in the remodeling process by producing collagen and other proteins that form a matrix and replace dead cardiomyocytes [143, 172]. This increased collagen deposition ultimately leads to interstitial fibrosis and the formation of the myocardial scar in the infarct area [173]. Using connexins, myofibroblasts form gap junctions with each other and cardiomyocytes [174]. Being nonexcitable cells, the myofibroblasts, lying between cardiomyocytes and expanding the ECM, will create gaps between the myocytes which may result in impulse conductivity problems such as arrhythmias [175, 176].

2.5.2. Implicated proteins and anti-fibrotic therapy—Myofibroblasts are activated by different proteins and cytokines such as TGF-β, Angiotensin-II, and TNF-α. In the cardiac tissue, TGF-β stimulates proliferation, migration, and differentiation of fibroblasts into myofibroblasts, thereby considered the top regulator of the fibrotic response after MI [177]. Antagonizing TGF-β in the early stage after MI might exacerbate ECM degradation and promote LV dilation [178], while antagonizing it in the late stage might be more beneficial to prevent fibrosis and adverse remodeling in noninfarct regions. The administration of c-Ski, an endogenous inhibitor of TGF-β, helps inhibit fibroblast differentiation into myofibroblasts, which might limit fibrosis and adverse remodeling after MI [179]. IL-6 promotes fibroblast proliferation, but reduces collagen synthesis and induces MMP secretion. IL-1β and TNF-α inhibit fibroblast proliferation and collagen synthesis and increase MMP levels [177]. Recent studies have suggested an important role for the

WNT/FZD pathway in regulating myofibroblast migration and differentiation [180-182]. The administration of an FZD receptor antagonist improves cardiac function after MI [182]. Angiotensin-II induces fibroblast proliferation and differentiation, with ACE and angiotensin-II receptors being expressed actively by myofibroblasts after MI [183]. ACE inhibitors have been part of the standard of care for heart patients for a long time, as they are associated with reducing TGF-β levels and fibrosis [184, 185]. The β-adrenergic sympathetic system is an important regulator of cardiac function and because of the massive loss of cardiomyocytes after MI, the system's activity increases with β2-adrenoceptor receptors dominating cardiac fibroblasts [177]. β-blockers have been shown to inhibit fibroblast proliferation [186]. Also, relaxin reduces fibroblast differentiation and proliferation, thereby preventing cardiac fibrosis [59]. Statins such as simvastatin can suppress human myofibroblast proliferation [187]. Simvastatin is also shown to reduce fibroblast α-SMA expression and that effect is abolished with TGF-β administration [188].

The fibrotic tissue that develops in the infarct area also expands to the noninfarct regions of the left and right ventricles driven by the excess collagen deposition accompanied by distorted crosslinking of collagen fibers [189]. This results in reduced compliance and increased stiffness of the heart muscle, thus leading to CHF [173]. Some studies argue in favor of some myofibroblast presence stressing on their roles as providers of contractile force across the ECM and wound-healing mediators [97, 165]. Therefore, it is important for anti-fibrotic therapies to be employed in late stages and prioritize the prevention of ECM deposition and fibrosis in noninfarct regions. This brings to attention the importance of optimizing doses and spatiotemporality of injected agents, because early excessive degradation of the ECM and suppression of collagen synthesis in the infarct region can contribute to LV dilation and possible rupture. The right balance is needed for proper repair and functional recovery.

2.5.3. Reprogramming cardiac fibroblasts into myocytes—Because replacing millions of lost cardiomyocytes is a difficult task and in order to counter the negative effects of interstitial fibrosis, the idea of turning a portion of endogenous cardiac fibroblasts into functional cardiomyocytes is quite intriguing. Recently, a new therapeutic approach that aims to reprogram and convert fibroblasts into cardiomyocyte-like cells emerged [12, 190]. The introduction of combinations of cardiac transcription factors to fibroblasts such as GATA4, Mef2c, Tbx5, HAND2 and/or microRNAs such as miR-1, miR-133, miR-208, miR-499 have shown potential to activate cardiac gene expression and directly convert fibroblasts from different sources into cardiomyocyte-like cells [191-194]. Additionally, blocking JAK/ STAT and WNT signaling pathways have been suggested to generate cardiomyocytes from fibroblasts [195]. However the reprogramming efficiency remains low.

In infarcted mouse hearts, gene delivery of combinations of the above-mentioned transcription factors led to the generation of myocytes from endogenous cardiac fibroblasts, which seemed to integrate and form gap junctions with pre-existing myocytes after several weeks. This therapeutic intervention in turn reduced infarct scar size and improved heart function [194, 196]. It is possible that other cell types within the heart such as progenitor cells and ECs might undergo reprogramming to adopt myocyte-like phenotypes. Introducing VEGF alongside cardiac transcription factors was found to enhance heart function and

reduce fibrosis more than the transcription factors alone, showing that revascularization mediated by VEGF can improve the survival of cardiomyocytes, new and preexisting, and add to the overall therapeutic benefit [196]. Using lentiviruses, the introduction of relevant miRNAs to infarcted mouse hearts led to the reprogramming of cardiac fibroblasts into cardiomyocytes [193].

It is important to develop the necessary tools that can increase the yield and efficacy of cell reprogramming. For instance, reprogrammed cells would be more beneficial if they were able to proliferate and couple electromechanically with the preexisting myocytes as well, in order to preserve proper contractile and conductive function. However, cell reprogramming should be performed under tight controls, so that abnormalities like cardiac arrhythmias do not develop. Also, specific targeting of cardiac fibroblasts with reprogramming factorsis important so that off-target fibroblasts will not be affected. Deeper understanding of the molecular mechanisms underlying cell reprogramming would help advance the therapeutic approaches based on this new technology.

2.6. Electrical conduction abnormalities after MI

Proper electrical conduction is necessary for optimal cardiac output. Calcium plays an important role in the contractile function of the heart. Following an action potential, the rush of calcium into the cytosol of a cardiomyocyte via its respective channels induces adenosine triphosphate (ATP) hydrolysis, which in turn drives the interaction between actin and myosin and causes the cardiomyocytes to contract [197]. It has been reported that patients with dilated cardiomyopathy show an impaired uptake of calcium, thus compromising the heart's contractile function [198, 199]. The ischemic environment causes oxidative stress and elevation of intracellular calcium levels that affect the survival and function of cardiomyocytes [200, 201]. Therapeutic interventions could benefit from the use of calcium channel blockers which block the cardiomyocyte L-type calcium channels [202] to reduce excessiveness in the heart's contraction and conduction velocity.

3. Proteins and protein-based therapies: importance and advantages

3.1. Physiological roles of proteins and the microenvironment

Proteins including GFs, morphogens, hormones, cytokines, chemokines, antibodies, transcription factors, and enzymes are very important in cell signaling, function, and behavior (Table 1). Proteins transmit signals that trigger various cellular processes between cells of same and different types, their ECM, and between different tissues and organs. Identifying the target cells is important because distinct types of cells can have different responses to the same protein. Equally important is the determination of the proteins of interest in the process of tissue regeneration. Proteins can initiate different processes such as proliferation, migration, differentiation, apoptosis, growth, and adhesion by binding to their specific receptors expressed by the target cells (Table 1). This receptor binding can almost exclusively occur when proteins are in their soluble form having been secreted by cells or released from the ECM by enzymes and proteases.

3.1.1. Role of protein concentration and gradient formation—The effects exerted by proteins depend on their concentration in the cellular microenvironment, thereby influencing the expression of their receptors and the levels of other proteins, whose secretion and effects can be either antagonized or promoted [203]. For example, depending on the concentration of VEGF in the microenvironment, angiogenesis can be inadequate, normal, or aberrant and excessive [204]. Different cellular effects of HGF have been observed depending on its concentration, level of activation, and receptor expression [85]. A threshold concentration of TGF-β can change the molecular signal from growth to apoptosis [205]. Also, the concentrations of signaling proteins and the expression of their receptors are time-dependent and change at different stages of repair and regeneration. There are temporal differences in the presence of certain proteins and the expression of their receptors during events such as angiogenesis, inflammation, cardiac remodeling, and bone repair suggesting their physiological roles might be limited to certain stages.

