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REVIEW

Cardiovascular regenerative therapeutics via synthetic paracrine factor modified mRNA

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Abstract The heart has a limited capacity for regeneration following injury. Recent strategies to promote heart regeneration have largely focused on autologous and allogeneic cell-based therapy, where the transplanted cells have been suggested to secrete unknown paracrine factors that are envisioned to promote endogenous repair and/or mobilization of endogenous heart progenitors. Here, we discuss the importance of paracrine mechanisms in facilitating replication of endogenous epicardial progenitor cells in the adult heart and signaling their subsequent reactivation and de novo differentiation into functional cell types such as endothelial cells and cardiomyocytes. Moreover, we discuss the use of a novel modified RNA technology in delivering such therapeutic paracrine factors into myocardium following injury. These studies suggest that modified mRNA may be a valuable experimental tool for the precise in vivo identification of paracrine factors and their downstream signaling that may promote heart repair, cardiac muscle replication, and/or heart progenitor mobilization. In addition, these studies lay the foundation for a new clinically tractable technology for a cell-free approach to promote heart regeneration.
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Introduction

Currently, heart disease is the leading cause of mortality in the industrialized world, partly due to the inability of the heart to regenerate in adults; and also the dietary and life style changes leading to a marked increase in the incidence of type II diabetes (for review, see Armstrong et al., 2013; Carvalheira et al., 2013; Ptaszek et al., 2012). Treatment options primarily address symptomatic manifestations; and current therapeutics can only delay the progression of heart failure until potential orthotopic heart transplantation is available (Ptaszek et al., 2012). Nevertheless, given the shortage of donor hearts for transplantation, there is an urgent need for novel therapies to repair severely diseased hearts. To address this issue, there is a growing interest in three research areas for heart regeneration including the use pluripotent stem cells (PSCs) for cell replacement therapy (for a review, see Addis and Epstein, 2013; Lui et al., 2012; Matsa et al., 2014; Vunjak-Novakovic et al., 2011; Xu et al., 2012), direct reprogramming of cardiac fibroblasts into myocardium in vivo (Fu et al., 2013; Ieda et al., 2010; Qian et al., 2012), or replication and reactivation of endogenous quiescent cardiovascular progenitor cells for differentiating into functional blood vessels and de novo cardiac muscle (Chong et al., 2011; Smart et al., 2011; Zangi et al., 2013). In this review, we focus on reactivation of our endogenous regenerative capacity through paracrine mechanisms, and describe a new technological platform via synthetic modified mRNAs to express these factors in vivo in the heart following myocardial infarction.

Paracrine mechanisms: lessons from cell-based therapies

Over the last decade, there has been a growing research interest in cell-based therapies with the hope of improving heart functions and attenuating adverse left ventricular (LV) remodeling in both ischemic and non-ischemic cardiomyopathy. Particularly, stem cells and progenitor cells which have the potential to self-renew and differentiate into functional cardiac muscle are attractive candidates for these purposes. The concept of cell-based regeneration has proven successful in clinical practice for over 50 years (Soiffer, 2008), in which patients receive umbilical cord blood hematopoietic stem cells which replenish their entire repertoire of immune cells following bone marrow transplantation. To date, in cell-based studies, skeletal myoblasts (Taylor et al., 1998), bone marrow-derived mononuclear cells (Balsam et al., 2004; Murry et al., 2004), bone marrow- (Silva et al., 2005) or adipose- (Cai et al., 2009; Valina et al., 2007) derived mesenchymal stem cells, CD34$^+$ hematopoietic stem cells (Botta et al., 2004; Wang et al., 2010), CD133$^+$ endothelial progenitor cells (Stamm et al., 2007; Voo et al., 2008) and c-kit + (Kajstura et al., 2005; Limana et al., 2005) or Sca-1$^+$ (Wang et al., 2006) cardiac progenitor cells have been introduced into the damaged heart; however, results from all these pre-clinical and clinical trials remain ambiguous and therapies are yet to be proven conclusively effective (for a review, see Sanganalmath and Bolli, 2013). While transdifferentiation of non-cardiac cells into cardiomyocytes and vascular cells following transplantation into the damaged heart remains controversial (Balsam et al., 2004; Murry et al., 2004), recent studies have also challenged whether adult cardiac progenitor cells can robustly regenerate heart muscle in vivo in both experimental model systems (van Berlo et al., 2014) and clinical studies (Nowbar et al., 2014).

Recently, human embryonic stem cells (hESCs) or induced pluripotent stem cells (hiPSCs), by virtue of their ability to self-renew and differentiate into almost all cell types of the body, have also been directed to differentiate into cardiomyocytes for transplantation studies (Cai et al., 2007; Caspi et al., 2007; Chong et al., 2014). Human ESC-derived cardiomyocytes were shown to attenuate LV remodeling and improve LV systolic function in rat hearts following myocardial infarction (MI) (Cai et al., 2007; Caspi et al., 2007). More recently, human ESC-derived cardiomyocytes were also demonstrated to generate extensive vascularized cardiac muscle in the infarcted hearts of non-human primates (Chong et al., 2014). Despite the advantage of using hESCs or hiPSCs to generate large numbers of human cardiomyocytes for clinical transplantation, these cells were quite diverse in terms of atrial/ventricular electrophysiological properties, as well as being partially mature and, therefore, proarrhythmic (Chong et al., 2014), resembling fetal-like rather than adult cardiomyocytes (for a review, see Lui KO et al., 2013). In addition, it is impossible to purify cardiomyocytes from human pluripotent stem cell systems without genetic markers and cardiomyocyte-specific cell surface markers that would allow complete purification have not been identified. Moreover, the success of cell-based therapies depends very much on the route of delivery, dosage and frequency of cell administration, and the degree of engraftment, long-term survival, lineage commitment and integration with the host myocardium (for a review, see Vunjak-Novakovic et al., 2011; Sanganalmath and Bolli, 2013). The optimal protocol is yet to be determined to gain the maximum benefits from transplanting each of the different cell types into the damaged heart.

