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Review Article

The dynamic facets of the cardiac stroma: from classical markers to omics and translational perspectives

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Abstract: Cardiac stromal cells have been long underestimated in their functions in homeostasis and repair. Recent evidence has changed this perspective in that many more players and facets than just "cardiac fibroblasts" have entered the field. Single cell transcriptomic studies on cardiac interstitial cells have shed light on the phenotypic plasticity of the stroma, whose transcriptional profile is dynamically regulated in homeostatic conditions and in response to external stimuli. Different populations and/or functional states that appear in homeostasis and pathology have been described, particularly increasing the complexity of studying the cardiac response to injury. In this review, we outline current phenotypical and molecular markers, and the approaches developed for identifying and classifying cardiac stromal cells. Significant advances in our understanding of cardiac stromal populations will provide a deeper knowledge on myocardial functional cellular components, as well as a platform for future developments of novel therapeutic strategies to counteract cardiac fibrosis and adverse cardiac remodeling.

Keywords: Cardiac fibroblasts, cardiac stromal cells, fibroblast markers, cardiac fibrosis, heart failure, cardiac remodeling, single-cell sequencing, omics data

Introduction

Stromal cells have been long underestimated in their functions in multiple tissues. A classical view of poorly specialized filler cells has been the reference until recently. Now the scenario is much different, even in proper connective tissues, such as the derma, where multiple populations of fibroblasts have been identified with very different behaviors, particularly concerning tissue repair and fibrosis mechanisms [1, 2]. The same change of perspective has affected the heart and its stromal populations, where many more players and facets than just "cardiac fibroblasts" have entered the field.

Several stromal populations have been described in the mammalian heart, with specific homeostatic roles, particularly concerning the synthesis and maintenance of the extracellular matrix (ECM), and the trophic support to other specialized cells, such as endothelial cells or cardiomyocytes. Many membrane and intracellular markers have been associated to each specific stromal phenotype, although with much overlap, and often lacking a unique consensus on the panel to be used to define and/or distinguish a single cell type (**Table 1**). This complex scenario implicitly suggests the existence of blurred lines separating distinct cell types or subpopulations, and that at least some of those populations may as well be different functional manifestations of a number of cell types much smaller than those described in the literature. Therefore, the field still needs to investigate phenotypes, markers, and functions thoroughly and comparatively.

The cardiac stromal compartment possesses a fundamental role in physiopathology due to its

Table 1. A summary of cardiac stromal cell markers identified through the years, and sorted as classical or derived from novel omics approaches

	Classical Markers			New Omics Markers
	Cell surface and Intracellular	Extracellular	Transcriptional	Cell surface, Extracellular & Transcriptional
Unactivated Fibroblasts	PDGFRα, DDR2, CD29, CD49e, CD51, CD73, CD90, CD105, Sca1, Vimentin, Filamin A	Collagens, TNC	GATA4, GATA6, Nkx-2.5, Hand2, Tbx18, Tbx20, Tcf-21, WT1	DCN, ELN, GSN
Activated Fibroblasts	↑CD105, CD90, ↓PDGFRα, ↑Vimentin, ↑DDR2, α-SMA	POSTN, Collagens	↓Tcf21, ↑Tbx18, ↑Wt1	Wisp1, Ckap4
Myofibroblasts	↑CD105, ↓PDGFRα, ↓DDR2, ↑α-SMA	↑↑Collagens	↓Tcf21	MYH11, FAP
Matrifibrocytes				CHAD, COMP
Pericytes	PDGFRα, PDGFRβ, NG2, CD146, CD73, CD90, CD105, CD271, SM-MHC, α-SMA			NCAM2, CD38, CSPG4, ABCC9, KCNJ8
Telocytes	CD34, CD117, PDGFRα, PDGFRβ, Vimentin			
Mesenchymal & Progenitor cells	CD51, CD105, CD73, CD90, Sca1, PDGFRα, Filamin A, Vimentin	Collagens	Islet-1, Tbx5, GATA4, Nkx-2.5, MEF2C, Tcf21, NANOG, OCT-4	CD38, ICAM2, Caecam1, CD36, CD93, CD322, KITL, JAG2, VEGF-C

many roles in ECM remodeling, fibrosis, angiogenic signaling, and crosstalk with the immune compartment. Multiple studies have demonstrated its pivotal role in the pathogenesis and outcome of several diseases, thus suggesting that its targeting may be highly effective for novel therapeutic strategies against cardiac diseases, particularly those involving fibrosis and adverse remodeling. In this review, we are presenting an overview of cardiac stromal cell types and functional states described in the literature (excluding the immune compartment), and the markers used to identify them in different physiological and pathological conditions. Moreover, we are discussing some perspectives on the possible exploitation of stromal cells as mediators or targets of novel therapeutic approaches for the treatment of cardiac diseases.

Cardiac fibroblasts and their classical markers

Fibroblasts have classically been studied and described as a unique cell type with a standard phenotype, independently of tissue origin, whose only function was to synthesize and remodel the ECM. This reductionist view has been challenged in the last decade, particularly by recent single-cell transcriptomic data, demonstrating high phenotypic heterogeneity and plasticity of fibroblasts, both under homeostatic and pathological conditions. In this regard, it is now clear that cardiac fibroblasts (CFs) do not represent a mono-dimensional population within the heart whose only role is to support cardiomyocytes and regulate ECM turnover. Instead, many studies have highlighted the diversity of cells listed as CFs with diverse localization and specialized properties, in both humans and other species. Mature fibroblasts are interspersed in the myocardium, and are able to maintain ECM homeostasis. In humans they are strongly positive for transcription factor 21 (Tcf21) [3], platelet-derived growth factor receptor alpha (PDGFR-α) [4], discoidin domain containing receptor 2 (DDR2) [5, 6], and vimentin [7]; conversely, they do not express alpha smooth muscle actin (α-SMA) and Periostin (POSTN). Mature fibroblasts have a low level of proliferation, but after injury they can rapidly proliferate and become activated fibroblasts, characterized by increased expression of Tcf21, PDGFR-α, POSTN, collagens, cell cycle genes, DDR2, and vimentin (as will be more accurately described in a dedicated paragraph below). After activation, a small proportion of these cells differentiate into myofibroblasts, which express α-SMA and produce collagens, while displaying reduced expression of Tcf21 and PDGFR-α.

