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From dissection of fibrotic pathways to assessment of drug interactions to reduce cardiac fibrosis and heart failure

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A B S T R A C T

Cardiac fibrosis is characterized by extracellular matrix deposition in the cardiac interstitium, and this contributes to cardiac contractile dysfunction and progression of heart failure. The main players involved in this process are the cardiac fibroblasts, which, in the presence of pro-inflammatory/pro-fibrotic stimuli, undergo a complete transformation acquiring a more proliferative, a pro-inflammatory and a secretory phenotype. This review discusses the cellular effectors and molecular pathways implicated in the pathogenesis of cardiac fibrosis and suggests potential strategies to monitor the effects of specific drugs designed to slow down the progression of this disease by specifically targeting the fibroblasts.

1. Introduction

Heart failure (HF) is a clinical condition in continuous growth, due to the increased age of the population. It is characterized by a very poor prognosis without therapy and with high economic burden for the health system (Ziaean and Fonarow, 2016). HF was defined as a “complex clinical syndrome that results from structural and/or functional impairment of ventricular filling or ejection of blood” (Writing Committee, et al., 2013). Prognosis of HF with reduced (HFrEF) or preserved ejection fraction (HFpEF) strictly depends on age, cardiovascular risk factors, and several comorbid conditions, such as diabetes mellitus, hypertension and coronary heart disease (Lehrke and Marx, 2017). These comorbidities, occurring in both HF types, but slightly more severe and frequent in HFpEF (Maeder et al., 2018), could induce alterations in the myocardial structure and impairment in cardiac cell functions, activating intracellular signalling pathways that lead to inflammation and matrix remodelling (Paulus and Tschope, 2013). While cardiac hypertrophy and interstitial fibrosis favour the development of HFpEF, HFrEF is more related to acute loss of cardiomyocytes, resulting from myocardial ischemia, and is associated with adverse cardiac remodelling (Ge et al., 2019). Cardiac fibrosis is a maladaptive condition, characterized by excessive deposition of extracellular matrix (ECM) proteins by cardiac fibroblasts (CFs). This limits heart muscle functions and accelerates the progression to heart failure (Jellis et al., 2010). While fibrosis is commonly associated with ischemic injury, it is active also in the absence of ischemia, such as hypertensive heart disease and hypertrophic cardiomyopathy (Travers et al., 2016). Fibrosis can be reparative or

reactive. In case of a transmural myocardial infarction, fibrotic scar prevents myocardial rupture; however this increases ventricular stiffness and leads to a reduced contractility (Swynghedauw, 1999). Cardiac fibroblasts are the main players in this pathological setting. Physiologically, these cells are responsible for ECM homeostasis, thus providing a structural support for cardiomyocytes and allowing a correct distribution of mechanical load throughout the myocardium (Souders et al., 2009). In the presence of pro-inflammatory cytokines and pro-fibrotic stimuli, CFs differentiate into myofibroblasts, becoming more proliferative and exhibiting increased contractile, migratory and secretory properties (Souders et al., 2009). In particular, activated myocardial fibroblasts remodel cardiac interstitium by increasing secretion of ECM-degrading metalloproteinases (MMPs) and collagen turnover (Garoffolo et al., 2020). As described before, this adaptive process is initially necessary to maintain the structural integrity of the heart. However, in advanced phases, fibrosis leads to adverse changes in the ventricular structure and compliance, culminating in heart failure. In the present contribution, we will describe the main signalling pathways driving pathologic programming of cardiac fibroblasts programming and some of proposed treatments, together with potentially new strategies for monitoring the efficacy of anti-fibrotic treatments.

2. Cardiac myofibroblast activation and inflammation: what are the main signalling players involved?

Several sources of CFs have been identified in the adult heart. Depending on the pathological stimulus, CFs could derive from the

