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REVIEW

Fibroblast-mediated intercellular crosstalk in the healthy and diseased heart

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Cardiac fibroblasts constitute a major cell population in the heart. They secrete extracellular matrix components and various other factors shaping the microenvironment of the heart. In silico analysis of intercellular communication based on single-cell RNA sequencing revealed that fibroblasts are the source of the majority of outgoing signals to other cell types. This observation suggests that fibroblasts play key roles in orchestrating cellular interactions that maintain organ homeostasis but that can also contribute to disease states. Here, we will review the current knowledge of fibroblast interactions in the healthy, diseased, and aging heart. We focus on the interactions that fibroblasts establish with other cells of the heart, specifically cardiomyocytes, endothelial cells and immune cells, and particularly those relying on paracrine, electrical, and exosomal communication modes.

Keywords: aging; cardiomyocytes; cardiovascular disease; electrical coupling; endothelial cells; exosomes; fibroblasts; heart; immune cells; paracrine signaling

Cardiac fibroblasts constitute a major cell population in the heart defined by their capacity to generate extracellular matrix (ECM). This cell type is crucial for the maintenance of the cardiac structure and the mechanical properties of the heart. Although, historically, the identification of a fibroblast population in heart has proven challenging, because lineage-specific markers for fibroblasts remains controversial [1], the epicardial transcription factor 21 (Tcf21) and the platelet-derived growth factor receptor alpha (PDGFRα) are commonly used to identify quiescent fibroblasts in the heart [2,3] (Fig. 1). Despite the lack of definitive markers, fibroblasts can be in general identified by the lack of basement membrane, presence of multiple elongated cellular processes, extensive rough endoplasmic reticulum, and abundant granular cytoplasmic material [4]. It is important to note that other cells in the heart, such as endothelial cells [5] or pericytes [6], can produce ECM and alterations observed in these cells may lead to cell states that can phenotypically and functionally overlap with fibroblasts; thus, exclusion criteria need to be carefully considered to properly define cardiac fibroblasts.

Initial studies estimated cardiac fibroblast number between 27% and 50% of total cells in mouse and rat ventricles, respectively [7,8]. Refinements like the optimization of tissue digestion protocols or the use of different cardiac fibroblast-specific mouse lines have allowed to revisit the cardiac tissue composition and establish that fibroblasts, resident adventitial and

Abbreviations
ECM, extracellular matrix; FGF, fibroblast-derived growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIMF, hypoxia-induced mitogenic factor; LIF, leukemia inhibitory factor; MHC, major histocompatibility complex; MIF, macrophage migration inhibitory factor; MMPs, metalloproteases; PRRs, pattern recognition receptors.

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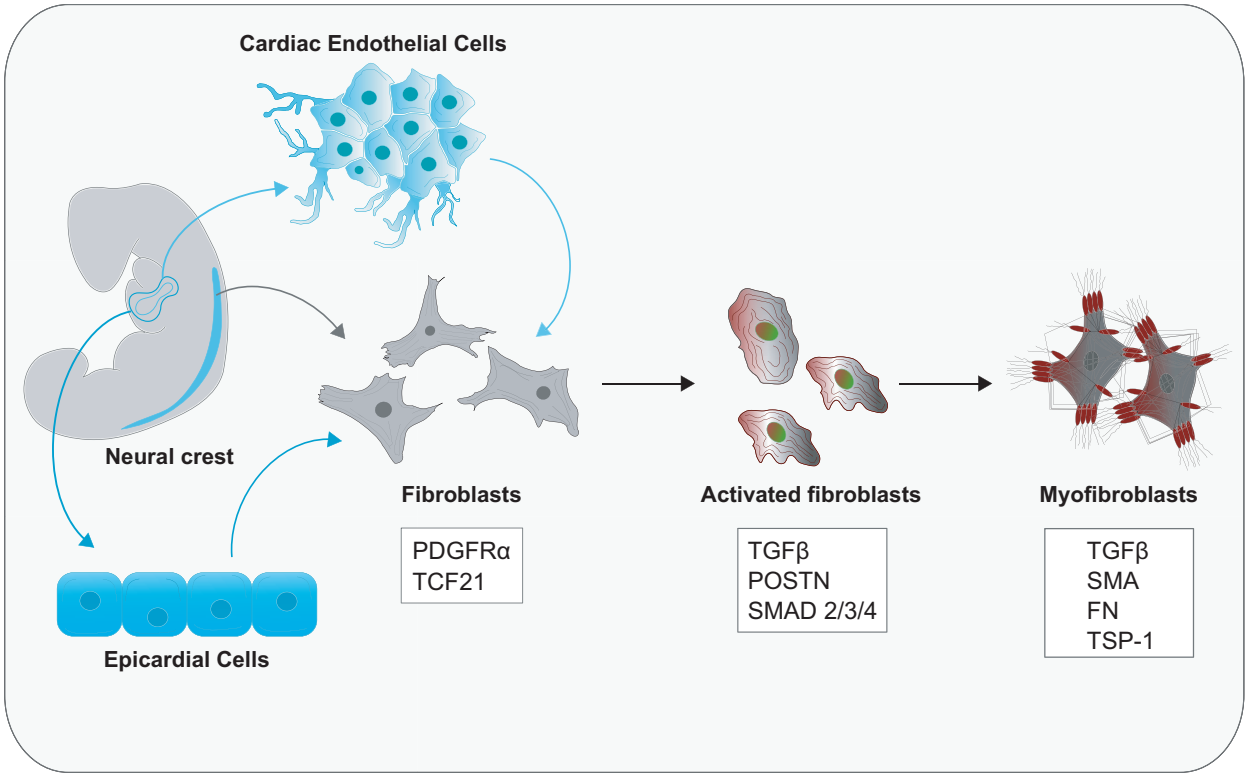


Fig. 1. Origin and diversity of cardiac fibroblasts. During embryonic development, cardiac fibroblasts (identified by the expression of PDGFRα, TCF21 [2,3]) arise from cardiac endothelial cells, the epicardium, and the neural crest [2,11,12,19]. Upon stimulation by inflammatory or profibrotic cytokines (e.g., TGFβ), cardiac fibroblasts transit to an activated state, which can be identified using marker genes and regulators such as TGFβ, POSTN, SMAD 2/3/4 [24,25,27–29], and can further differentiate to myofibroblasts that show a strong contractile and matrix-secretory phenotype [30].

interstitial, contribute to approximately 10% of all cells [9]. Nevertheless, the study of fibroblast biology remains crucial for the correct understanding of cardiac health, disease, and aging.

