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REVIEW ARTICLE

New Strategies to Enhance Myocardial Regeneration: Expectations and Challenges from Preclinical Evidence

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Abstract: Nowadays, cardiac regeneration is an emerging topic in the cardiovascular field because of the compelling need for effective therapies for repairing or replacing cardiac tissue damaged by pathological or physiological conditions. Indeed, irreversible myocardial remodeling which follows acute myocardial infarction represents a serious burden of this century. In this context, a great improvement in pharmacological and interventional techniques is accompanied by a big challenge of cardiac regenerative medicine. In the last 20 years, several clinical trials tried to investigate the role of different types of stem cells in promoting cardiac repair. However, the promising results obtained in the preclinical trials have not yet been reproduced in patients. Thus, the development of novel strategies to improve stem cell efficiency became imperative. Here, an overview of the more recent cell types proposed for cardiac regeneration is presented, together with the most interesting approaches to enhance cell regenerative potential as well as cell-free approaches.

Keywords: Myocardial infarction, cardiac regeneration, stem cells, preclinical model, cell therapy, clinical translation.

1. INTRODUCTION

Although cardiovascular mortality has decreased over time due to the advancements in reperfusion and medical therapies, cardiovascular diseases (CVD) still constitute a serious clinical and social concern worldwide. As indicated by the Heart Disease and Stroke Statistics report, CVD accounted for more than 17 million deaths per year in 2016 and the mortality rate is expected to increase by 35% by 2030 due to the growing prevalence of advanced aged population [1]. Despite substantial advances in pharmacological and interventional techniques aiming to contain myocardial necrosis and fibrotic scar tissue formation in the context of acute myocardial infarction (MI), the irreversible myocardial damage most commonly results in post-infarction left ventricular (LV) remodeling and heart failure (HF) in the long-term. Hence, novel therapeutic strategies are evermore needed for the management of CVD.

In this regard, cardiac regenerative therapy is promising in repairing or replacing cardiac tissue in damaged and aged hearts. Over the years, the quest to restore compromised function and regenerate lost tissue in the failing heart led to the development of cell-, gene- and tissue engineering-based therapeutics.

A number of clinical trials have been conducted in the last 20 years aiming to investigate the contribution of a variety of cell lineages to promote cardiac repair. At present, no specific cell type has proved to be uniquely superior for cardiac regenerative purposes [2]. More importantly, it is well known that the physiologic and pathologic conditions of donor patients impact the regenerative potential of stem cells for cardiac transplantation [3, 4].

To complicate matters, the impressive results observed in the preclinical trials were inconsistent or modest when translated in patients [5]. Indeed, the considerable improvements of LV function observed in rodents (ranging between 10 to 40%) were only modest in large animals (about 7-9%) and almost negligible in patients (2-4%) [6].

It is, therefore, essential to develop novel strategies that might improve the therapeutic response after stem cell administration. Many efforts are being made in this direction, leading to the discovery of new possible cell-based therapies and technologies that have already been tested in preclinical models or that, at the moment, are still in a more immature phase of development with only *in vitro* demonstration of their potential [7-9]. In addition, new strategies to increase

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the capacity of stem cells to reach the site of interest, survive in the long term, and integrate into the damaged tissue (both structurally and functionally) have been studied. Finally, yet importantly, more recent evidence in this research field has been driving paradigm shifts in the pharmacotherapy of degenerative and ischemic heart diseases, leading to the emerging area of cell-free approaches.

In this review, it is aimed to give an overview of the new challenges in regenerative medicine that can lead to the identification of more effective therapies for treating incurable heart diseases in the near future. In particular, the review deals with novel cell-based therapeutics, strategies to improve the delivery, retention, engraftment and survival of cell products and cell-free approaches (Fig. **1**).

2. NEW STEM CELL TYPES

Recent studies have suggested the use of pericytes as a promising candidate for cardiac cell therapy. Pericytes are mural microcirculation cells capable of inducing angiogenesis, regulating blood flow and differentiating towards mesenchymal lineage. Furthermore, these cells demonstrated a particular resistance to hypoxic insult and, therefore, were proposed as good candidates in the context of ischemic environments [10]. There are different types of pericytes classified based on the sampling source. In particular, cardiac, skeletal muscle and adventitia pericytes have been tested in preclinical models of MI in mice. Cardiac and skeletal muscle pericytes proved to differentiate into cardiac cells and to promote angiogenesis *in vivo* [10], while adventitia pericytes, combined with cardiac stem cells (CSC), exhibited a synergic effect in terms of infarct size reduction, vascular proliferation and angiogenesis [11]. Interestingly, the dominant effect of this combination approach was explained with complex interactions of paracrine factors secreted by the two cell populations [11]. Unfortunately, such positive findings were not confirmed when translated in large animal models, both after autologous transplantation and xenotransplantation [12, 13]. Differences in phenotypic characterization, isolation, expansion and purification protocols may be at least partially responsible for the lack of consistency in preclinical studies. However, given the high pro-angiogenic paracrine activity of pericytes, future applications can involve cell-free approaches based on their exosomes [14].

Likewise, synthetic stem cells have been proposed based on the fact that cells act primarily through paracrine mechanisms. They are generated by the encapsulation of the cell secretome using a biocompatible polymer which is then covered by the original cell membrane. So far, synthetic stem cells have been successfully fabricated from cardiosphere-derived cells (CDC) and bone marrow (BM)-derived mesenchymal stem cells (MSC). Consistently, they were observed to express surface antigens similar to the cell population from which they originated, and exhibited a comparable profile of paracrine factors. When administrated to infarcted hearts in mice, synthetic stem cells showed angiogenic properties and the ability to preserve the vital myocardium, in-

