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REVIEW

View from the heart: cardiac fibroblasts in development, scarring and regeneration

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ABSTRACT

In the adult, tissue repair after injury is generally compromised by fibrosis, which maintains tissue integrity with scar formation but does not restore normal architecture and function. The process of regeneration is necessary to replace the scar and rebuild normal functioning tissue. Here, we address this problem in the context of heart disease, and discuss the origins and characteristics of cardiac fibroblasts, as well as the crucial role that they play in cardiac development and disease. We discuss the dual nature of cardiac fibroblasts, which can lead to scarring, pathological remodelling and functional deficit, but can also promote heart function in some contexts. Finally, we review current and proposed approaches whereby regeneration could be fostered by interventions that limit scar formation.

KEY WORDS: Fibroblasts, Regeneration, Scarring, Heart

Introduction

Over the past decade, regenerative medicine has focused on the discovery and understanding of stem cells for cell or tissue replacement strategies. Although much progress has been achieved to this end, many limitations still exist with regard to translating results into therapy. Specifically for the heart, various endogenous types of stem and progenitor cells have been described, mostly based on mouse genetic studies. However, it has become clear that although these cell types may exist, they do not provide a robust and reliable source of cells for the replacement of lost muscle mass following acute or chronic injury. The community has now turned its attention to the modulation of differentiated cell types of the heart, such as cardiomyocytes, fibroblasts and endothelial cells, to ameliorate the outcome of heart failure.

This Review focuses on recent advances in our understanding of heart fibrosis, one of the main pathological features of heart failure. In a homeostatic heart, the extracellular matrix (ECM) turnover is low. Following an insult, cell death and inflammatory infiltration lead to the activation of fibroblasts, which adopt a myofibroblast phenotype. These cells proliferate, acquire a migratory smooth muscle cell-like behaviour and increase secretion of ECM components, mainly collagen. Excess deposition of ECM leads to the development of fibrous tissue, considered 'reparative' fibrosis (Weber et al., 2013). This is necessary to maintain the integrity of the myocardial wall following the death of muscle cells depleted of oxygen and nutrients. Persistent myofibroblast activity compromises heart function because it increases stiffness and disrupts the electrical properties of the heart muscle, a process normally referred to as adverse or pathological remodelling. Ultimately, persistent fibrosis results in heart dilation and failure.

Despite the role of fibroblasts in causing fibrosis, this cell type has long been neglected and is often described simply as 'biological glue' for body tissues. However, fibroblasts have recently reached centre stage in regenerative medicine due to their capacity to be reprogrammed into alternative cell lineages (Fu et al., 2013; Ieda et al., 2010, 2009; Nam et al., 2014, 2013a; Qian et al., 2013, 2012; Takahashi et al., 2007; Takahashi and Yamanaka, 2006), their capacity to interact with other cell types in their microenvironment (Kim et al., 2015), and their ability to modulate disease processes (Furtado et al., 2014a; Takeda et al., 2010). As cardiac fibroblasts play a prominent role in heart scarring, it is essential to understand and control the activity of these cells in order to develop efficient treatments for heart failure. Current anti-fibrosis therapies are inefficient and non-specific (Brown et al., 2005), mostly due to confusion over the identity of fibroblasts and our inability to modulate them in a tissue-specific manner (Box 1).

If heart regeneration is to be achieved, then a balance must be struck between the replacement of lost cells and the formation of scar tissue. The current paradigm surrounding the field of cardiac regeneration is to promote cardiomyocyte hyperplasia (growth through proliferation) as opposed to hypertrophy (growth in size, as commonly seen in pathological settings), as well as to reduce fibrosis, which significantly impairs heart function. This Review will discuss the origin of heart fibroblasts, their genetic programme and how they contribute to heart development, homeostasis and disease. Although this Review will focus primarily on cardiac fibroblasts, cardiomyocytes and fibroblasts are intimately interconnected and successful regeneration can only be achieved if both cell types are appropriately manipulated to preserve architecture in the tissue. Therefore, we will also discuss current advances in the modulation of cardiomyocyte activity.

What are cardiac fibroblasts and where do they come from?

The definition of a fibroblast is somewhat vague and outdated. Fibroblasts are loosely defined based on their capacity to secrete ECM and to adhere to a substrate *in vitro*, and on their interstitial location, where they constitute the mesenchymal component of organs (Souders et al., 2009). Progress to properly define fibroblasts has been hampered by the lack of appropriate tools: heart fibroblasts are still poorly characterised with regard to their molecular properties, compartmentalisation (atria, ventricles, septal, valvular, and so on), and function during various stages of heart development, homeostasis, injury and repair. Many of the proteins used to detect fibroblasts are either non-specific or only identify subpopulations, as opposed to the whole fibroblast pool. This is the case for smooth muscle actin, which marks smooth muscle cells, pericytes and myoepithelial cells; vimentin, which marks endothelial cells, smooth muscle cells, myoepithelial cells and

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Box 1. Anti-fibrosis therapies currently used in the clinic

Current anti-fibrosis therapies generally target fibrotic pathways and their components that are expressed systemically, therefore affecting multiple tissues in addition to the target tissue. This lack of specificity is neither ideal nor effective for treating fibrosis in an organ-specific manner, such as that which occurs in the heart after injury. A number of novel drugs are currently under clinical trial for fibrosis (as noted below), but still the target pathways are generic and open to systemic adverse effects. Given that cardiac fibroblasts display a unique molecular profile compared with non-cardiac fibroblasts, it might be possible to modulate fibrosis in a heart-specific manner in the future by targeting molecules and pathways that are uniquely expressed in these cells.

Compounds	Targets	Processes
AMD3100*	Cxcr4 antagonist antibody	Recruitment of monocytes/macrophages
Bosentan and others‡	Endothelin 1 receptor blocker	Modulation of myofibroblast activity, blood pressure control
Captopril, Benazepril, Enalapril and others (ACE inhibitors)	Angiotensin converting enzyme (ACE)	Modulation of myofibroblast activity, blood pressure control
Dasatinib‡	c-Abl (Abl1), c-Kit, Src	Modulation of myofibroblast activity
Imatinib‡	c-Abl, c-Kit, PDGFR	Modulation of myofibroblast activity
Losartan, Irbesartan, Amlodipine and others	Angiotensin I and II receptor blockers	Modulation of myofibroblast activity, blood pressure control
Metelimumab (CAT-192)‡	Anti-TGFβ1 monoclonal antibody	Modulation of myofibroblast activity, blood pressure control
Macrolide antibiotics‡	Fli1	Immune response regulators (anti-inflammatory)
Nilotinib*	c-Abl, c-Kit, PDGFR	Modulation of myofibroblast activity
Nintedanib‡	PDGFR, VEGFR, FGFR	Modulation of myofibroblast activity
Pirfenidone‡	TGFβ, TNFα and other pathways	Modulation of myofibroblast activity, anti-inflammatory, anti-oxidative stress
Simvastatin, Lovastatin, Atorvastatin and other statins	Inhibitors of hydroxymethylglutaryl-coenzyme A reductase	Cholesterol lowering, modulation of myofibroblast activity
Sorafenib‡	PDGFR, VEGFR	Modulation of myofibroblast activity
Tocilizumab*	Antibody against interleukin 6 receptor	Immune response regulators (anti-inflammatory)