Cardiac fibroblasts are of heterogeneous origin, arising from different sources in the heart including the epicardium, the neural crest, and the endothelium (Fig. 1). Nonetheless, it has been reported that this developmental heterogeneity does not predict different pathological responses of cardiac fibroblasts [10]. Several reports have shown that cardiac fibroblasts originate from the epicardium, the mesothelium that covers the heart [11,12]. In the mouse, cellular progenitors of fibroblasts have been described to invade the myocardium from the epicardium around embryonic day 13.5 [13,14] and are characterized by the expression of Tcf21 [15], Wt1 [16], and Tbx18 [17]. It is important to note that the epicardium can give rise to different cell types in the heart [11,17,18] and that the heterogeneous gene expression levels of the above markers has been linked to the specification into different lineages. In particular, the expression of Tcf21 in a subset of epicardial cells is crucial for their differentiation into fibroblasts [14].

The neural crest is comprised of an heterogeneous population of migratory cells that originate from the dorsal part of the neural tube and give rise to a variety of cell types in different organs [19], including cardiac fibroblasts [20]. The neural crest plays a crucial role in the development of the outflow region of the heart [21], giving rise to valve mesenchyme. Neural crest-derived fibroblasts, identified using the *Pax3-Cre* transgenic model [22], have been detected within the myocardium, predominately in the right atrium [10].

Finally, the embryonic endothelium constitutes a third source of cardiac fibroblasts [2] and gives rise to 10–20% of the fibroblasts resident in the heart, in particular those located in the ventricular septum and right ventricle [10,23].

Cardiac fibroblasts are a plastic cell population capable of activation and differentiation after injury to the heart, being this ischemic, mechanical, or

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inflammatory (Fig. 1). Activation results in increased ECM deposition and leads to fibrosis. Lineage tracing, and later single-cell RNA sequencing have revealed that activated fibroblasts expresses high levels of Periostin (Postn) [24,25]. Furthermore, targeted ablation of Periostin in activated fibroblasts resulted in reduced fibrosis, although it induced localized cardiomyocyte hypertrophy [25]. Activated fibroblasts express several matrix metalloproteases (MMPs) that degrade the ECM and mediate the activation of TGFβ, thus paving the way for pathological fibrosis. The cytokine TGFβ, the best characterized fibrogenic growth factor, plays a crucial role in fibroblast activation [26]. It induces the phosphorylation of SMAD2 and SMAD3, which subsequently translocate to the nucleus together with SMAD4 to promote the expression of fibroblasts activation-specific target genes [27–29].

After activation, cardiac fibroblasts additionally transdifferentiate into myofibroblasts in a step-wise way with intermediate phenotypes [30]. The appearance of myofibroblasts is a hallmark of the cardiac fibrotic response [31]. Myofibroblasts are characterized by the expression of alpha smooth muscle actin (αSMA) in stress fibers and are able to exert contractile force [25,32]. Furthermore, myofibroblasts change their ECM profile expressing molecules like Fibronectin (FN) [33] or Thrombospondin-1 (TSP-1) [27]. This transdifferentiation can work in both directions, as myofibroblasts can differentiate back to quiescent fibroblasts once the fibrotic scar has been stabilized [34]. After injury, myofibroblasts migrate to the site of injury in response to the secretion of different growth factors and hormones, such as TGFβ [35], PDGF [36], Angiotensin II [37], Aldosterone [38,39] or Endothelin-1 [40], and others, where they orchestrate pathological remodeling [32,41].

Several nonfibroblast cells have been reported to give rise to activated fibroblasts or myofibroblasts upon cardiac injury. Bone marrow-derived cells, perivascular cells or endothelial cells have all been reported to be the primary source of newly activated fibroblasts and myofibroblasts in the heart after injury. However, these findings remain controversial as recent reports using highly refined genetic markers have not confirmed these results (reviewed in [42]). Nonetheless, single-cell RNA sequencing has recently shown that endothelial cells transiently acquire mesenchymal fibroblast-like features after myocardial infarct [43].

Fibroblasts have a high capacity to modulate and affect the environment that surrounds them. Below, we will explore the interaction of fibroblasts with the other cells in the heart.

Communication between fibroblasts and cardiomyocytes

Cardiomyocytes are the cardiac-specific contractile units, accounting for 65% to 80% of the volume of the adult mammalian heart [44,45]. In the healthy heart, cardiomyocytes are organized in layers of aligned cells in a complex anisotropic structure, with distinct mechanical and biochemical interactions guiding cardiomyocyte organization and homeostasis [46–48]. The communication between cardiomyocytes and other cardiac cell types, especially cardiac fibroblasts, plays a crucial role in regulating cardiomyocyte function in particularly upon acute or chronic stress conditions. Interestingly, transcriptomic profiling has revealed more similarities between cardiac fibroblasts and cardiomyocytes than between other cell types [49]. Furthermore, specific age- and disease-dependent signatures occur with a similar kinetic in cardiac fibroblasts and cardiomyocytes, suggesting an interrelated stress response and possibly close communication between these two cell types [50]. The interactions between cardiac fibroblasts and cardiomyocytes involve paracrine signaling [51] (Fig. 2A), mechanical stimuli [52] (Fig. 2B), electrotonic coupling via gap junctions [48,53] (Fig. 2C), and exosome-mediated crosstalk [54] (Fig. 2D).