Below is a brief presentation of the above-mentioned classical markers that are widely used to characterize the dynamic shift of mature fibroblasts into myofibroblasts, as we will explain later. The related intracellular pathways are depicted in **Figure 1**.

● Tcf21 encodes for a transcription factor of the basic helix-loop-helix family, which is meso-

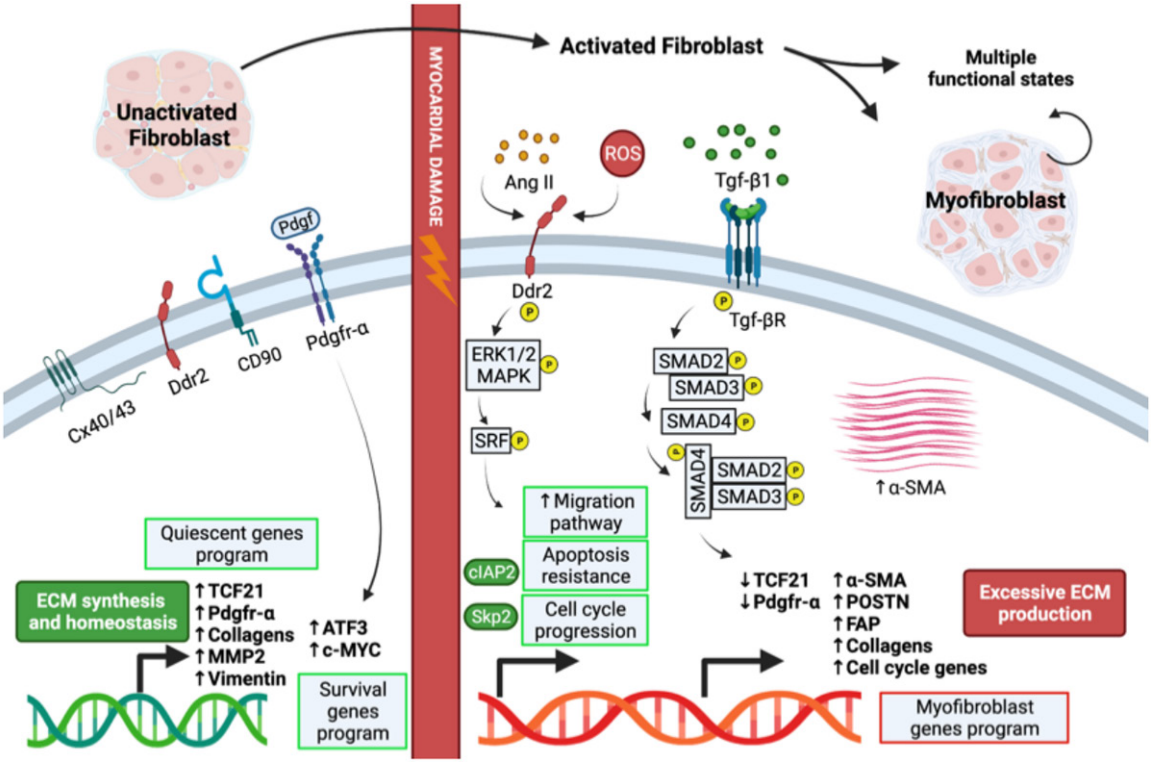

Figure 1. The phenotypic shift of fibroblasts into myofibroblasts. Depiction of signaling pathways and functions involved in the activation of fibroblasts and differentiation into myofibroblasts. Image was created with the Biorender software. Cx40/43: connexin 40/43. MMP2: matrix metallo-proteinase 2. ATF3: activating transcription factor 3. c-MYC: cellular myelocytomatosis oncogene product. Ang II: angiotensin II. ROS: reactive oxygen species. Erk1/2: extracellular signal-regulated kinase 1/2. MAPK: mitogen-activated protein kinase. SMAD: small mother against decapentaplegic. cIAP2: cellular inhibitor of apoptosis 2. SKP2: S-phase kinase associated protein 2. SRF: serum response factor.

derm specific and expressed in the embryonic epicardium. It plays a crucial role in regulating cell differentiation and cell fate specificity through epithelial-to-mesenchymal transition (EMT) during cardiac development, but it is still active in adult resident CFs [3].

● PDGFR-α encodes for a cell surface tyrosine kinase receptor for members of the platelet-derived growth factor family. PDGFR-α signaling directs migration and differentiation of epicardial-derived fibroblasts during heart development [8]. In the adult heart, multiple studies have established that PDGFR-α signaling controls CFs proliferation and activation. Moreover, human CFs require PDGFR-α signaling for survival [4].

● DDR2 encodes for a member of the discoidin domain receptor subclass of the receptor tyrosine kinase protein family. It is expressed on the surface of cells of mesenchymal origin. DDR2 mediates a variety of cell functions, including growth, migration, differentiation, EMT, and is associated with the fibrotic process. The DDR2 receptor is also present in myofibroblasts, therefore it cannot be used to distinguish between cell sub-types unless a combination of markers is used. Moreover, there are controversial results in the literature regarding the abundance of DDR2^{+} fibroblasts [9].

● Vimentin is a type III intermediate filament protein that is expressed in multiple cells. It plays a significant role in maintaining cell shape and integrity of the cytoplasm, and in stabilizing cytoskeletal interactions. Vimentin is commonly used as a CFs marker because it labels cells with great sensitivity, however it is not specific. In fact, vascular smooth muscle cells (V-SMCs), endothelial cells, and macrophages also express vimentin [10].