The formation of a protein gradient enables the cells to detect directional and spatial cues, so as to respond to the protein signal. The diffusion rate, receptor binding, size, half-life, ECM affinity, and secretion or inhibition of a protein are all factors that determine the formation of a gradient and its steepness [206-208]. It was demonstrated that cells can modify their receptors through endocytosis and reorient itself towards the direction of a chemoattractant [209, 210]. This directed migration of cells requires a concentration gradient to effectively guide the cells towards the target site, and the threshold of the concentration gradient might differ from one chemoattractant to another depending on the signaling cascades it activates. For instance, NGF can stimulate extracellular-signal-regulated kinases (ERK) activity at 30% lower concentration threshold than epidermal GF (EGF) [211]. The direction of axonal growth can be affected by different chemical gradients of NGF and laminin [208]. A density gradient of Arg-Gly-Asp (RGD)-containing peptides can direct the alignment of fibroblasts [212].

3.1.2. Effect of biomechanics and architecture on protein behavior—The ECM is comprised of architectural, mechanical, and molecular components responsible for the structural integrity of tissues and transfer of information and signals between cells, tissues, and organs (Fig. 5). The signaling cascades can be triggered to activate cellular processes and regulate cell behavior by the binding of ECM proteins and polysaccharides to integrins on the cell surface [143]. ECM glycosaminoglycans (GAG) such as heparan sulfate contribute to the formation of protein gradients by facilitating the interaction between GFs such as VEGF and FGF and their receptors (Fig. 5). This prolongs the duration of GF signaling by protecting them from proteolytic degradation, thus rendering their actions on processes like proliferation, migration, and differentiation more effective [213].

The mechanics of the ECM influenced by traction forces, shear stresses, fluid flows, and others can affect the behavior of cells and tissues and how they respond to protein signals [214]. Abnormal matrix synthesis or degradation can have dire consequences on the cells of a mechanically stressed tissue. Mechanical forces can induce the release of proteins and work in conjunction with them to remodel the ECM or affect cell behavior. For instance, fibroblast differentiation into myofibroblasts needs both TGF-β and mechanical stress [215]. Vascular SMCs are triggered to express various differentiation markers in response to the

cyclic stretching of arterial walls [216]. Cells in a constrained collagen matrix generate different contractile forces depending on the stimulation of different GFs, while their responses are similar in a floating collagen matrix [217, 218]. In cases of laminar, pulsatile, and steady blood flow, resultant shear stresses modulate endothelial cell function, phenotype, gene and protein expression in a different way than when the flow is disturbed [216]. The mechanics of the microenvironment help determine the fate of cells when stimulated by proteins, including apoptosis, growth, differentiation, migration, and contraction. Cell behavior is also dependent on the structural organization of the ECM. It has been shown that interactions between cells, ECM, and signaling proteins can differ between two-dimensional (2D) and three-dimensional (3D) architectures [219]. Cells in a 3D microenvironment enjoy the ability to spread, attach, cluster ligands, change integrin and receptor expression, and perform chemokinesis or chemotaxis more effectively.

Therefore, the process of tissue repair and regeneration depends in a collective fashion on a complex network of signaling proteins that are present in specified concentrations and spatiotemporal gradients in the wider context of the ECM microenvironment with its mechanics and architecture. All of these parameters and aspects are crucial when designing therapeutic strategies to treat cardiovascular diseases.

3.2. Advantages and challenges of protein-based therapy

Exogenous proteins can be produced at high yields in a cost-effective manner with the aid of recombinant DNA and phage display technologies. Proteins can also be stabilized for relatively long periods, thus offering off-the-shelf availability. Additionally, protein administration can be potentially regulated spatially and temporally with specific doses used. Moreover, protein therapies offer the advantage for enhanced targeted interventions with the ability to elucidate mechanisms of action and regulatory molecular pathways involved [24, 37, 220]. The exogenous administration of therapeutic proteins can be utilized to supplement inadequate endogenous levels or replace defective proteins. They can also be used to upregulate or downregulate other molecules or to trigger certain cellular processes and activate specific molecular pathways (Fig. 6).

Because proteins play a central role in the process of tissue repair and regeneration, strategies to exogenously administer proteins of interest that have the potential to repair and restore normal function are continuously developed and improved (Table 1). No protein therapy has made it to the cardiovascular market and achieved clinical use yet [37]. Therapies that were dependent on bolus administration of proteins showed some efficacy in improving the function of ischemic hearts in animal models [20, 24]. However, in clinical trials, such method of administration proved ineffective and results were generally disappointing. For instance, VEGF, FGF-2, HGF, EPO, GM-CSF, and NRG-1 therapies did not demonstrate consistently significant improvements in revascularization and myocardial function compared to placebo in Phase I and II clinical trials, despite being tolerable and reasonably safe at different doses used [9, 24, 31, 38]. This is likely due to the drawbacks of bolus injections and the use of single proteins to repair tissues that likely require the complex signals and cooperation of many proteins. Proteins, administered by bolus injections, have poor retention at the target tissue because they are diluted and diffuse away

quickly. In addition, soluble proteins are highly unstable and typically have short half-lives because they are prone to proteolytic degradation and enzymatic deactivation. High doses are often needed to induce small therapeutic benefit, and such high systemic levels of proteins can be potentially toxic [220].

Thus, in order to make a breakthrough in the field of protein-based therapy for cardiac regeneration, it is logical to use multiple proteins that have different functions to address different challenges. Equally important is the use of controlled delivery systems that can present these proteins in a bioactive form spatiotemporally per their physiological cues. Such presentation allows, as close as possible, the recapitulation of the natural microenvironment of a healthy functional heart. Developing such sophisticated strategies might yield a comprehensive cardiac repair and regeneration process for MI patients (Fig. 6).

4. Controlled release systems: importance and potential in cardiac repair

4.1. Development and characterization of properties

Controlled release systems provide an exciting potential to overcome the challenges posed by bolus administration of proteins for cardiac repair after MI. These systems can be designed to protect, control, sustain, and localize the delivery of proteins to the ischemic heart muscle (Fig. 7, Fig. 8). In addition to the potential protein-mediated therapeutic benefits, some delivery systems are based on biomaterials that can also provide mechanical support and reduce adverse LV remodeling [221]. The main challenges that face the development of effective delivery systems include the optimal combination of proteins and the ability to control their concentration and spatiotemporal gradients upon delivery. Ideally, a controlled delivery vehicle would serve as a depot that provides physiological cues of crucial proteins needed for proper tissue regeneration, thus assuming the essential role of the ECM in protection, stabilization, regulation of activity, control of concentration, and spatial translocation of proteins within the myocardium (Fig. 7).

Biomaterials used in the development of controlled release systems can be natural or synthetic polymers. Natural polymers include fibrin, collagen, gelatin, alginate, chitosan, hyaluronic acid, heparin, and others [24, 222]. These natural materials are appealing because they can be easily recognized by the natural microenvironment, thereby reducing potential immunogenicity or toxicity. In addition, they can be degraded by endogenous enzymes. However, their mass production can be costly and the modification of their mechanical and chemical properties can be challenging. On the other hand, synthetic polymers include poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), polycaprolactone (PCL), poly-L-lactide (PLLA), and others [24, 222]. These synthetic materials offer the advantage of easier tailoring of material properties to fit the specialized needs of biomedical applications. Moreover, synthetic materials can be typically produced in a cost-effective fashion and in large quantities. Major challenges to the successful and effective use of synthetic materials include biocompatibility and biodegradability. They need to be non-toxic and tolerated by the immune system. The material should completely degrade and resorb into the body or excrete from the body. They may also need to mimic the behavior of natural materials in strength, compliance, stiffness, porosity, among other properties (Fig. 7).