Fibroblast-cardiomyocyte cell communication in the diseased heart

Cytokines, hormones, and growth factors

Paracrine signaling was investigated as a common route of communication between cardiac fibroblasts and cardiomyocytes in the heart, allowing for indirect crosstalk without direct cell to cell contacts [55,56]. Several paracrine signaling pathways impact both, cardiac fibroblasts activation to myofibroblast triggering fibrosis, and cardiomyocyte function. Angiotensin II (Ang II) and the cytokine TGFβ were among the first paracrine effector identified in the context of cardiac fibrosis. cardiac fibroblasts are a major source of the hormone Ang II [57], which has an important role in ventricular remodeling after cardiac injury [58,59]. Ang II also has more direct arrhythmogenic actions by inducing cardiomyocyte swelling [60], which is known to impair the gap junction permeability in adult rat ventricular cardiomyocytes [61]. Inhibition of Ang II generation or downstream interference with the Ang II receptors prevents cardiac hypertrophy and interstitial fibrosis and normalizes intercellular communication under disease states [62,63]. TGFβ is induced under

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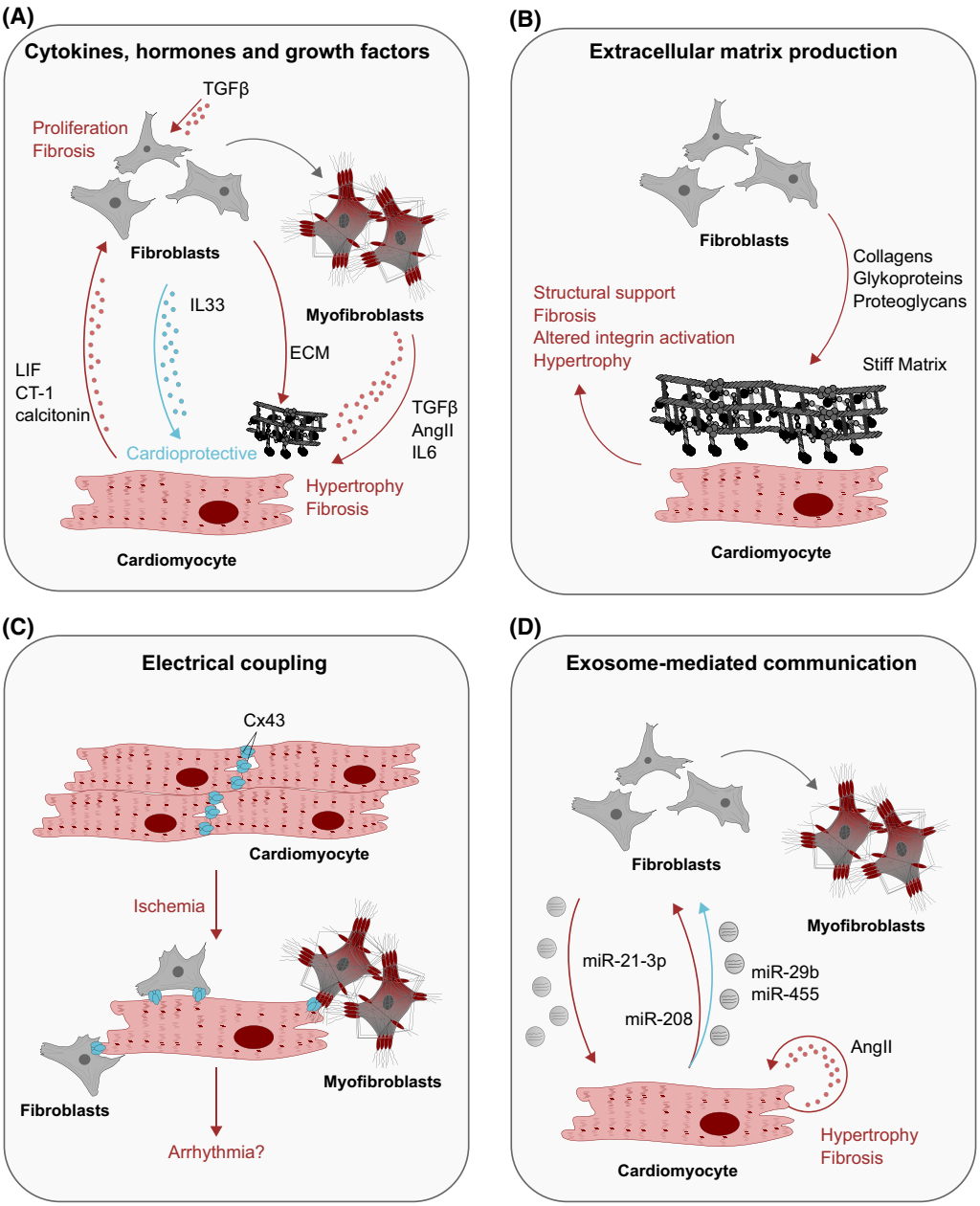


Fig. 2. Fibroblast–cardiomyocyte crosstalk in diseased and aged heart. (A) Crosstalk by paracrine signals and cytokines. Upon TGFβ-stimulation cardiac fibroblasts differentiate to myofibroblasts, which release paracrine factors such as TGFβ, AngII, and IL6 that induce cardiomyocyte hypertrophy and cardiac fibrosis [51,57,67,68]. Fibroblast-derived factors might also be cardiac protective, as IL33 is attenuating hypertrophy in cardiomyocytes [71]. Cardiomyocytes can also signal back to fibroblasts by releasing LIF, CT-1, and calcitonin, which stimulates fibroblast proliferation and matrix protein production [75–77]. (B) Crosstalk via extracellular matrix components. Upon injury, cardiac fibroblasts increase the secretion of matrix components, such as collagens, proteoglycans, and other glycoproteins. The increase in matrix components grants cardiac stability but also makes the extracellular environment more rigid, which alters cardiomyocyte integrin-signaling and mechanosensing [85]. (C) Crosstalk via electric coupling. Cardiomyocytes are electrically coupled via gap junctions (Connexin 43, Cx43) that reside within the intercalated disks [91,92]. Under ischemic conditions, Cx43 might translocate from the intercalated disks at the polar ends of the cell to the lateral surfaces, therefore increasing the likelihood of cardiomyocyte to fibroblast/myofibroblast coupling associated with the risk of arrhythmia [93]. (D) Exosome-mediated communication. Fibroblasts can release miR-21-3p-laden exosomes. Cardiomyocytes take up such exosomes and through the action of miR-21-3p AngII is released thereby leading to cardiomyocyte hypertrophy and fibrosis [99]. Cardiomyocytes can signal back to fibroblasts and exert both fibrotic and protective effects. By releasing miR-208-carrying exosomes, cardiomyocytes can stimulate the fibroblast-to-myofibroblast transition [101]. In turn, miR-29b and miR-455-laden exosomes may attenuate fibrosis [103].