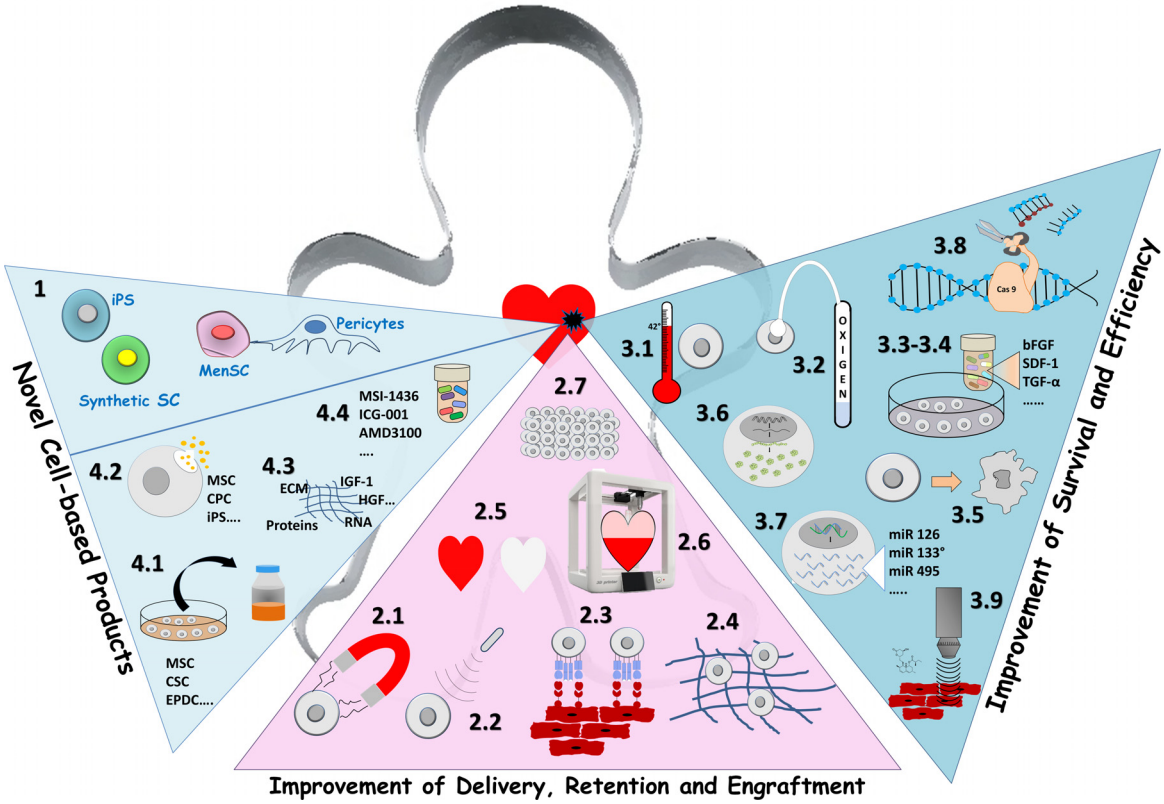

Fig (1). Visual index of the review. From the left to the right 2. New stem cell types; 5. stem cell-free approches; 3. Improvement of stem cell delivery, retention and engrafment; 4. Improvement of stem cell survival and efficiency. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

creasing the cardiac function and mitigating the remodeling of LV [15, 16]. Additionally, the maintenance of the naïve cell immune privilege, together with their high stability to cryopreservation, makes this technology attractive for application with different cell types in different organs [15, 16].

In the last 10 years, menstrual blood-derived endometrial stem cells (MenSC) have been proposed for cardiac regeneration [17, 18] because of their easy and abundant obtainment by non-invasive methods. More importantly, MenSC exhibited high transdifferentiation capacity and multiple paracrine effects in animal models of MI. In addition, a comparative study on MenSC and BM-MSC showed that the *in vivo* transplantation of MenSC after induced MI significantly enhanced LV fractional shortening and fibrosis area after 2 weeks and demonstrated a superior activity in improving cardiac function after the induction of MI in rodents compared with BM-MSC [19]. Nevertheless, further investigations into large animals need to be performed to better define the optimal dosage and delivery approach and to augment our knowledge concerning the precise mechanism of action of these cells.

Since the first generation of induced pluripotent stem cells (iPS) by Takahashi and Yamanaka [20], several investigators oriented their research to differentiate these cells into cardiomyocytes (iPSdCM) [21-23]. Although iPSdCM have a more immature phenotype than adult cardiomyocytes, positive outcomes were achieved after their injection into the infarcted heart of primates [24]. They showed the ability to efficiently integrate into the host tissue, leading to improvements in cardiac function and LV remodeling at 12 weeks after transplantation. On the other hand, a transient but significant increase of ventricular tachycardia was observed in the iPSdCM-treated group as compared to the vehicle control [24]. Besides arrhythmogenic issues, additional problems of iPSdCM, such as genomic instability [25] and immunogenic capacity [26], still need to be addressed before their translation in the clinic.

3. IMPROVEMENT OF STEM CELL DELIVERY, RETENTION, AND ENGRAFTMENT

The effectiveness of cell therapy is, in large part, related to the proper administration method which should safely guarantee adequate cell retention into the myocardium. Two main methods of delivery have been applied so far: the systemic (intravenous or intracoronary) and the local (intramyocardial) injection. Generally, intramyocardial injection is preferred both in preclinical and clinical trials and, although more invasive, it allows a greater recovery of the cells at the site of interest and a lesser shedding in non-target tissues. Notwithstanding, the engraftment of cells in the ischemic tissue continues to remain very inefficient. Indeed, it is estimated that more than 90% of the cells are already washed-out in the first two hours after delivery due to the high perfusion of the heart [27], and only a percentage between 0.1 and 10% of the remaining cells continues to survive for more than a few weeks after the transplantation [5]. In this view, it is crucial to develop other methods ensuring proper cell delivery, retention and engraftment. To date, the most promising approaches for the delivery involve magnetic-, ultrasound- and bispecific antibodies-based targeting, while biomimetic, decellularized and 3D printed tissue scaffolds, but also scaffold-free constructs, are currently being studied to increase cell retention and engraftment into the host tissue.

3.1. Magnetic Cell Targeting

A method for increasing and driving cell delivery consists of the labeling of cells with particles responsive to a magnetic field and the guidance of magnetically-labeled cells to the target tissue. In more detail, Huang *et al.* demonstrated that the targeting of MSC with superparamagnetic oxide nanoparticles can increase the retention of transplanted cells by 2.7-2.9 fold following the induction of MI in rats [28]. Yet, this approach led to an attenuation in LV remodeling and an increase in LV ejection fraction (LVEF) 3 weeks after surgery. Magnetic targeting was also tested in CDC by means of ferumoxytol nanoparticles, feraheme, demonstrating a 3 fold increase in cell retention after transplantation in a mouse model of MI [29]. This strategy ultimately resulted in decreased LV remodeling, increased LVEF and angiogenesis 3 weeks after the treatment.