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activation and proliferation of cells with pericyte characteristics (Kramann et al., 2015), from cells undergoing endothelial-mesenchymal transition (Zeisberg et al., 2007), and cells recruited from the epicardium (Smart et al., 2007). Endothelial cells of coronary vasculature are able to acquire fibroblast-like phenotype and migrate into the interstitium, where they contribute to the fibrotic response (Zeisberg et al., 2007). In addition, pericytes resident in perivascular niche of large arteries and arterioles, respond to heart injury by increasing proliferation and differentiating into myofibroblast-like cells, participating actively to cardiac fibrosis (Kramann et al., 2015). Other possible fibroblasts precursors might be the so called 'fibrocytes', hematopoietic-derived cells (e.g. monocytes/macrophages), which under particular conditions, may account for up to 60% of all fibroblasts within the site of cardiac injury and are involved in collagen production and scar formation (van Amerongen et al., 2008). During cardiac development, the epicardium gives rise to cardiac fibroblasts by epicardial-mesenchymal transition (Gessert and Kuhl, 2010). Indeed, epicardial cells acquire mesenchymal phenotype and migrate into the developing ventricle (Gessert and Kuhl, 2010). A similar process seem to occur also after cardiac injury (Zhou et al., 2011). Myocardial infarction increases epicardial cell proliferation and differentiation in mesenchymal cells, promoting expression of fibroblast and smooth muscle cell markers in the epicardial region (Russell et al., 2011). The problem of myofibroblasts origin is related to the problem of their identification. Unlike quiescent fibroblasts, myofibroblasts express contractile microfilaments, cell-to-matrix attachment receptors and intercellular adherens and gap junctions (Hinz et al., 2007). Of particular interest are the α -smooth muscle actin (α -SMA), the classically employed marker for myofibroblast identification (Shinde et al., 2017), and Thy-1, a membrane protein used for inverse recognition, being expressed in fibroblasts but not in differentiated myofibroblasts (Koumas et al., 2003). Although several cell types may be implicated in fibrotic remodelling of the heart, directly by producing ECM proteins or indirectly by secreting fibrogenic mediators, the myofibroblast conversion of CFs remains one of the key cellular events involved in cardiac fibrosis. Indeed, myofibroblasts contribute to several processes of cardiac remodelling, including inflammation, proliferation, ECM remodelling, and scar deposition and maturation. Interestingly, while in the early phases post injury, CFs acquire a pro-inflammatory phenotype with secretion of TNF α , Interleukin-1 β (IL-1 β) and IL-6 (van Nieuwenhoven and Turner, 2013) they later acquire a more anti-inflammatory and pro-reparative functions, secreting transforming growth factor- β (TGF- β), to promote scar tissue formation (van Nieuwenhoven and Turner, 2013).

2.1. Inflammation

During the pro-inflammatory phase, CFs secrete cytokines, such as TNF α , IL-1 β and IL-6, which recruit numerous inflammatory cells at the site of injury and affect directly CFs phenotype. In particular, IL-1 β and TNF α strongly increase cardiac fibroblast migration by activating ERK1/2 MAPK signalling cascade (Mitchell et al., 2007). Indeed, IL-1 β increases fibroblast expression of cell adhesion molecules, thus promoting migration into the zone of injury (Kacimi et al., 1998). Therefore, IL-1 β -dependent activation of ERK and JNK MAPK kinases is also subject to an increased expression of metalloproteinase-9 and enhanced collagen turnover (Fig. 1) (Xie et al., 2004). Cardiotrophin-1, a member of IL-6 family, is upregulated in the infarct zone post-MI in rats and mediates CF proliferation by increasing DNA synthesis (Freed et al., 2003). In addition, this cytokine, beyond its hypertrophic effect on cardiac myocytes (Fukuzawa et al., 2000), influences the synthesis and secretion of collagen by myofibroblasts (Freed et al., 2005). This suggests a potential role of this protein in the scar formation and maturation by stimulating the repopulation of the infarct scar associated with ECM remodelling (Freed et al., 2005). Since inflammation has a critical role in the fibrotic response to the cardiac injury, inhibition of these inflammatory mediators may be therapeutically relevant.

2.2. Transforming growth factor- β

TGF- β is the most extensively studied mediator of fibroblasts activation during the anti-inflammatory cardiac healing phase (Bujak and Frangogiannis, 2007), and it is also involved in fibrotic processes in various organs, such as liver and kidney (Leask and Abraham, 2004). In CFs, TGF- β is synthesized and secreted as part of the large latent complex (LLC), providing a reservoir of latent TGF- β 1 in the ECM. This complex can be proteolytically cleaved, and activated by integrin-mediated traction force (Sarrazy et al., 2014). The release of TGF- β from its latent state includes not only the cleavage of LLC by proteases, such as plasmin and MMP-2/9 (Annes et al., 2003), but also through direct binding to latency associated protein (LAP) through thrombospondin, a matricellular protein involved in fibrotic response to the injury (Garoffolo et al., 2020). Av β 5 and α v β 3 integrins, expressed at high levels in fibrotic zones of the myocardium, are also able to activate TGF- β by inducing conformational changes in the latent complex (Sarrazy et al., 2014). Inhibition of these integrins reduces the ability of CFs to differentiate into myofibroblasts (Sarrazy et al., 2014). All these evidences may open the way to novel therapeutic strategies to inhibit specifically TGF- β 1 activation in CFs.