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diseased conditions in fibroblasts and other cardiac cell types and acts as the main driver for myofibroblast activation [64,65]. TGFβ-dependent activation of cardiac fibroblasts to myofibroblasts induces cardiac fibrosis, cardiomyocyte hypertrophy while reducing action potentials and conductivity in neighboring cardiomyocytes [51].

Moreover, pro-inflammatory cytokines, especially interleukin-6 (IL-6), are important mediators in the communication process. IL-6 is a pleiotropic cytokine that has distinct biological functions in the heart [66], with crucial contributions to aging- and disease-dependent processes including cardiomyocyte hypertrophy and fibrosis [67,68]. Enhanced secretion of IL-6 by cardiac fibroblasts effects multiple crosstalk pathways by inducing myofibroblast proliferation and differentiation, cardiomyocyte apoptosis, and angiogenesis of endothelial cells [69]. Recently, Kumar *et al.* reported that the hypoxia-induced mitogenic factor (HIMF) acts as an upstream regulator of IL-6 in cardiomyocyte to cardiac fibroblast paracrine crosstalk [70].

Other fibroblast-derived cytokines may also elicit beneficial effects. Thus, interleukin 33 (IL-33), which is produced by fibroblasts inhibits cardiomyocyte hypertrophy and fibrosis after pressure overload, suggesting that IL-33 may function as a paracrine signal produced by fibroblasts to modulate cardiomyocyte responses to hypertrophic stimuli [71]. Nevertheless, multiple other cardiac cell types can contribute to the secretion of the paracrine factors as discussed above in a complex network of heterocellular crosstalk.

Wnt signaling plays a major role in cardiac fibroblast–cardiomyocyte crosstalk. Inhibition of Wnt signaling globally after myocardial infarction *in vivo* reduced fibrosis, ameliorated cardiomyocyte recovery, and improved cardiac function [72,73]. However, cardiomyocyte-specific blockade of Wnt signaling induced a contradictory response, by increasing fibrosis and impairing cardiomyocyte recovery after myocardial infarction [74]. Taken together, these observations demonstrate that Wnt signaling is a major regulator of cardiac fibroblast–cardiomyocyte communication, with distinct responses in global versus cardiomyocyte-specific inhibition.

In addition, cytokines and hormones can also be produced by cardiomyocytes and signal to fibroblasts. For example, leukemia inhibitory factor (LIF) and cardiotrophin-1 (CT-1) can regulate fibroblast proliferation and cardiomyocyte hypertrophy likely amplifying the injury response. LIF induces hypertrophy in cardiomyocytes while inhibiting myofibroblast activation and collagen deposition *in vitro* [75]. CT-1 enhances cardiomyocyte hypertrophy but promotes fibroblast proliferation [76]. In addition, atrial cardiomyocytes produce the hormone calcitonin that induces neighboring collagen-producing cardiac fibroblasts to stimulate proliferation and further secretion of ECM proteins [77]. Interestingly, this study demonstrated that human patients with atrial fibrillation showed increased levels of myocardial calcitonin compared to healthy individuals, suggesting that this paracrine crosstalk mechanism could have therapeutic implications [77].

Extracellular matrix

The mechanical properties of the cardiac tissue environment are mainly regulated by the ECM, which surrounds individual cardiomyocytes and provides structural support for cardiomyocyte organization within the myocardium [78]. With cardiac fibroblasts regulating stiffness of the cardiac microenvironment, they also regulate the mechanical stimuli on the neighboring cardiomyocytes. Two distinct models can be used to describe the cardiomyocyte mechanosensing machinery: The localized model suggests stretch signals near the plasma membrane, whereas the decentralized model considers force generation at the cell surface, which is then transmitted to other parts of the cell [79]. In addition to structural support, physical ECM–cardiomyocyte interactions have been shown to provide a strong mechanical link from the intracellular contractile apparatus to the surrounding ECM, an interaction established by the dystrophin glycoprotein complex [80]. The transmembrane protein dystroglycan connects the cytoskeleton of the cardiomyocytes to the ECM, contributing to cardiac homeostasis [81]. Apart from electrical conductivity, various studies demonstrated that culturing cardiac fibroblasts or cardiomyocytes on stiffer substrates results in increased expression of cell-cell contact proteins. Pulsatile stretch induces an upregulation of adhesion proteins, such as N-cadherin, in both cardiac fibroblasts and cardiomyocytes [82,83].

Fibroblast-derived extracellular matrix proteins may also control cardiomyocyte responses to injury resulting in reparative or regenerative processes [84]. Interestingly, embryonic fibroblasts express a pattern of extracellular matrix proteins that induce cardiomyocyte proliferation by activating β1-integrin signaling [85]. This activity was lost in adult heart-derived fibroblasts, suggesting that changes in the fibroblast-cardiomyocyte communication may contribute to the loss of regenerative capacities in the adult heart. Furthermore, in a model of pressure overload, activated cardiac fibroblast exhibit a protective role by preserving the ECM network, thereby blocking inflammation and decreasing cardiomyocyte injury,

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by Smad-mediated pathway that suppresses matrix-degrading proteins [86].

