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Biomarkers for the identification of cardiac fibroblast and myofibroblast cells

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Abstract
Experimental research has recognized the importance of cardiac fibroblast and myofibroblast cells in heart repair and function. In a normal healthy heart, the cardiac fibroblast plays a central role in the structural, electrical, and chemical aspects within the heart. Interestingly, the transformation of cardiac fibroblast cells to cardiac myofibroblast cells is suspected to play a vital part in the development of heart failure. The ability to differentiate between the two cells types has been a challenge. Myofibroblast cells are only expressed in the stressed or failing heart, so a better understanding of cell function may identify therapies that aid repair of the damaged heart. This paper will provide an outline of what is currently known about cardiac fibroblasts and myofibroblasts, the physiological and pathological roles within the heart, and causes for the transition of fibroblasts into myoblasts. We also reviewed the potential markers available for characterizing these cells and found that there is no single-cell specific marker that delineates fibroblast or myofibroblast cells. To characterize the cells of fibroblast origin, vimentin is commonly used. Cardiac fibroblasts can be identified using discoidin domain receptor 2 (DDR2) while α-smooth muscle actin is used to distinguish myofibroblasts. A known cytokine TGF-β_1 is well established to cause the transformation of cardiac fibroblasts to myofibroblasts. This review will also discuss clinical treatments that inhibit or reduce the actions of TGF-β_1 and its contribution to cardiac fibrosis and heart failure.

Keywords Cardiac fibroblast · Cardiac myofibroblast · Biomarkers · Alpha-smooth muscle actin · Heart failure

Introduction

Cardiovascular disease (CVD) remains one of the leading causes of death and accounts for 32% of the deaths worldwide [1]. There are many different types of CVD; however, heart failure (HF) is of concern due to its rise in many Western countries around the world. Heart failure is a complex form of CVD as it involves numerous pathways within the body. Structural changes known collectively as cardiac remodeling occurs during the clinical course of HF. Cardiac remodeling is defined as adaptive or maladaptive changes in the structure of the heart in response to chronic hemodynamic load and/or cardiac injury and includes changes in mass, shape, and dimensions of the heart which can affect its ability to function properly [2, 3].

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Fibroblast cells are associated with myocardial remodeling, which occurs after a cardiac event such as a myocardial infarction. After an ischemic event, the remodeling process occurs rapidly within the first few hours after the infarct and continues to progress [2]. Throughout the years, the cause of heart failure was often thought to involve mostly the death of cardiomyocytes and associated remodeling; however, the role of the cardiac fibroblasts has more recently emerged in the scientific literature. Traditionally, cardiac fibroblasts were postulated to perform a structural role within the heart and were therefore often ignored in research. Over the years, however, interest in cardiac fibroblast research has risen as they were found to be involved in heart failure [4]. Cardiac fibroblasts have the ability to transform into the myofibroblast phenotype which are the cells implicated in the progression to heart failure [5]. Cardiac myofibroblasts arise after injury to the heart and its products contribute to fibrosis [6]. While it has been recognized that fibroblasts can transform into myofibroblast cells following conditions of cardiac stress, an ability to identify these cells has primarily focused on the expression of smooth muscle actin [6, 7]. This review will outline the role of both the cardiac fibroblast and myofibroblasts, examine

all reported known and possible biomarkers that could be implemented to identify the cardiac fibroblasts and delineate them from myofibroblast cells, and discuss the potential clinical targets associated with the TGF-β_1 mediated transformation of fibroblasts and cardiac fibrosis.

Cardiac fibroblasts

Cardiac tissue is comprised of cardiac myocytes and non-myocyte cells. Previous studies have found that the population of myocytes and non-myocytes can vary among different species [8]. In normal healthy adult rat and human hearts, the cardiac myocyte is accountable for approximately 30–40% of tissue mass while non-myocyte cells account for the remaining 60–70% tissue mass [4, 8–11]. In mice, previous research using fluorescence-activated cell sorting (FACS) analysis indicates that the adult mouse heart contains approximately 56% myocytes and 27% fibroblasts [12]. Non-myocyte cells consist primarily of cardiac fibroblasts; however, endothelial or vascular smooth muscle cells also exist, but in a comparatively smaller population compared to fibroblasts [13]. Cardiac fibroblasts are found throughout the heart and bridge the gaps that occur between the myocardial tissue layers. Morphologically, fibroblasts are flat, spindle-shaped cells with many processes stemming from the body of the main cell. A distinctive characteristic of cardiac fibroblasts is the lack of a basement membrane, which is a defining feature that distinguishes it from other cardiac cells [8, 13]. Fibroblasts also tend to display a prominent Golgi apparatus and extensive rough endoplasmic reticulum particularly when in an active state [13]. Fibroblasts are classified active or inactive based on their morphological appearance.