It has been proposed that the beneficial effects of cell-based therapies might be mediated via several direct and indirect mechanisms, including recruitment of endogenous progenitors, differentiation into functional cardiomyocytes and vascular cells, induction of angiogenesis, promotion of perfusion, reduction of fibrosis and inhibition of apoptosis (Vunjak-Novakovic et al., 2011; Sanganalmath and Bolli, 2013; Loffredo et al., 2011). These repair processes are, in fact, mediated by proteins and small molecules and, therefore, replenishing the damaged heart with therapeutic paracrine factors in vivo may be sufficient to directly activate repair mechanisms, opening a new avenue to regenerative medicine without cellular transplantation (for a review, see Green and Lee, 2013). To search for these therapeutic paracrine factors, we can learn from the ischemic heart which secretes "Mayday" signals (e.g. stromal cell-derived factor-1) for recruiting progenitor cells that express the homing receptor (e.g. CXCR4) and home to the peri-infarct zone (Segers et al., 2007); or from studies using heterochronic parabiosis, a surgical technique in which joining of two mice leads to a shared circulation, that identified rejuvenative circulating factors (e.g. growth differentiation factor 11) secreted by the young, healthy mice which could improve cardiac function in old mice with age-related cardiac hypertrophy (Loffredo et al., 2013). Moreover, we may also learn from a growing human fetal heart to identify paracrine factors (e.g. vascular endothelial growth factor, VEGF) responsible for proliferation of endogenous cardiac

progenitor cells (Lui et al., 2013; Qyang et al., 2007) and their subsequent differentiation into different cellular lineages of the heart, namely cardiomyocytes, and smooth muscle and endothelial cells.

Lineage-mapping studies: searching for therapeutic paracrine factors during cardiogenesis

Over the past four decades, hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO) (Nemir et al., 2012) have been commercially available for clinical use to treat granulocytopenias, anemia, and neutropenia in patients with malignancies or congenital bone marrow failure (Groopman et al., 1989; Vose and Armitage, 1995). Indeed, there is a tightly-regulated network of hematopoietic growth factors or cytokines secreted by immune cells, including G-CSF, M-CSF, GM-CSF, EPO, stem-cell factor (SCF), interleukins, interferons and tumor necrosis factor. These paracrine factors are involved in self-renewal, mobilization and differentiation of hematopoietic stem cells, and in maturation and activation of their differentiated myeloid or lymphoid cells to maintain proper functions of the immune system.

Similar to hematopoietic stem cells, multipotent cardiovascular progenitor cells are also capable of differentiating into different cellular lineages of the heart such as cardiomyocytes, pacemaker cells, smooth muscle cells and endothelial cells during mammalian cardiogenesis. Recent advances in the cre/lox technology enable us to identify distinct cardiovascular progenitor cells and label their derivatives in murine lineage-tracing models. The heart develops from defined multipotent cardiovascular progenitor cells located at the first heart field marked by expression of Tbx5 and Nkx2.5 (Herrmann et al., 2011); the second heart field marked by expression of Isl1 (Qyang et al., 2007; Laugwitz et al., 2005; Moretti et al., 2006); and the epicardium and epicardium-derived progenitor cells (EPDCs) are marked by expression of WT1 (Smart et al., 2011; Zangi et al., 2013; Zhou et al., 2008) (Fig. 1). These progenitors are capable of differentiating into the three major cellular lineages of the heart including cardiomyocytes, and smooth muscle and endothelial cells as demonstrated by lineage-tracing experiments. Despite the discovery of these multipotent cardiovascular progenitor cells, which are capable of further differentiating into mature cardiac muscle with intact calcium dynamics and action potentials (Laugwitz et al., 2005), clinical application of the embryonic $Tbx5^{+}$, $Nkx2.5^{+}$ or $Isl1^{+}$ cardiovascular progenitor cells for autologous cell-based therapy is limited by their absence in the adult heart. In the normal adult heart, there is an endogenous pool of $WT1^{+}$ EPDCs, albeit the numbers are very low (Smart et al., 2011; Zangi et al., 2013; Zhou et al., 2011). The $WT1^{+}$ EPDCs readily differentiate into cardiac fibroblasts and smooth muscle cells, but have limited capacity to form endothelial cells, and make little, if any, contribution to cardiomyocytes in both the normal and infarcted adult hearts (Smart et al., 2011; Zangi et al., 2013; Zhou et al., 2011). Additional factors such as thymosin beta-4 (Smart et al., 2011) and $VEGF_A$ (Zangi et al., 2013) have been used to amplify the endogenous $WT1^{+}$ EPDCs and activate their cardiac differentiation potential following MI.