Electrical coupling

In addition to their role as main drivers of fibrotic processes by exacerbated collagen deposition and myocardial stiffening, cardiac fibroblast can couple through gap junctions with each other and with cardiomyocyte, maintaining the scar regions electrically conductive [87]. Although activated myofibroblasts can have a positive impact on conduction across disarranged cardiomyocyte networks [88], computer-modeling and experimental studies demonstrated their detrimental effects on cardiac function by directly depolarizing cardiomyocyte, decreasing action potential conduction and promoting ectopic electrical activity [89,90]. Gap junctions are the main mechanism of electrotonic coupling in the heart, with connexin 43 (Cx43) being the most prominent gap junction protein expressed in both cardiomyocytes and cardiac fibroblasts [91,92]. During ischemic conditions, cardiomyocytes undergo structural remodeling, with Cx43 translocating from the intercalated disks at the polar ends of the cell to the lateral surfaces, therefore increasing the likelihood of cardiomyocyte to myofibroblast coupling associated with the risk of arrhythmia [93]. Increased expression of Cx43 in cardiac fibroblasts after cardiac injury was associated with augmented coupling with neighboring cardiomyocytes [94]. In contrast, cardiomyocytes from injured hearts had reduced Cx43 levels, which has been shown to result in slowed conduction velocity and thereby contributing to the risk for arrhythmias [95]. This study was supported by *ex vivo* experiments where isolated myofibroblasts from infarcted hearts cocultured with cardiomyocytes decreased the conduction velocities and duration of the action potential [55]. The differential regulation of Cx43 expression in cardiac fibroblasts and cardiomyocytes was proposed to be mediated via the activation of β-adrenergic receptors upon cardiac injury, which results in reduced Cx43 expression in cardiomyocytes, but increased expression in fibroblasts [96]. Such interaction can be studied in heterocellular cardiac tissue models allowing to quantify electric interactions between fibroblasts and cardiomyocytes *in vitro* by cell type-specific optogenetic manipulation of membrane potential [97]. Funken *et al.* demonstrated that under basal conditions, fibroblast membrane potential alterations had minor effects on cardiomyocytes. However, after TGFβ1 stimulation and differentiation of fibroblasts toward myofibroblasts, fibroblast-specific depolarization or hyperpolarization directly modulated cardiomyocyte membrane potential and accelerated or blocked spontaneous beating, respectively. Together, multiple studies have demonstrated the role of electrical coupling of activated myofibroblasts and cardiomyocyte in aging and disease [98], but the extent of cardiac fibroblast–cardiomyocyte interactions in the healthy heart and the role they play in the maintenance of electrophysiological conduction remains to be elucidated.

Exosomes

Finally, exosomes are well recognized to play essential roles in mediating intercellular crosstalk. They transport molecules that regulate the physiological and pathophysiological processes of the recipient cells [54]. Analysis of the exosome content of cardiac fibroblast by RNA sequencing showed a high abundance of the 3′-p passenger 'star' strand of the profibrotic microRNA miR-21 in exosomes of Ang II-treated cardiac fibroblast. miR-21-3p containing exosomes were shown to be taken up by cardiomyocytes via a endocytosis-dependent manner and augments cardiomyocyte miR-21-3p expression, which reduces the expression of cardiomyocyte-specific structural proteins, potentially contributing to cardiomyocyte hypertrophy [99]. Additionally, other reports confirmed exosome-mediated interaction and show that cardiac fibroblast-derived exosomes augment Ang II production in cardiomyocytes resulting in the induction of cardiac pathological hypertrophy [100]. On the other hand, cardiomyocyte-derived exosomes also can control the function of cardiac fibroblast, as demonstrated by the induction of fibrosis and myofibroblast activation via cardiomyocyte-derived, miR-208 loaded exosomes [101]. However, cardiomyocyte-derived exosomes may also exhibit anti-fibrogenic roles by attenuating fibrosis and activating angiogenesis via Hsp20 [102], miR-29b, and miR-455 [103]. Further studies are needed to identify the specific mechanisms of crosstalk between cardiac fibroblasts and cardiomyocytes via miRNA-loaded exosomes, especially in homeostasis versus disease conditions [54].

Fibroblast–cardiomyocyte communication in the aged heart

Fibroblasts contribute to age-associated alterations in the heart, and cardiac fibrosis is a hallmark of the aging heart. It is not entirely clear whether fibroblasts are the primary triggers of aging or if they are involved secondary to senescence-associated changes in the cardiac microenvironment. Fibroblasts are activated during aging and showed the most profound change in gene expression as compared to other cell types of the aging mouse heart [104]. Aged fibroblasts are characterized by changed expression patterns of inflammatory, extracellular matrix organization angiogenesis, and osteogenic genes [104].

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This affects the microenvironment and possibly may contribute to increased substrate stiffness. Since increased substrate stiffness triggers contractile dysfunction associated with telomere shortening one may speculate that changes in the extracellular matrix environment by aged heart fibroblasts may support a phenotype of accelerated aging [105]. Moreover, studies demonstrated that aging of the heart is accompanied by disturbed expression patterns and distribution of Cx43, with age-dependent decrease of Cx43 [106], which may be linked to increased risk for arrhythmic events [107].

Communication between fibroblasts and endothelial cells

Fibroblasts are closely interacting with endothelial cells in the heart [108]. Endothelial cells form the inner surface of the cardiac chambers and line the entire macro- and microcirculatory system that supplies the heart with blood. The vasculature thereby ensures tissue oxygenation and supply of nutrients. In addition, endothelial cell-derived paracrine signals, so-called 'angiocrine mediators', contribute to the vascular niche, which control organ homeostasis, repair, and regeneration in various organs such as liver, lung, and bones [109]. However, less is known whether and how such angiocrine mediators control cardiac disease and aging and to what extend cardiac fibroblasts are involved in maintaining or disturbing the vascular niche.

By controlling the extracellular matrix and by providing growth factors, fibroblasts play a critical role in the growth and stabilization of capillaries. Most prominently, fibroblast-derived growth factors (FGF) are well-known pro-angiogenic factors. While the effect of other mesenchymal cells, such as mesenchymal stromal cells, have been extensively studied and various studies demonstrated that they can be used as cell therapeutic strategy to provide pro-angiogenic factors, matrix proteins, and exosomes [110–112], the specific interactions of intrinsic cardiac fibroblasts with endothelial cells in the tissue has not been deeply explored. Interestingly, in silico prediction of cellular communication based on single-nuclei-RNA-sequencing data sets of healthy hearts revealed that endothelial cells show a high number of incoming signals arising from fibroblasts both in the healthy as well as in the diseased and aging heart [104].