This approach offers the advantage of being non-toxic and, therefore, can be applied to a variety of cell types; however, it needs further bench work to optimize cell targeting and to proceed with studies on large animal models.

3.2. Ultrasound-Mediated Delivery

Another innovative technique to improve cell delivery is the loading of microbubbles on the cell product surface combined with the use of ultrasound (US). Specifically, the US is used to burst the microbubbles causing site-specific delivery of the bioactive materials. The primary effect of the US-targeted microbubble destruction is the improvement of myocardial permeability along with the stimulation of the damaged heart and blood vessels to secrete paracrine factors [30]. As a result, Tong and collaborators demonstrated an increase in homing efficiency and consequent increase in cardiac function and neoangiogenesis after the transplantation of BM-MSC conjugated with nitric oxide microbubbles in a mouse model of MI [31]. Similarly, Chang *et al.* studied the effect of US-mediated microbubble destruction in combination with intracoronary transplantation of BM cells in a canine model of MI. They demonstrated an increase in cell homing in the target zone, which determined the reduction of infarct size and the improvement of cardiac function [32]. Equally promising results were observed by Woudstra *et al.,* who targeted adipose-derived MSC (ADMSC) by coupling microbubbles with targeted CD90 antibody conjugated to the microbubble shell. This novel technique, called StemBells, resulted in improved LV function 5 weeks after intravenous injection in a rat model of MI [33].

3.3. Bispecific Antibodies

Another effective method for targeting cells is the use of bispecific antibodies (BiAbs) which are artificial proteins able to simultaneously bind two different antigens.

In murine preclinical models, an engineered cross-linked BiAb that contemporaneously binds CD45 antigen and anti-rat myosin light chain, typically expressed in infarcted myocardium, was designed to efficiently target human $CD34^{+}$

cells [33]. The arming of $CD34^+$ cells with the above construct determined cell delivery improvement and LV function amelioration 5 weeks after MI [34]. Similarly, the BiAb strategy was used to arm BM-derived Lin^+/Sca^+ murine cells with a cross-linked antibody that bound c-kit on the cells and vascular cell adhesion protein 1 on injured myocardial cells [35]. After the induction of MI, Lin^+/Sca^+ cells were highly retained in the injured myocardium showing comparable positive results by either using direct intramyocardial or intravenous injection [35].

Using a similar principle of cell manipulation, Tang and collaborators [36] fused platelet nanovesicles onto CSC surface membranes to take advantage of the natural injury-targeting power of platelets, thus ultimately enhancing the targeting of CSC toward the lesion site. As a result, this bond showed a higher retention of CSC and a reduction of infarct size in a rat ischemia/reperfusion model. These data were further confirmed in a porcine model of MI [36]. In essence, the advantages of this approach are the absence of genetic manipulation and the potential applicability to any type of cell. It is worth noting that this specific tool can also be used to further investigate basic stem cell biology.

3.4. Biomimetic Scaffolds

Biomimetic scaffolds are polymeric 3D platforms used to protect cells from the hostile environment and prevent their loss immediately after the transplantation. In general, biomimetic scaffolds are designed to mimic the flexibility of cardiac tissue, allowing physiological electrical propagation and release of oxygen and nutrients to the inserted cells.

There are two categories of biomaterials: the first concerns the injection of a liquid matrix within the heart that immediately solidifies after transplantation while the second comprises the construction of a cell-matrix patch before transplantation with a structure similar to the target tissue.

The liquid matrix approach determines cell encapsulation with the intent to increase the engraftment of cells in the host tissue and consequently to improve the therapeutic performance [37]. With this particular approach, biocompatible and biodegradable substances of natural (e.g. fibrin hydrogel, alginate, collagen, silk, chitosan, matrigel, hyaluronic acid) or synthetic origin (poly L-lactic acid, polylactic co-genic acid, nanofibers, polycaprolactone) have been applied [37]. The liquid matrices composed of natural compounds are based on proteins with a structure similar to the native extracellular matrix (ECM), having a resorption rate compatible with native ECM replacement [37]. Natural-based liquid matrices have been shown to promote the engraftment of transplanted cells with the consequent increase in neovascularization and LVEF in mouse models of MI [38, 39]. Although they possess low immunogenic activity and high capacity for cell adhesion, natural-based matrices showed inadequate mechanical strength and structural sensitivity that make them difficult to manipulate outside the body [37]. To overcome these drawbacks, synthetic compounds were proposed. These injectable carriers are characterized by easy manipulation due to the predictable mechanical and chemical properties. On the other hand, they may induce an immune response and undermine cell survival [37]. In a recent study, human CSC were safely and effectively encapsulated in thermosensitive nanogel (poly(N-isopropylacrylamine-coacrylic acid) or P(NIPAM-AA)). After 3 to 4 weeks of the induction of MI, the injection of encapsulated CSC led to the preservation of cardiac function and the reduction of the scar size both in mice and pigs [40].

Of note, biomaterials can also be strengthened with molecules, such as insulin growth factor-1 (IGF-1), stromal cell-derived factor-1 (SDF-1), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), to further boost the activity of liquid matrices [41-43].

Likewise, cardiac patches showed interesting results. Initially, single-layer patches loaded with MSC were generated to promote the repair of infarcted heart, increasing neovascularization and decreasing fibrosis in preclinical murine models [44, 45]. Nevertheless, other studies showed that single patches did not efficiently promote cell survival after the transplant due to the lack of blood supply to the cells [46]. For these reasons, other cell types, such as endothelial cells, pericytes, iPS, and embryonic stem cells (ESC), were introduced into the new cardiac patches. This new generation of multi-layer cardiac patches contains microvessels composed of endothelial cells able to integrate with the host tissue leading to increased capillary density [9, 47]. However, cardiac performance improvement resulting from this approach remains insufficient [48]. Other groups tested the generation of human cardiac muscle patches composed of fibrin scaffolds containing cardiomyocytes, smooth muscle cells, and endothelial cells differentiated from human iPS and combined with IGF or not [49]. Ultimately, this approach, applied in a model of MI in pigs, resulted in improved cardiac performance, reduced hypertrophy and cardiac apoptosis and was not associated with an increase in arrhythmias despite the 10% rate of engraftment at 4 weeks [50].