There are various methods for protein encapsulation in a delivery vehicle, which ultimately determine the release rate of these proteins. Physical entrapment requires the mixing of proteins with polymers before they gel or solidify. The crosslinking density and structural stability of the polymeric network determine the encapsulation efficiency and release of the protein via diffusion and/or degradation mechanisms depending on the size of the protein relative to pore sizes of the matrix [223]. Proteins can be immobilized to the matrix using electrostatic interactions, ionic and hydrogen bonds, and covalent attachment (Fig. 8). These methods can employ macromolecules and polymers such as heparin, heparan sulfate, hyaluronic acid, PEG, MMP linkers, and functional groups such as carboxyl, amino, and hydroxyl groups [207, 220]. Some encapsulation methods include steps of leaching, use of organic solvents, processing at high temperatures, freeze-drying, and chemical modification which can be harmful to the stability and bioactivity of the proteins [220]. Therefore, it is essential to employ techniques that prevent potential denaturation and deactivation of proteins, so that they can perform their intended activity upon release from the delivery system (Fig. 7). By modifying polymerization conditions, composition, stoichiometry, functional groups, and other tunable parameters, natural and synthetic polymers can form different kinds of injectable matrices for the controlled delivery of proteins that can be implemented in cardiac repair strategies (Fig. 8). Different kinds of release profiles can be achieved depending on the type and property of a delivery vehicle (Fig. 9). Injectable delivery platforms such as hydrogels, micro- and nanoparticles, coacervates, peptide nanofibers, and liposomes are discussed in the next sections.

4.2. Hydrogels

Hydrogels are made through physical or chemical crosslinking of polymers to create hydrophilic networks swollen by water (Fig. 8) [3]. They are often biocompatible, can be made to have soft tissue-like elasticity and permeability. Certain hydrogels can be injected into the body through minimally invasive techniques. The water content of hydrogels can help reduce interfacial tension with other tissue fluids allowing gas permeation and small compound diffusion. They typically have burst releases of embedded proteins and can sustain their release for short periods (Fig. 9). Gaining a better control over the release kinetics and the tailoring of hydrogel mechanical and chemical properties are areas of continued investigation. For instance, the mechanical properties of hydrogels based on natural materials can be enhanced by conjugating inhibitors or crosslinkers that reduce hydrolysis. Immobilizing affinity groups on hydrogels can strengthen their binding of proteins and prolonging their release. Moreover, biodegradability of synthetic hydrogels can be improved by introducing proteolytic sequences in their synthesis, while injectability can arise through crosslinks triggered by in vivo stimuli. Gelatin and chitosan hydrogels have been used to deliver proteins such as FGF-2 and EPO to induce revascularization and cardiac repair [224, 225]. Fibrin gels have been utilized to release angiogenic factors and increase the formation of microvessels [226]. Collagen gels containing TIMP-1 and TIMP-3 can improve cardiac function and reduce adverse remodeling after infarction [97]. Alginate hydrogels have been used for sequential delivery of proteins such as IGF-1 followed by HGF or VEGF followed by PDGF to improve ischemic heart function [45, 88]. Recently, hyaluronic acid-based hydrogel loaded with SDF-1α and angiogenic peptide Ac-SDKP was delivered to the infarcted myocardium improving ejection fraction, stem cell recruitment,

and angiogenesis [130]. The delivery of TIMP-3 using a hyaluronic acid hydrogel improves ejection fraction and reduces ventricular dilation, LV wall stress, MMP activity, inflammation, and infarct size in a porcine model [165]. An ECM-derived hydrogel releasing an engineered HGF fragment demonstrated cardiomyocyte protection and downregulation of pro-fibrotic markers in vitro, and improved cardiac function and angiogenesis in vivo [227]. PEG-based hydrogels have been used to treat infarcted hearts and deliver single or multiple proteins such as VEGF, HGF, and IGF-1 which reduced scar burden and enhanced heart function [228-230].

4.3. Micro- and nanoparticles

Micro- and nanoparticles are injectable small particles often produced from polymers, functionalized to target specific injury sites, and control the release of embedded bioactive molecules like proteins which can be dissolved within, entrapped, encapsulated, or adsorbed (Fig. 8). Because of their small size, micro- and nanoparticles are injectable and can diffuse and accumulate in different tissues. The particle size also plays an important role in the release rate of encapsulated proteins because of changing surface-to-volume ratios and the ability of cells to endocytose them (Fig. 9) [231]. The loading of proteins into micro- and nanoparticles usually requires the use of relatively harsh conditions and organic solvents that put the proteins at risk of denaturation and loss of bioactivity [220]. Such conditions prompted the utilization of surfactants, carrier proteins, and sugars as stabilizers during the process of protein encapsulation in a bid to minimize potential loss of protein bioactivity [232]. PLGA is one polymer that has shown a lot potential in controlled delivery because of its high biocompatibility and safety. It is also FDA-approved for various medical applications. PLGA microparticles have been used to deliver SDF1-α, thus increasing the extent of stem cell recruitment in vitro [129]. Delivering VEGF to the ischemic heart using PLGA microparticles induces angiogenesis and reduces LV wall thinning and adverse remodeling [233]. In another study by the same group, these microparticles are used to co-deliver FGF-1 and NRG-1 to the heart after MI which improved cardiac function, revascularization, cardiomyocyte proliferation, progenitor cell homing, and reduced infarct size and fibrosis [121]. IGF-1 delivered by PLGA nanoparticles can significantly improve Akt activation and ejection fraction and reduce apoptosis and infarct size in mice hearts [86]. Heat shock protein 27 (HSP27)-loaded PLGA microparticles inside an alginate hydrogel improved cardiac cell protection under hypoxia [234]. PLGA microparticles loaded with milrinone have also been used to improve ejection fraction and reduce inflammation in a rodent MI model [235]. Micro- and nanoparticles based on other biomaterials such as porous silicon, silica, lecithin, pluronic, and dextran have been recently investigated for delivery of proteins to repair the infarcted myocardium [236-239].

4.4. Coacervates

Complex coacervates are a new class of drug delivery vehicles [240]. They can be formed by the mixing of oppositely charged polyelectrolytes resulting in aggregates of colloidal droplets held together by electrostatic attractive forces and apart from the surrounding liquid (Fig. 8) [241, 242]. The coacervation process leads to phase-separation of a polymer-rich liquid phase from a polymer-poor one. The coacervate droplets exist in dynamic equilibrium, thus reducing their likelihood of aggregation in response to ionic concentration

or temperature changes in their environment. The stability of ionic coacervates is an area that needs improvement especially for a systematic delivery route when blood carries the coacervate [241, 242]. Heparin-based coacervates are able to encapsulate heparin-binding proteins with high efficiency, protect them from proteolytic degradation, prolong their bioactivity, and sustain their release over time (Fig. 9) [242]. There are a number of ways to control the formation of coacervates and their release kinetics such as altering the molecular weights of the polyelectrolytes, their charge density, the stoichiometric ratio of positively- and negatively-charged polymers, pH, salt concentration, and others [241-243]. Our group has utilized the coacervation process between a polycation poly(ethylene arginyl aspartate diglyceride) (PEAD) and heparin to control the delivery of proteins for various biomedical applications such as therapeutic angiogenesis, wound healing, cardiac repair, and bone regeneration [240]. Heparin, the most negative natural polymer in the body, binds over 400 proteins and peptides, many of which have important biological functions. The heparin-binding domain of many proteins contains basic amino acid residues such as lysine and arginine, which are important for inter-molecular interaction and downstream signaling [244]. In our coacervate system, heparin is non-covalently immobilized within the complex by electrostatic interactions, which can guarantee the preservation of its natural bioactivity. The polycation PEAD is designed specifically for protein delivery. It is biodegradable with minimal cytotoxicity. With regard to cardiac repair, we showed that Shh delivered by the coacervate is cardioprotective and can improve vascularization and heart function in rodents after MI [95, 245]. In addition, FGF-2 coacervate and FGF2+IL-10 coacervates significantly improved cardiac function and reduced scar burden in a mouse MI model [246, 247]. In a combinatorial approach, we used fibrin gel and the coacervate to achieve sequential delivery of VEGF and PDGF-BB to the ischemic heart of rats (Fig. 10). VEGF released within one week, while PDGF release was sustained for at least 3 weeks in vitro (Fig. 10B). This sequential release approach significantly enhanced heart function as evident by the significant improvement in fractional area change, a measurement of cardiac contractility (Fig. 10C). We also demonstrated enhanced angiogenesis and cardiomyocyte survival, and reduced fibrosis, inflammation, LV wall thinning, scar expansion, and granulation after MI (Fig. 10D) [48].