● Alpha-SMA (αSMA) is a member of the highly conserved actin family of proteins, which plays

a key role in cell motility, structure, and integrity. It is a classical marker used to distinguish mature fibroblasts from activated fibroblasts and myofibroblasts. However, this actin isoform is also expressed in V-SMCs and pericytes (see also dedicated paragraph). α-SMA is not expressed under homeostatic conditions, but it is up-regulated in response to pro-fibrotic and hypertrophic stimuli in the human heart [11].

● POSTN is a transforming growth factor-beta 1 (TGF-β1)-inducible secreted extracellular protein that plays essential roles in wound healing, ECM deposition, CFs activation and proliferation, and tissue fibrosis. POSTN is highly expressed during cardiac development, but its expression is reduced in un-activated fibroblasts. However, it is up-regulated after injury, such as myocardial infarction; therefore it represents a consensus marker of activated fibroblasts and myofibroblasts [12].

Studies in multiple species, particularly in mice, have shown that, in addition to the above-mentioned classical markers, CFs share the expression of many common fibroblast markers, such as collagens 1α1/1α2, filamin A, and Tenascin C (TNC). In addition, they are characterized by significant heterogeneity of cell surface receptors: virtually all cells are positive for CD29, CD49e, CD51, while a vast majority express CD90 and stem cell antigen 1 (Sca1) [13]. CFs express many other cardiogenic transcription factors, such as Tbx18, Tbx20, GATA4/6, Hand2 and Nkx-2.5 (**Table 1**; **Figure 2**), also involved in cardiomyocyte (CM) development and function. Expression programs reveal a heterogeneous landscape of CFs, partially due to their regional specification. Tbx20 is among the highest and most consistently expressed genes in fibroblasts of all cardiac compartments. Furtado et al. have demonstrated that this transcription factor plays a key role in the development and maturation of both myocardial and non-myocardial compartments [13]. The expression of epicardial genes Tcf21 and Wt1 (particularly in ventricular or atrial CFs, respectively) also endorses the epicardial origin of most CFs. Overall, Tcf21, Wt1, and Tbx18 are transcription factors expressed during embryonic development that regulate the fate of epicardial cells and their differentiation towards various lineages, including fibroblasts. In the adult murine heart, instead, Tcf21 is expressed at baseline also by some perivascular and interstitial cells. Cardiac injury, though, increases the expression of Tcf21, first in the epicardial region and then in the myocardial interstitium where fibrosis is induced [8]. Therefore, tissue damage leads to the expression of Tcf21, Wt1, and Tbx18 in different subepicardial mesenchymal populations, recapitulating what happens during embryonic development [14].

Activated fibroblasts and myofibroblasts

The activation of $Tcf21^{+}$ fibroblasts following cardiac injury is functional to their differentiation into myofibroblasts, typically identified by the expression of α-SMA and their localization within the scar site [15]. In a mouse model of myocardial infarction, activated fibroblasts upregulate proliferation and migration pathways, as well as cytoskeletal and ECM-modifying genes; then this expression profile is down-regulated few weeks after injury. For some classical markers (e.g., Tcf21, PDGFR-α) the specific time-course of gene expression modulation remains partly debated, with some authors reporting downregulation instead of upregulation [16]. Other genes typical of bone, connective tissue, cartilage, and tendon development or processing, characterize this activated state as well. Induction of the myofibroblast phenotype, instead, has been associated to a multitude of stimuli, and the key effector in this process is the cytokine TGF-β1 (**Figure 1**). Moreover, myofibroblast activation is a hallmark of several cardiovascular diseases, as these cells are responsible for the excessive deposition of ECM proteins, and are the primary drivers of cardiac fibrosis.

Although α-SMA is a key marker of myofibroblast differentiation (**Figure 1**), Fu et al. have reported that its expression is extinguished 14 days after myocardial infarction in a mouse model, suggesting that myofibroblasts do not represent a permanent differentiation state [16]. Nevertheless, α-SMA expression persists in these cells. Myofibroblasts initially localize around the damaged area with long cytoskeletal extroflexions. As they downregulate αSMA expression, they reorganize with a linear shape and lose processes. This suggests that α-SMA may regulate the structural organization of myofibroblasts, which initially surround the damaged regions with a network of filaments. During the scar maturation, myofibroblasts lose