3.5. Decellularization/Recellularization Technology

Whole organ engineering starting from allogeneic or xenogeneic corpse sources is a very promising field for the future treatment of CVD in which an organ transplant is required. Decellularization is an alternative method to generate a biological scaffold capable of guiding cell adhesion, proliferation and differentiation during tissue recellularization [51]. It is a process that involves the removal of cells from their ECM, keeping the integrity of the external structure intact, and enabling the seeding of new cells (recellularization). Decellularization was tested for the first time in 2008 by Ott and collaborators using a rat heart [52]. This process allows to obtain 4 intact chamber structures and valve geometry that can be recellularized with different cell types of the heart (neonatal cardiomyocytes, iPSdCM, MSC, fibroblasts, endothelial and smooth muscle cells) to recover a new contractile structure [52, 53]. Furthermore, the decellularized scaffold can be conjugated to chitosan obtaining a scaffold that is able to transmit both chemical and mechanical signals of the original myocardial tissue guiding the growth of cardiac progenitor cells (CPC) efficiently [54]. Notably, decellularization was also achieved in large animal models [55] and in the human heart [53].

3.6. Three-Dimensional Organ Printing

Three-dimensional bioprinting is a technique of combining different cell types and growth factors encapsulated within biomaterials. Through a layer by layer process, it is possible to generate a 3D construct that mimics the characteristics of the native tissue [51]. In this setting, CPC printed on hydrogel and gelatin/hyaluronic acid patches were tested for the treatment of cardiac-induced ischemia in mice, demonstrating the ability of the constructs to promote vascularization leading to an increase in cardiac performance [56, 57].

3.7. Scaffold-Free Sheet

Similarly, the cell sheet technique is an alternative strategy to deliver a greater number of cells based on the building of a 3D scaffold-free sheet for cardiac tissue. Using a scaffold-free approach, it is possible to overcome the inflammatory reaction and formation of fibrous tissue caused by scaffold degradation. Cell sheets are obtained using specific temperature-sensitive cell culture surfaces that allow 2D cell layers to be detached as intact confluent sheets and stacked to produce a 3D cardiac construct [37]. With this technique, it was possible to generate cell sheet starting from ADMSC [58], rat neonatal cardiomyocytes [59], iPSdCM [21, 60], endothelial and vascular mural cells [61]. In infarcted rat hearts, cell sheets demonstrated the induction of angiogenesis, the reduction in ventricular dilation progression and the improvement of cardiac function [58]. Furthermore, sheets derived either from neonatal rat cardiomyocytes or multiple lineage cardiac cells from iPS showed the ability to connect with the vasculature of the host, integrating with the native myocardial tissue through the expression of connexin-43 [59, 60]. In addition, the pedicle omental flap technique associated with cell sheets proved to further increase graft efficiency due to the enhanced survival of transplanted cells [46, 62].

4. IMPROVEMENT OF STEM CELL SURVIVAL AND EFFICIENCY

Another emerging option to improve survival and therapeutic activity of transplanted stem cells came from the approaches based on non-genetic and genetic modifications [3, 63]. The first approach encompasses different pretreatment strategies spanning from heat shock, hypoxic or irradiation treatment to the use of pharmacological agents and growth factors/cytokines [27, 63]. The second strategy comprises the genetic modification of stem cell products, applying, for example, protein overexpression and the editing or silencing of genes [27, 63].

4.1. Heat Shock

The effect of heat shock in protecting the heart tissue was reported in a preclinical rabbit model of myocardial injury in the early ’90s [64]. However, this application in the field of cardiac regenerative medicine has been proposed only in the last few years [65]. In 2014, Feng *et al.* suggested heat shock as a novel exploitable approach to potentiate cell-based therapy for ischemic heart disease [66]. Specifically, BM-derived Sca-1^+ stem cells were incubated at 42°C from 15 minutes up to 3 hours and transplanted in a mouse model of MI. This method reduced cardiomyocyte apoptosis and fibrosis and ameliorated heart functionality up to 4 weeks after injection. The regulation of the triangle Heat Shock Factor 1/miR-34a/Heat Shock Protein 70 was described as the key mechanism underpinning the effect achieved *in vivo* [66].

4.2. Hypoxic Pretreatment

The hypoxic cell pretreatment was proposed as an adjunctive cell preconditioning method. Basically, the rationale behind this approach was to stimulate cell defense mechanisms by the exposure of cells to low oxygen levels. The available preclinical reports employed different hypoxia degrees (ranging from 0.5 to 3% O_2) and duration (from few hours to days) [67]. However, despite the heterogeneity of the protocols used, these studies consistently reported that after hypoxia preconditioning cells were less susceptible to the action of reactive oxygen species and expressed higher levels of C-X-C chemokine receptor type 4 (CXCR4), which was directly involved in cell homing [68-70]. Moreover, oxygen deprivation determined the activation of AKT and MAPK signaling pathways which in turn regulated the release of pro-survival and pro-angiogenic factors directly involved in cardiac regeneration (*e.g*. hypoxia-inducible factor 1, angiopoietin 1, erythropoietin and VEGF) [69, 71, 72]. All these mechanisms have been found to determine the improvement in cell migration and engraftment and, definitively, in cardiac function *in vivo*. Specifically, the stimulation of different cell populations, such as MSC and CDC, with low oxygen levels before the transplantation was found to increase LV function and angiogenesis near the infarcted zone, both in mouse and pig models of MI [67, 68, 73, 74]. Moreover, reduced collagen deposition was observed in the infarcted hearts of mice after the administration of preconditioned CDC sheets [74].