Fibroblast-endothelial cell communication in the diseased heart

Particularly after myocardial injury or stress, myofibroblasts are the most ligand providing cell type to signal to endothelial cells [113]. Especially, a distinct type of activated fibroblasts that expressed Wnt inhibitory factors (WntX-fibroblast) was detected in the border zone at day 3 postinfarction and was proposed to interfere with cardiac endothelial cells. This 'WntX-fibroblast' population expressed paracrine factors, such as Wif1, Timp3, Ptn, Mdk, Apoe, Fbln1, Igf1, and Rspo3, which were corresponding to endothelial-expressed receptors [113]. All these factors are known to regulate angiogenesis [114–117], which suggests a potential role of the subpopulation of WntX-fibroblasts in regulating the revascularization of the border zone upon infarction. While most of the expressed genes are pro-angiogenic, some inhibit vessel growth and may also have a negative impact on the heart. For example, Wif1 inhibits tumor angiogenesis via interfering with Wnt and Vegf pathways [118,119] (Fig. 3A). Especially, after myocardial infarction *Wif1* expression was found to be induced in cardiomyocytes, but not in fibroblasts or endothelial cells and its deletion was reported to further induce abnormal chamber remodeling upon myocardial infarction in mice [120]. By contrast, cardiomyocytes-specific *Wif1* induction rather caused dilated cardiomyopathy *in vivo* [121], indicating the need of a well-balanced *Wif1* expression to positively contribute to cardiac repair. However, to what extent the newly identified WntX-fibroblast population plays a role in cardiac remodeling, is still debatable. In advanced postinfarct cardiac remodeling, myofibroblasts further contribute to the fibroblast-endothelial crosstalk by expressing matrix proteins like Postn, Fn1, and Col8a1 (Fig. 3A). These factors can interact on endothelial cell expressed receptors and might thereby regulate endothelial cell adhesion and angiogenesis [113]. Of note, such bioinformatical approaches ignore anatomical and spatial information, hence immunofluorescence detections were used to confirm the close proximity between *Pdgfra*+ fibroblasts and *CD31*+ endothelial cells [113]. Despite the histological proximity, robust experimental approaches proofing that the specific fibroblast subpopulations and fibroblast-derived factors control endothelial cell phenotypes and functions are still lacking. However, first *in vitro* approaches revealed that fibroblast not only secrete paracrine factors, but also release microRNA-loaded exosomes to communicate with endothelial cells. In a profibrotic setting, it was shown that mouse cardiac endothelial cell function is impaired when cultured with exosomes that were isolated from TGFβ-pretreated fibroblasts [122]. These findings are of potential interest since the up-take of fibroblast-derived miR in endothelial cells might change their phenotype toward mesenchymal cells.

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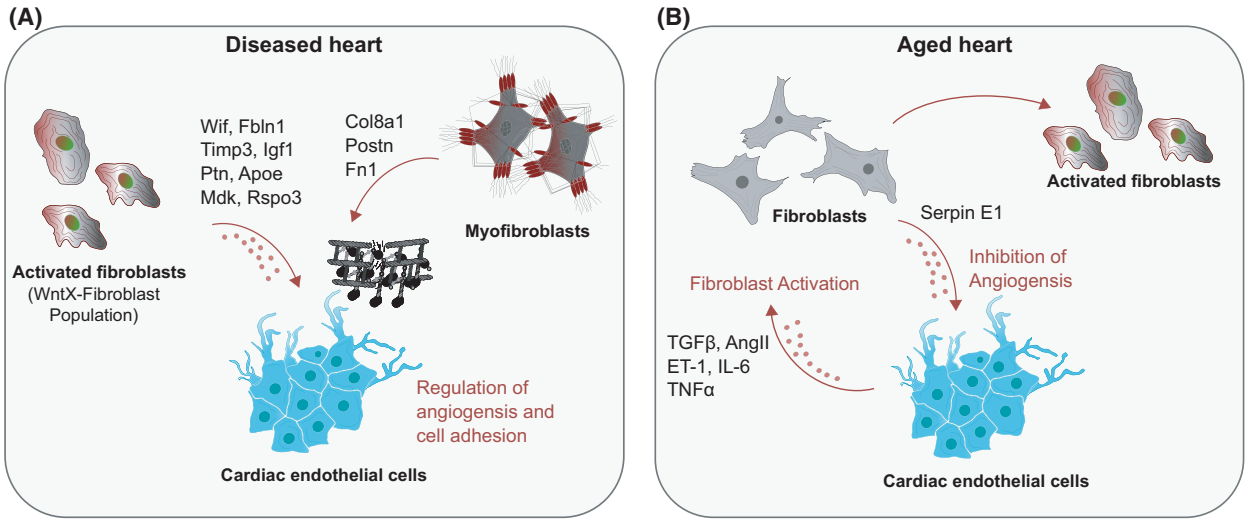


Fig. 3. Fibroblast-endothelial cell crosstalk in diseased and aged heart. (A) In the diseased heart, a Wnt-expressing subpopulation of activated fibroblasts (WntX-Fibroblast) releases paracrine factors, such as Wif1, Timp3, Ptn, Mdk, Apoe, Fbln1, Igf1, and Rspo3, that control angiogenesis [113]. In addition, myofibroblasts release matrix proteins that further promote angiogenesis and endothelial cell adhesion [113]. (B) In the aging heart, endothelial cells secrete pro-inflammatory and profibrotic factors (ET-1, angiotensin II, TGFβ, TNFα, and IL-6) that provide a stimulatory environment for fibroblast activation [126–128]. Cardiac fibroblasts also signal back to endothelial cells. By secreting Serpin E1, they control angiogenesis in the aging heart [104].