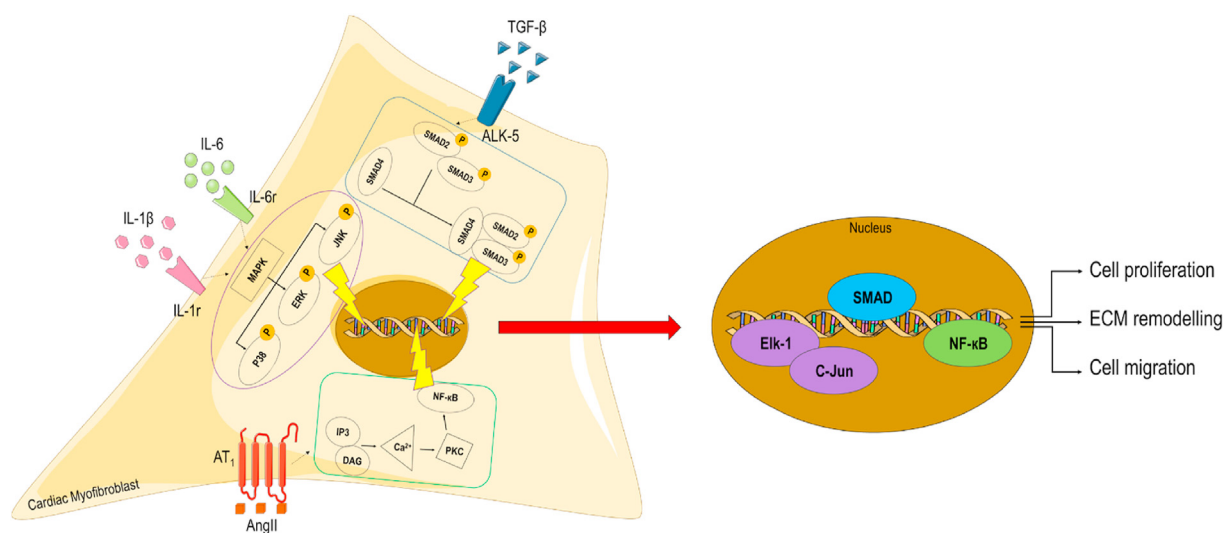


Fig. 1. Schematic representation of the main intracellular signalling pathways that participate to myofibroblast transdifferentiation of cardiac cells, one of the key events involved in cardiac fibrosis.

The pro-fibrotic activities of TGF- β 1 are mediated by its cellular receptors. In particular, binding of active TGF- β to the TGF- β receptor type II (TGF- β -RII) leads to the phosphorylation and recruitment of TGF- β -RI, also known as Activin receptor-like kinase (ALK) 5. This complex, in turn, phosphorylates Smad2/3, which bind Smad4 and translocate into the nucleus to regulate the expression of pro-fibrotic genes (Fig. 1) (Bujak et al., 2007). The implication of TGF- β /Smad signalling in post-infarction ventricular remodelling is highlighted by results obtained in Smad3-null mice (Bujak et al., 2007). In particular, these mice showed a marked reduction in ventricular dilatation and diastolic dysfunction due to the lack of interstitial fibrosis and ECM remodelling (Bujak et al., 2007). Indeed, collagen content within the infarct region in Smad3-null mice was substantially lower than in wild-type mice, again suggesting that collagen deposition is a Smad3-dependent activity.