*Approved for other diseases with only experimental evidence of anti-fibrotic activity demonstrated to date.
‡Under clinical trial for fibrosis (sourced at Clinicaltrials.gov).

pericytes; collagens, which mark osteoblasts and chondrocytes; discoidin domain receptor 2, which marks smooth muscle cells, hepatic stellate cells and endothelial cells; fibroblast specific protein 1 (also known as S100a4), which marks monocytes, smooth muscle cells and carcinoma cells; and CD90 (Thy1), which marks leukocytes, endothelial cells and other cell types (Krenning et al., 2010). For cell surface analysis, mEF-SK4 are so far the most reliable antibodies when used in combination with CD31 (Pecam1) and CD45 (Ptprc) to exclude endothelial and hematopoietic cells (Pinto et al., 2015). Mouse reporter lines, such as collagen1-GFP (Moore-Morris et al., 2014) and PDGFRα$^{GFP/+}$ (Pinto et al., 2015), have also proven useful, although it is still not clear whether these reagents uniformly label the whole fibroblast pool. Further studies are still required to clarify this issue.

In an adult homeostatic heart, fibroblasts are found in the cardiac skeleton and within the myocardial interstitium (see Fig. 1). The cardiac skeleton is a connective tissue structure that forms the valvular components of the heart and also connects them to the septa. Although fibroblasts are an integral part of this heart component, they are also abundant in the muscle compartment (Fig. 2A). In this microenvironment, fibroblasts are highly connected with other cell types, including the endothelial cells that make up the microvasculature, resident myeloid cells and, more importantly, cardiomyocytes. These interconnections are important to maintain the homeostatic balance of the heart.

Recent advances in the field have demonstrated that cardiac fibroblasts in the resting heart are generated during embryonic development by two major distinct compartments: the endocardium and the epicardium (Ali et al., 2014; Moore-Morris et al., 2014) (Fig. 1). The endocardium provides the endothelial lining and microvasculature of heart chambers, while the epicardium envelops the heart as its outermost layer. Both compartments undergo epithelial-to-mesenchymal transition (EMT) during embryonic development to generate fibroblasts (Fig. 1). The use of lineage tracers in mouse has revealed that most fibroblasts isolated from the adult heart in homeostasis are of epicardial origin: 80% of cells are labelled by the epicardial lineage tracer Wt1-cre or Tbx18-cre, whereas only 16% are labelled by the endocardial lineage tracer Tie2 (Tek)-cre or Nfatc1-cre (Moore-Morris et al., 2014). These findings were confirmed by Ali and colleagues, who showed similar endocardial and epicardial contributions using Tie2-cre and Tbx18-cre (Ali et al., 2014). The latter group also demonstrated that a minority of fibroblasts located specifically in the great vessels within the outflow tract region of the heart, originated from a Pax3-cre fraction, which confirms the existence of neural crest-derived fibroblasts in the adult heart. In addition, using a pathological stimulus, in this case pressure overload using transverse aortic constriction, both groups found that newly formed fibroblasts were generated through the proliferation of pre-existing fibroblasts, as opposed to *de novo* EMT (Ali et al., 2014; Moore-Morris et al., 2014). These findings revolutionised the field, as they showed that fibroblasts are formed in an organ-specific manner and may therefore be tailored to the function of the organ in which they are embedded.

The contribution of fibroblasts derived from hematopoietic cells was excluded by the studies of Moore-Morris et al. (2014) and Ali et al. (2014). Here, transplantation and parabiosis studies in which the bone marrow or hematopoietic stem cells were labelled with fluorochromes (Ali et al., 2014), as well as experiments in which cells were labelled with the bone marrow marker Vav-cre (Moore-Morris et al., 2014), showed no contribution of the cells to the cardiac fibroblast population. These findings were also confirmed

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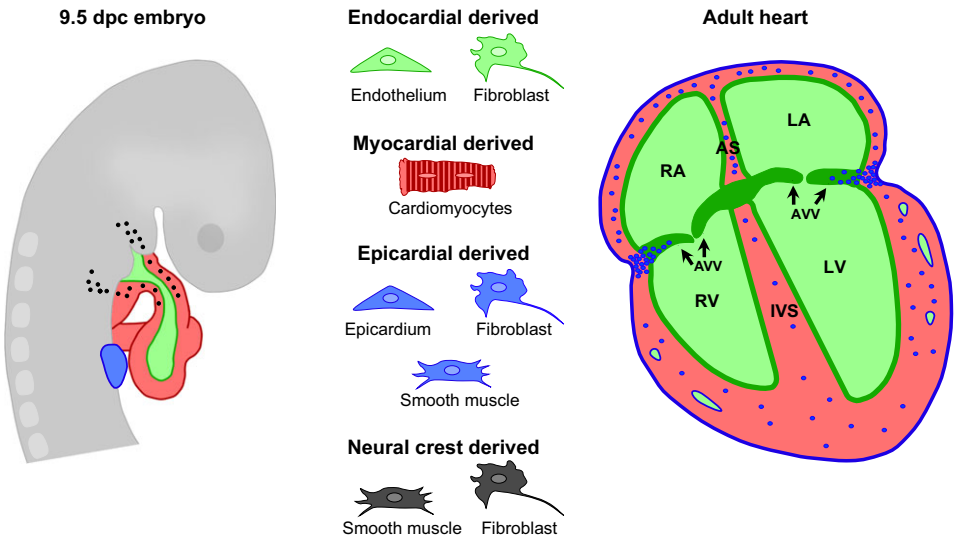

Fig. 1. Embryological origin of cardiac fibroblasts. Cardiac fibroblasts are generated during embryonic development (left) from the endocardium (green) and epicardium (blue) through epithelial-to-mesenchymal transition and persist to adulthood (right). A minor contribution from the neural crest to the fibroblast pool (black) has also been defined using genetic labels and is found in the outflow tract region of the heart (not shown in the adult heart). The endocardial component is also responsible for the formation of the endothelial lining of cardiac chambers and vasculature (dark green in the adult heart). The epicardium forms the outermost layer of the heart (blue in the adult heart), as well as interstitial fibroblasts and smooth muscle cells. A contribution of the epicardial-derived cells to the coronary endothelium has also been described (Katz et al., 2012). AS, atrial septum; IVS, interventricular septum; AVV, atrioventricular canal valves; LA, left atrial chamber; LV, left ventricular chamber; RA, right atrial chamber; RV, right ventricular chamber; dpc, days post coitum.

after transverse aortic constriction, eliminating hematopoietic-derived fibroblasts as a contributing source of the cardiac fibroblast population following pressure overload. These recent findings are in contrast to earlier experiments by several groups that reported the presence of circulation-derived fibroblasts in the heart after insult (Haudek et al., 2006; van Amerongen et al., 2008).