In addition to EPDCs, endogenous c-kit^{+} cells have been reported as cardiovascular progenitor cells capable of generating the three major cellular lineages of the heart (Beltrami et al., 2003; Ellison et al., 2013). Since the c-kit^{+} cardiovascular progenitor cells can be purified by flow cytometry and are present in both adult and infarcted hearts, various clinical trials have been conducted to investigate their potential in treating damaged tissues after MI (Sanganalmath and Bolli, 2013; Bolli et al., 2011; Chugh et al., 2012); however, the cardiac differentiation potential of the c-kit + cells has been recently challenged by new findings derived from a murine lineage-tracing study (van Berlo et al., 2014). Molkentin and colleagues generated two reporter systems where Cre or a tamoxifen-inducible MerCreMer protein was targeted to the *Kit* locus to permanently mark and trace the cell-fate of c-kit^{+} cells (van Berlo et al., 2014). In contrary to previous reports which suggested the cardiac differentiation potential of c-kit^{+} cells (Beltrami et al., 2003; Ellison et al., 2013), Molkentin and colleagues have demonstrated that the endogenous c-kit cells in the heart, while readily able to generate cardiac endothelium, rarely (0.027%) contributed to cardiomyocytes (van Berlo et al., 2014). Moreover, their contribution in cardiac repair as reported in recent clinical trials has also been challenged (Nowbar et al., 2014). Therefore, more lineage-mapping studies and careful interpretation are required to determine, unequivocally, the cardiac differentiation potential of the c-kit^{+} cells following MI particularly in both preclinical and clinical settings. Altogether, these studies will be important to verify the therapeutic potential of the c-kit^{+} cells for autologous cell-based therapy, and provide new insights into regenerative cardiovascular therapeutics.

During normal aging or following myocardial injuries such as MI, replacement of cardiomyocytes occurs at a very low rate and is mediated by de novo differentiation of endogenous cardiovascular progenitor cells (Smart et al., 2011; Zangi et al., 2013; Zhou et al., 2008; Hsieh et al., 2007), and is in contrast to the replication of pre-existing cardiomyocytes seen in rodent models which occurs in the first few days of life (P1–P4) (Li et al., 1996; Porrello et al., 2011; Naqvi et al., 2014) or during adolescence (P14–18) (Naqvi et al., 2014) driven by thyroid hormone (T3). Cardiosphere-forming cells (CDCs), found in human percutaneous endomyocardial biopsies, are capable of differentiating into cardiomyocytes (Smith et al., 2007); and adoptive transfer of cardiosphere-derived cells (CDCs), expanded in vitro, into human infarcted hearts during a recent randomized phase-I clinical trial led to reduced scar size, increased viable myocardium and improved regional LV function (Makkar et al., 2012; Malliaras et al., 2014a). While the cardiovascular progenitor cells might harness great potential for autologous cell-based therapy, the molecular pathways that underpin development of various multipotent cardiovascular progenitor cells are still unclear. Gaps in our understanding of paracrine signaling responsible for expansion of cardiovascular progenitor cells in vivo and subsequently for making their cell-fate decisions have presented substantial barriers to heart regeneration. Since one of the major mechanisms by which transplanted cells (e.g. CDCs) function is through paracrine signaling (e.g. VEGF (Chimenti et al., 2010), hepatocyte growth factor/HGF (Chimenti et al., 2010), insulin-like growth factor-1/IGF-1 (Chimenti et al., 2010) and stromal cell-derived factor 1/SDF-1 (Malliaras et al., 2014b)), targeting expansion and myocardial differentiation of cardiovascular progenitor cells