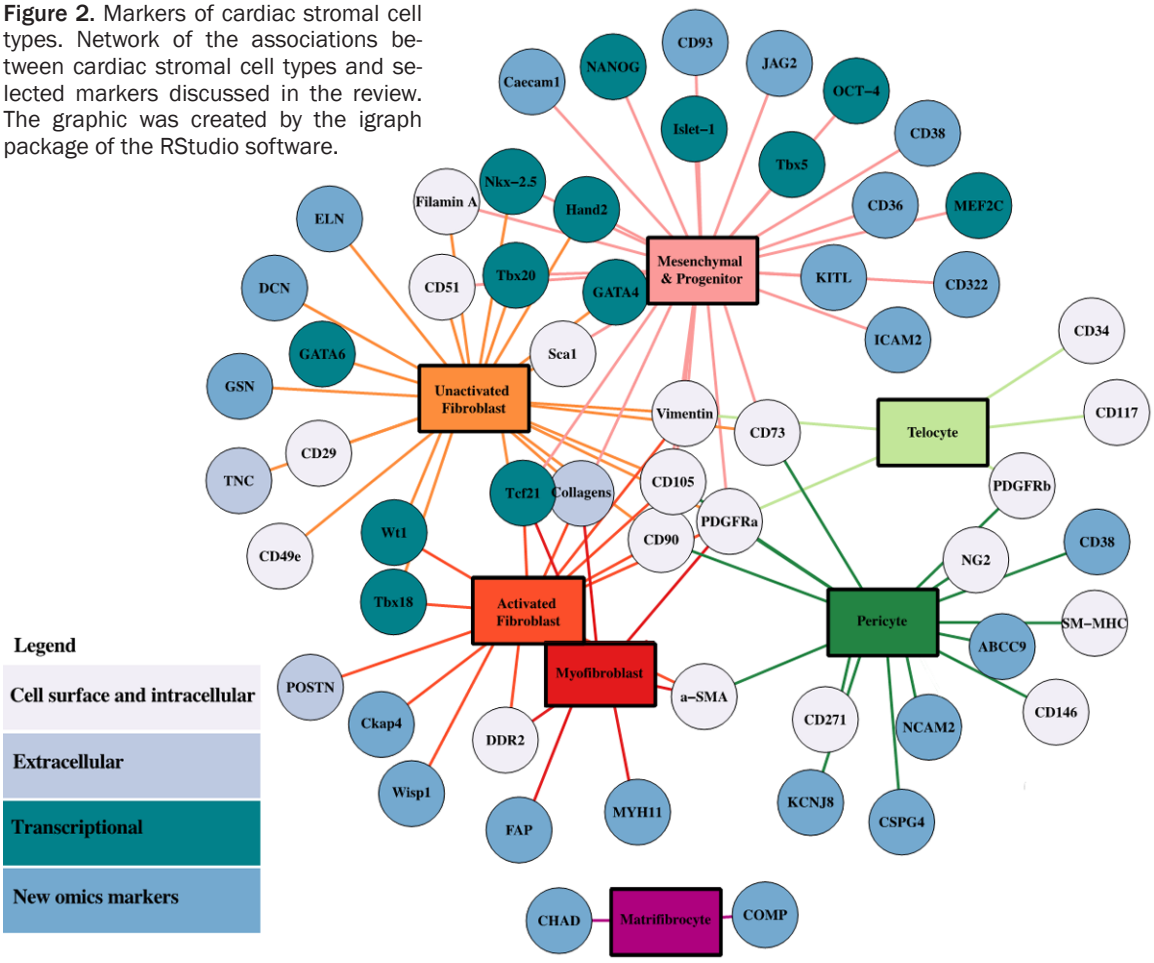

Figure 2. Markers of cardiac stromal cell types. Network of the associations between cardiac stromal cell types and selected markers discussed in the review. The graphic was created by the igraph package of the RStudio software.

this phenotype, as collagens gradually become fully supportive of wall integrity. In fact, Fu et al. have shown that blocking collagen maturation leads to persistence of α-SMA expression in these cells [16].

Thus, fibroblasts (Tcf21^{+}), activated fibroblasts (POSTN^{+}), and myofibroblasts (α-SMA^{+}) persist long term within the scar, but myofibroblasts are only a transient differentiated state. Interestingly, Fu et al. have recently identified a further population of cells that becomes detectable after scar formation in humans and mice, and named it "matrifibrocytes" [16] (**Figure 2**). They appear to differ from myofibroblasts, although closely related to them. In fact, these cells may have the functional role of maintaining the integrity of the mature scar, as they seem to be a more suitable cell type for this environment, expressing unique ECM proteins from dense connective tissues, most likely to confer increased mechanical features to the healing tissue.

Interestingly, Braitsch et al. [8] have demonstrated different expression profiles for the key markers Tcf21, Wt1, and Tbx18 in response to different injuries: for example, ischemia induces all three epicardial progenitor markers associated to epicardial fibrosis; instead, Tcf21 is mostly induced in perivascular fibrosis after pressure overload, but interstitial fibrosis is nonetheless associated to all three markers in different injury models. These transcription factors are not co-expressed with the myofibroblast marker α-SMA, suggesting that they are only activated in differentiated fibroblasts during fibrosis. Moreover, another different profile is associated with chronic heart injury, such as that induced by prolonged angiotensin II exposure, and associated with the upregulation of many fibrosis-related genes in CFs, such as collagen isoforms Col1a1, Col1a2, Col3a1, and Col8a1, fibronectin (Fn1), connective tissue growth factor (CTGF), insulin-like growth factor 1 (IGF-1), protein-lysin 6-oxidase (Lox), and TGF-β1. Moreover, other specific markers, such as

α-SMA, DDR2, POSTN, PDGFR-α, S100 calcium binding protein A4 (S100a4), and CD90 are strongly upregulated after injury with a well detectable increase also in the proportion of expressing cells (**Table 1**; **Figure 2**).

Cardiac mesenchymal and progenitor cells

The presence of resident cardiac mesenchymal stem cells (recently more cautiously renamed as mesenchymal stromal cells, C-MSCs) has been hypothesized under the quest for resident regenerative cells in adult tissues. Indeed, the C-MSC niche has been described as a reservoir of mesenchymal stem cells and tissue-specific progeny residual from the embryonic development of the heart [17]. Specific criteria have been used through the years for the identification of MSCs, regardless of the tissue of origin. Together with the ability to differentiate towards the three mesodermal lineages (adipocytes, chondrocytes, osteoblasts), and being clonogenic and negative for hematopoietic lineage markers (i.e. CD45, CD34, CD14, CD11b), C-MSCs are largely positive for Endoglin-CD105, the GPI-anchored surface proteins CD73 [18], and CD90 (**Table 1**; **Figure 2**). These phenotypic characteristics appear to be similar in all MSCs, although Kang et al. have demonstrated that the percentage of $CD90^+$ cells is reduced by approximately 40% in human C-MSCs compared to bone marrow-derived mesenchymal cells (BM-MSCs). However, it has been shown in animal models that the CD90-negative fraction of C-MSCs possesses stronger cardiovascular trophic functions due to greater production of growth factors (such as HGF, VEGF, and bFGF) compared to $CD90^+$ cells [19], and that the $CD90^+$ fraction is closer to a fibrotic-prone cell type. Another important membrane protein, the integrin alpha v (CD51), has been reported to mark specifically resident C-MSCs in the heart of postnatal mice [20].