4.5. Other delivery systems

A few other delivery systems have been utilized in protein delivery to the ischemic heart (Fig. 8). Lipid-based vehicles have been developed for use in cardiac repair, however challenges posed by liposomes such as instability and interaction with circulating lipoproteins are still being addressed [239, 248]. Anti-P-selectin-conjugated liposomes loaded with VEGF were delivered to the infarcted myocardium leading to an improved systolic function and fractional shortening [249]. Self-assembled peptides, formed by alternating hydrophilic and hydrophobic domains of oligopeptides, are another platform used for delivery of proteins (Fig. 8) [250]. They have been used to deliver IGF-1, PDGF-BB, FGF-2, VEGF, and others to improve cardiac function post infarction [251-254].

4.6. A clinical and market perspective on protein delivery systems

Following the limited success of protein-based therapies in the clinic, there has been a rising interest in the development of more effective methods of administering proteins to the target

tissues. Commercially marketed protein-containing products such as Regranex and InFUSE have not been fully adopted in regenerative medicine because of safety and efficacy concerns. Much testing and validation need to be performed before successful adoption of protein delivery systems in the market. In particular, in vivo studies are necessary to develop appropriate administration methods and demonstrate safety and efficacy of the encapsulated proteins in inducing the desired response. Proving safety, scalability, reproducibility, ease of manufacturing, cost-effectiveness, biocompatibility, and biodegradability are all factors that can help push controlled release systems past clinical trials and pave the road towards full adoption in the clinics. There is a long way to go and many hurdles to overcome, but the potential market for therapeutic proteins for the heart is just beginning to open up and has a huge growth potential. This motivates many researchers to improve the controlled protein delivery field and race to clinical success.

5. Conclusions and future directions

Current treatments only defer further cardiac damage and dysfunction rather than restore the normal function of the heart. Given the limited potential of the adult mammalian heart to repair and regenerate on its own after MI, and the identification of favorable proteins that are able to induce cardiac protection, repair, and regeneration, protein therapeutics remain a hopeful, feasible, and effective path for future treatments of ischemic heart disease, even though the road towards clinical translation is still filled with obstacles.

Further understanding and elucidation of molecular mechanisms of myocardial tissue repair and regeneration will contribute to the development of more effective treatment strategies. Working on multiple aspects such as revascularization, remodeling prevention, and cardiomyogenesis is posed to be a more promising approach towards full recovery and comprehensive regeneration of the infarcted myocardium than single-focus approaches. Therefore, the decision on which proteins to combine to address many of the aspects discussed in this review is important. Additionally, the ability to design therapeutic strategies that can mimic the natural regenerative microenvironment is a key determinant of successful repair process after MI.

The notion of recapitulating the normal physiology of the heart environment can be facilitated and potentially achieved through the utilization of efficient, targeted, biocompatible, and tightly controlled protein delivery systems that can support the bioactivity, stability, and retention of released proteins at the target site. Proteins are not meant to be available at any time or any place during the repair process. Delivery and release kinetics of proteins need to be tightly regulated spatially and temporally, so that their physiological concentrations, gradient formations, and biological cues are optimal for preservation and regeneration of the myocardium. Because hundreds of millions of cardiomyocytes are lost after an ischemic insult, it is extremely hard to replace such a vast amount of lost tissue. Many researchers support the idea of combination therapy that combine the use of cell and protein therapies. Such an approach needs to clearly identify the source and quantity of cells to use. If the cells become fully differentiated into functional adult cardiomyocytes that are electromechanically coupled with the rest of the heart muscle, it will be a powerful way to regenerate the damaged heart.

The complexity of biological systems makes it difficult to integrate all the aspects of tissue repair and regeneration. Systems biology approaches can potentially help combine massive experimental data with computational modeling to design highly effective strategies. The physiology and pathology of the heart are intrinsically complex, thus it is indispensable to design strategies that can yield substantial therapeutic benefit and pave the way to full clinical adoption in the cardiovascular market. As we expand our knowledge, decipher more experimental data, and utilize more advanced technological tools, establishing a cure for ischemic heart disease could be within our reach in the foreseeable future.

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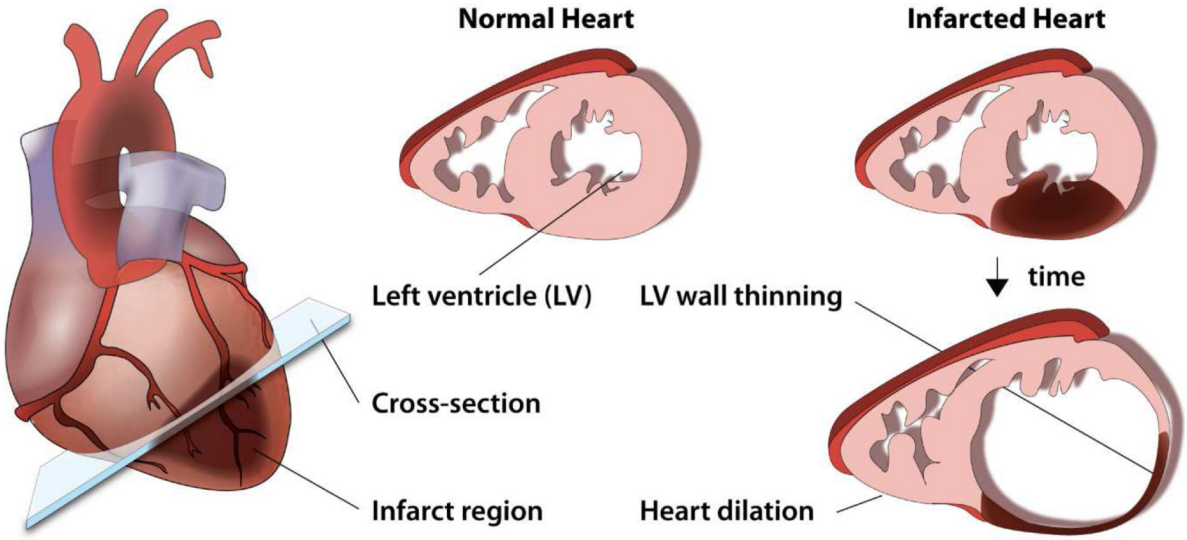

Fig. 1.

Myocardial infarction (MI) causes severe damage and adverse remodeling in the left ventricle (LV) myocardium, leading over time to LV wall thinning and dilation and ultimately progressing to contractile dysfunction and heart failure.