Fibroblast–endothelial cell communication in the aged heart

Similar to pathophysiological states, cardiac aging is also associated with fibroblast activation and fibrosis [123], as well as vascular remodeling [124]. Aged heart-derived endothelial cells were found to switch their basement membrane from a laminin β2 to a laminin β1 rich matrix in both cardiac aging and disease. *In vitro* studies show that this change in matrix proteins has an autocrine influence on endothelial cells and control cell adhesion, autophagy, and inflammation [125]. Since laminins are also critical regulators of fibroblasts (at least in the lung), one may consider also an interaction with laminin β1 and β2 on fibroblasts. Advanced age and cardiac disease are additionally associated with an increased expression of endothelial-derived pro-inflammatory factors, such as ET-1 [126], Angiotensin II [127], TGFβ, TNFα and IL-6 [128] (Fig. 3B). These factors can create a profibrotic environment, stimulating fibroblast activation and hence vascular fibrosis, although fibroblasts show an age-related decline in their response to growth factors such as TGFβ and EGF [129,130] (Fig. 3B).

Recent studies confirm bioinformatic predictions, suggesting that fibroblasts signal back to endothelial cells in the aging heart: aged heart fibroblast express angiogenesis-controlling genes including Serpin E1 (also known as plasminogen activator inhibitor-1 (PAI-1)) (Fig. 3B). Supernatant of fibroblasts isolated from aged hearts, which were enriched in Serpin E1, impaired endothelial cells *in vitro* [104]. Since Serpin E1 was identified to be a senescence-associated secretory factor [131,132], a potential role for senescent fibroblasts in mediating endothelial impairment in the aging heart should be additionally considered in this context. However, one should note that fibroblast senescence may also beneficially affect injury response in the young mouse heart since it limits the expansion of profibrotic fibroblasts [133]. In addition, in neonatal mice, fibroblast senescence was reported to rather support heart regeneration upon myocardial infarction, as fibroblast proliferation and thereby fibrosis were reduced [134]. Therefore, targeting fibroblast senescence as therapeutic strategy may be considered with caution.

Communication between fibroblasts and immune cells

Under homeostatic conditions, nonmyocytes in the heart mostly exhibit quiescent phenotypes, but during exposure to stress by aging or disease, a variety of cell types change their phenotype and contribute to remodeling processes. Here, various distinct immune cell types, such as T lymphocytes, monocytes, and macrophages, infiltrate into the myocardium and have diverse spatially and temporally regulated functions

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[135,136]. Recently published single-cell RNA-sequencing data of the human heart demonstrated that immune cells constitute between 5% and 10% of all cardiac cell types, revealing various immune cell states with distinct clusters [44]. In the same study, bioinformatically predicted cell-cell interactions identified specific crosstalk patterns between cardiac fibroblasts and immune cells. Here, monocyte-derived and antigen-producing macrophages were predicted to crosstalk with cardiac fibroblasts via CD74-MIF, an interaction contributing to fibrosis if inhibited [137] (Fig. 4A). Additionally, a recently published bioinformatic cell-cell communication study of the heart showed that cardiac M1 macrophages and myofibroblasts exhibited a large number of outgoing communication signals [113]. Other cardiac fibroblasts–immune cell interactions demonstrated that upon cardiac injury myofibroblasts secrete the factor GM-CSF instructing resident macrophages to recruit other inflammatory cells and triggering myocardial inflammation [138,139] (Fig. 4B). Moreover, cardiac fibroblasts modulate inflammatory processes at various levels by interfering with chemotaxis, infiltration, and migration of inflammatory cells. In recently conducted studies, cardiac fibroblasts have been demonstrated to be directly involved in responding to damage by pattern recognition receptors (PRRs), which in turn causes a feed-forward inflammatory response via NF-κB, MAPK8, and p38 stress signaling pathways triggering the release of pro-inflammatory cytokines [140,141] (Fig. 4B).

Additionally, the crosstalk between cardiac fibroblasts and immune cells was shown to occur in bidirectional fashion, with cardiac infiltrating Ly6C^{hi} macrophages mediating the overexpression of profibrotic genes, stimulating cardiac fibroblast proliferation and collagen production [142] (Fig. 4C). Macrophages also communicate via the release of exosomes containing miRNAs, specifically miR-155, which regulates inflammation and cardiac injury upon myocardial infarction by transfer of miR-155 to cardiac fibroblasts via macrophage derived exosomes [143]. Another miRNA, miR-21, has recently been shown to modulate macrophage–cardiac fibroblast crosstalk by regulating cardiac macrophages in their paracrine, profibrotic secretome toward cardiac fibroblasts, controlling cardiac remodeling, and function [144]. Recent studies highlight the different roles of tissue-resident macrophages versus circulating bone marrow-derived monocytes. Dick *et al.* [145] reported that within six months, 80% of the resident CCR2+ macrophages and about 25% of the CCR2-MHC-II^{hi} macrophages were replaced by cells derived from circulating monocytes, whereas CCR2-MHC-II^{lo} showed little replacement. It would be interesting to explore how these different populations interact with fibroblast after cardiac injury.

Moreover, neutrophils are among the first immune cells infiltrating into the damaged myocardium. Activated neutrophils release oxygen species (ROS) generated via the NADPH oxidase, exerting profibrotic processes in the myocardial environment [146,147] (Fig. 4C). Although it is challenging to exclude confounding effects of ROS released by other cardiac cell types, it has been shown that NADPH oxidase 2 (Nox2)-deficient mice show an attenuated fibrotic response under pressure overload, also confirmed by reduction of collagen and MMP-2 production [148]. Neutrophils also communicate via the release of granules containing myeloperoxidase, MMP, elastase, cathepsins, and others. These granules can degrade connective tissue released by activated cardiac fibroblasts during fibrosis [149]. Several other immune cells, for example, eosinophils, modulate cardiac function upon injury. After myocardial infarction, eosinophil counts within the heart are increased in the infarct region, and they exhibit an important cardioprotective function in protecting cardiomyocytes from apoptosis and blocking cardiac fibrosis by inhibiting TGF-β-induced cardiac fibroblast activation [150] (Fig. 4C). The trafficking of eosinophils to the heart was also investigated in myocarditis, showing that high levels of eotaxin (CCL11) in fibroblasts attracts eosinophils, which expressing the eotaxin receptor CCR3 [151]. Here, fibroblasts are the main source of CCL11 in the heart, underlining the central role of cardiac fibroblasts in regulating cardiac inflammation via paracrine signaling [151]. Together, these studies demonstrate a close bidirectional crosstalk between immune cells infiltration and myofibroblast activation.