Studies have also correlated the multipotency and self-renewing features of MSCs with the expression of typical transcription factors of embryonic stem cells, such as Nanog, Oct-4, and Sox-2 [21]. High expression of these latter has been described both in C-MSCs extracted from human aborted fetuses and rat heart tissue [22], while C-MSCs derived from adult cardiac tissue display expression of NANOG, but not OCT-4 and SOX-2 [23].

Positivity to a single marker is not sufficient to define C-MSCs, but characterization of this cell type is still uncertain in the literature. Many recognized markers expressed in C-MSCs are expressed in other cardiac cells, such as cardiac progenitor cells (see paragraphs below), fibroblasts, and pericytes (**Figure 2**), although these cell types seem to have different functional potential in myocardial homeostasis and repair.

Under the same quest for resident reparative cells, cardiac progenitor cell (CPC) populations within the adult mammalian heart have been described in the last two decades with a high translational interest for regenerative purposes. One of the main surface markers used for the isolation of such a population in mice is Sca1 [24]. Typical features of $Sca1^+/CD31^-$ CPCs are high clonogenic efficiency [25], a primitive undifferentiated phenotype, long term proliferation, and the ability to differentiate into different cardiac lineages in vitro, such as smooth muscle and endothelial cells [26]. The $Sca1^+/CD31^-$ population expresses Nanog and the telomerase reverse transcriptase (TERT), two genes associated with pluripotent phenotypes and not expressed by differentiated fibroblasts (**Figure 2**) [27]. In addition, CPCs express the embryonic heart markers Islet-1 (ISL-1) and TBX5 [28], as well as cardiac-specific transcription factors GATA-4, Nkx-2.5, and MEF2C (**Table 1**). Conversely, they are negative for markers of mature cardiomyocytes, such as cardiac α-myosin heavy chain (α-MHC) [28]. Therefore, these cells have cardiac-specific features, but do not display markers of neither mature cardiomyocytes, activated fibroblasts or myo-fibroblasts, although similarities may be recognized in the profile of un-activated CFs (**Figure 2**), or in subpopulations with anti-fibrotic features, as will be further discussed below.

Fate mapping assays have revealed that virtually all Lin^- (hematopoietic lineage)/$Sca1^+$ cells derive from $Mesp1^+$ precursors [25], suggesting a mesodermal origin with a possible pro-epicardial contribution [29]. Interestingly, the PDGFR-α^+/$Tcf21^+$ fraction also seems to overlap with the so-called side population (SP) dye-efflux phenotype, which is a widely described functional criteria used to identify adult CPCs [30]. Indeed, the $Sca1^+$ population is characterized by a cardiogenic signature and enrichment

for stemness-associated markers (e.g. Abcg2, Abcb1b, Klf4). Overall, CPCs identified by the SP phenotype can be defined as PDGFR-α$^+$/Tcf21$^+$ cells, and this expression profile defines more exactly a population enriched for a cardiogenic signature that can be purified by isolation of the PDGFR-α$^+$/CD31$^-$ population from Lin$^-$/Sca1$^+$ cells. Consistently, based on single cell analysis, Lin-/Sca1$^+$/Tcf21$^+$/PDGFR-α$^+$/CD31$^-$ cells show an enrichment for GATA4/6, Mef2c, Hand2, and Tbx5/20, regardless of their SP status, while the Sca1$^+$/PDGFR-α$^-$ pool displays vascular/endothelial features [25].

Another functional criterion used to isolate primitive undifferentiated stromal cells with CPC features is a combination of the explant culture technique (i.e., the classical culture to isolate fibroblasts) with a selection step for spontaneous spheroid growth, which is a widely recognized assay for primitive undifferentiated phenotypes [31-35]. CPCs isolated with this protocol are highly clonogenic, display a mesenchymal signature (CD45$^-$/CD105$^+$) and are largely Sca1$^+$/CD31$^-$, in combination with the expression of several cardiogenic and pluripotent markers (e.g., GATA4, Nkx2.5, Oct4), and with being negative for α-SMA and DDR2. Interestingly, a transcriptomic study on CPCs isolated from the adult human heart has demonstrated very high similarity between human CPCs isolated by different criteria (e.g., Sca1$^+$ versus spheroid selection) [36].

Overall, considering the recurrent overlap of markers, it cannot be excluded that, at least to some extent, C-MSCs, CPCs, un-activated CFs or CFs with an anti-fibrotic signature, may represent different functional states of the same resident stromal pool with a surprising degree of plasticity (**Table 1**; **Figure 2**). Several studies have reported that CPC populations can shift in the relative expression of markers associated with the myofibroblast phenotype or cardiac fibrosis, in response to different stimuli [37-42]. These phenotypic shifts seem to exert a biological effect mostly through altered paracrine properties of the cells [43, 44] that can indeed propagate anti-fibrotic [45] and anti-inflammatory signals by intercellular communication when maintained in their best reparative phenotype. For example, the relative abundance of cells expressing CD90 (i.e., a marker associated in situ with the activation of CFs and fibrotic processes in the heart [8]) has been negatively correlated with beneficial paracrine profiles, as well as clinical data on reduced functional recovery of the heart after cell transplantation [46, 47].

Pericytes

In addition to the stromal phenotypes described so far, other specific non-immune cell types are described in the myocardium. Among them, pericytes are a peculiar cell type which is present in all vascularized organs, anatomically defined as perivascular cells that closely surround endothelial cells in capillaries and microvessels. They are involved in different physiological and pathological functions, including the regulation of blood pressure, tissue healing and scarring, and are well described also in the murine cardiac interstitium [48]. The consistent expression of pericyte markers by human myocardial perivascular cells surrounding microvessels and capillaries has been demonstrated, including neuron-glial antigen 2 (NG2), CD146, α-SMA, smooth muscle myosin heavy chain (SM-MHC), PDGFR-β and PDGFR-α (**Table 1**) [49]. Different combinations of co-expressed markers (e.g., NG2 and CD146, CD146 and α-SMA, CD146 and SM-MHC) in ventricular pericytes have been described based on the localization around microvessels of different sizes. For example, PDGFR-β can be detected on all myocardial pericytes, while expression of PDGFR-α can be found on nearly all pericytes surrounding microvessels, but not on those surrounding capillaries [50]. Furthermore, studies have reported that perivascular cells share the expression of several mesenchymal markers, such as CD146, CD73, CD90, CD105, CD271, and NG2 (**Figure 2**) [51].