TGF- β can activate also other signalling cascades, including ERK, JNK, MAPK, which in turn regulate Smad-independent TGF- β responses (Derynck and Zhang, 2003). Usually, the activation of these intracellular pathways promotes TGF- β 1 expression, thereby amplifying the TGF- β response (Yue and Mulder, 2000), or induces epithelial-mesenchymal transition (Bakin et al., 2002). Finally, TGF- β can activate Rho-like GTPases, such as RhoA, Rac and Cdc42 (Edlund et al., 2002). These proteins are mainly involved in TGF- β -induced changes in cytoskeletal organization and epithelial-mesenchymal transition. In particular, activation of Rac1 and RhoA is important also to promote CF migration because is required for rapid membrane ruffling and lamellipodial formation (Edlund et al., 2002). In conclusion, TGF- β signalling participate to matrix deposition during fibrotic process, by promoting the transcription of ECM genes via Smad dependent/independent activities. In particular, while Smad3 is essential for the induction of pro-fibrotic genes, such as collagen I and connective tissue growth factor (CTGF) (Holmes et al., 2001), the transcription of fibronectin requires JNK signalling (Hocevar et al., 1999).

2.3. Renin angiotensin system

The renin-angiotensin-aldosterone system (RAAS) plays a critical role in cardiac remodelling. Angiotensin II (AngII), the central product of RAAS system, is considered a potent pro-fibrotic molecule (Sopel et al., 2011). Indeed, it has been demonstrated that cardiac fibroblasts are activated in response to hypertrophic stimuli, including AngII, becoming more proliferative and increasing the production of ECM proteins (Craib et al., 1994). In particular, neonatal and adult rat CFs express AT1 receptors for AngII and not AT2 that are more expressed by cardiac myocytes and other cell types in the heart (Sadoshima and Izumo, 1993). Tissue stiffening and ECM remodelling following myocardial injury can stimulate the release of AngII from cardiomyocytes stores, or can contribute to the activation of AT1 receptors in CFs (Zou et al., 2004). The functions of AngII in CFs are pleiotropic, involving several effectors. For example, AT1 receptor stimulates phospholipase C activity, which, through inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), leads to calcium mobilization from internal stores and activation of protein kinase C (PKC). PKC plays a key role in mediating NF- κ B signalling pathway (Fig. 1) (Schorb et al., 1993; Ji et al., 2016 #1416). Translocating into the nucleus, NF- κ B promotes the transcription of pro-inflammatory genes and cell proliferation (Ji et al., 2016). AngII is able also to stimulate ECM remodelling within the site of injury, increasing the release of latent TGF- β 1 from CFs (Dostal et al., 1996). In particular, this promotes the synthesis of collagen I α , suggesting a possible participation of AngII in the scar formation (Dostal et al., 1996). Finally, AngII mediates also the inflammatory response of CFs through TGF- β /Smad3-dependent signalling (Ma et al., 2012). In particular, the activation and expression of TGF- β stimulate the release of IL-6, a cytokine known to participate actively to the myofibroblast trans-differentiation (Ma et al., 2012).

2.4. RhoA-MRTF-SRF signalling pathway

As described above, after MI, CFs differentiate into myofibroblasts, a contractile cell population that display smooth muscle-like features and are considered to be primarily responsible for myocardial fibrosis and remodelling. The transcription factor Serum Response Factor (SRF) is involved in the regulation of smooth muscle specific genes via binding to the sequence termed a CArG box or serum response element (SRE) (Pipes et al., 2006). SRF regulates gene transcription through the association with specific co-activators, myocardin and myocardin-related transcription factors (MRTF-A/MAL and MRTF-B) (Pipes et al., 2006). Rho-GTPases are also involved in the regulation of SRF via their ability to induce actin polymerization (Miralles et al., 2003). Indeed, alteration in actin dynamics are necessary to activate SRF, but the mechanism by which Rho-actin signalling regulates this process is still unknown. It has been shown that Rho-dependent actin polymerization induces the nuclear translocation of MRTF-A, a SRF coactivator (Miralles et al., 2003). SRF- MRTF-A interaction controls the expression of a fibrotic gene program in CFs, including genes related to ECM remodelling and smooth muscle cells differentiation (Small et al., 2010). In keeping with these results, mice deficient for MRTF-A are protected from cardiac fibrosis and scar formation following MI, even after AngII infusion (Small et al., 2010). SRF-mediated CF differentiation occurs in a Rho/ROCK dependent manner: Y-27632, a ROCK inhibitor, blunted the nuclear localization of MRTF-A, also in response to pro-fibrotic stimuli, such as TGF- β , preventing CFs differentiation (Small et al., 2010). These evidences suggest also a possible implication of cell mechanosensing in the onset of cardiac fibrosis. As we have already discussed in previous contributions (Garoffolo et al., 2020; Garoffolo and Pesce, 2019), CF activation might be affected also by mechanical cues, such as mechanical stretch and matrix stiffness, which are able to induce a F-actin cytoskeletal rearrangement inside the cell, necessary for the activation of intracellular signalling cascades leading to pro-fibrotic CF phenotype. In particular, increased matrix hardening in the infarct zone of the myocardium induces smooth muscle α -actin fiber formation and collagen production in CFs, two important traits of activated myofibroblasts (Herum et al., 2017a,b). In agreement with these results, inhibition of MRTF-A signalling determined by pharmacological inhibition of mechano-dependent transcription factor yes-associated protein (YAP), led to reduction of cardiac fibrosis in an *in vivo* model of myocardial infarction (Francisco et al., 2020).