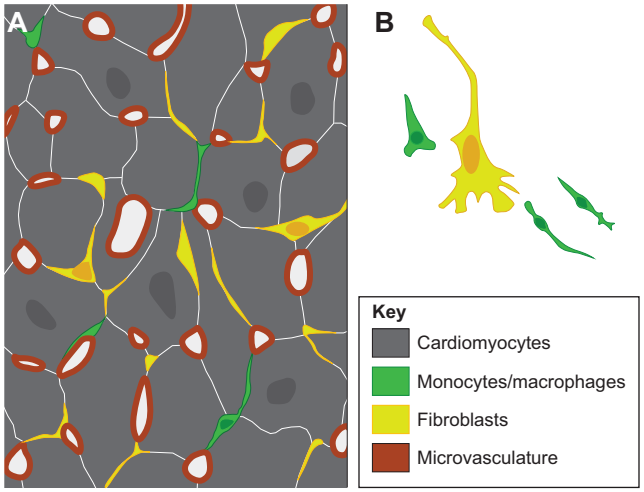

Fig. 2. Fibroblasts, cardiomyocytes and immune cells in the homeostatic heart. (A) In a normal heart, interstitial fibroblasts (yellow), cardiomyocytes (dark grey) and myeloid cells (green) interact with one another in their microenvironment to maintain a homeostatic balance. Other resident cell types, such as pericytes and smooth muscle cells, are not depicted. (B) Cardiac fibroblasts and myeloid cells display almost indistinguishable morphological characteristics in the interstitial space of the homeostatic heart; however, they exhibit very different shapes and sizes after isolation and adhesion to plastic (cells are drawn to scale).

These cells are commonly referred to as fibrocytes and carry a hematopoietic signature ($CD45^{+}$) (Abe et al., 2001; Zeisberg and Kalluri, 2010). Myofibroblasts and fibrocytes of hematopoietic origin have been described in the scar area following myocardial infarct or ischemia/reperfusion injury (Haudek et al., 2006; van Amerongen et al., 2008); however, these cells have so far been poorly characterised. It has recently been demonstrated that immune cells infiltrate the heart early in development (Epelman et al., 2014) and reside in the organ during homeostasis (Pinto et al., 2012). Heart-resident monocytes/macrophages, for example, are abundant and can display a morphology that is highly similar to that of interstitial fibroblasts in the heart tissue (Fig. 2A), although *in vitro* plated cardiac fibroblasts and monocytes differ dramatically in size and morphology (Fig. 2B). High-throughput profiling of these cells revealed expression of CD45 and an anti-inflammatory M2 macrophage profile. These resident cells also express collagen (Pinto et al., 2012). As immune cells are abundant in heart injury sites in the first week after infarction, it remains to be addressed whether fibrocytes and macrophages have been interchangeably described owing to their similar morphology and localisation or if they are in fact distinct cell types.

Gene expression signature of cardiac fibroblasts

In an attempt to better characterise the cardiac fibroblast, our group profiled gene expression patterns in short-term cultures of murine adult cardiac fibroblasts as compared with a non-organ cell source, in this case tail fibroblasts (Furtado et al., 2014a). We discovered a remarkable cardiogenic identity for cardiac fibroblasts isolated from the adult heart in homeostasis (Fig. 3A). The gene expression profile was characterised by the presence of various transcription factors that are seminal for heart formation during embryonic development and have been strongly implicated in congenital heart disease (Butler et al., 2010; Garg et al., 2003; Granados-Riveron et al., 2012; Kirk et al., 2007; Misra et al., 2012; Posch et al., 2010; Qiao et al., 2012; Schlesinger et al., 2011; Smemo et al., 2012). Among

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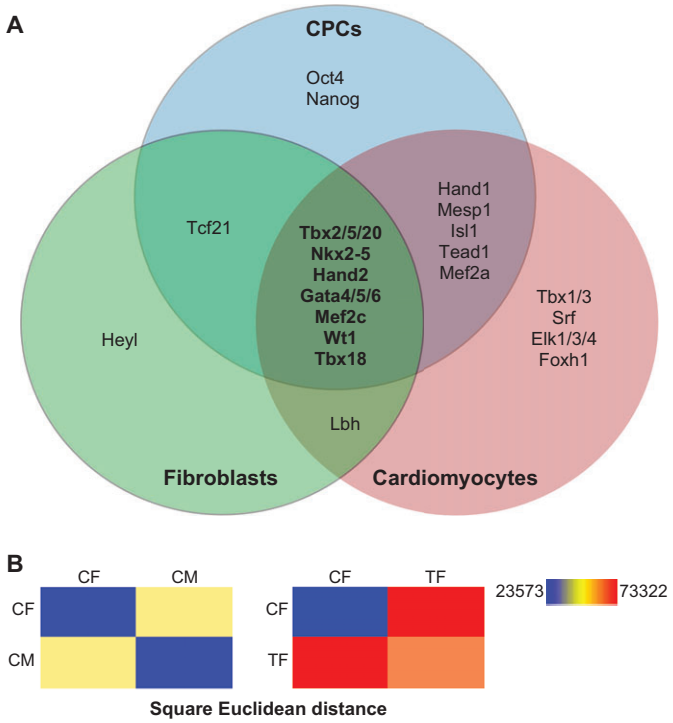

Fig. 3. Cardiogenic signature of heart fibroblasts. (A) Core cardiogenic transcription factors previously described as expressed in cardiac progenitor cells (CPCs; blue) or fibroblasts (green) isolated from the adult mouse heart. Such factors are part of the heart development network (red) found in developing and/or adult cardiomyocyte subpopulations (Ali et al., 2014; Bollini et al., 2011; Bouveret et al., 2015; Briegel and Joyner, 2001; Furtado et al., 2014a,b; Moore-Morris et al., 2014; Noseda et al., 2015). Factors shown in bold are shared among all three cell types. (B) Global normalisation strategies for systematic transcriptome-wide comparison between three cell types, namely cardiac fibroblasts (CF), cardiomyocytes (CM) and tail fibroblasts (TF), taken from two microarray datasets (Fu et al., 2013; Furtado et al., 2014b). Datasets were transformed and normalised such that all samples have identical statistical distributions. Heat map plot shows pairwise square Euclidean distances between the CF and CM populations, or between the CF and TF populations. The CM/CF distance is smaller (blue to yellow; closer in colour) than the CF/TF (blue to red; furthest in colour), suggesting that CFs are transcriptionally more similar to CMs than to CFs.