With the advent of single cell transcriptomics [52], it has been possible to analyze the different patterns activated by pericytes in various conditions. As demonstrated by Litvinukova et al., pericytes also express ABCC9 and KCNJ8, and segregate into several clusters: pericytes resident in ventricles express adhesion molecules (e.g., NCAM2, CD38, and CSPG4) that are involved in microvascular morphogenesis and endothelial cell cross-talk. Conversely, other clusters identified are atria-enriched pericytes, or pericytes with cardiomyocyte features, and so-called stromal pericytes. This latter represents a transitional state between pericytes and endothelial cells [53].

Telocytes

Telocytes represent a stromal population with a typical morphology, including a small cell body and very long and thin moniliform processes, named telopodes. Thanks to these features, they are able to build a 3D physiological network throughout the whole stromal space, in order to communicate with each other and with neighboring cells. In addition, they exert physiological roles such as the release of growth factors, guiding cardiac progenitors during organogenesis. They also play a role in response to pathological states, enhancing angiogenesis, cardiomyocyte renewal, and improving cardiac function [54]. These cells are described in multiple sites in the myocardium, suggesting their significant contribution to cardiac homeostasis and repair mechanisms [55]. Several studies have elucidated their markers profile, that includes CD34, c-kit/CD117, PDGFR-α and β, and Vimentin (**Table 1**; **Figure 2**) [56-58]. It was demonstrated that telocytes are implicated in intercellular communication through direct gap junctions or extracellular vesicle release [59]. In particular, the vesicles released can transfer macromolecular signals to adjacent cells to stimulate neovascularization in the infarcted myocardium in mice and rats [60]. Other studies have reported that telocytes play a protective role also by secreting VEGF, expressing angiogenic-associated microRNAs, and establishing direct nano-contacts with newly derived endothelial cells at the border zone of myocardial infarction [59].

An "Omics" perspective on the cardiac stroma

Single cell transcriptomic studies on cardiac interstitial cells have confirmed phenotypic plasticity of the cardiac stroma, whose transcriptional profile is dynamically regulated in homeostatic conditions and in response to external stimuli, with different populations and/or functional states that appear either in homeostasis or disease. In fact, it is known that the heart cellular composition changes during pathological stress, and genome-wide expression analysis specifically occurring within each cell type during cardiac stress is providing unprecedented knowledge on cell dynamics in injury and repair. Dissociated cardiac muscle has been used by Tucker et al. [61] for single-nucleus sequencing (sn-RNA-seq) on nuclei derived from the 4 chambers of the normal human heart of transplant donors, revealing 9 major cell types and 20 subclusters of cell types. Cellular subclasses included 3 fibroblast subsets which constitute 32.4% of observed cells, and display common markers of the fibroblast lineage, such as Decorin (DCN) and Elastin (ELN). The authors identified clusters characterized by upregulation of fibrosis-associated genes, such as NOX4, IGF1, ADAMTS4, VCAN, and AXL. However, as expected in healthy myocardial tissue, they did not identify a subcluster of canonical activated fibroblasts defined by the expression of classical markers of activation (POSTN), myofibroblasts transition (MYH11, fibroblast activation protein-FAP), or transformation into matrifibrocytes (CHAD, COMP) (**Figure 2**) [61].

Similarly, Litviňuková and colleagues have identified 7 subclusters of CFs combining single cell (sc)-RNA-seq and snRNA-seq data from human healthy transplant donors [52]. In detail, CF compartments share the expression of three markers: DCN, which regulates collagen fibrillogenesis [62], Gelsolin (GSN) [63], and PDGFR-α [4]. However, inside this population the authors found three subclusters displaying different properties: the first has higher expression of genes involved in ECM homeostasis; the second has higher expression of cytokine receptors, such as oncostatin M receptor (OSMR) and interleukin 6 receptor subunit alpha (ILST6); the third is defined by the expression of TGF-β signalling responsive genes (POSTN, TNC, and FAP) thus displaying features of activated CFs (**Table 1**; **Figure 2**) [64]. In this context, fibroblast activation could be the result of age-related changes in cardiac physiology, which lead to progressive dominance of fibrotic remodeling circuits. Interestingly, in both studies, fibroblast clusters display chamber specific distribution across the heart, likely related to their diversity in developmental origin and specialized functions. Together, these single cell analyses of healthy human heart provide fascinating information to deepen our understanding of cardiac physiology in homeostatic conditions and normal aging.

According to another single-cell dual-omics approach on mouse cardiac tissue, where transcriptome and epigenome of cardiac non-myocytes were described, CFs can be subdi-

vided into three distinct populations. Each has specific functional states related to cellular response, cytoskeleton organization, and immune response. The three CF sub-types showed a specific distribution, with known markers expressed at comparable levels (e.g., Tcf21, PDGFR-α, Col1a1, and Col3a1). Gene enrichment analysis identified Hsd11b1 and Gfpt2 as the representative marker genes for the differential states described by the authors [65]: state 1 with high levels of Hsd11b1, Inmt, and Cxcl14; state 2, with high levels of Gfpt2, pi16, and Uap1 gene expression; state 3 with the features of fibrocytes (that is a mesenchymal cell type arisen from monocyte precursors). The abundance of each CF population varies after cardiac injury. In fact, early after ischemia, the infarct area is colonized by Hsd11b1^{+} and Gfpt2^{+} cells (state 1 and state 2 CFs), with a peak at 7 days post MI for state 3 fibroblasts. Thus, the proportion of cells changes dynamically during time after MI, suggesting a different function for each CF population upon injury [65].