Cardiac fibroblasts have structural, biochemical, mechanical, and electrical conductance roles within the heart. Fibroblasts contribute to cardiac development, myocardial structure, cell signaling, and electro-mechanical function in both healthy and diseased myocardium [4, 14]. It is well established that cardiac fibroblasts are involved in the homeostasis of the extracellular matrix (ECM). This is achieved by maintaining a balance between the synthesis and degradation of certain factors such as cytokines, growth factors, and matrix metalloproteinases (MMPs) which contribute to ECM homeostasis [4]. The maintenance of the ECM is crucial as it provides a scaffold for all cardiac cells and also acts as an electrical buffer by separating the atria and ventricle to enable proper cardiac function [15, 16]. Fibroblasts are also able to secrete non-rigid collagenous extracellular matrix such as type I and type III collagen. They have the ability to proliferate and migrate to the site of injury while also producing large amounts of collagen to repair damaged tissue [17].

Cardiac fibroblasts are electrically unexcitable cells; however, they provide an important contribution to the electrophysiology of the heart [18, 19]. The fibroblast is also seen as an insulator in terms of myocardial electrical signaling and can contribute to arrhythmias in disease states when cardiac fibroblasts proliferate [10]. Cardiac fibroblasts are known to be non-excitable cells and were initially thought to only serve a structural role; however, more recently, there has been evidence suggesting that cardiac fibroblasts can have a role in the electrical function [20]. Fibroblasts have been reported to form gap junctions and communicate with other fibroblast and cardiomyocytes through electrical interactions [21]. Previous research utilized immunohistochemical techniques to study the structure and spread of Lucifer yellow dye to evaluate the functionality of intercellular coupling between fibroblasts and fibroblast to myocytes. Findings have shown fibroblasts express both connexion (Cx) 40 and Cx45 to form gap junctions. Cx40 is reported to be present between fibroblast cells while Cx45 is found where fibroblasts interact with myocyte cells [22]. This study has also revealed that fibroblasts can act as a conductive pathway between the myocytes to form bridges for electrical communications [22]. Kohl and colleagues [23] observed that fibroblasts act as current sinks where they slow the generation of intrinsic excitation in the sinoatrial node. In ventricular tissue, fibroblast sinks may also lead to unidirectional block of conduction [23]. The block in the electrical conduction can either be long- or short-term depending on the remodeling dynamics of the heart, heart rate, mechanical load, and disease progression [23]. There has been suggestion that fibrosis of the heart (increased collagen production) can interfere and alter the cardiomyocyte architecture through the loss of the cell-to-cell gap junction pathway (collagen blocks these pathways) and ultimately lead to atrial fibrillation [20].

Cardiac myofibroblast cells

The study of myofibroblast biology in specific organs such as in the heart is an important but relatively understudied area. Myofibroblasts have been shown to be associated with hypertrophic fibrotic scar formation in many cardiac injury models. It is well established that the treatment of fibroblasts with transforming growth factor beta (TGF-β) induces fibroblast differentiation into myofibroblasts [24–26]. Myofibroblasts are large cells with ruffled membranes and contain a highly active endoplasmic reticulum. Myofibroblasts are not a component of normal cardiac tissue and appear only following cardiac injury [27]. A key distinctive feature that differentiates myofibroblasts from fibroblasts and other cardiac cells is the presence of the alpha smooth muscle actin (α-SMA) [28, 29]. α-SMA expression in myofibroblasts is increased in fibrotic hearts that have been exposed to pressure/volume overload or found in infarct scar tissues postmyocardial infarction [26]. Similar to fibroblasts, myofibroblasts are also non-excitable cells and are not directly involved in conduction after a myocardial infarction. Instead, myofibroblasts can intercalate

themselves between cardiac myocytes and can hinder their ability to distribute electrical conduction evenly, thus leading to arrhythmias [27].