the transcription factors found in our analysis, Tcf21 (epicardin) and Wt1 are both epicardial markers. Other factors include the muscle marker Mef2c and the more heart-specific Hand2, T-box family members (Tbx2, Tbx5 and Tbx20) and GATA family members (Gata4, Gata4, Gata6), all of which are found in both cardiomyocytes and cardiac progenitor cells (CPCs). Importantly, the short-term culture strategy employed in our study allowed the exclusion of contaminant RNAs from cardiomyocytes, which are normally found in harsh heart tissue dissociation conditions. This was supported by the lack of any significant signal for structural cardiomyocyte genes, such as troponin T2 (*Tnnt2*) and myosin heavy chain (*Myh6*, *Myh7*). The strongest signature found in heart fibroblasts was for the transcription factors Gata4 and Tbx20, which immunofluorescence and single-cell qPCR showed were both ubiquitously expressed in this population, as opposed to the heterogenic expression found for the other factors. This signature was conserved in human cardiac fibroblasts and throughout heart compartments such as the atria and ventricles, demonstrating an evolutionary conservation of the cardiogenic profile (Furtado et al., 2014a). These findings define an organ-specific genetic programme for cardiac fibroblasts. Further studies will determine the usefulness of this programme in the development of organ-tailored anti-fibrosis therapies.

The overlap of gene expression signatures between cardiac fibroblasts and cardiomyocytes suggests that the cardiac fibroblast may in fact be primed for transdifferentiation through the cardiogenic programme and might be the ideal cell source for therapeutic manipulation. Indeed, the transcription factor combination Mef2c, Gata4 and Tbx5 (± Hand2), all of which are present in the cardiac fibroblast population, has been successfully used to reprogram mouse and human fibroblasts into cardiomyocytes (Ieda et al., 2010; Nam et al., 2014, 2013a,b; Qian et al., 2013, 2012) (Fig. 3A). Interestingly, the efficiency of direct conversion of tail and cardiac fibroblasts into cardiomyocytes is similar, suggesting that the presence of cardiogenic factors alone is not sufficient for cardiomyocyte reprogramming (Zhou et al., 2015). Indeed, this must be the case, otherwise cardiac fibroblasts would naturally convert into cardiomyocytes. It is possible that strong repressors are present in the cardiac fibroblast population, thereby blocking the fibroblast-to-cardiomyocyte conversion, but so far this issue has not been addressed.

By normalising 12 transcriptomic profiles from two datasets, bioinformatics analysis demonstrated that the cardiac fibroblast is transcriptionally more similar to the cardiomyocyte than to fibroblasts of unrelated sources, in this case tail fibroblasts, as judged by the pairwise Euclidean distances (Fig. 3B) (Fu et al., 2013; Furtado et al., 2014b). This reinforces the fact that the cardiac fibroblast is a specialised heart cell type and not a generic mesenchymal cell. Their genetic signature underscores the need for careful consideration when demonstrating the conversion of cardiac fibroblasts into cardiomyocytes. Phenotyping approaches must involve not only the observation of well-organised structural sarcomeric markers, but also functional assays, in which the action potential of differentiated cardiomyocytes can be fully assessed.

Demystifying cardiac progenitor cells

Many endogenous CPC types have been described over the past decade. Most of these cells have been subselected based on cell surface markers, subjected to long-term culture conditions to evaluate stemness and differentiation potential, and further characterised in heart homeostasis and disease scenarios. Some of the commonly used CPC markers include c-Kit (CD117) (Beltrami et al., 2003), Sca1 (Ly6a) (Oh et al., 2003) and breakpoint cluster region pseudogene 1 (BCRP1) (Asakura and Rudnicki, 2002; Oh et al., 2003). Other defined populations include the cardiospheres (CDCs) (Messina et al., 2004) isolated from mouse or human heart biopsies, so named due to their capacity to grow as adherent clusters *in vitro*, and Islet1 (Isl1)-positive cardioblasts (Laugwitz et al., 2005). More recently, epicardial-derived cells (EPDCs) with cardiomyocyte differentiation potential (Chong et al., 2011; Smart et al., 2011) have been identified. These cells can be isolated based on the expression of the genetic markers Wt1 or Pdgfrα (Chong et al., 2011). The peptide thymosin β4 (Tβ4) was found to restimulate Wt1 expression in adult endogenous murine epicardial cells, which could then be mobilised and reprogrammed to give rise *de novo* to cardiomyocytes (Smart et al., 2011). Pdgfrα^{+} cells were considered analogous to mesenchymal stem cells (MSCs) and found to express Sca1, as well as other MSC markers.

The unravelling of a cardiogenic signature for adult fibroblasts, as discussed in the previous section, carries profound implications for the field of regenerative medicine. CPCs and cardiac fibroblasts

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share many cardiogenic transcription factors (Fig. 3A). Moreover, both cell types can be forced to transdifferentiate directly into cardiomyocytes *in vitro* and *in vivo*, albeit at low efficiency. It has been demonstrated that EPDCs (van Wijk et al., 2009), such as fibroblasts, are generated within the heart field during mouse embryonic development by the same progenitors as cardiomyocytes. These progenitors then undergo divergent differentiation pathways to form specific cell types of the heart, such as endothelial cells, epicardial cells, muscle cells and fibroblasts. It is therefore not surprising that cardiogenic transcription factors are expressed in fibroblasts. These findings further raise the question of whether CPCs and fibroblasts are in fact the same cell type. In addition to all the above-mentioned CPCs, pericytes with MSC activity have also been reported (Caplan, 2008). As the name suggests, pericytes are perivascular cells embedded in the basement membrane of the microvasculature in many organs, including the heart (Armulik et al., 2011). Molecularly, pericytes are characterised by the expression of melanoma cell adhesion molecule (Mcam; also known as CD146), chondroitin sulphate proteoglycan 4 (Cspg4; also known as NG2), Pdgfrα, Pdgfrβ and smooth muscle actin/myosin (Sma/Smm) (Armulik et al., 2011; Chen et al., 2015), most of which are also present in the fibroblast/myofibroblast pool. Pericytes isolated from the heart have been shown to differentiate into cardiomyocytes *in vitro*, like other CPCs (Chen et al., 2013, 2015). Although their embryological origin is still under discussion, cardiac pericytes have a potential ontogeny in the epicardium of the heart (Armulik et al., 2011). The same controversy surrounding MSCs applies here: are pericytes another fully defined cell type or in fact a subpopulation of the fibroblast pool?