In another study, dissociated mouse hearts were analyzed by single cell transcriptomic, and 12 distinct cell clusters expressing known markers of major cell types were identified. The clusters comprised endothelial cells, fibroblasts, granulocytes, pericytes, SMCs, as well as lymphocytes, dendritic cell (DC)-like cells, and Schwann cells [66]. In this work the authors again confirmed the presence of a cell subpopulation (fibrocytes) [67] expressing intermediate levels of canonical genes corresponding to both fibroblasts (Col1a1, PDGFR-α, Tcf21) and macrophages/leukocytes (Fcgr1, Cd14, Ptprc). Given the overlap of known markers, a strategy was designed to discriminate pericytes, SMCs, Schwann cells, and fibroblasts, by identifying genes with higher expression in one of these cell types. For example, by using staining for the mesenchymal marker ITGA7 and gene expression data, as well as transgenic reporter mouse strains, fibroblasts could be distinguished from mural perivascular cells using mEF-SK4 as a secondary marker, thus proposing a new specific marker to distinguish PDGFR-α^{+} CFs from mural cells [66].

Transcriptomic studies have also enhanced our understanding of CF functions. Interestingly, the analysis of possible cellular interactions identified fibroblasts as the most trophic cell population with connections to many cell types. For example, the expression of colony stimulating factor 1 (CSF1) and IL34 signal through the CSF1 receptor are essential are essential factors for macrophage growth and survival. Fibroblasts also express growth factors NGF, VEGFA, IGF1, and FGF2, which support neurons of the autonomous nervous system, endothelial cells, and mural cells [68, 69]. Thus, CFs appear to establish networks that support not only cardiomyocyte survival and define cardiac ECM, but can also modulate the immune response and support cardiac innervation.

Recent omics studies have allowed us to deepen the understanding of classical markers, as well. Using the PDGFR-α-GFP reporter mouse line, the resident fibroblast population has been divided into two major sub-populations after sc-RNA seq: Sca1^{high} (F-SH) and Sca1^{low} (F-SL), both expressing canonical fibroblast markers such as PDGFR-α, DDR2, and Col1a1 (**Table 1**). F-SH and F-SL show distinct adhesive and secretory phenotypes, highlighting the likely functional differences between them [70], although both representing populations of quiescent un-activated CFs. In addition, a novel activated fibroblast population, expressing a strong anti-Wingless-related integration site (WNT) transcriptome signature (F-WNT-X) was identified in healthy hearts, as well as after MI [70]. WNT plays complex roles in cardiac biology and disease, impacting immune, vascular, and pro-fibrotic pathways [71, 72]. F-WNT-X stromal cells uniquely expressed Wif1, encoding a canonical and non-canonical WNT signaling antagonist [73, 74], acting on multiple pathways such as CTGF and VEGF, whose regulation is important for efficient cardiac repair (**Figure 1**) [75-77]. In addition to WIF1, F-WNT-X cells showed upregulation of other WNT and TGF-β pathway antagonists, overall making these cells paracrine mediators of an anti-WNT/CTGF/TGF-β signaling, essential for anti-fibrotic cardiac repair. Strategies for the enhancement and potentiation of this stromal population after cardiac injury could be of high interest for translational purposes.

In the same study, analysis in injured hearts has revealed a high complexity of signaling, and stromal cells plasticity has shown its importance in driving cardiac tissue response

to injury. When mice were subjected to MI, fibroblast populations showed a dynamic change in time and space. At day 3 post-MI both F-SH and F-SL were significantly diminished, apparently converting into an activated state (F-Act) defined as $POSTN^{+}Acta2^{negative\text{-}low}$, with recruitment and activation of cells from areas outside the infarct, then restored by day 7 post-MI [70]. F-Act share some transcriptional features with myofibroblasts, such as activation of collagens and genes associated with wound healing (**Figure 1**), but they appear more related to resident fibroblasts than to myofibroblasts. These cells showed indeed active proliferation in the first week after MI, consistent with the known proliferation peak observed in CFs [78, 79]. Analysis of cells isolated from the infarcted mouse heart revealed the presence of three fibroblast clusters appearing at day 3 post-MI. Cells from two of the three clusters were characterized by the relatively high expression of POSTN, Wisp1, and TNC, classically associated with fibroblast activation [80, 81], and were specifically present only after injury [82]. In addition to these known fibrosis markers, the gene cytoskeleton associated protein 4 (Ckap4) was found upregulated specifically in post-MI activated fibroblasts. Ckap4 is a trans-membrane protein and its function in CFs is unknown. Increased Ckap4 expression is specific for the stressed heart and overlaps with vimentin, a marker for CFs in the ischemic heart. Preliminary evidence showed that in activated fibroblast CKAP4 functions to decrease the expression of genes indeed related to activation, making this protein a possible new important modulator of this process [82].