A newly discovered role for the cardiac myofibroblast was reported by Nakaya and colleagues [30], who found that myofibroblasts are able to efficiently engulf dead cells after a myocardial infarction. Fluorescently marked apoptotic cells were used to assess the uptake of the fluorescence by the cardiac myofibroblasts and macrophages in mice. Previous research has shown that the apoptotic cells that are engulfed by phagocytes transition from phase-bright to phase-dark. This research also reported that a phase-bright to phase-dark transition was seen to illustrate engulfment of apoptotic cells by both macrophages and more interestingly cardiac myofibroblasts. It was also reported that these myofibroblast cells attained anti-inflammatory like properties upon the engulfment of dead cells including a decrease in LPS induced IL-6 and an increase in TGF-β [30].

Canonical and non-canonical pathways in fibroblasts

The canonical Wnt signaling system controls the proliferation and differentiation of both embryonic and mature cardiac myocytes. In the heart, both the positive and negative Wnt pathways contribute to cardiac remodeling [31]. The glycoprotein Wnt3a was shown to cause the differentiation of mouse embryonic fibroblast to myofibroblast cells which is dependent on β-catenin. β-catenin is a component of the canonical pathway (Wnt/ β-catenin pathway), and Wnt3a deregulates the TGF-β and SMAD2 signaling pathways [32]. In mouse embryonic fibroblast cells, the c-Jun N-terminal kinases (JNK) and focal adhesion kinase (FAK) pathways have been implicated in the TGF-β-induced conversion of fibroblast to myofibroblast cells [32, 33].

The canonical pathway, also known as the Wnt/ β-catenin pathway, is a conserved pathway involved in cell fate determination, cell migration, cell polarity, neural patterning, and organogenesis during embryonic development [34, 35]. Wnt proteins are glycoprotein growth factors that play a role in embryonic development [34]. These proteins bind to the N-terminal extracellular cysteine-rich domain of the Frizzled (Fz) receptor family [34]. The Fz protein has seven transmembrane protein similar structures to G-couple protein receptors. It has also been found that the Wnt pathway requires other co-receptors to activate this signaling cascade. When a protein binds to the Wnt receptor complex, a signal is transduced towards the Disheveled protein (Dsh/Dvl) [34]. From there, the Wnt signal can be separated into three cascades: canonical (Wnt/ β-catenin pathway), planar cell polarity, and Wnt/Ca^{2+} pathways [34]. The Wnt pathway is regulated through a number of Wnt antagonists which are secreted in the ECM [34].

The Wnt/Fz pathway has been established to be involved in cardiac fibrosis. In vitro, cardiac fibroblasts are regulated by the Wnt/Fz pathway [36]. Different combinations of Wnt and Fz stimulate various responses. This was observed when WNT3A/FZD1 overexpression increased collagen-I expression and WNT5A/FZD1 decreased collagen-I expression. In general, cardiac fibroblasts increasing WT1 demonstrated an increase in ECM secretion and proliferation of cells [36]. In terms of cardiac myofibroblast cells, an increase in the FZD2 Wnt receptor postmyocardial infarction has been reported [36]; however, the canonical pathway in myofibroblasts remains to be fully studied. The non-canonical pathway has been shown to induce mitogen-activated protein kinases (MAPK) such as c-Jun N-terminal kinase (JNK) and p38 [6]. TGF-β-activated kinase (TAK) 1 is also involved in the non-canonical pathway and has been known to contribute to cardiac hypertrophy and heart failure [6, 37]. There is also growing evidence indicating that the non-canonical pathway may be more heavily involved in the cardiac remodeling process [6].

Contributors to cardiac fibroblast transformation into myofibroblast cells

Only a few factors have been identified that independently induce cardiac fibroblast differentiation. Evidence has shown that fibroblasts convert to proto-myofibroblast cells (an intermediate cell) following mechanical stress [27]. Proto-myofibroblast cells exhibit stress fibers and the unique cell marker such as a splice variant of fibronectin called the fibronectin ED-A (extradomain A) [38]. Further stimulation of the proto-myofibroblast by the cytokine TGF-β causes the proto-myofibroblast to progress towards the myofibroblast cell phenotype, cells which can contribute to cardiac remodeling associated with heart failure. Other causes and conditions that influence the transformation of fibroblasts include myocardial infarction, inflammation, reactive oxygen species, changes in mechanical tension, aging, and seeding isolated cardiac cells at low density as seen in Fig. 1.