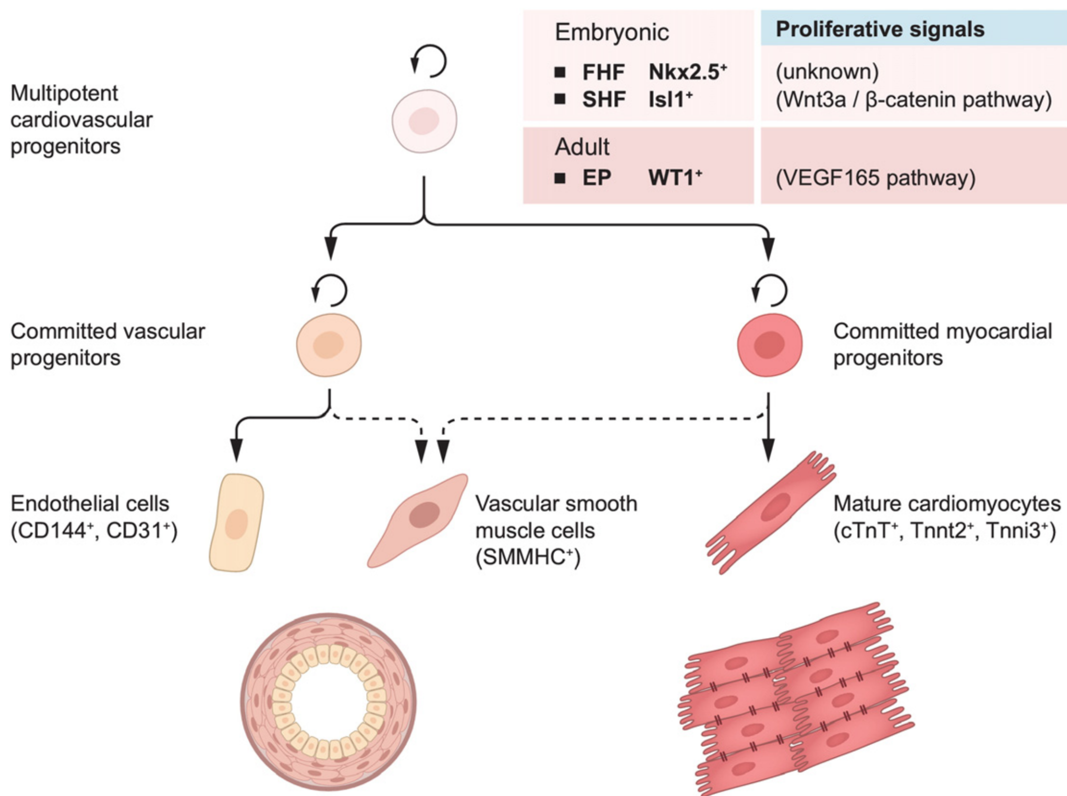

Figure 1 A cellular hierarchy of heart cell lineage diversification from embryonic and adult multipotent cardiovascular progenitors. This provisional schematic fate map of cardiac progenitors is based on currently available data from mouse and human ES cells with in vivo lineage-tracing results. The self-renewing, multipotent cardiovascular progenitors derived from both embryonic first (FHF, marked by Nkx2.5) and second (SHF, marked by Isl1) heart field and adult epicardium (EP, marked by WT1) can give rise to the self-renewing, committed vascular and myocardial progenitors. The committed vascular progenitors further differentiate into $CD144^{+}CD31^{+}$ endothelial cells; whereas the committed myocardial progenitors differentiate into $cTnT^{+}$, $Tnnt2^{+}$ and $Tnni3^{+}$ cardiomyocytes. It is still unclear whether there is a defined population of committed smooth muscle progenitors or whether the vascular $SMMHC^{+}$ smooth muscle cells are differentiated from the vascular or myocardial progenitors.

once we understand the underlying signaling mechanism might provide an alternative strategy for promoting heart regeneration.

The paracrine hypothesis for cell-based therapy is the concept that transplanted cells induce myocardial repair by releasing signals (e.g. cytokines, chemokines, growth factors, possibly exosomes and microparticles) into the injured myocardium, which, in turn, promote a number of repair processes including replication and activation of endogenous progenitor cells, neovascularization, inhibition of apoptosis, inhibition of hypertrophy, and favorable alterations of extracellular matrices (for a review, see Vunjak-Novakovic et al., 2011; Sanganalmath and Bolli, 2013). Collectively, these actions result in enhanced LV function, improved perfusion, reduced fibrosis and myocardial repair (Gnecchi et al., 2008).

$VEGF_{165}$: one of the many paracrine factors and signaling molecules that drives cell-fate specification of cardiovascular progenitor cells

Our group has utilized human fetal hearts and a lineage-tracing, Isl1−cre knock-in hESC line as tools to study the developmental programs during human cardiogenesis and to examine the paracrine signaling mechanisms involved in self-renewal (Qyang et al., 2007) and lineage specification (Lui et al., 2013; Bu et al., 2009) of Isl1-expressing human cardiovascular progenitor cells. We have reported previously that Wnt/beta-catenin signaling is a major component by which cardiac mesenchymal cells modulate self-renewal and inhibit differentiation of Isl1-expressing cardiovascular progenitor cells isolated from human fetal hearts (Qyang et al., 2007). Moreover, Isl1-expressing cardiovascular progenitor cells derived from hESCs are capable of differentiating into cardiomyocytes and smooth muscle cells without added paracrine signaling (Lui et al., 2013; Bu et al., 2009), albeit with low efficiency.

Paracrine signaling is required to drive vascular endothelial cell specification of Isl1-expressing cardiovascular progenitor cells. In order to identify paracrine signals responsible for driving vascular cell-fate, we have performed quantitative gene expression profiling and identified $VEGF_{165}$ as the most abundantly expressed paracrine factor in human fetal heart vessels compared with non-cardiac, human umbilical cord blood vessels (Lui et al., 2013). Replication of the human cardiovascular progenitor cells is observed when cultured in the presence of $VEGF_{165}$ (Lui et al., 2013). In addition, $VEGF_{165}$ alone is sufficient to drive vascular specification of the Isl1-expressing human cardiovascular progenitor cells away from cardiomyocyte or smooth muscle cell fate. These Isl1 lineage-derived

endothelial cells resemble Isl1-expressing endothelial intermediates located at the outflow tract of early human fetal hearts, characterized by coexpression of Isl1 and vascular markers including CD144 and vWF.