4.3. Preconditioning with Pharmacological Agents

Alternatively, the drug-mediated preconditioning was proposed as an effective method to produce cells more prone to replace damaged cardiac tissue [63]. Specifically, in the literature, it was reported that the treatment of ADMSC with sildenafil before the injection determined the release of basic fibroblast growth factor (FGF-b) and VEGF, which helped to reduce fibrosis and ameliorate cardiac function in a MI mouse model [75]. Improvements in cardiac function, together with reduced fibrosis, have also been observed in rats after the pharmacological pretreatment of MSC or endothelial progenitor cells (EPC) with trimetazidine or diazoxide, respectively [76, 77]. Another important effect exerted by pharmacological preconditioning is the ability of some drugs to activate anti-apoptotic pathways. For example, Khan *et al.* showed that MSC pre-treatment with 8-pCPT-2'-O-Me-cAMP (CPT) increased cell survival and adhesion in a rat model of MI through the activation of Rap1 pathway [78]. In the same line, the preconditioning with AM1241, a potent and selective agonist of cannabinoid receptor type II, protected ADMSC from oxidative damage via Stat3 activation with a final improvement in cardiac function in a mouse model of MI [79]. Finally, although it has been shown *in vitro* that the myogenic differentiation ability of stem cells can be increased by 5-azacytidine [80, 81], this treatment was not effective in reducing the extent of infarction and improving the cardiac function when applied *in vivo* [82, 83].

4.4. Application of Growth Factors or Cytokines

As drug-mediated preconditioning, the culture of stem cells with growth factors or cytokines *in vitro* mimicking a damaged heart has the aim to influence their functionality in such a harsh environment [27]. Interestingly, the addition of SDF-1 in the culture medium of BM-MSC before the injection in a rat model of MI strongly increased the homing and pro-angiogenic ability of the cells. This consequently determined fibrosis and infarcted area reduction and improvement of cardiac functionality [84]. Similar effects were also observed after MSC preconditioning with transforming growth factor-alpha (TGF-α) in a rat model of MI [85]. Interestingly, in the latter case, the effects seemed to be mediated by the ability of preconditioned cells to reduce the pro-inflammatory environment in the infarcted myocardium [85].

4.5. Apoptotic Cells and Their Secretome

In 2005, an unusual hypothesis postulated the apoptotic cells and their secretome as useful cell-free tools for cardiac tissue repair [86, 87]. Based on the idea that about 5-25% of transplanted cells are already apoptotic, and thus able to attenuate inflammatory reaction by the modulation of TGF-β and IL-10, apoptotic peripheral blood mononuclear cells (PBMC) were injected in rats after MI [88]. This study showed that the suspensions of apoptotic PBMC, but not viable PBMC or vehicle, prevented LV remodeling by increasing elastin expression in cardiac scar tissue [88]. Another experiment used the culture supernatants derived from irradiated PBMC, named APOSEC, for the injection both in rat and porcine models of MI [89, 90]. Both the animal studies reported an attenuation in myocardial remodeling and infarct size mediated by the activation of pro-survival signaling cascades.

4.6. Protein Overexpression

The overexpression of bioactive molecules, normally released by engrafted stem cells, has been explored as a reliable tool to support the survival of transplanted cells. This approach has been mostly carried out by viral transduction, an efficient procedure which is tricky to translate in the clinical setting due to its intrinsic safety risk [91, 92]. The most interesting targets for this tool are proteins that possess anti-apoptotic, pro-angiogenic and, in general, cardioprotective activity. In particular, the anti-apoptotic IGF-1 was overexpressed in ADMSC before the transplantation in a rat model of MI, obtaining an increase in LVEF 6 weeks after the MSC injection despite an improvement in transplanted cell survival was not recorded [93, 94]. Similarly, Jackson *et al.* found a significant amelioration in myocardial regeneration due to the *in vivo* injection of CSC overexpressing IGF-1, which also displayed decreased apoptotic events [95]. Another paracrine factor of interest for cardioprotection is HGF because of its ability to enhance cell survival, angiogenesis and cardiomyocyte proliferation as observed after the injection of HGF-transduced MSC in a mouse model of MI [96]. Then, the possible synergistic effect of IGF-1 and HGF overexpression in cardiac regeneration was also evaluated. Interestingly, the IGF-1 and HGF co-expression in ADMSC decreased inflammation, improved angiogenesis but induced only moderate amelioration of cardiac function in an MI porcine model [97].

Stem cell viability has been also found to be positively influenced by the overexpression of Bcl-2, a cell-survival protein best known for its roles in inhibiting apoptosis that once transduced in MSC inhibited apoptosis and improved heart function [98]. Interestingly, the integrin β1 overexpression also showed to facilitate the proliferation of BM-MSC under oxygen-glucose deprivation condition and to regulate the expression of Caspase-3, Bax, Bcl-2, focal adhesion kinase, and integrin-linked kinase. Li *et al.* demonstrated that integrin β1 overexpression in BM-MSC increased the survival after 1 week of transplantation in a MI rat model by inhibiting the apoptosis of both transplanted BM-MSC and cardiomyocytes through adhesion-mediated cell survival signaling. After 4 weeks, the authors also observed a significant improvement in heart function compared to the control group [99]. On the same line, Mao *et al.* showed that the overexpression of integrin-linked kinase in MSC, injected in a porcine MI model, reduced apoptosis and fibrosis and increased cardiomyocyte proliferation and angiogenesis [100]. In addition, MSC engineered for Akt overexpression, alone or in combination with Angiotensin I, showed enhanced cell survival and regenerative capacity *in vivo* [101, 102].

Finally, CSC overexpressing SDF-1α determined a higher efficiency in restoring cardiac function and in limiting cardiac scar development, by acting on cardiomyocyte proliferation and BM-derived cell recruitment in mice previously subjected to experimental infarction [103].

4.7. Gene Silencing: The Use of miRNAs

A safer approach to improve the cardiac regenerative potential of stem cells involves the use of miRNAs to modulate the paracrine activity. Ham *et al.* showed that the transfection of let-7b into human MSC increased cell survival by targeting caspase-3, thus suppressing apoptosis and autophagy. Moreover, the transfected cells, when transplanted into a rat model of ischemia/reperfusion injury, promoted angiogenesis and ameliorated LV function and infarct area [104].