3. Defining pathway-specific treatments, route of administration and monitoring criteria for next generation treatments of cardiac fibrosis

As discussed above, numerous signalling pathways implicated in cardiac fibrosis may be targeted, leading to potential treatments overlap for early pathological activation of CFs, as well as the onset/progression of cardiac ECM remodelling. In order to obtain indications on the most efficiently druggable pathways, it is necessary not only to define the principal intracellular molecular players, but also to uncover and contextualize their potential adverse interactions. In this regard, new insights on the pathophysiological process of cardiac fibrosis may derive from a more accurate definition of the cellular populations that could dynamically contribute to the different stages of the pathology. In fact, as discussed in various contributions (Shaw and Rognoni, 2020; Soliman and Rossi, 2020), CFs are intrinsically heterogeneous cells, whose definition only on the basis of cellular markers may be difficult (Shinde and Frangogiannis, 2017). The new possibility offered by the evolution of next generation sequencing, e.g. the single-cell RNA sequencing, revealed recently that different CF subtypes might play different roles during the wound healing process after MI (Gladka et al., 2018; Ruiz-Villalba et al., 2020). In particular, these studies identified a

sub-population of CFs that responds to myocardial infarction, characterized by a specific transcriptomic signature, and in particular by expression of *Cthrc1* (collagen triple helix repeat containing 1), a gene linked to collagen biosynthesis and deposition (Pyagay et al., 2005). *Cthrc1*⁺ cells are almost exclusively located in the infarct and the border zones, and represent the final stage of the injury-activated CFs mainly involved in the reparative processes (Pyagay et al., 2005). Indeed, in mice deficient for *Cthrc1*, after MI, a decrease in collagen deposition in LV was observed and this caused ventricular rupture and increased mortality (Pyagay et al., 2005). These evidences highlight the importance to identify specific cellular populations acting in myocardial remodelling and define their transcriptional signatures, to increase the specificity of the treatments to target pathways in selected cell subpopulations.

A viable possibility to increase the selectivity of pharmacological treatments for cardiac fibrosis is the refinement of advanced imaging protocols to detect cardiac fibrosis at its early states and monitor its progression. Several systems might amenable to this aim, such as for example, by cardiac magnetic resonance (CMR) protocols (Karamitsos et al., 2020). One of CMR applications in particular, named cardiac diffusion tensor imaging (DTI), is a well-established quantitative method based on the possibility to detect the vectors of water diffusion inside the myocardial tissue (Basser et al., 1994). Scalar parameters obtainable from DTI, such as the fractional anisotropy, the mean, the secondary and the tertiary diffusivity, can be correlated to the collagen content and fibres orientation (Abdullah et al., 2014) thereby enabling dynamic description of the spatial and temporal evolution of the fibrotic process (Chen et al., 2003; Wu et al., 2007). These findings point out a potential role of this technique not only to better characterize the disease, but also to monitor with higher precision the therapeutic efficacy of pathway-specific drugs with non-invasive imaging.

Several difficulties have limited also the development and the design of new pharmacological therapies, due to the complexity of the cell types and the signalling pathways involved. Some clinical data have shown benefits on cardiac fibrosis with RAAS inhibitors. Indeed, although they are not approved for cardiac fibrosis therapy, inhibitors of angiotensin signalling, such as angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs), have demonstrated significant efficacy in the treatment of heart failure, reducing fibrosis not only in animal models, but also in humans (Diez et al., 2002; Yang et al., 2009). While ACE inhibitors block the conversion of inactive AngI into active AngII, ARBs prevent the binding of AngII to its receptors. Losartan, one such ARBs, reduces cardiac fibrosis preventing endothelial-mesenchymal transition and collagen synthesis by affecting TGF- β -induced phosphorylation of ERK (Oliveira-Junior et al., 2014). Therefore, Losartan reduces also the expression of inflammatory markers inactivating NF- κ B signalling pathway (Miguel-Carrasco et al., 2010). Recent studies have showed that the treatment with ACE inhibitors, within the first phases of heart failure, is able to attenuate significantly hypertrophy and prevent contractile dysfunction and fibrosis (Brooks et al., 1997). However, recent clinical trials in heart failure patients with preserved ejection fraction did not demonstrate the same benefits (Massie et al., 2008).