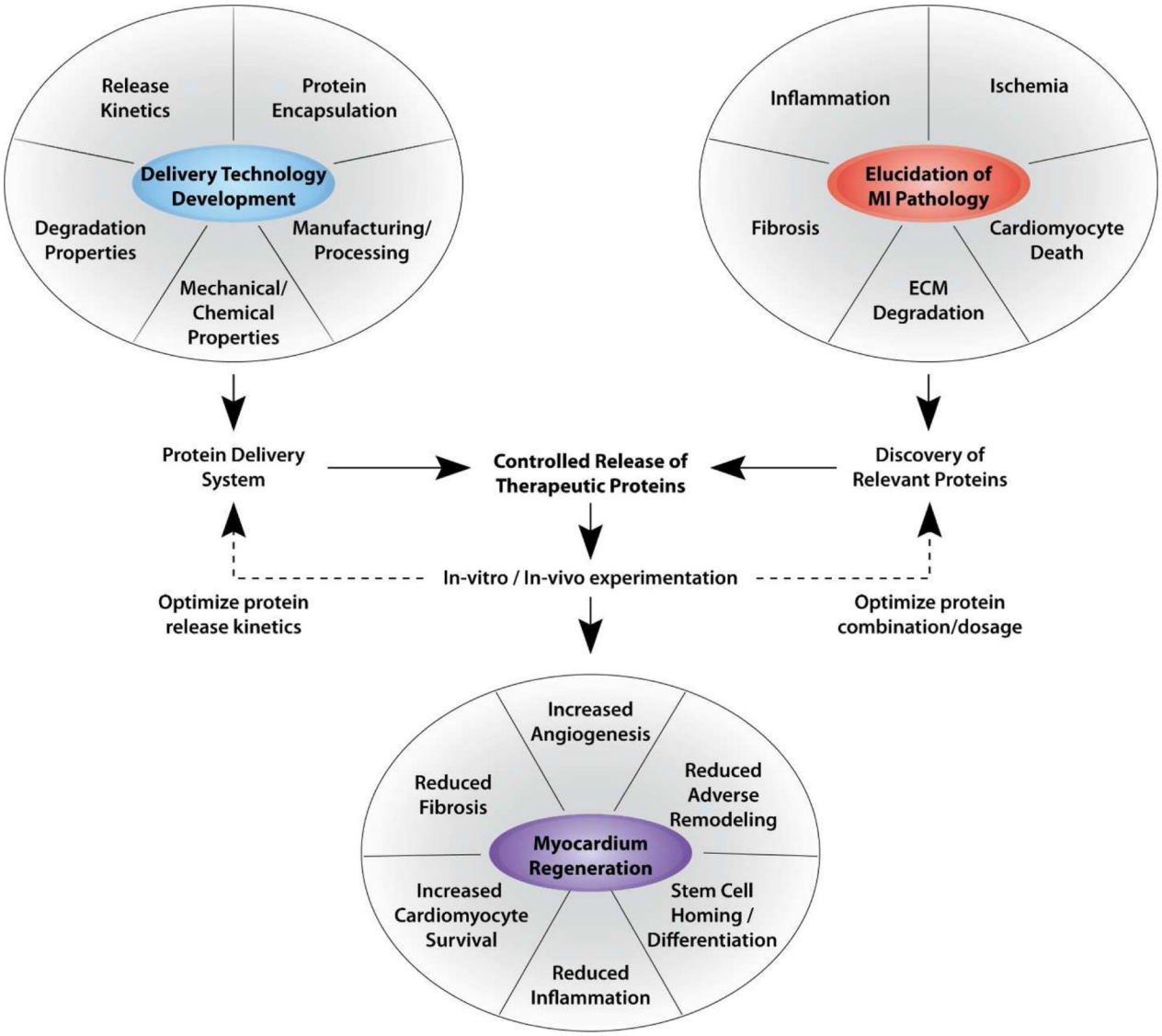

Fig. 2.

Schematic of a protein therapy design. An effective therapy requires the elucidation of the pathological changes after MI, leading to the identification of involved proteins. It is also essential to develop a proper delivery technology that can encapsulate proteins of interest and deliver them in a physiologic manner. The optimized strategy can potentially counter or reverse the pathological progression and trigger the repair and regeneration mechanisms in the heart.

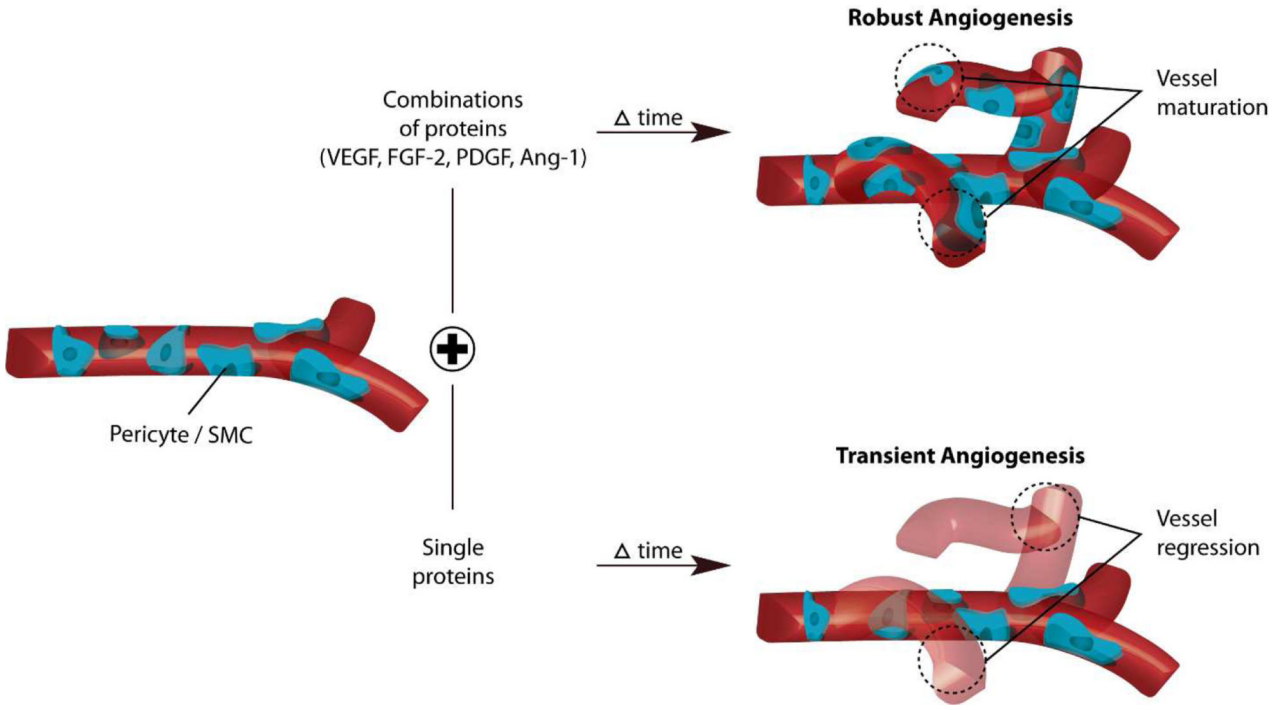

Fig. 3.

Fate of angiogenesis induced by combination or single protein therapies. A combination therapy that employs proteins involved in triggering angiogenesis (i.e. VEGF, FGF-2) in combination with proteins involved in stabilizing new blood vessels by pericytes (i.e. PDGF, ANG1), is more likely to induce a robust angiogenesis process forming mature and stable vasculature. Single protein therapies might lead to a transient angiogenesis process with new blood vessels prone to regression due to lack of stability and maturity provided by pericytes.