The contribution of CPC types to myocardial renewal is surrounded with controversy regarding reproducibility among groups and true cardiomyocyte differentiation potential (Bolli et al., 2011; The Lancet Editors, 2014; van Berlo et al., 2014; Zhou et al., 2012). In fact, although initially described by Chong and colleagues as a CPC, human PDGFRα^{+} cells were later found to only contribute to smooth muscle and endothelial cells *in vitro* using differentiation assays in human cells (Chong et al., 2013). Mouse genetic tracing studies have shown that Pdgfrα is essential for the formation of epicardially derived fibroblasts during embryonic development (Smith et al., 2011). We have further found that cardiac fibroblasts isolated from the adult murine heart display high levels of Pdgfrα, as well as other MSC surface markers, such as Sca1, CD44 and CD90 (Furtado et al., 2014a). Moreover, these markers were not specific to cardiac cells, but were also found in fibroblasts isolated from the mouse tail. These findings demonstrate that the field lacks a comprehensive global analysis of various subselected populations in relation to the total mesenchymal population present in the adult heart. It also raises the question as to whether MSCs and fibroblasts are indeed different cell types, or, at a minimum, if the endogenous role of CPCs is in fact to give rise to interstitial fibroblasts under homeostatic and/or disease scenarios. Our data have further indicated that at least the fibroblast fraction expressing Sca1 and a periostin transgene (*Periostin*$^{cre/+}$*;Rosazsgreen*$^{f/+}$*;Sca1*$^{+}$) does not significantly contribute to newly formed cardiomyocytes after myocardial infarction (Furtado et al., 2014a). Although much work is still required to elucidate the role of CPCs in animal models and human patients, it is clear that none of these CPC populations has so far convincingly proved to replenish the cardiomyocyte pool in injury settings (Bolli et al., 2011; Malliaras et al., 2012, 2014, 2013; Yacoub and Terrovitis, 2013).

The role of cardiac fibroblasts in modulating heart function

Cardiac fibroblasts are abundant in the valvular apparatus, cardiac skeleton and myocardial interstitium of the heart. Despite their significant contribution to these structures, the putative role of fibroblasts in heart formation and function has long been neglected. It has been postulated that the cardiac fibroblast might be linked to hypertrophic cardiomyopathies (HCMs) (Olivotto et al., 2009). A proportion of patients with HCM carry mutations unlinked to cardiomyocyte structural genes; however, the same cohort also presents myocardial disarray, fibrosis, valve abnormalities and microvascular remodelling (Olivotto et al., 2009), suggesting that these types of pathology are directly linked to congenital problems with EPDC deployment.

The EPDC transcription factor Tcf21 is found in the mesenchymal component of many organs, including lungs and kidney. *Tcf21* knockout mice die perinatally of respiratory insufficiency, which is likely to be caused by the severe lung malformation seen in mutant embryos (Quaggin et al., 1999). Confounded by its many sites of expression in the body, the role played by epicardially derived Tcf21 in heart development remains to be properly addressed. Tcf21 is essential for the formation of cardiac fibroblasts (Acharya et al., 2012). Indeed, full genetic ablation of *Tcf21* leads to loss of cardiac fibroblasts in the mouse embryonic heart, causing highly hypoplastic ventricular chambers. Using an inducible *Tcf21* conditional knockout model (*Tcf21*^{iCre}), the same group confirmed the role of Tcf21 in the generation of cardiac fibroblasts, but did not address the efficiency of *Tcf21*^{iCre} in completely removing the fibroblast population (Acharya et al., 2012). It also remains unclear whether the ventricular hypoplasticity observed in full knockout animals is compatible with birth. We have further demonstrated that hearts lacking Tbx20 in the fibroblast compartment (*Periostin*$^{cre/+}$*;Tbx20*$^{flox/flox}$) show ventricular septal defects, hyperplastic valves and hypoplastic compact myocardium (Furtado et al., 2014a) (Fig. 4A). Moreover, mutant hearts showed upregulation of Bmp10, a trabeculation marker, corroborating abnormal myocardial formation. These malformations did not cause full embryonic lethality, as many animals survived to adulthood and showed normal heart function in homeostasis, seemingly due to partial penetrance of periostin-cre in deleting *Tbx20* in the fibroblast compartment (Furtado et al., 2014a). Further clues to the importance of fibroblasts for cardiomyocyte biology can be obtained from the work of Ieda et al. (2009). Using an *in vitro* co-culture system, this group has demonstrated that embryonic fibroblasts are capable of inducing cardiomyocyte proliferation, while adult fibroblasts induce cardiomyocyte hypertrophy. In summary, the combined data obtained so far point to an important role for fibroblasts in heart development, which is still underappreciated owing to the limited capacity of tools available for genetic analyses. In order to fully appreciate the role played by cardiac fibroblasts in development and disease, new reagents that can specifically and fully label the fibroblast compartment of the heart must be generated. Nevertheless, the suggestion that HCM can be linked to a congenital defect in EPDC deployment is reasonable (Olivotto et al., 2009).

In adulthood, cardiac fibroblasts are considered foes of regeneration. Their importance for pathological disease remodelling is undeniable, as various disease settings have diffuse or localised fibrosis as a main feature (Berk et al., 2007; Daskalopoulos et al., 2014; Segura et al., 2014). The fibrotic process causes phenotypic changes in cardiac fibroblasts: cells acquire a myofibroblast fate through the overexpression of cytoskeletal smooth muscle actin and secretion of pro-inflammatory cytokines (Lajiness and Conway,

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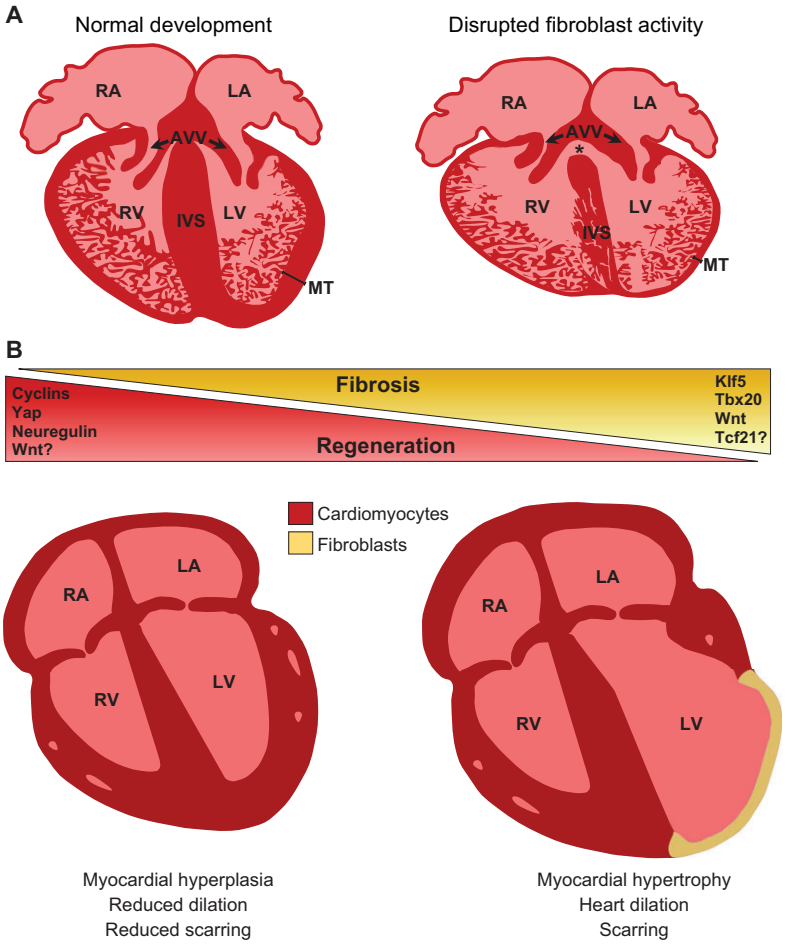