Summary and outlook

While fibroblasts have been historically mainly viewed as collagen-producing cells that induce cardiac fibrosis, technological improvements allowing for a detailed analysis of cell types and subsets of cells are giving novel insights into the crosstalk between the cells in the heart. In response to injury, stress or aging, fibroblasts become activated and change the microenvironment around them by secreting ECM, cytokines, growth factors, and other mediators of intercellular communication such as exosomes. These changes in the environment critically contribute to cardiovascular disease and dysfunction. Learning more about these communication pathways, but more specifically investigating subsets and states of fibroblasts, may lead to

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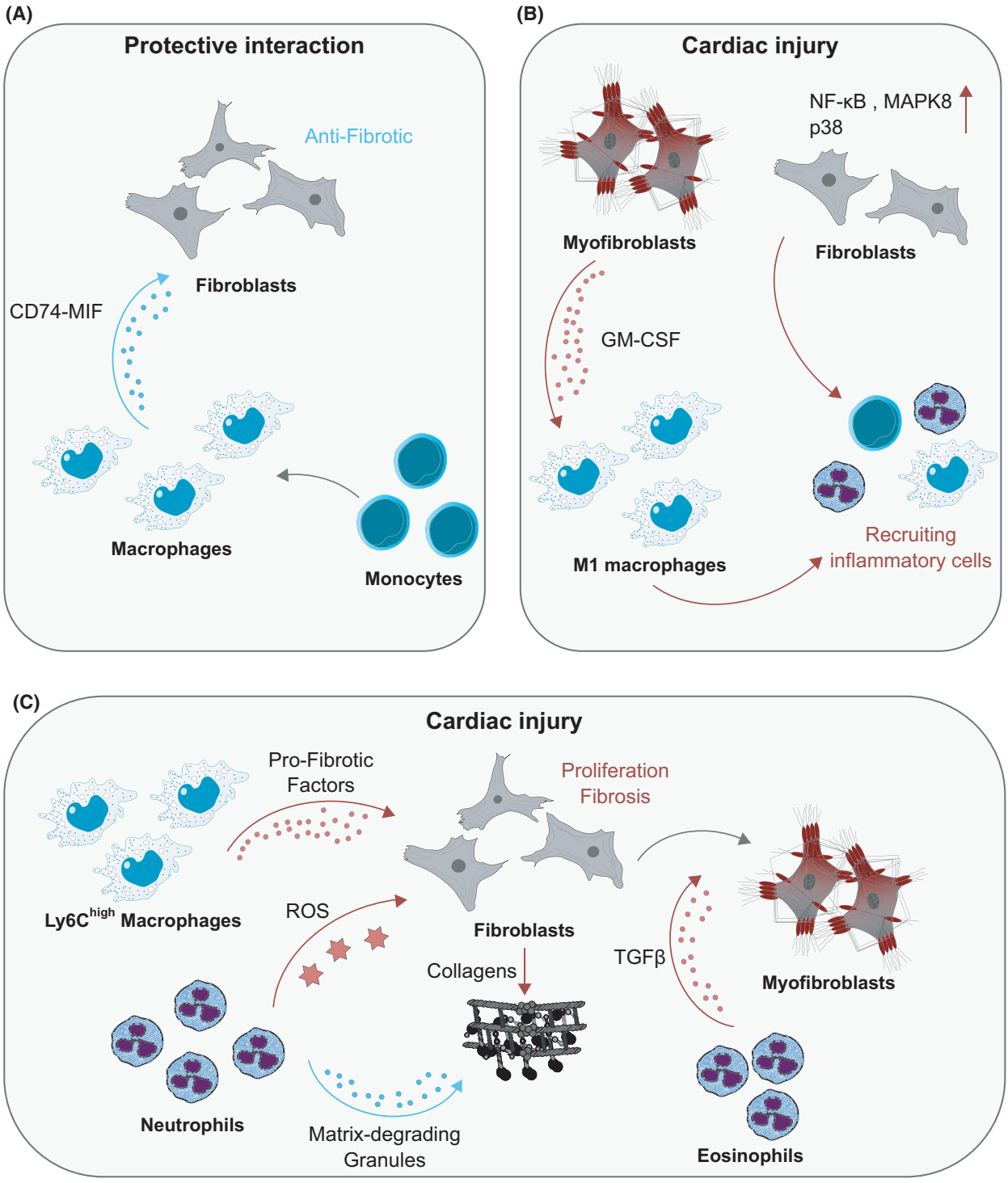


Fig. 4. Fibroblast–immune cell crosstalk in diseased and aged heart. (A) Protective interaction. Monocyte-derived macrophages release CD74-MIF and thereby attenuate fibroblast activation [137]. (B) In the diseased heart, myofibroblasts secrete GM-CSF, which stimulates macrophages to recruit inflammatory cells [138,139]. In addition, NF-κB, MAPK8, and p38 activation induce fibroblasts to recruit further inflammatory cells [140,141]. (C) Ly6C^{high} macrophages release profibrotic factors that activate cardiac fibroblasts and drive their conversion into myofibroblasts [142]. Neutrophil-derived ROS and eosinophil-derived TGFβ further activate fibroblast-to-myofibroblast differentiation [146,147]. Neutrophils can also act in an antifibrotic manner by releasing granules that contain matrix metalloproteases to degrade excessive collagen deposits [149].

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the identification of novel possible interventional strategies to combat cardiovascular disease. Searching for secreted factors, for example, by using secretome mouse models allowing cell type-specific tracking of released proteins [152], may also lead to the identification of circulating biomarkers of fibroblast activation for identification of patients at risk of worsening cardiac function and chronic fibroblast activation.

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