TGF-β

TGF-β is a cytokine that contributes to cell function through the regulation of inflammation, extracellular matrix deposition, cell proliferation, differentiation, and growth [39, 40]. There are three identified isoforms of TGF-β: TGF-β1,2 and 3 [39, 40]. The most common isoform of TGF-β is TGF-β1, whereas the other isoforms are limited to specific cells and tissues. TGF-β is found as a latent form in tissues [39], and more specifically, platelets are believed to be one of the largest suppliers of latent TGF-β1 [41]. Latent TGF-β comprises a dimeric complex where it has a C-terminal comprising mature

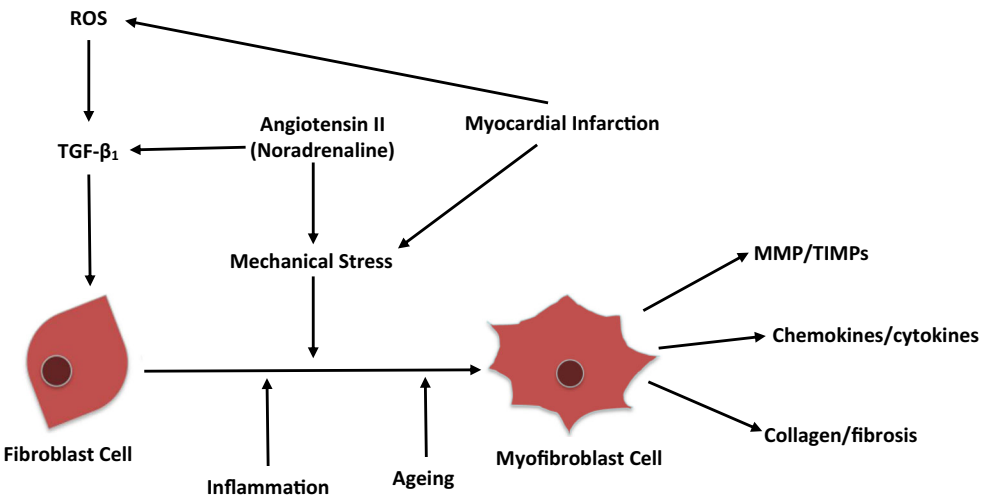

Fig. 1 A diagram of reported factors that influence the transformation of cardiac fibroblast to myofibroblast cells

TGF-β and a N-terminal pro-domain containing LAP (TGF-β latency-associated peptide) which acts to prevent TGF-β from interacting with its receptors [39]. Proteolytic cleavage of the LAP from TGF-β by an extracellular protease such as plasmin or a plasma membrane bound furin liberates TGF-β and enables it to bind to its receptors [39]. TGF-β can also be activated through a receptor mediated activation process through receptors that bind latency-associated peptide (LAP) [42]. LAP binding allows the cells to hold latent TGF-β on their surface to assist other cells to distribute active TGF-β in a paracrine/autocrine approach [42].

TGF-β1 is well established as the growth factor that stimulates the transformation of fibroblasts to myofibroblasts. However, other compounds can modulate the actions of TGF-β1 during this process. Connective tissue growth factor has been reported to promote TGF-β1-induced fibroblast transformation. However, connective tissue growth factor cannot replace TGF-β1 as α-SMA expression remained unaffected by the sole administration of recombinant connective tissue growth factor to corneal fibroblast cell cultures [43]. Galactin-3 is also a novel TGF-β1 co regulator, where in hepatic stellate cells, it is essential for fibroblast transformation. In experimental kidney, liver, and lung fibrosis, galactin-3 has been reported to be upregulated [44, 45]. The exact mechanism and relationship between galactin-3 and TGF-β1 is not yet fully understood [45]. There are also growth factors which have been reported to antagonize the actions of TGF-β1 in fibroblast cells. A recombinant protein of the cytokine interleukin-1 (IL-1), a known inflammatory mediator, has been reported to inhibit TGF-β1-induced α-SMA expression in cultured dermal fibroblasts [46]. The overexpression of intracellular IL-1 receptor antagonists have also been shown to have inhibitory effects on TGF-β1, thus reducing fibroblast differentiation to myofibroblasts [46]. In dermal fibroblast cells, IFN-γ, a T cell cytokine, has also shown to reduce α-SMA expression in fibroblast cultures. IFN-γ activates the Y box binding protein (YB-1) repressor protein which translocates to the nucleus to the nucleus to interfere with the Smad3-mediated transcription of TGF-β1-induced genes such as the α2(I) pro-collagen gene (COL1A2) which is responsible for generating type I collagen [44]. The YB-1 box can also upregulate inhibitory Smad 7 expression [44]. Only a few factors have been identified that independently induce fibroblast differentiation. Interleukin-6 (IL-6) is another factor that has been recognized to induce fibroblast transformation into myofibroblasts. IL-6 null mice have been reported to demonstrate impaired cutaneous wound healing and decreased fibroblast differentiation at unchanged levels of TGF-β1 [47]. Proteases such as MMP 2, MMP 9, and plasmin have been reported to activate TGF-β [48].