Fig. 4. Role of fibroblasts in development and regeneration. (A) Disruption of fibroblast activity during embryonic development leads to a heart with smaller ventricular chambers, in which myocardial thickness (MT) is reduced (see bars in ventricular myocardium showing MT) (Furtado et al., 2014a). These hearts also display septation defects, such as interventricular septum (IVS) defect (asterisk) and immature atrioventricular valves (AVV, arrows). These defects are related to atrioventricular canal cushion malformations. (B) Healing in the heart depends on the balance between regeneration (red) and fibrosis (yellow). Scarring, a product of poor regenerative capacity, leads to dilation and myocardial wall thinning following myocardial infarction. To achieve healing, cardiomyocyte hyperplasia should be favoured at the expense of fibrosis. Current putative genetic strategies to promote a favourable regenerative balance promote cardiomyocyte hyperplasia (cyclins, Yap, neuregulin) or reduction in fibrosis (Tbx20, Klf5, Wnt). Tcf21 is likely to play an important role in fibrosis, although the role of this factor in an injury setting is yet to be fully addressed. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.

2014; Leask, 2010; Segura et al., 2014). Fibrosis also causes exacerbated collagen deposition, which increases myocardial stiffness. Since fibroblasts are capable of coupling through gap junctions with each other and with cardiomyocytes, scar areas are considered electrically conductive (Kakkar and Lee, 2010; Kohl and Gourdie, 2014). Indeed, cardiac fibroblasts express a broad range of ionic channels and calcium-handling proteins and are capable of transducing electrical information received from the microenvironment (Camelliti et al., 2005). These conductive properties have prompted a modern interpretation of the scar as a living structure. Scars are also capable of generating arrhythmias through conduction re-entries to areas of living myocardium (Kohl and Gourdie, 2014). Therefore, the contribution of cardiac fibroblasts to decreased myocardial function in pathological settings is manyfold.

In order to promote a favourable outcome for myocardial remodelling during regeneration, a healthy balance between the extent of fibrosis and the presence of viable cardiomyocytes must be achieved. A conditional knockout strategy to delete the transcription factor Klf5 in cardiac fibroblasts nicely illustrates this phenomenon (Takeda et al., 2010). Mouse hearts lacking fibroblast-derived Klf5 showed a reduction in cardiac hypertrophy in response to moderate pressure overload – an improved response compared with the wild-type cardiac fibroblasts. Thus, under these moderate pressure conditions, the cardiac fibroblast appears to have been acting detrimentally. However, when subjected to high-pressure overload, knockout hearts developed severe heart failure, suggesting that the cardiac fibroblast is also cardioprotective. The same group further demonstrated that Klf5 drives expression of Igf1, which has been shown by many groups to positively impact on myocardial growth and survival (Santini et al., 2007; Troncoso et al., 2014). Our group has noted a beneficial effect of impaired cardiac fibroblast activity on myocardial regeneration through the deletion of the cardiogenic transcription factor Tbx20 (Furtado et al., 2014a). Following ligation of the descending left coronary artery to induce myocardial infarction, hearts lacking Tbx20 in the fibroblast compartment showed thicker scars and reduced dilation.

Another family of factors involved in fibroblast function include the large Wingless (Wnt) family of lipophilic proteins, which is composed of 19 ligands and ten Frizzled receptors, as well as various co-receptors, transducers and inhibitors. The role played by Wnts in heart development is well defined. Wnt signalling inhibition is essential for heart formation in early embryonic development (Guan and Hasenfuss, 2013). Many components of this pathway are expressed in the adult homeostatic heart, while several others are upregulated in ischaemic heart injury (Deb, 2014). Wnts exert pleiotropic effects in heart regeneration. This is most likely due to the complexity of pathway components and cell populations in the heart, which can respond to the same signal in multiple different ways.

In general, strategies involving the use of Wnt antagonists throughout myocardial remodelling after infarct exert beneficial effects on myocardial function due to the modulation of fibroblast activity and reduction of scarring (Barandon et al., 2003, 2005; He et al., 2010; Laeremans et al., 2011; Matsushima et al., 2010; Saraswati et al., 2010). Paradoxically, however, removal of the

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antagonist of Wnt signalling, secreted frizzled-related protein 2 (Sfrp2), caused an effect opposite to that which normally occurs when modulating other components of the pathway. *Sfrp2* knockout mice showed reduced fibrosis and a significant increase in cardiac performance, as measured by an improvement of ejection fraction 2 weeks after infarction. Sfrp2 expression is dramatically increased in infarcted hearts within 1 week, a time when fibroblast activity is maximised (Kobayashi et al., 2009). Biochemical studies further demonstrated that this amelioration was due to a cross-talk between Sfrp2 and the BMP pathway, through which Sfrp2 controls collagen processing by modulating Bmp1 activity (Kobayashi et al., 2009). This phenotype could be explained by many factors, including inhibitor concentration, physiological differences between mice and rats and/or possible combinatorial effects of the different signalling inputs of both Wnt and BMP. In addition, overexpression of Sfrp1 solely in cardiomyocytes induced larger infarcts and deteriorated function, suggesting that Wnt inhibition in fibroblasts and cardiomyocytes might have opposing effects on heart physiology (Barandon et al., 2005). Although the role of Wnt signalling in cardiomyocytes remains to be properly addressed *in vivo*, genetic manipulation of the downstream transducers β-catenin or glycogen synthase kinase 3β specifically in cardiomyocytes has been shown to modulate the hypertrophic response (Antos et al., 2002; Baurand et al., 2007). However, it is not clear whether this is achieved through Wnt signalling or cross-talk with other pathways (Haq et al., 2003).