By recapitulating the developmental and/or therapeutic role of paracrine factors in the clinic, one might be able to bypass cell therapy by introducing therapeutic paracrine factors directly into the ischemic heart. Indeed, various growth factors have been investigated in clinical trials for their potential in myocardial repair (Table 1), which fall into several functional categories, including those involved in promotion of angiogenesis (e.g. EPO (Silverberg et al., 2001)), fibroblast growth factor 2/FGF2 (Simons et al., 2002) and VEGF (Gao et al., 2010; Henry et al., 2003)); induction of mobilization and activation of the endogenous, quiescent progenitor cells (e.g. G-CSF (Abdel-Latif et al., 2008)); inhibition of apoptosis (e.g. EPO (Silverberg et al., 2001)), FGF2 (Simons et al., 2002) and VEGF (Gao et al., 2010; Henry et al., 2003)) and promotion of cardiomyocyte replication (e.g. growth hormone (Tritos and Danias, 2008) and neuregulin-1 (Jabbour et al., 2011)). Although these trials have given novel insights into development of regenerative therapeutics, it is still unclear whether these paracrine factors can provide a long-term benefit to patients following such treatments. In general, there is a lack of statistically significant difference in functional outcomes (e.g. improvement in myocardial perfusion or increase in left ventricular ejection fraction/LVEF) in the treatment groups compared to the placebo groups; and a lack of consistency between repeated trials using the same paracrine factors.

There are possible complications associated with paracrine factor-based therapy such as the limited half-life and systemic release of the introduced proteins, desensitization of the responding cells, and impaired capability of the failing heart to reawaken endogenous regenerative mechanisms. In short, given that many of the paracrine signaling systems operate on multiple cell types of the heart, it is important to note that therapeutic paracrine signaling should be localized to stimulate only the target cells. Moreover, prolonged expression might likely lead to unwanted side effects; therefore, more studies are required to determine delivery methodologies with the appropriate duration of expression. It is also important to examine the effects of paracrine factors in aged recipients with more severely damaged myocardium, or even in a diabetic background which is also clinically relevant.

Modified $VEGF_{165}$ mRNA: a delivery platform for therapeutic paracrine factors in vivo

Indeed, various phase-I/II clinical trials with $VEGF_{165/121}$ for promoting revascularization in the ischemic heart have been conducted by intracoronary, intravenous or intramyocardial

Table 1 Summary of paracrine factors used in clinical trials for myocardial repair.

Factor	Phase	Patient type and no.	Results	Ref
rhEPO	Randomized, controlled trials	Chronic heart failure, n = 32	An increase of 5.5% and a decrease of 5.4% in LVEF in the treatment and control groups, respectively.	Silverberg et al. (2001)
rhFGF2	Phases I and II	Coronary artery disease, n = 337	No improvement in exercise tolerance or myocardial perfusion at day 180. Significant reduction in angina was observed at day 90 but not at day 180 compared to that of the placebo group.	Simons et al. (2002)
rhG-CSF	Randomized, controlled trials	Acute MI, n = 385	Increased EF in patients with early MI (4.65%, $p < 0.0001$) or with mean baseline EF < 50% (4.73%, $p < 0.0001$) compared to that of the controls.	Abdel-Latif et al. (2008)
rhGrowth hormone	A mixture of controlled and uncontrolled trials	Congestive heart failure, n = 212 in 14 studies	Improved LVEF by 4.3% compared to that of the controls. An increase in left ventricular mass and wall thickness was observed. There was no examination in development of arrhythmias.	Tritos and Danias (2008)
rhNeuregulin-1	Phase I	Chronic heart failure, n = 15	Increased LVEF (4–12%, $p < 0.001$) at day 12–84 post infusion with neuregulin-1 compared to controls. Adverse side effects were reported.	Jabbour et al. (2011)
	Phase II	Chronic heart failure, n = 44	No statistically significant difference in % LVEF compared to that of the placebo group.	Gao et al. (2010)
rhVEGF	Phase I (VIVA trial)	Coronary artery disease, n = 178	Low-dose rhVEGF did not have any effect by day 60 after infusion; high-dose rhVEGF improved angina by day 120 compared to that of the placebo group.	Henry et al. (2003)
	Phase I	Coronary artery disease, n = 14	Improved myocardial perfusion at rest in a dose-dependent manner.	Braitsch et al. (2013)

Abbreviations — rh: recombinant human protein; LVEF: left ventricular ejection fraction.

injection of recombinant protein (Henry et al., 2003; Hendel et al., 2000; Sato et al., 2001), naked cDNA (Losordo et al., 1998; Stewart et al., 2009), non-viral plasmid (Losordo et al., 1998; Stewart et al., 2009) or adenoviral plasmid (Hedman et al., 2003; Stewart et al., 2006); however, the improvement in myocardial perfusion and LV function was inconsistent between these trials (Table 2, for a review, also see Hinkel et al., 2011; Formiga et al., 2012; Simon-Yarza et al., 2012). The failure in achieving consistency in the recombinant $VEGF_{165}$ protein trials could be attributed to the very short half-life of VEGF in plasma (about 30 min in humans) (Carmeliet et al., 1999), a lack of controlled release, and off-target side effects associated with systemic delivery. The studies highlight the difficulty in achieving a therapeutically relevant and durable dose for a lasting effect in revascularization using recombinant $VEGF_{165}$ protein. Recently, biodegradable scaffolds including hydrogel (Gao et al., 2011), collagen (Wu et al., 2011) or self-assembling peptide nanofibers (NF) (Lin et al., 2012) have been implanted to increase the retention of VEGF in the infarcted heart and the use of NF could even prolong the release of VEGF for up to 14 days (Lin et al., 2012). Indeed, injection of the VEGF protein with these biodegradable scaffolds shows better improvement in vascularization and LV function following MI (Gao et al., 2011; Wu et al., 2011; Lin et al., 2012). More studies are still required to determine the risk of immune rejection, fibrosis, formation of leaky blood vessels and edema following the implantation.