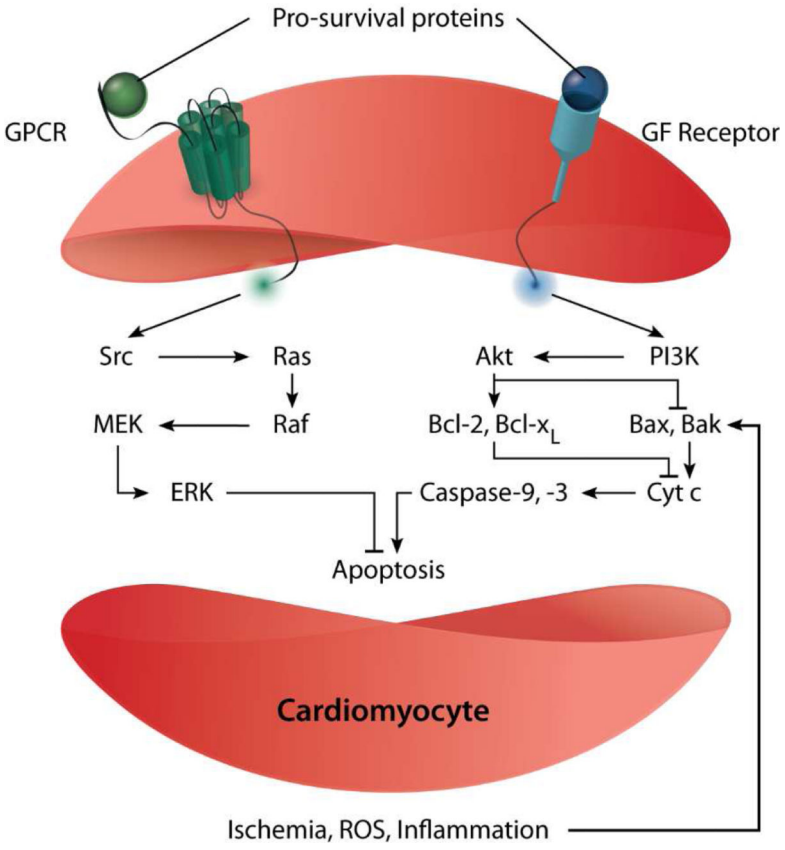

Fig. 4.

Ischemia, reactive oxygen species (ROS), and inflammation can trigger pro-apoptotic protein signaling (Bax, Bak) and inhibit anti-apoptotic protein signaling (Bcl-2, Bcl-x_L) within cardiomyocytes leading to release of cytochrome c and activation of caspases causing apoptosis. Pro-survival proteins that bind to their respective receptors on the myocyte surface can trigger PI3K/Akt and Ras-Raf-MEK-ERK pathways anti-apoptotic molecular pathways to prevent cell death.

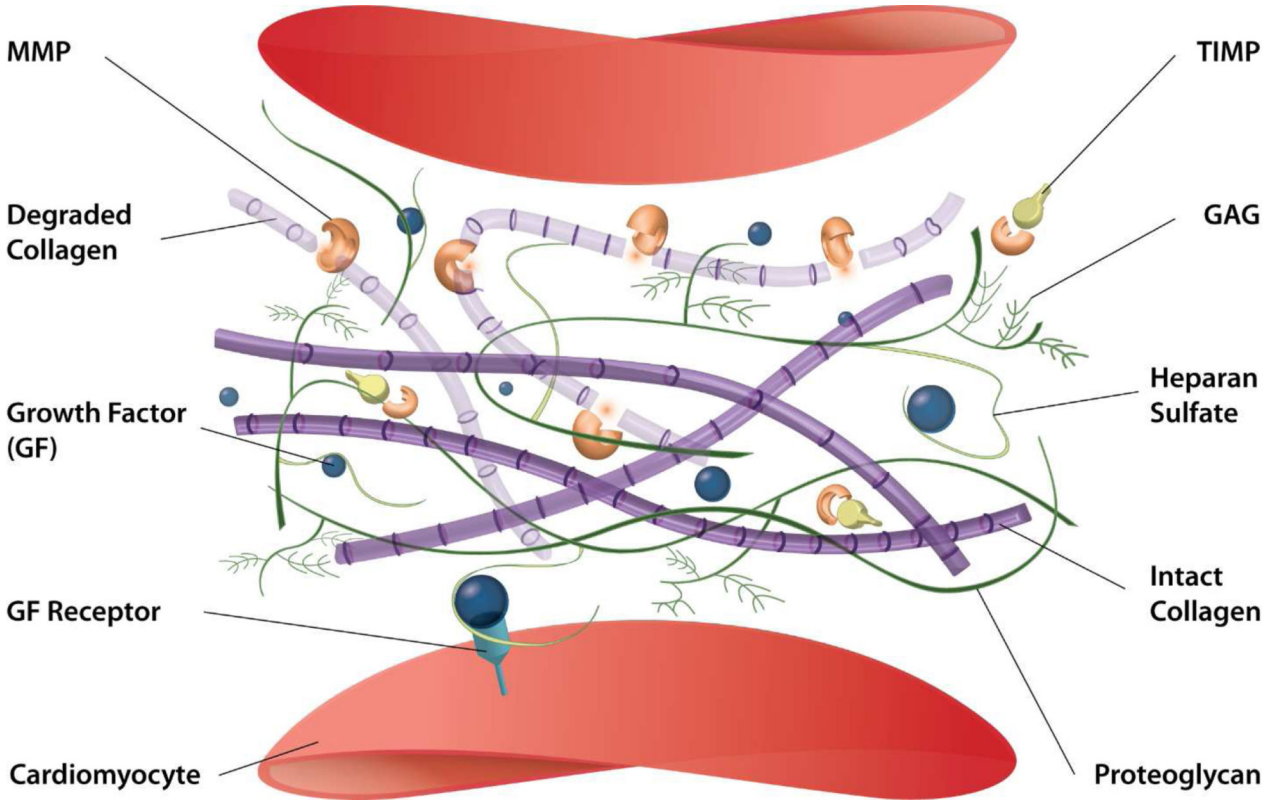

Fig. 5.

The myocardial extracellular matrix (ECM) serves as the base that connects cardiomyocytes, provides structural stability, and enables the transmission of chemical signals and contractile forces. The ECM contains structural proteins such as collagen and elastin, proteoglycans such as heparan sulfate, and adhesive glycoproteins such as fibronection and laminin. The ECM composition and orientation are strictly regulated in a healthy myocardium mainly by matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). TIMPs can help reduce early ECM degradation after MI alongside GFs involved in promoting cell survival, cardiomyogenesis, and angiogenesis.

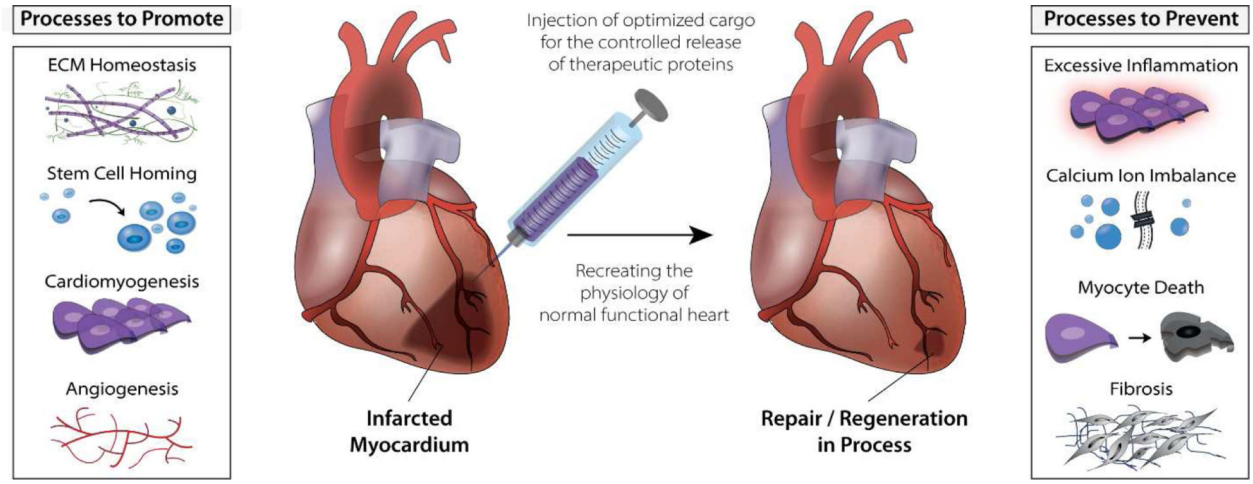

Fig. 6.