Among RAAS effectors, the mineralocorticoid aldosterone is able to directly stimulate collagen synthesis or inhibit collagenase activity in adult rat cardiac fibroblasts (Brilla et al., 1994), which express high affinity corticoid receptors for aldosterone (Brilla, 2000). Aldosterone increases AngII receptors in the myocardium and in this way potentiates the fibrogenesis effects of this pathway (Lijnen and Petrov, 1999). Treatment with aldosterone receptor antagonist, spironolactone, prevents myocardial fibrosis in either rat model of left ventricle hypertrophy and arterial hypertension (Brilla et al., 1993). It has been demonstrated that eplerenone, a selective aldosterone blocker, in combination with best medical therapy including ACE inhibitors or ARBs, reduced cardiovascular mortality and improved survival and hospitalization rates in patients with acute myocardial infarction complicated by left ventricular dysfunction and heart failure (Pitt et al., 2003). These findings suggest a direct interaction between aldosterone and cardiac fibroblasts in

mediating cardiac fibrosis and the combined therapy with RAAS antagonists could represent a valid therapeutic treatment to contrast cardiac fibrosis and prevent heart failure.

As discussed above, cardiac inflammation has an important role in the pathological progression of cardiac fibrosis. For this purpose, several new therapeutic strategies are emerging in this direction. First successful results in this field came from Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trail. In this study, canakinumab, a monoclonal antibody targeting IL-1 β , lowered the inflammation burden in high-risk patients with atherosclerotic disease who had survived a MI (Ridker et al., 2017). Although the mechanism by which neutralizing IL-1 β produced a cardiovascular benefit is still unknown, it is possible to speculate that targeting IL-1 β prevents the recruitment of inflammatory cells at the site of injury, inhibits metalloproteinase activity and pyroptosis in leukocytes and resident cells, thus preventing the inflammatory response to infarction (Sager et al., 2015). In addition, IL-1 induces uncoupling of the β -adrenergic receptor from adenylyl cyclase and from calcium channels, thus reducing the responsiveness of the cardiomyocytes to contractility and inducing systolic dysfunction (Chung et al., 1990). Genetic deletion of IL-1 receptor was protective in an animal model of MI, as shown by reduction of infarct size and left ventricle impairment (Abbate et al., 2011). Cardiac inflammation is not only characterized by elevated levels of pro-inflammatory cytokines, but it is also associated with higher circulating levels of natriuretic peptides that contribute to vasodilation, natriuresis and diuresis, as compensatory responses to increased cardiac wall stress (Volpe et al., 2016). Given the anti-inflammatory properties of these hormones, it has been postulated that cardiac inflammation may trigger natriuretic peptides release (de Bold, 2009; Tomaru Ki et al., 2002). Indeed, pro-inflammatory cytokines such as TNF- α and IL-1 β , promoted brain natriuretic peptides (BNP) synthesis and secretion by neonatal rat cardiomyocytes (de Bold, 2009). In a rat animal model, atrial natriuretic peptide (ANP) prevented AngII-induced myocardial remodelling and myocyte hypertrophy by attenuating inflammation (Fujita et al., 2013). In particular, ANP reduced infiltration of macrophages and suppressed tenascin-C-mediated inflammatory/fibrotic signalling cascade (Fujita et al., 2013). In this way, natriuretic peptides could represent good candidates for both diagnosis and treatment of cardiac fibrosis. All these data suggest that targeting cardiac inflammation can confer modest cardiovascular benefit in very high risk patients.