Although the concept of direct interference with the genetic and molecular foundation of cardiac cells is simple and elegant, myocardial gene transfer is difficult to achieve as a clinical reality. While the use of certain vectors ensures durable release of VEGF without genome integration, these approaches are limited by low gene transfer efficiency (Hinkel et al., 2011). Improved $VEGF_{165}$ expression with adenoviral vectors leads to more robust neovascularization; but also contributes to untoward effects, including potential genomic integration, systemic inflammation against the viral vectors (Wright et al., 2001), local edema (Rutanen et al., 2004) or angiomas (Schwarz et al., 2000) as a result of prolonged exposure to $VEGF_{165}$. These side effects might have masked the therapeutic benefit induced by $VEGF_{165}$. Therefore, better delivery technologies with more regulated spatial and temporal expression of therapeutic gene products are needed.

To address issues associated with non-viral and viral plasmid-mediated gene therapies, we (Zangi et al., 2013; Lui et al., 2013) and others (Kariko et al., 2005, 2011; Kormann et al., 2011; Mandal and Rossi, 2013; Warren et al., 2010) have utilized modified mRNAs (modRNAs) as a non-immunogenic tool to deliver proteins of interest into mammalian cells with high efficiency. Since the immune system has a crucial role in guarding against infections by detecting microbial metabolism, both DNA and RNA can activate dendritic cells of the innate immunity through recognition by Toll-like receptors (TLRs). In translational studies, nucleoside modifications are, therefore, needed to ensure escape from immune surveillance. It has been reported that replacement of cytidine with 5-methyl-cytidine and uridine with pseudouridine suppresses RNA recognition by dendritic cells via TLRs 3, 7 and 8 (Fig. 2) (Kariko et al., 2005). Therefore, incorporation of modRNA both reduces innate immune activation and increases efficiency for mRNA translation (Kariko et al., 2011) (Fig. 2). Recently, it has been demonstrated that twice weekly application with an aerosol containing surfactant protein B (SP-B) in the form of modRNA restored 71% expression of the wildtype SP-B protein in vivo and prolonged survival of mice with a lethal congenital lung disease attributed to SP-B deficiency (Kormann et al., 2011). In addition to the added stability of modRNAs compared to mRNAs, the non-integrating nature of modRNAs also allows transient expression of proteins which, if prolonged, might generate side effects. For instance, high doses of $VEGF_{165}$ can lead to formation of leaky blood vessels (vascular hyperpermeability) (Nagy et al., 2012) and hypotension (excessive release of nitric oxide) (Yang et al., 2002); therefore, the use of VEGF modRNA could be safer than the use of integrating vectors.

The identification of $VEGF_{165}$ as a cell-fate switch in determining vascular specification of the human Isl1-expressing cardiovascular progenitors has led to pioneering work in using $VEGF_{165}$ modRNA as a therapeutic paracrine factor for driving heart regeneration (Zangi et al., 2013; Lui et al., 2013). Direct injection of a single paracrine factor such as $VEGF_{165}$ in the form of modRNAs not only leads to replication and reactivation of the endogenous, quiescent $WT1^+$ adult epicardial cells in the infarcted myocardium, but also directs differentiation of these cells away from a fibroblastic, scar-forming cell fate (Zhou et al., 2008) and toward vascular and myocardial cell fates (Zangi et al., 2013) (Fig. 3). Such an approach stimulates the endogenous regenerative capacity of an infarcted adult heart by limiting pathological remodeling, effecting a significant improvement in heart function including increased ejection fraction, reduced fibrosis and prolonged survival (Zangi et al., 2013). Nevertheless, future studies are still needed to determine whether the modRNA technology is a safer and more efficacious approach compared to other systems previously employed in pre-clinical and clinical studies for delivering paracrine factors and signaling molecules.

Future perspectives

The emerging approaches that utilize synthetic, chemically modified mRNA as paracrine factor therapeutics for regenerative cardiology could also be a paradigm for regeneration in other tissues and organs. Indeed, in addition to the heart (Zangi et al., 2013; Zhou et al., 2011), it has also been shown that vascular endothelium-derived paracrine factors play an important role in regeneration of liver (Ding et al., 2010), lung (Ding et al., 2011) and pancreas (Brissova et al., 2014): In the liver, liver sinusoidal endothelial cells secrete HGF and Wnt2 which initiate hepatocyte replication and sustain liver regeneration following 70% of partial hepatectomy (Ding et al., 2010); in the lungs, pulmonary capillary endothelial cells secrete VEGF, fibroblast growth factor (FGF) and matrix metalloproteinase (MMP) 14 that induce replication of epithelial progenitor cells for alveologenesis following unilateral pneumonectomy (Ding et al., 2011); in the pancreas, increased VEGF-A secretion in beta cells induces an initial beta cell loss but subsequent beta cell replication (Brissova et al., 2014). Therefore, the in vivo expression of libraries of paracrine factors in the form of modRNA could be informative for identifying known and unknown paracrine factors for a diverse group of solid organ degenerative

Table 2 Summary of methodologies for VEGF delivery in myocardial repair.