Another study showed a massive increase in VEGF expression and secretion after the transfection of miR-146, miR-126, or miR-377 in MSC before their injection in a rat model of MI. As a result, the higher release of VEGF promoted angiogenesis, diminished fibrosis, and ameliorated LVEF [105]. MSC engineered for miR-133a or miR-301a showed higher pro-survival and homing activity, as well as improved cardiac function, when injected in a preclinical rat model of infarcted heart [106, 107].

Conversely, antagomiR is another useful approach for ameliorating cardiac regenerative performance. Liang *et al.* showed that the inhibition of the anti-angiogenic miR-495 in human iPS enhanced the acquisition of an endothelial phenotype *in vitro* and their pro-angiogenic activity in a MI mouse model [108].

4.8. Gene Editing by CRISPR/Cas9 and TALEN Technologies

Despite genetic modification has clearly proved to increase stem cell regenerative potential, the translation of this technique in clinics is strongly debated and limited due to the

possible side effects related to the stable DNA integration into the cell genome, basically concerning oncogene activation. Interestingly, two novel strategies for gene editing emerged: the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) 9 (CRISPR-Cas9) and the Transcription activator-like effector nuclease (TALEN). Both the technologies permit a reduction in undesired oncogene activation [63]. Indeed, these approaches allow precise insertion of therapeutic genes into the stem cell genome without side effects on neighboring cells [63]. To the best of our knowledge, no preclinical study concerning the use of CRISPR-Cas9 with a regenerative purpose in the myocardial infarction model has been published until now. On the contrary, the TALEN application has been exploited to obtain a more efficient adeno-associated virus integration site 1. Luo *et al.* demonstrated that iPSdCM engineered with this method were able to maintain transgene expression after injection into the infarcted heart of mice for at least 7 weeks [109]. More recently, the TALEN application has been used to modify human iPSdCM to create an engineered functional ventricular heart tissue in a dish [110]. Specifically, a selection marker (e.g. neomycin or enhanced GFP) was inserted after the locus of myosin light chain 2 in human iPS to ameliorate the iPSdCM purification. Interestingly, selected iPSdCM have demonstrated to form a compact tissue-like structure when cultured with MSC on a decellularized heart ECM [110].

4.9. Tissue Preconditioning and Reprogramming

On the other hand, the host tissue can also be preconditioned to improve its responsiveness to stem cell delivery. The preconditioning could be accomplished through the administration of pharmacological treatment or by physical cues to modify the transplant site [63]. This novel concept has been already tested at the preclinical level demonstrating higher cell engraftment and survival [63, 111, 112]. As an example, treatment with statins promoted BM- and ADMSC survival and therapeutic effects on ischemic myocardium in preclinical models [113, 114]. Of interest, the low-energy shock waves have been used to induce the up-regulation of SDF-1 and other chemoattractants in the ischemic tissue to enhance the homing of transplanted cells and thus improve neovascularization of the damaged tissue [115]. Another interesting target used to precondition the host tissue is agrin, a component of the mice ECM able to confer the myocardial regenerative capacity to neonatal mice [116]. Of note, the *in vivo* administration of agrin has been found to promote myocardial regeneration in an adult mouse model of MI [116].

5. STEM CELL-FREE APPROACHES

Accumulating evidence suggests that the therapeutic effect of stem cells in the context of ischemia diseases is, in large part, mediated by the release of paracrine factors [117]. Starting from this viewpoint, many research groups in this field have shifted their efforts to study the use of stem cell-conditioned medium (CM) or derived exosomes as a source of a broad array of paracrine factors. Stem cell-free approaches also include the development of scaffold-free constructs and small molecules able of activating endogenous regenerative processes.

5.1. Conditioned Media

It is known that, given a specific stem cell population, CM can contain different types and percentages of small proteins, cytokines and chemokines, soluble molecules and extracellular vesicles (*i.e.* exosomes and microvesicles) [117, 118].

At the preclinical level, the intravenous and intracoronary administration of CM derived from human MSC determined a marked reduction in myocardial nuclear oxidative stress with respect to non-CM or saline and was associated with a 60% reduction in infarct size [119].

Consistently, paracrine factors secreted by human CSC exhibited a cardioprotective role (especially the extracellular vesicle compartment), leading to increased cardiac function after the induction of MI in mice [120]. Furthermore, human epicardial-derived cells (EPDC)-CM showed a beneficial effect in maintaining vascular integrity following ischemia/reperfusion injury [121]. In particular, the authors found that the HGF/IgG complex in human EPDC-CM was the key player in the promotion of endothelial cell survival.

Recently, Bollini's group described that CM and extracellular vesicles derived from human amniotic fluid-derived cells could improve cardiac repair and prompt cardiac regeneration by modulating endogenous reparative mechanisms [122]. More interestingly, CM exerted higher pro-angiogenic potential while extracellular vesicles improved cardiac function, thus suggesting their preferential application according to the specific therapeutic necessity [122]. Further investigations are needed to define whether the whole CM or a selected active component is more useful for cardiac regenerative purposes.

5.2. Exosomes

In the field of cell-free regeneration, exosomes have proved to be promising for cardiac regeneration [123]. At present, they are isolated from the extracellular vesicle compartment of different stem cell-conditioned media including MSC, CPC, ESC, iPS and EPC [124-128]. In preclinical MI models, exosomes have been instrumental for increasing homing, retention and engraftment of co-transplanted cells, and also for ameliorating the performance of the infarcted heart, reducing infarct size, inflammatory response, oxidative stress, fibrosis, activating survival signals and stimulating neovascularization [127, 129-131]. Regarding the delivery route, both intracoronary and intramyocardial injections were used; however, intramyocardial administration was proven to be more effective when CDC-derived exosomes were injected in pigs [127].

Exosomes contain a variety of molecules and factors such as lipids, proteins, RNAs and miRNAs that can influence and reprogram host cells. Among them, miRNAs demonstrated a key role in the therapeutic effect of exosomes. For example, it has been reported that miR294 contained in the ESC exosomes was able to increase cardiac function after the induction of MI by promoting the survival and proliferation of host CPC [132]; whereas miR181b derived from CDC exosomes and miR22 from MSC exerted their effects on rat, pig and mouse models of MI by conferring cardiopro-

tective property to macrophages phenotype and by reducing cardiomyocytes apoptosis, respectively [133, 134].