Modulation of cardiomyocyte activity

One of the main obstructions to heart regeneration is the terminal differentiation of adult cardiomyocytes, which do not significantly proliferate throughout the life span of mammals and cannot be efficiently replaced after a pathological insult (Ahuja et al., 2007; Bergmann et al., 2009; Kajstura et al., 2010; Li et al., 1996). Consequently, cardiac fibrosis becomes a necessary evil, as necrotic myocardial tissue has to be replaced. Fibrotic deposition leads to the scarring of dead myocardial areas, but does not replace muscular contraction strength or electrophysiological properties. It is therefore imperative to concomitantly modulate fibrotic activity and replace dead cardiomyocytes to achieve successful regeneration. There are multiple approaches to this task: it might be possible to derive *de novo* cardiomyocytes from other cell types, such as pluripotent stem cells, and deliver these to the heart. Alternatively, endogenous cardiomyocytes might be mobilised to undergo proliferation, thus replacing lost ones. The merits and caveats of each of these approaches are discussed below.

Delivery of *in vitro* derived cardiomyocytes to the adult heart after injury represents an attractive possibility for replacing lost muscle cells. Embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) differentiation protocols have achieved remarkable improvements in terms of the yield and quality of cardiomyocytes (Kattman et al., 2011); however, many hurdles remain to be addressed in order to gain therapeutic value. For example, none of the dish-derived cardiomyocytes are capable of achieving an 'adult' phenotype (Feric and Radisic, 2016; Veerman et al., 2015). Current protocols provide cells that retain a fetal or neonatal phenotype at most, and such cells are unlikely to be up to the task of contributing to the highly demanding job of an adult pumping heart. Morphologically, they are smaller, resemble embryonic cardiomyocytes and lack organised sarcomeric structure. Metabolically, ESC-derived cardiomyocytes also behave as fetal cells, displaying small mitochondria that lack complexity, in that the cristae are not well formed, as well as large glycogen deposits of over 30% of cell volume, as opposed to less than 2% in adult cardiomyocytes. Functionally, spontaneous automaticity, gap junctions, electrophysiology and calcium-handling properties are also different. In terms of gene expression, ESC-derived cardiomyocytes generally show less than 50% of mRNA message when compared with adult heart tissue. Although these cells are relevant for drug discovery studies and even disease modelling, the extent to which they can fully integrate into and repair the adult organ is still controversial. There have been multiple attempts to inject ESC-derived cardiomyocytes into the adult primate heart (Blin et al., 2010; Chong et al., 2014). The study by Blin and colleagues injected cardiovascular progenitor cells [Oct4 $(Pou5f1)^+$, SSEA-1 $(Fut4)^+$, $Mesp1^+$ cells] into infarcted Rhesus monkey hearts. These cells engrafted to ∼20% of the scar areas, but additional experiments to determine the quality of grafts were not performed. In the most recent study (Chong et al., 2014), an extremely high number of cells (1 billion) were injected into the adult heart of pigtail macaques following myocardial infarction through ischemia reperfusion injury. The study used seven macaques of various ages and gender, in which two animals were used as sham controls. Out of five macaques injected with cells, all showed ESC-derived cardiomyocyte integration to infarct areas at various ratios, and grafted cells showed electrical coupling with host myocardium, so far not achieved by any other study. However, problems with the experimental design were encountered in the study, stirring controversy in the community. These include the number of treated animals, lack of age/gender matching, variable infarct sizes, the presence of arrhythmias and a lack of proper evidence of cardiomyocyte integration, among others. The conclusion is that, so far, this approach is neither safe, due to risk of arrhythmias and thrombi formation, nor feasible, as extremely high amounts of cells would have to be injected into a human patient and consistent integration into myocardium or improvement of heart function cannot be assured (Anderson et al., 2014).

Rather than injecting cardiomyocytes into the adult heart, it might be possible to mobilise endogenous differentiated cardiomyocytes by stimulating their re-entry into the cell cycle. Remarkable progress has been achieved on this front in the past decade by forced expression of general cell cycle regulators in adult cardiomyocytes, including cyclin A2 (Chaudhry et al., 2004; Shapiro et al., 2014; Woo et al., 2006), cyclin D1/2/3 (Hassink et al., 2008; Pasumarthi et al., 2005; Soonpaa et al., 1997) and cyclin-dependent kinase 2 (Cdk2) (Liao et al., 2001). In all cases, reactivation of the cell cycle regulator was achieved in rodent or porcine hearts using transgenic or viral approaches. Cardiomyocyte-specific *Cdk2* transgene overexpression is among the first strategies to show increased DNA synthesis and proliferation index in adult murine hearts (Liao et al., 2001). However, challenge to transgenic hearts using pressure overload caused maladaptive hypertrophy in transgenic animals, suggesting that this approach might not be pro-regenerative. Alternative approaches used cyclin A2, which is normally downregulated after birth coinciding with cardiomyocyte cell cycle arrest in the postnatal period (Chaudhry et al., 2004). Forced constitutive cyclin A2 expression in murine cardiomyocytes using the MHC promoter led to enlargement of adult hearts, accompanied by cardiomyocyte mitosis, as assessed by phospho-histone H3 staining. Woo and colleagues further demonstrated that adenoviral injection of cyclin A2 into the border zone of infarcted rat hearts increased cardiomyocyte proliferation, as measured by PCNA antibody staining, as well as improved heart function (Woo et al., 2006). The study of Woo and colleagues was followed up by adenoviral injection of cyclin A2 into infarcted porcine hearts

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(Shapiro et al., 2014), a model physiologically closer to humans. The porcine model confirmed findings previously described for rodents, as virus-transduced hearts showed improved function, increased cardiomyocyte mitosis as assessed by Ki67 antibody staining, increased cardiomyocyte numbers and decreased fibrosis. The authors further claimed that full cytokinesis was achieved in cyclin A2-infected adult porcine cardiomyocytes *in vitro*, although these findings have not been confirmed in the infarcted hearts. Overexpression of cyclin D2 using the cardiomyocyte-specific MHC promoter has also shown an improvement in the regenerative response (Hassink et al., 2008; Pasumarthi et al., 2005), although cyclins D1/3 were not useful in heart insult situations. Cyclin D2 transgenic murine hearts responded to isoproterenol-induced hypertrophy, pressure overload, myocardial cauterisation and infarct by increasing DNA synthesis and cardiomyocyte numbers, and, in the latter case, by reduction in infarct size. The combined data point to cyclins A2 and D2 as the most promising cell cycle regulators for possible therapeutic use. Although these studies provide useful insights, it remains unclear whether re-entry into the cell cycle in each case was accompanied by complete cell division or only nuclear division. Nevertheless, these studies demonstrate the capacity of cell cycle regulators to improve myocardial regeneration following ischemic events.