There are also approaches to influence myofibroblast activation by blocking TGF- β or Smad3 signalling. A first example is the use of TGF- β neutralizing antibodies, which do not improve cardiac functions but increase mortality and left ventricular dilatation (Frantz et al., 2008). Inhibitors of TGF- β receptors are currently under investigation as potential anti-fibrotic therapies due to their abilities to block TGF- β signalling activity, leading to the attenuation of left ventricular remodelling and expression of collagen after MI (Tan et al., 2010). However, long-term inhibition induces cardiac toxicity, thus limiting its clinical application (Herbertz et al., 2015). All these evidences suggest that directly targeting TGF- β might not be applicable clinically. In this regard, the two most promising agents are pifrenidone and tranilast, which may inhibit pro-fibrotic TGF- β functions in the infarcted myocardium, such as ECM deposition (Edgley et al., 2012).

One possible explanation of the limited efficacy of the therapies for heart failure may be also the conventional administration of drugs via systemic delivery. In this respect, a localized delivery of anti-fibrotic drugs in the infarcted area could avoid side effects and may increase drug bioavailability. One possible solution to achieve local delivery of drugs in the remodelling myocardium could be the use of injectable biomaterials such as hydrogels with anti-fibrotic and anti-inflammatory properties (Deng et al., 2015). For example, in rat chronic myocarditis model, hydrogels containing hepatocyte growth factor reduced cardiac fibrosis by suppressing TGF- β -dependent collagen synthesis and activating metalloproteinases to increase ECM degradation (Nakamura et al., 2005; Nakano et al., 2014). In addition to their anti-fibrotic effects,

hydrogels showed also pro-angiogenic and tissue regenerative abilities, although the time of the injection limits hydrogel therapeutic efficacy (Yoshizumi et al., 2016). Nanoparticle (NP)-based drug delivery could finally represent a promising therapeutic solution. In particular, lipid NPs (e.g., PEGylated lipid NPs) could be very promising for the treatment of cardiac fibrosis for they ability to deliver both hydrophilic and lipophilic substances (Saludas et al., 2018). Several micellar and liposomal formulations are up to date already approved by FDA as drug delivery system for the treatment of other clinical conditions. Studies reported that in both acute and chronic MI, micelles and liposomes are able to permeate the entire infarct area and to reduce infarct size in animal models (Dong et al., 2017; Paulis et al., 2012). In this framework, particularly interesting appears the possibility to ‘functionalize’ NPs for cargo delivery in selected populations of the heart such as inflammatory cells and myofibroblasts. An interesting example was the design of nanoliposomes coated with hyaluronic acid (HA) to selectively target the monocytes/macrophages (expressing the HA receptor CD44) recruited in the ischemic myocardium at early stages following infarction (Ben-Mordechai et al., 2017). Another possible solution for targeting macrophages is represented by the delivery of apoptotic-mimicking particles. One example is phosphatidylserine-presenting liposomes, since macrophages recognize apoptotic cells by exposed phosphatidylserine (Harel-Adar et al., 2011 #1481). This prevents the secretion of pro-inflammatory cytokines, resolving inflammation and eliciting infarct repair (Harel-Adar et al., 2011). A possible implementation in this approach may be to include in nanoparticles also iron oxides, quantum dots, radioactive nanoparticles as cell trackers to identify implanted cells in animal models of myocardial infarction (Provenzale, 2006). Nanoparticles have been reported also as tools in detecting markers of cardiac injury. An example is the dual gold NP conjugate-based lateral flow assay for the detection of Troponin I in serum samples of patients with MI. This one-step and rapid low-cost system is 100-fold more sensitive than the conventional method (Choi et al., 2010). The most common use of NPs in cardiac preclinical studies is in the area of magnetic resonance imaging (MRI) to enhance the contrast in the targeted structures. Superparamagnetic iron oxide NPs are able to discriminate normal from infarcted myocardium using high field MRI (Chapon et al., 2003); paramagnetic quantum dots allow the detection of angiogenic activity in the infarcted heart using an *in vivo* molecular MRI (Oostendorp et al., 2010). The possibility, finally, to design ‘smart’ nano-vehicles containing drugs and *in vivo* ‘tracers’ offers the possibility to monitor with multimodal imaging the homing of the delivered nanoparticles and the therapeutic effects in the specific sites of drug delivery (Merinopoulos et al., 2020).