Additionally, exosomes can be loaded with different cargoes, such as siRNA, miRNA or other molecules of interest [9, 135]. Of note, Vandergriff and co-workers reported that by the use of a targeting peptide, cardiac homing peptide, to target intravenously-infused exosomes to the infarcted heart, there was a significant improvement in the outcomes with reduced fibrosis and scar size and increased cellular proliferation and angiogenesis [136]. Moreover, exosomes derived from MSC and treated with SuxiaoJiuxin protein increased cardiomyocyte proliferation by the upregulation of histone 3 lysine 27 in HL-1 cardiomyocytes [137].

5.3. Scaffold-Free Constructs

Other acellular strategies were tested for the treatment of ischemic conditions. Platelet nanocell, that incorporates both prostaglandin E2-modified platelet membrane and CSC-secreted factors, reduced cardiac remodeling, increased cardiomyocyte proliferation, and promoted angiogenesis and the activation of endogenous progenitor cells in a mouse ischemia/reperfusion model [138]. Moreover, alginate hydrogel proved to be efficient in preventing cardiac dysfunction both in recent and old infarcts in rats [139]. In addition, fibrin-specific poly nanogels led to a significant increase of LVEF at 2 and 4 weeks following MI, along with the decrease of infarct size and fibrotic marker expression [140].

Differently from cell-based therapy, acellular heart repair products are also interesting for the ideally simplest and fastest path to clinical use. In this perspective, cardiac patches containing acellular substances such as proteins, RNA or ECM, but not live cells, have been introduced to treat MI [9]. In this way, it was possible to deliver the recombinant follistatin-like 1 protein through collagen patches in the infarcted myocardium of mice and pigs to promote the re-entry of pre-existing cardiomyocytes into the cell cycle and increase both cardiac function and animal survival [141]. Cardiac patch derived from decellularized ECM without the addition of biologics demonstrated to promote cardiac function restoration by itself, through the recruitment of resident progenitors ($GATA4^+$/$c\text{-}kit^+$) in the presence of both recent and chronic myocardial scars [142]. Following infarction, the administration of injectable hyaluronic acid hydrogel combined with miR-302 led to the decrease of LV volume, increase of LVEF and fractional shortening 1 month after the injection [143]. Interestingly, to overcome the problem of open-chest surgery for cardiac patch placement, researchers developed a spray-paintable, polymerizable biomaterial, named platelet-fibrin gel. This new biomaterial, integrated with several regenerative stem cell factors, including VEGF, IGF-1, HGF, TGF-β, and PDGF, was used for cardiac repair in animal studies. The studies demonstrated that platelet-fibrin gel efficiently released the regenerative growth factors in the first two weeks after being sprayed and effectively preserved cardiac function and reduced scar fibrosis *in vivo* [144].

Since the major patch disadvantage remains the invasiveness of the implant procedure (which generally requires an open-chest surgery), the spray patch not only paves the way to use stem cell factor cocktails for cardiac regenerative therapy but also demonstrates a new drug delivery approach for potential minimally invasive patch transplantation [144].

5.4. Small Molecules

The development of small molecules capable of activating endogenous regenerative processes offers the potential advantage of using substances that, differently from cellular, protein or nucleic acid-based agents, are physically and chemically defined. Of note, they can be produced with reproducible quality with a lack of ethical concerns and are likely to have lower treatment costs [145]. Moreover, another striking advantage is their lack of immunogenicity compared with cell-based or protein therapeutics. Nevertheless, small molecule discovery and development have been constrained by the limited understanding of the signals and mechanisms controlling tissue homeostasis and the endogenous processes of tissue repair and regeneration after injury.

Small molecules have the potential of targeting all the endogenous tissue regeneration sources represented by somatic cells and the tissue-specific (adult) stem and progenitor cells, of which they can potentiate self-renewal, proliferation, differentiation toward specific cell lineage, mobilization from niche, migration homing and engraftment into the injury site. Nowadays, very few studies have highlighted the potential of these approaches that rely on the success of *in vitro* experiments and animal models of regeneration where potential targets can be identified and developed.

In the context of cardiac regeneration, the screening of ESC models identified small molecules with regenerative potential, such as bone morphogenetic protein (BMP), TGF-β and Wnt inhibitors [145-148]. Wnt inhibitors are the best candidates for *in vivo* regeneration as Wnt signaling has been shown to maintain embryonic cardiac progenitors in the proliferative state [147], whereas their inhibition drives progenitor differentiation towards cardiomyocytes. So far, two small molecule inhibitors of Wnt pathways, pyrvinium and ICG-001, have been proposed as drug candidates to reduce the adverse effects of myocardial infarction [149, 150]. However, although these molecules represented a *proof-of-principle* of Wnt inhibitors as future drugs for cardiac repair after injury, further studies are necessary to better characterize the underlying mechanisms. Moreover, since pyrvinium showed severe toxicity in animal models [149], the same studies should be repeated with the different drug-like Wnt inhibitors now available [151].

Drugs developed to improve self-renewal by epigenetic landscape modulation such as histone deacetylases inhibitors, DNA methyltransferase [152, 153], and drugs designed to ameliorate mobilization, homing and engraftment of hematopoietic stem cells (HSC) (e.g. SB-247464, AMD3100 and DPP-4 inhibitors) can also be included in the category of small regenerative molecules. HSC is known to participate in the regeneration of non-hematopoietic tissues such as myocardium after ischemic damage [154]. Mobilized HSC are thought to home to the site of injury where engraft and differentiate to regenerate the tissue or alternatively provide paracrine instructions for tissue generation. SB-247464 is a mimetic of granulocyte-colony stimulating factor (G-CSF) that, by activating G-CSF-receptor and disrupting CXCR4/SDF-1 axis, has a dominant role in promoting HSC

mobilization [155]. Notably, this effect is further boosted by the synergistic action of CXCR4 antagonists, such as AMD3100 (Plerixafor). Plerixafor used in combination therapy with G-CSF or SB-247464 increases HSC yields for autologous transplantation [156, 157]. Currently, numerous second-generation CXCR4 antagonists have been developed with some already in clinical trials [158, 159]. Similarly, even if in contradiction with the mechanisms described above, small molecules, such as diprotin A and sitagliptin, have also been developed to strengthen the CXCR4/SDF-1 interaction and improve homing and engraftment of HSC in the injury site. These molecules, originally designed as type 2 anti-diabetic drugs, pharmacologically inhibit the membrane-bound dipeptidyl peptidase 4 (DDP4) enzyme involved in SDF-1 degradation. The final result in murine models was a local increase of SDF-1 tissue levels that favored HSC homing and engraftment [160].