Repair and regeneration of the infarcted myocardium can be driven by delivery of proteins that address MI pathologies. To treat MI, a therapy needs to promote ECM homeostasis, stem cell homing, cardiomyogenesis, and angiogenesis, and prevent excessive inflammation, calcium imbalance, cardiomyocyte death, and fibrosis. Processes needed to be promoted or prevented after MI can have temporal differences. Some such as ECM homeostasis and calcium balance need to happen early on, while others such as fibrosis prevention should happen later. Injecting a protein delivery system carrying specific proteins of interest and delivering them per their physiologic cues offers the potential to trigger repair and regeneration signaling cascades leading to the restoration of a functional myocardium.

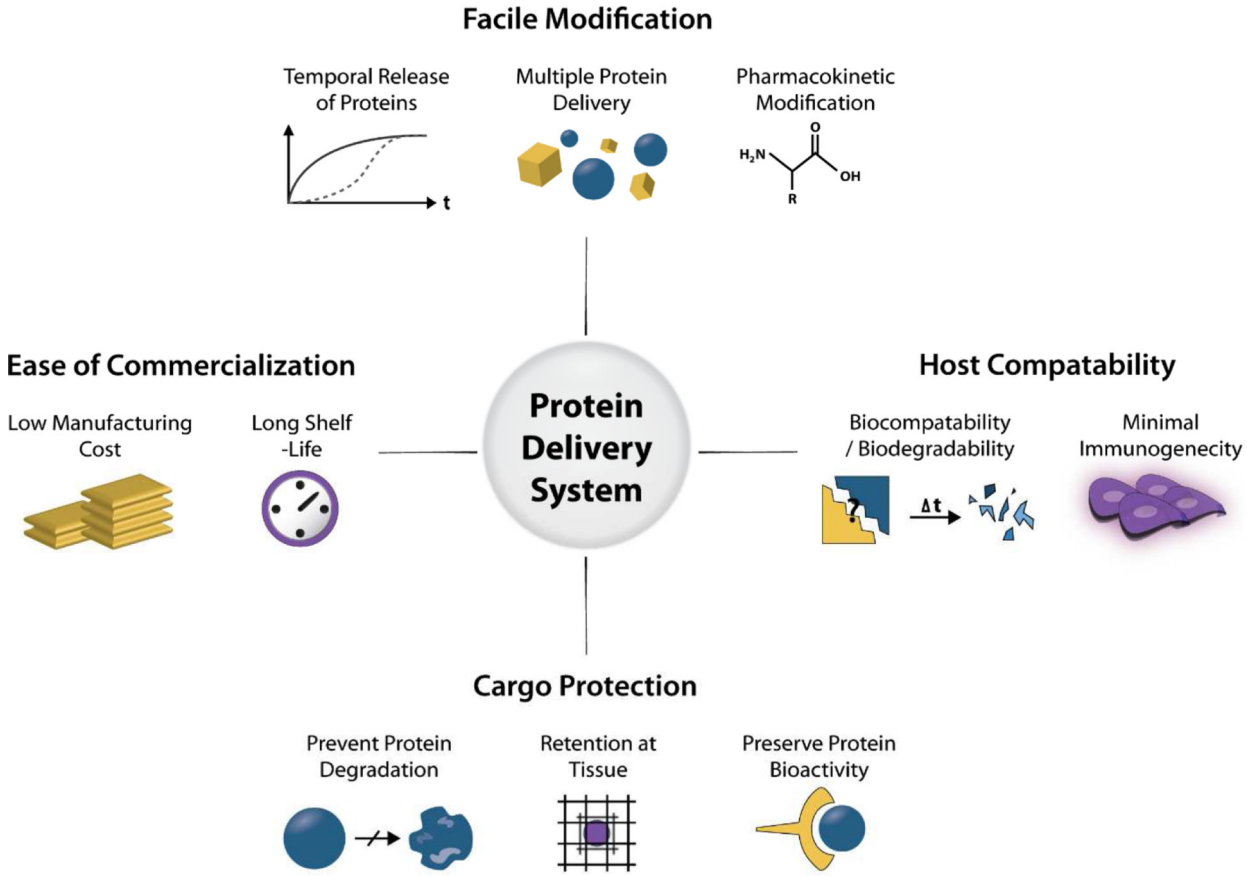

Fig. 7.

Desirable properties of an effective protein delivery system. Practically, it may be difficult to satisfy all of the desirable properties and a balance has to be made based on cost and resources.

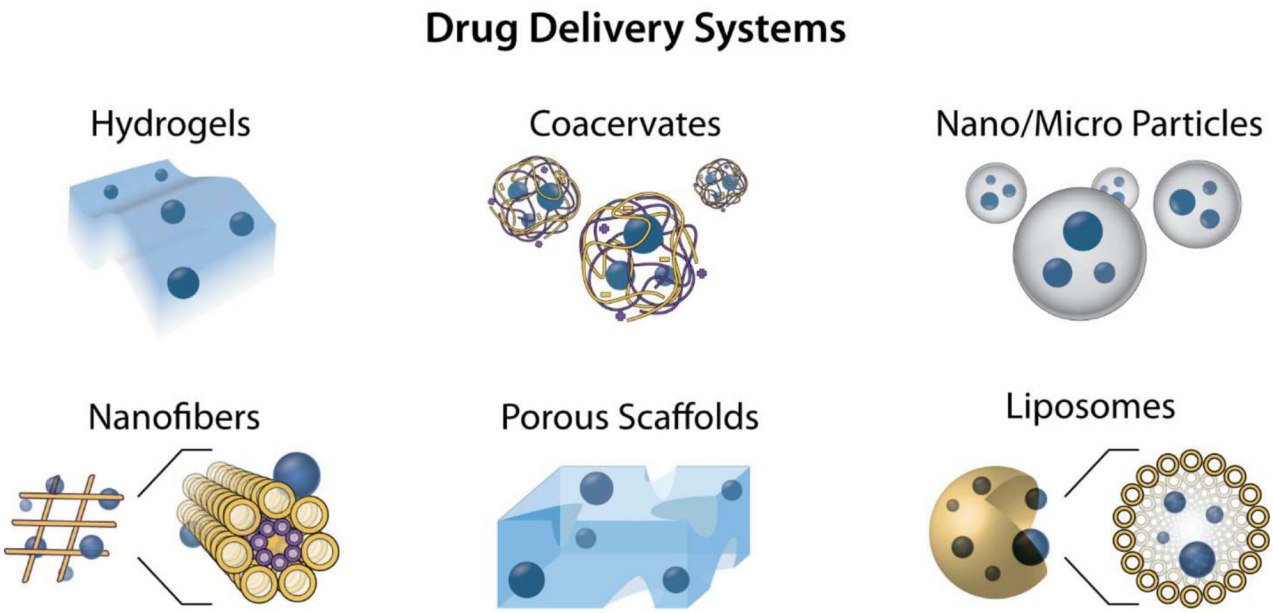

Fig. 8.

Commonly used and developed drug delivery systems include hydrogels, nano/micro particles, coacervates, self-assembled nanofibers, porous scaffolds, and liposomes. The structural, mechanical, and chemical properties of these systems can be modified to control the release kinetics of cargo.