Another aspect limiting the development of new therapeutic strategies to take into consideration is the drug-drug interaction. Drug-drug interaction (DDI) can occur when two or more drugs co-administered to a patient cause combined responses, with positive or negative therapeutic effects. This problem arises from the introduction of several new drugs into clinical use, although the prevalence, risks and natural history of the diseases associated with their use are not still well defined. Evaluating DDI and clinical risks preclinically is important to not to affect the beneficial effects of pharmacological treatment. There are many drugs with synergistic or additive therapeutic effects. For example, a synergistic attenuation of myocardial fibrosis was observed in spontaneously hypertensive rats treated with combined Benazepril, an ACE inhibitor, and candesartan, an ARB (Meng et al., 2009). In this case, even if separate treatment with ACE inhibitor or ARB has produced significant therapeutic outcomes, the combined use of the two drugs induced additive protection against myocardial fibrosis (Meng et al., 2009). In particular, the combined treatment decreased TGF- β and Smad3 protein levels, preventing the intracellular signaling transduction and producing an anti-fibrotic effect. In addition to pharmacological treatments, also transplantation of combined cell types may have an adjuvant effect in myocardial repair. For example, while inflammatory macrophages

contribute to the initiation and propagation of adverse cardiac remodelling after infarction, macrophages with anti-inflammatory phenotype participate actively to repair process and wound healing (Medzhitov and Horng, 2009). Bone marrow derived-stem cells are able to modulate macrophages toward an anti-inflammatory phenotype (Cho et al., 2014). Combined transplantation of macrophages and bone marrow derived-stem cells, both harvested from the same donor, induced significant functional and structural improvements in MI rat model (Lim et al., 2018). This suggests that the combination of different autologous cell populations could represent an optimal adjuvant strategy to enhance stem cell therapy. Another important problem is related to the cardiotoxicity of several drugs. One example is the anticancer drugs, such as anthracycline-based chemotherapy, used for the treatment of several tumor types or, as an adjuvant in breast cancer treatment (Mackey et al., 2013), which was found to cause cardiotoxicity, especially in younger subjects (Moudgil and Yeh, 2016). Cardiotoxic effects of anthracycline drugs, such as Doxorubicin (Dox), have been attributed to i) cardiomyocyte apoptosis due to DNA damage and formation of reactive oxygen species and ii) cardiac fibroblast activation with consequent increase in ECM protein production and cell proliferation (Levick et al., 2019). This leads to cardiac fibrosis and left ventricle dysfunction. There are a few strategies to prevent cancer treatment-induced cardiotoxicity. Dox induces DNA damage through topoisomerase 2 β (Top2 β), which causes DNA double-strand breaks and changing into the transcriptome, leading to mitochondrial dysfunction (Vejpangsa and Yeh, 2014). Since Top2 β is the only Top2 expressed in the heart, one possible strategy may be to inhibit this enzyme and thus prevent cell death in cardiomyocytes (Lyu et al., 2007). In alternative, Dox-induced cardiac dysfunction might be prevented by the treatment with desacyl ghrelin, which has anti-apoptotic and antioxidative effects on cardiomyocytes (Baldanzi et al., 2002; Garcia and Korbonits, 2006). In addition, this compound was able to attenuate collagen deposition and thus prevented myocardial fibrosis induced by Dox in mouse model (Pei et al., 2014). Another example is that of the morphine, commonly used for pain management, which was shown to induce cardiac interstitial fibrosis and cardiomyocyte hypertrophy, and to increase the risk of myocardial infarction (Gaweda et al., 2020). To overcome these shortcomings, Pramipexole, a dopamine receptor D3 agonist, used as an adjunct therapy with morphine, abolished adverse cardiac effects. This suggests that this drug, prescribed clinically for neurologic disorders, can protect against morphine-induced cardiac remodelling (Gaweda et al., 2020). Taken together, all these evidences highlight the importance of the screening and, in some cases, the withdrawal of therapies in order to prevent patients from developing severe cardiac complications, even if many of these therapies are necessary for their substantial symptomatic relief.

4. Conclusions

In this review, we describe in details the relevance of various intracellular signalling pathways that could be druggable to prevent development of cardiac fibrosis, focusing mainly on the specific role of cardiac fibroblasts in disease pathological setting. We have contextualized some of the advantages offered by new gene expression analysis tools, allowing dissecting the specific role of defined cellular subsets involved in pathology setting, and finally discussed some of the perspectives offered by targeted gene delivery using nanotechnology, taking into consideration also problems related to drug-drug interactions and cardiotoxicity. These efforts will have to be conveyed into more integrated approaches to combat with system-level power the increasing burden of cardiac fibrosis and heart failure.

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Gloria Garoffolo and Maurizio Pesce conceived the manuscript and wrote the paper.

Declaration of competing interest

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