Nature	Format	Route	Preclinical or clinical?	Strength	Weakness	Ref
Protein	Recombinant protein	Intracoronary	Phase I and II trials	Safe; local delivery; perfusion improvement at high doses	Short-lived; single dose; lack of controlled release	Henry et al. (2003) and Hendel et al. (2000)
	Recombinant protein	Intravenous	Phase I and II trials	Safe	Short-lived; off-target side effects; no improvement in perfusion	Henry et al. (2003) and Sato et al. (2001)
	Recombinant protein with hydrogel	Intramyocardial	Preclinical studies	Biodegradable; increased retention of cells and proteins; increased angiogenesis; improved LV functions	Immunogenic; side effects such as fibrosis, leaky vessels or edema were not determined	Gao et al. (2011)
	Recombinant protein with collagen patch	Intramyocardial	Preclinical studies	Biodegradable; increased retention of cells and proteins; increased angiogenesis; improved LV functions	Immunogenic; side effects such as fibrosis, leaky vessels or edema were not determined	Wu et al. (2011)
	Recombinant protein with nanofibers	Intramyocardial	Preclinical studies	Biodegradable; increased retention of cells and proteins; prolonged release up to 14 days; increased angiogenesis; improved LV functions	Immunogenic; risk of fibrosis	Lin et al. (2012)
DNA	Naked cDNA or non-viral plasmids	Intramyocardial	Phase I and II trials	Low cost; durable expression; non-integrating	Lack of controlled release; immunogenic; inconsistent results from improvement in angiogenesis or LV functions	Losordo et al. (1998) and Stewart et al. (2009)
	Viral plasmids (adeno-associated or lentiviral)	Intramyocardial	Phase I and II trials	Long-term expression; high transduction efficiency; tissue-specific	Lack of controlled release; random genome integration; immunogenic/presence of neutralizing antibodies; inconsistent results from improvement in angiogenesis or LV functions	Hedman et al. (2003) and Stewart et al. (2006)
RNA	modRNA	Intramyocardial	Preclinical studies	Non-integrating; non-immunogenic; more stable; localized; highly efficient; controlled release; progenitors activation; increased angiogenesis; improved LV functions	Cost; short-term expression	Zangi et al. (2013)