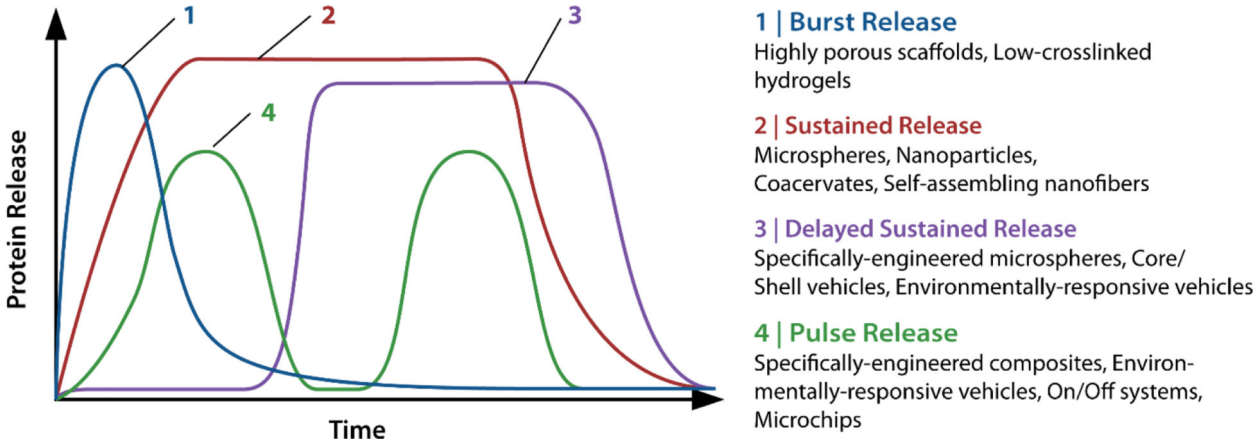

Fig. 9.

Different release profiles can be attained by different controlled release systems. The rate and style of release over a certain period can be controlled by changing the design and chemical and mechanical properties of the delivery vehicle.

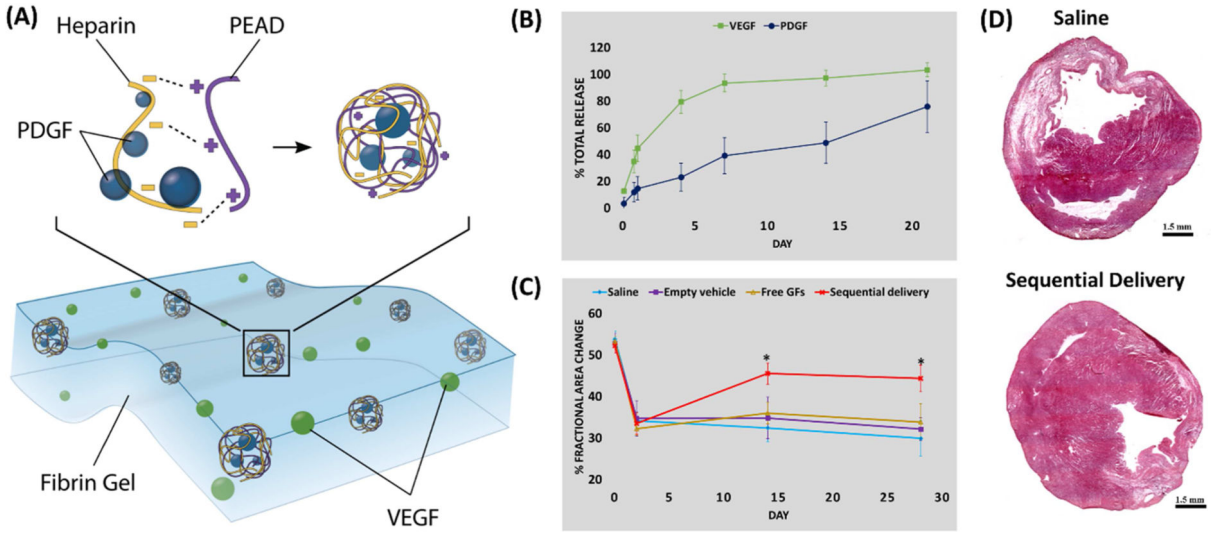

Fig. 10.

(A) A coacervate can be self-assembled by mixing PEAD and heparin. Embedding VEGF in a fibrin gel and PDGF in a coacervate that is distributed in the same gel leads to a (B) sequential release of VEGF followed by PDGF. (C) Sequential delivery of VEGF and PDGF significantly improves cardiac function in rats compared to saline, empty delivery vehicle, and free proteins. (D) Hematoxylin and eosin (H&E) staining shows significant damage of heart morphology, dilation, wall thinning, scar expansion, and granulated tissue in saline control compared to significant reduction of damage due to sequential delivery after MI.

Table 1

Major proteins of interest with functions related to cardiac tissue that should be either promoted or antagonized after MI to induce proper cardiac repair and regeneration.

Protein	Abbreviation	Main cardiac functions	References
Insulin-like growth factor-1	IGF-1	Anti-apoptotic; promote stem cell growth and differentiation	[30], [84], [126], [133]
Hypoxia-inducible factor-1α	HIF-1α	Trigger release of angiogenic growth factors and nitric oxide	[15], [19-21]
Transforming growth factor-β	TGF-β	Upregulate fibroblast proliferation, migration, and differentiation; trigger myofibroblast activation	[168], [170], [172], [207]
Sonic hedgehog	Shh	Induce cardiac morphogenesis; anti-apoptotic	[23], [91]
Fibroblast growth factor-1	FGF-1	Initiation of angiogenesis; stimulate cardiomyocyte proliferation	[21], [23], [113], [229]
Fibroblast growth factor-2	FGF-2	Initiation of angiogenesis; upregulate proliferation, migration, and survival of endothelial cells; induce chemotaxis; anti-apoptotic	[19-21], [23], [25], [93]
Vascular endothelial growth factor-A	VEGF-A	Initiation of angiogenesis; upregulate proliferation, migration, and survival of endothelial cells ; trigger nitric oxide release	[19-23]
Platelet-derived growth factor-BB	PDGF-BB	Upregulate proliferation and chemotaxis of pericytes; promote angiogenesis and neovessel maturation; anti-apoptotic	[20], [30], [42], [249]
Angiopoietin-1	Ang-1	Promote stabilization of endothelial cell-pericyte interactions and vessel maturation	[41], [44]
Angiopoietin-2	Ang-2	Promote destabilization of endothelial cell-pericyte interactions and vessel extravasation	[41], [44]
Hepatocyte growth factor	HGF	Angiogenic, anti-apoptotic; chemotactic on stem/progenitor cells	[33], [34], [126], [219]
Granulocyte colony-stimulating factor	G-CSF	Chemotactic on stem/progenitor cells; anti-apoptotic; angiogenic	[31], [85], [125]
Stromal cell-derived factor 1-α	SDF1-α	Chemotactic on stem/progenitor cells; angiogenic	[35], [36], [90], [121], [220]
Erythropoietin	EPO	Chemotactic on stem/progenitor cells; angiogenic; anti-apoptotic	[86-89], [128], [217]
Thymosin-β4	Thymosin-β4	Chemotactic on stem/progenitor cells; angiogenic	[30], [127]
Neuregulin-1	NRG-1	Induce cardiomyocyte proliferation; anti-apoptotic	[109-112]
Matrix metalloproteinase-2	MMP-2	Promote extracellular matrix (ECM) degradation by catalyzing the proteolysis of ECM proteins	[65], [135], [145]
Matrix metalloproteinase-9	MMP-9	Promote extracellular matrix (ECM) degradation by catalyzing the proteolysis of ECM proteins	[65], [135], [145]
Tissue inhibitor of metalloproteinases-1	TIMP-1	Inhibit MMP activity and ECM degradation; anti-apoptotic	[93], [152], [154]
Tissue inhibitor of metalloproteinases-3	TIMP-3	Inhibit MMP activity and ECM degradation; anti-apoptotic; anti-inflammatory	[93], [144], [151], [153], [154], [181]
Interleukin-10	IL-10	Anti-inflammatory; anti-apoptotic	[71], [72]
Interleukin-1β	IL-1β	Pro-inflammatory; induce MMP activity	[60], [143], [168]
Interleukin-6	IL-6	Pro-inflammatory; upregulate fibroblast proliferation; induce MMP activity	[60], [168]
Tumor necrosis factor-α	TNF-α	Pro-inflammatory; pro-apoptotic; trigger myofibroblast activation; induce MMP activity	[60], [67], [68], [143]

Protein	Abbreviation	Main cardiac functions	References
Monocyte chemoattractant protein-1	MCP-1	Pro-inflammatory; angiogenic; chemoattractant	[29], [66], [132]