Tissue regeneration can be achieved not only by targeting stem cell function, but also by stimulating cell division of somatic cells within the tissue. Pro-proliferative agents are of particular interest for organs with very limited proliferative activity, such as the heart. Although such a therapeutic approach should be carefully evaluated for the risk of uncontrolled cell growth and tumor formation, boosting the proliferation as a means of tissue regeneration has led to direct *in vivo* experimentation that already yielded results with promising new small molecules, such as MSI-1436.

MSI-1436 is a naturally occurring aminosterol originally isolated from the liver of dogfish shark [161]. It is a potent and highly selective inhibitor of the ubiquitous protein phosphatase 1b (PTP1B) that acts *via* a non-competitive allosteric mechanism [162, 163]. PTP1B dephosphorylates and inactivates receptor tyrosine kinase (RTK) that has been activated by ligand binding-induced autophosphorylation [164]. RTK signaling has a key role in cell growth, differentiation, survival, wound repair process and metabolism, therefore, the inhibition of PTPB1 has been considered the major pharmacological target for the treatment of cancer, obesity and type 2 diabetes [164-166]. Numerous PTPB1 inhibitors have been developed including MSI-1436. MSI-1436 was tested in phase I clinical trials for type 2 diabetes treatment with promising results and good tolerance in the patients [ClinicalTrials.gov Identifier: ID NCT00509132, NCT00606112, NCT00806338, Genaera Corporation]. The molecule is now back in preclinical studies for its regenerative properties. In adult zebrafish, MSI-1436 showed to stimulate the regeneration of caudal fin and heart muscle following injury, without apparent tissue malformation or overgrowth suggesting that the molecule does not alter normal developmental processes and tissue homeostasis. The compound was also tested in the heart injury of adult mice that have limited regenerative capacity. The administration of MSI-1436 to adult mice after permanent coronary artery ligation increased the survival and heart function by 2-fold, increased cellular proliferation in the infarct border zone and reduced ventricular wall thinning by 4-fold compared with the control, reducing 53% of the infarct size [167]. The pro-regenerative effects of MSI-1436 seem to be mediated by the de-differentiation of precursor cells and/or the stimulation of cell division; however, an extensive additional *in vitro*/*in vivo* research is still required.

CONCLUSION AND FUTURE DIRECTIONS

Cardiac regenerative medicine remains a hot topic for clinicians and researchers, as it promises the replacement of lost myocardium and the stimulation of endogenous reparative mechanisms, thus preventing the progression of heart diseases. Indeed, a number of clinical trials of cardiac cell therapy have been conducted in the last 20 years, spurred by the impressive results obtained in animal models of myocardial injury. Unfortunately, accumulating evidence indicates that the beneficial effects in terms of cardiac function improvements are not fully translated to clinics.

As described above, major recognized challenges in this field are the identification of the optimal regenerative product (cells or molecules) to achieve a significant therapeutic effect along with the optimization of cell delivery approaches and, ultimately, the enhancement of retention, engraftment and survival of transplanted cells. To overcome these intrinsic limitations, cell-, gene- and tissue engineering-based therapeutics were improved over the last years and some of them either alone or in combination have entered the clinical stage, especially in the context of chronic HF. For example, a phase IIa clinical trial of 22 patients with nonischemic cardiomyopathy has been conducted by the administration of allogeneic ischemia-tolerant MSC (itMSC) or placebo [168]. itMSC therapy was safe, caused immunomodulatory effects, and ameliorated the patient's health status and functional capacity. Concerning the tissue engineering approach, a tissue patch resulting from the combination of allogeneic ESC-derived cardiac progenitor cells and fibrin was employed in patients with HF in the absence of safety concerns [169]. In addition, genetic modifications of the cells have already found an application in the clinic for the treatment of advanced HF, as recently reported by Gwizdala and collaborators, showing safety feasibility and effectiveness [170]. Finally, the iPSdCM cell sheet is planned to be tested in Japan for the application in HF repair [171].

LIST OF ABBREVIATIONS

ADMSC	=	Adipose-Derived Mesenchymal Stem Cells
BiAbs	=	Bispecific Antibodies
BM	=	Bone Marrow
BMP	=	Bone Morphogenetic Protein
CDC	=	Cardiosphere-Derived Cells
CM	=	Conditioned Medium
CPC	=	Cardiac Progenitor Cells
CSC	=	Cardiac Stem Cells
CVD	=	Cardiovascular Diseases
CXCR4	=	C-X-C Chemokine Receptor Type 4
ECM	=	Extracellular Matrix
EPC	=	Endothelial Progenitor Cells
EPDC	=	Epicardial-Derived Cells
ESC	=	Embryonic Stem Cells
FGF-b	=	Basic Fibroblast Growth Factor

HF	=	Heart Failure
HGF	=	Hepatocyte Growth Factor
HSC	=	Hematopoietic Stem Cells
IGF-1	=	Insulin Growth Factor-1
iPS	=	Induced Pluripotent Stem Cells
iPSdCM	=	Induced Pluripotent Stem Cell-Derived Cardiomyocytes
LV	=	Left Ventricular
LVEF	=	Left Ventricular Ejection Fraction
MenSC	=	Menstrual Blood-Derived Endometrial Stem Cells
MI	=	Myocardial Infarction
MSC	=	Mesenchymal Stem Cells
PBMC	=	Peripheral Blood Mononuclear Cells
PDGF	=	Platelet-Derived Growth Factor
SDF-1	=	Stromal Cell Derived Factor-1
TALEN	=	Transcription Activator-Like Effector Nuclease
TGF-α	=	Transforming Growth Factor-Alpha
TGF-β	=	Transforming Growth Factor-Beta
US	=	Ultrasound
VEGF	=	Vascular Endothelial Growth Factor

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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