More recently, the involvement of the Hippo pathway in organ size control has been revealed. Hippo is a tyrosine kinase receptor that phosphorylates the Yap1 transcriptional co-activator. Yap1 phosphorylation leads to transcriptional inactivation and consequent growth restriction. Genetic deletion of *Yap1* in cardiomyocytes leads to myocardial hypoplasticity and reduced proliferation during embryonic development (von Gise et al., 2012; Xin et al., 2013, 2011), or dilated cardiomyopathy, increased apoptosis and fibrosis (Del Re et al., 2013), as well as compromised regeneration after birth (Xin et al., 2013). Conversely, overactivation of Yap1 leads to enhanced proliferation (Xin et al., 2013, 2011), hyperplasia and increased regeneration (Xin et al., 2013), as well as cardiomegaly (von Gise et al., 2012). In adult hearts, modulation of components of the Hippo pathway favours beneficial remodelling following an ischemic event, increasing regeneration through reduction of scarring and improvement of functional parameters (Lin et al., 2014; Xin et al., 2013).

Growth factor signalling pathways are also implicated in cardiomyocyte proliferation and may be utilised to enhance regeneration following cardiac injury. Recently, a novel strategy for heart repair based on the Neuregulin pathway was reported (D'Uva et al., 2015). Neuregulin 1 is a growth factor that signals through tyrosine kinase receptors from the V-erb-b erythroblastic leukemia viral oncogene homologue family (ErbB2/3/4 in mice), and it is essential for myocardial formation during embryonic development (Gassmann et al., 1995; Lai et al., 2010; Lee et al., 1995). The ErbB2 receptor is downregulated after birth, coincident with the window in which cardiomyocyte proliferation ceases in mice (D'Uva et al., 2015; Porrello et al., 2011). Overexpression of ErbB2 in neonatal, juvenile and adult cardiomyocytes caused cardiomegaly, that is, an abnormal enlargement of the heart, in this case through both hypertrophy and hyperplasia (D'Uva et al., 2015). Using an inducible strategy, the same group further demonstrated that induction of ErbB2 expression after a heart insult, in this case myocardial infarction, led to a remarkable improvement in the regenerative capacity, presumably through cardiomyocyte proliferation. A second group (Polizzotti et al., 2015) further showed that administration of neuregulin 1 in neonatal mouse hearts or cardiomyocytes derived from paediatric patients also brings beneficial effects to myocardial function through cardioprotection (protection from cell death) and proliferation. Nevertheless, none of these experiments has demonstrated a lack of scarring in the adult heart, which is important since the activity of the fibroblast cells also needs to be addressed in order to achieve full regeneration (D'Uva et al., 2015; Lin et al., 2014; Polizzotti et al., 2015; Xin et al., 2013).

Perspectives from other organs

Fibrosis is a common cause of organ failure in many disease settings, including idiopathic pulmonary fibrosis (Spagnolo et al., 2015), liver cirrhosis (Schuppan, 2015), kidney disease (Ballermann and Obeidat, 2014) and systemic sclerosis (Ebmeier and Horsley, 2015), among others. It is predicted that nearly 45% of all deaths in the developed world are related to chronic fibrosis (Wynn, 2008). The initial trigger for fibrotic reactions normally includes inflammation, through which immune cells recruit fibroblasts to start proliferating and transforming into myofibroblasts. The fibrotic reaction has been extensively characterised in various organ models and seems to include common regulators, including cytokines such as the interleukins and TGFβ, chemokines such as monocyte chemoattractant protein 1 (MCP-1; also known as CCL2) and macrophage inflammatory protein 1α (MIP-1α; also known as CCL3), angiogenic and growth factors such as VEGF and PDGFα/β and the renin-angiotensin-aldosterone system (Wynn, 2008). While these and many other fibrotic regulators have been targets of anti-fibrosis therapies and can reduce morbidity and mortality, there is no current cure for this pathology (Rosenbloom et al., 2013). In addition, none of the current therapies targets a particular organ in a specific manner, since the drug effects often extend beyond the intended targets, or target generic molecular pathways used by many cell types. The challenge facing this field is the complexity of the fibrotic process in various organs. Although the final pathological outcome – activation of myofibroblasts and ECM deposition – is similar among organs, the aetiology and molecular mechanisms may in fact be quite specific, as highlighted for the heart in this Review. Therefore, it is imperative to understand fibrosis in an organ-specific manner. As with the heart, other organs may similarly display an organ-specific genetic programme for endogenous fibroblasts. Thus, specific targeting of these molecular pathways represents a promising strategy for treating fibrosis in an organ-specific manner, in order to avoid systemic complications and the inefficacy of current treatments.

Concluding remarks and future perspectives

It is an exciting time for the study of fibroblast biology. Particularly in the heart, the characterisation of the embryological origin of the cardiac fibroblast and the discovery of a fibroblast cardiogenic programme have been paradigm-shifting, demonstrating that the cardiac fibroblast is unique among fibroblasts, as well as being functionally relevant for both regeneration and pathological remodelling. With the rapid growth of high-throughput technologies and associated bioinformatics tools, a promising area for better understanding the cardiac fibroblast is the systematic construction and analysis of underlying genetic networks and regulatory pathways. These advances will potentiate future large-scale dissection of organ-specific pathways for novel anti-fibrosis strategies. In addition, fibrotic targets in the mouse need to be further validated in large animal models, such as sheep or pigs, to gain translational potential, considering cardiac physiological differences between mice and other mammals. The use of human

biopsies is also of vital importance to corroborate target expression before any therapeutic approach can be applied.

Although much progress has been made in understanding cardiac fibroblasts and fibrosis over the last couple of years, many questions remain unanswered. For example, what is the full contribution of the cardiac fibroblast in various disease settings, including congenital malformations and heart failure? What is the role of the cardiogenic profile in fibroblast biology and overall heart function, and are cardiac fibroblasts and cardiac MSCs in fact the same cell type? In order to answer these questions, murine genetic models will be of vital importance, as they provide ways to visualise and modulate specific genes or pathways involved in cardiac fibroblast biology. All knowledge generated so far suggests a synergistic relationship between cardiac fibroblasts and cardiomyocytes in homeostatic conditions (Fig. 4B). This relationship is broken by disease processes such as myocardial infarction, when triggers of cardiomyocyte death and fibroblast overactivity cause an imbalance that promotes myocardial hypertrophy and scarring, culminating in heart dilation and impaired function. This inter-relationship between cardiomyocytes and fibroblasts highlights the importance of modulating both compartments to achieve a pro-regenerative response. Signals that acutely trigger cardiomyocyte survival or modulate myofibroblast activity should be used in combination to promote cardiac regeneration and avoid heart failure.

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