CPC/MSC populations have also been investigated by omics approaches. A single-cell transcriptomic study of cardiac progenitors isolated through cardiospheres has showed that different functional subpopulations exist, which cooperate in heart muscle repair. In this population the expression of the Ly6a gene could evidence 30% of cells being $Sca1^{+}$ [83]. Cell-cell interactions can be mapped through the analysis of ligands and receptors expression by cell therapy donor and recipient cells, respectively, for a given signaling molecule [66]. Single cell transcriptomic data allowed the construction of a network reflecting the strength of ligand-receptor connections among CPC subpopulations. The number of connections from $Sca1^{+}$ to $Sca1^{-}$ cells was higher, specifically for angiogenesis related factors, suggesting that $Sca1^{+}$ CPCs behave as a signaling hub with pro-angiogenic signals. The cardioprotective function of CPCs has been also linked to GATA4 and β-catenin expression [84]. $Sca1^{+}$ cells show upregulated expression of GATA4 and downregulated expression of β-catenin. Finally, $Sca1^{+}$ cells show repair activity in infarcted hearts in vivo, a feature not shared with $Sca1^{-}$ cells, which however have a strong proliferative and angiogenic capacity in vitro [83]. The molecular phenotype of $Sca1^{+}$ resident CPCs was analyzed with a combined transcriptomic and proteomic approach. The data revealed that undifferentiated $Sca1^{+}$ cells express CD38 and CD105 surface markers, as well as others implicated in cell adhesion, such as Icam2, Ceacam1, CD36, CD93, and CD322 (**Table 1**; **Figure 2**). In addition, growth factors like KITL, JAG2, PDGF-β, and VEGFC showed higher expression in Sca1 progenitor cells [85], overall confirming the strong capacity of these cells to provide positive microenvironment cues for cardiac repair.

Targeting cardiac fibroblasts: a translational point of view

The detailed description of stromal cell functions and phenotypes in cardiac disease models might allow the design of targeted therapies against those cell types or functional states responsible to drive remodeling. In this view some therapeutic approaches are being developed to specifically interfere with possible detrimental actions of cardiac stromal cells in the injured cardiac muscle.

Pharmacological therapies

Renin-angiotensin-aldosterone system (RAAS) and TGF-β signaling are implicated in the activation of CFs (**Figure 1**) and the onset of cardiac fibrosis. Inhibition of these pathways using a pharmacological approach is of great interest for the treatment and prevention of cardiac fibrosis. The most studied anti-fibrotic drugs are RAAS inhibitors, which target angiotensin II thus reducing CF proliferation and collagen synthesis [86]. In the past years, several clinical studies have shown that RAAS inhibitors counteract cardiac fibrosis progression [87-89]. Recently, Garvin et al., using single-cell RNA sequencing, have demonstrated that transient ACE (angiotensin-converting enzyme) inhibitor

treatment in a spontaneously hypertensive rat model suppresses future fibrogenic capacity and heterogeneity of CF subpopulations [90]. However, inhibition of RAAS only modestly regresses cardiac fibrosis once consolidated, which persists in heart failure patients, indicating a need to develop novel antifibrotic therapies effective also at later time points of disease progression [91]. In this regard, TGF-β1 is another candidate target to treat cardiac fibrosis using a pharmacological approach. Inhibitors of TGF-β signaling have been extensively studied in animal models of fibrosis; however, translation of these findings into treatments for human cardiac diseases has been limited due to the broad range of responses to TGF-β1, and its role in tissue homeostasis. Nonetheless, a few TGF-β inhibitors are currently under clinical evaluation for the treatment of cardiac fibrosis [92].

In situ reprogramming

CFs have also been the target of the so called "direct reprogramming" strategy, which induces a partial dedifferentiation to a state plastic enough to allow the subsequent trans-differentiation into induced cardiomyocytes (iCMs), both in vitro and in situ. This could be an alternative therapeutic strategy to repopulate the myocardial scar with newly formed CMs after injury, thus reducing fibrosis in favor of regeneration. However, the optimal cocktail of transcription factors and/or microRNAs for CF-iCM reprogramming [93, 94] has yet to be identified, and new studies are required to accelerate the translation of these technologies to the clinic. Interestingly, the epigenetic profile of induced pluripotent cells generated from CFs appears to be more prone for differentiation towards the cardiac lineage, rather than cell types from heterotopic sources, suggesting that CFs could be a plentiful source of CMs for cell-based therapy or tissue engineering [95].

Specific targeting of CFs

Adverse remodeling might be counteracted by selective ablation of those specific cell populations (or functional states) mediating the fibrotic process (**Table 1**; **Figure 1**). Early evidence that this strategy might be useful to reverse fibrosis and ameliorate heart function after injury came from Kaur's study [96], where $POSTN^+$ activated fibroblasts were genetically ablated in animals engineered to express the diphtheria toxin receptor specifically in activated fibroblasts. In the setting of pressure overload and heart failure injury, the fibrotic burden was decreased without compromising scar stability, and cardiac function was improved by selective removal of pro-fibrotic $POSTN^+$ fibroblasts.

As previously mentioned, FAP expression also distinguishes activated fibroblasts and myofibroblasts from un-activated fibroblasts (**Table 1**; **Figure 1**). In fact, FAP is strongly expressed by CFs in response to acute myocardial infarction [97], thus representing a potential target to selectively hit pathological CFs. In this context, Epstein's group recently presented a very elegant experiment to demonstrate the efficacy of redirected chimeric antigen receptor (CAR)-T-cell immunotherapy to specifically target pathologic cardiac fibrosis. In detail, after CAR binding to FAP, CAR-T cells were able to cause cytotoxic killing of activated pro-fibrotic CFs, decreasing their number in the tissue. This specific ablation resulted in a significant reduction of cardiac fibrosis, and in the restoration of cardiac function in a mouse model of hypertensive cardiac injury and fibrosis [98]. Importantly, extensive analysis revealed no signs of toxicity in this model system, which is in agreement with previous mouse studies in which CAR-T cells against FAP have been used for cancer treatment [99].

Conclusions

The experimental evidence presented in this review on cardiac stromal cells highlights the plasticity and heterogeneity of this compartment, which had long been underestimated (**Figure 2**). Cardiac stromal cells act as a signaling hub, a support population for cardiomyocytes, and as a potent element of response to injury, which dramatically changes the muscular structure during heart failure progression. The availability of molecular and cellular markers, together with the new description of the stroma dynamics at single cell resolution, allows now a better description of the cellular mechanisms behind cardiac homeostasis and disease, and offers unprecedented information useful for the development of targeted therapies to counteract pathological myocardial remodeling.

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Disclosure of conflict of interest

None.

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