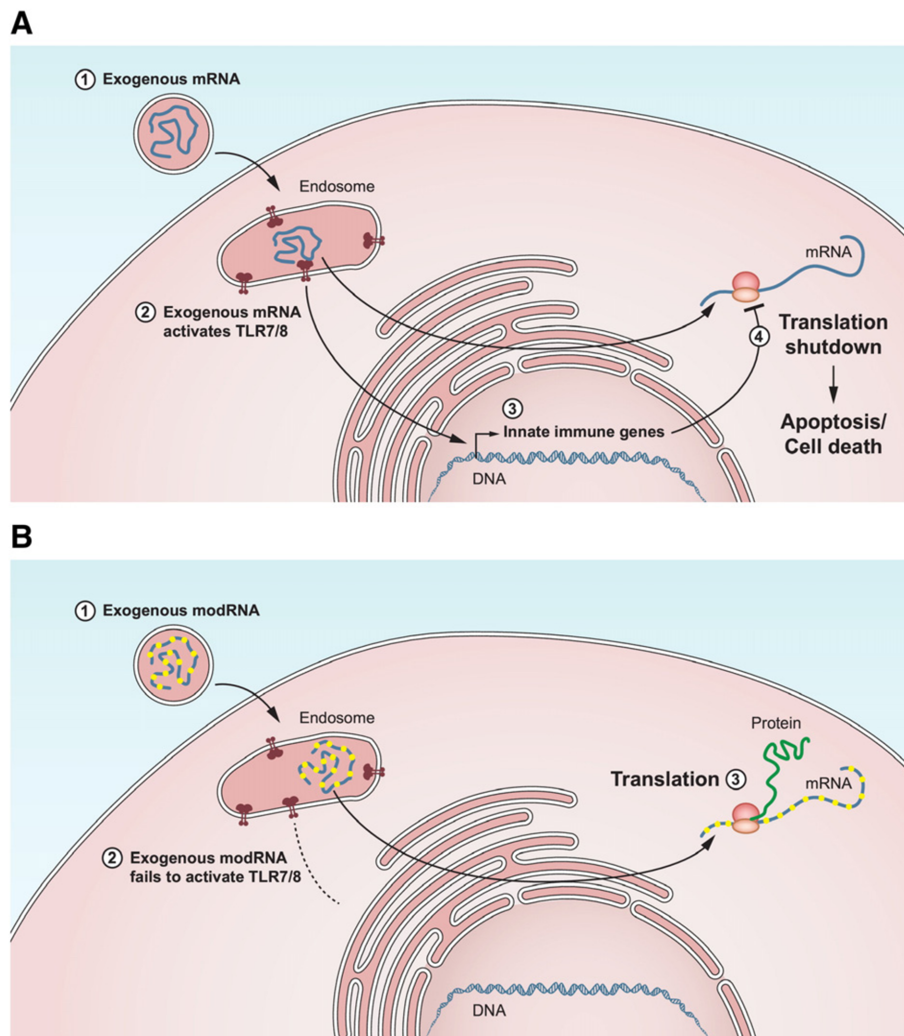

Figure 2 Modification of mRNA bypasses Toll-like receptor-induced apoptosis of transfected cells. (A) The exogenous mRNA that enters the endosome of the transfected cell can be recognized by Toll-like receptors 7 and 8 (TLRs 7 & 8). TLR recognition induces expression of genes involved in innate immunity such as type I interferons and RIG1, leading to a translational shutdown of proteins and apoptosis of the transfected cells. (B). A change in two ribonucleotides of mRNA (uridine replaced by pseudouridine and cytidine replaced by 5-methyl-cytidine) leads to a change in the secondary structure of mRNA, bypassing immune recognition through TLRs. The modified mRNA goes to the ribosomes and can be translated to proteins without eliciting immune response or compromising the genome of the transfected cells.

diseases. Specifically, promoting angiogenesis and revascularization via VEGF modRNA, and in addition other angiogenic paracrine factors, could also be an effective tool in enhancing regeneration in multiple organ systems following injuries.

Since the first and second heart field-derived cardiovascular progenitor cells do not exist in the adult heart, either under normal conditions or after MI (Sanganalmath and Bolli, 2013), it is unlikely that they could be expanded following myocardial injuries. It has been reported that several populations of cardiovascular progenitor cells exist in the adult epicardium, including WT1$^+$ (Smart et al., 2011; Zangi et al., 2013; Zhou et al., 2008), Tbx18$^+$ (Zhou et al., 2011; Cai et al., 2008) or Tcf21$^+$ (Braitsch et al., 2013) EPDCs, which contribute to scar formation and fibrosis following MI. Nevertheless, it is still not clear how to expand these endogenous quiescent progenitor cells and inhibit them from differentiating into cardiac fibroblasts. Therefore, harnessing paracrine signaling with the appropriate in vivo delivery strategy such as modRNAs might stimulate replication and differentiation of EPDCs into more functional cell types such as endothelial cells and cardiomyocytes for heart regeneration.

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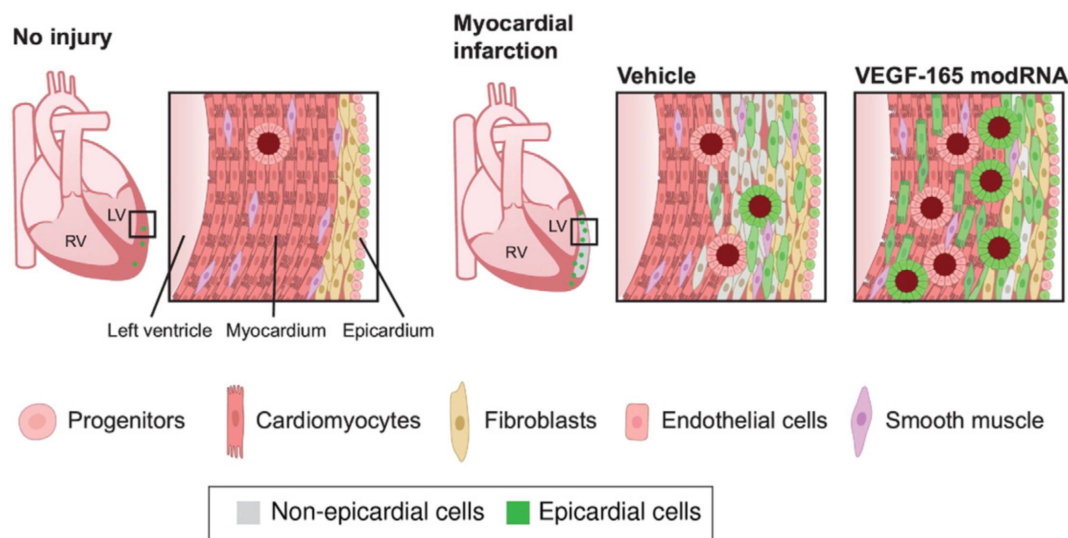

Figure 3 Cell fate switch of epicardial cells from the pro-fibroblastic to the cardiovascular lineage through overexpression of VEGF modified mRNA. In normal uninjured condition, there is a small population of the quiescent epicardial cells located in the epicardium of murine hearts which can be activated upon stimulation. In injured conditions such as myocardial infarction, a minimal replication of these progenitor cells is found but the majority of them differentiate into cardiac fibroblasts and might contribute to cardiac fibrosis (vehicle); however, there is a greater degree of replication of epicardial cells following myocardial injection of $VEGF_{165}$ modified mRNA (VEGF-165 modRNA). The amplified epicardial cells are activated and migrate into the myocardium where they differentiate into more functional cell lineages, away from the fibroblastic but toward the cardiovascular lineages including endothelial cells and cardiomyocytes. Therefore, neovascularization and improved heart functions are found in the $VEGF_{165}$ modified mRNA-treated group compared to the vehicle-treated group.

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