Activated TGF-β binds to heteromeric type I and II TGF receptors (TGFβRI and TGFβRII) which activates a cascade of intracellular signals through the phosphorylation of TGFβRI associated Smad2 and 3 [49]. The C-terminal serine residues are phosphorylated by TGFβRI kinase, activating Smad2 and 3 and allowing for the nuclear translocation and binding of protein Smad2 and protein Smad3 to the nuclear transcription factors (TF) and DNA to control the expression of downstream genes [49]. The activation of TGF-β dependent Smad2/3 pathway in rat cardiac fibroblast cells contribute to the development of fibrosis, where ECM proteins are produced and fibroblasts transform into myofibroblast cells [50]. TGF-β stimulation of fibroblast to myofibroblast conversion and its increased collagen production is irreversible even after the removal of TGF-β [51]. Interestingly, the suppression of α-SMA expression results in a reduced collagen gene expression [51].

Myocardial infarction

After a myocardial infarction, fibrotic tissues are formed to replace what has been lost following necrosis of the cardiomyocytes. The balance of this process is important as

a lack of fibrosis after a myocardial infarction can lead to wall thinning and ECM deficiencies, whereas the opposite can lead to excessive fibrosis and myocardial stiffness. The fibrotic response that occurs is classified into two categories including reparative and reactive fibrosis which are mediated by both the cardiac fibroblasts and myofibroblasts [52]. The process of reparative fibrosis involves the formation of scar tissue to prevent the rupturing of the ventricular wall while the reactive fibrosis can lead to exaggerated fibrotic response and impaired cardiac function. During a myocardial infarction, the oxygen depletion that occurs causes death of the cardiomyocytes which in turn stimulates a series of events in order to reduce the damage to the heart. In embryonic and neonatal rodents, they have the capability to regenerate an injured area within the heart by replacing it with a fibrin clot and temporary collagen based scar through inflammatory responses, rise of myofibroblasts, and neovascularisation [53]. However, in adult mammals, this ability is lost, and therefore, dead cells are replaced with permanent collagen scars. These scars are often inflexible and can cause the heart to pump inefficiently, which then leads to heart failure.

The processes in damaged tissue that occurs following a myocardial infarction can be divided into three phases: the inflammatory phase, proliferative phase, and the maturation phase. The inflammatory phase is initiated by necrosis occurring in the cardiomyocytes. As surrounding cells such as the interstitial fibroblasts, endothelial cells, and masts cells are much more resilient to ischemic injury in comparison to the cardiomyocytes, they are thought to be the effector cells that respond to injury to initiate inflammation [12]. The resident fibroblasts produce matrix metalloproteinases (MMPs) to breakdown the ECM to allow for cell migration to the site of injury.

In experimental models of myocardial infarction, levels of the pro-inflammatory markers such as TNFα, IL-1β, and IL-6 are increased, though their exact functional role is not known. However, IL-1 is thought to control fibroblast phenotype and delay its conversion to myofibroblast cells until dead cells are cleared and ready for new ECM [54].

As the transition occurs from the inflammatory phase towards the proliferative phase, inflammation is repressed by inhibitory molecules [55]. During the proliferative phase, cardiac fibroblasts become the dominant cell type. The fibroblasts are activated by reactive oxygen species (ROS) to enhance MMP activity and decrease ECM synthesis. Interestingly, however, in vivo studies have revealed that ROS stimulates collagen synthesis in rat cardiac fibroblasts. During the proliferative phase, many fibroblasts convert to myofibroblasts. The infarct border zone is infiltrated by myofibroblasts. Fibroblasts and myofibroblasts secrete ECM proteins in the infarct border zone and proceed towards the center of the injury site. In most species including rat, mouse, rabbit, and canine experiment models of myocardial infarction, the existence of myofibroblasts in the infarct zone is a prominent characteristic and is also present in human myocardial scars. Myofibroblasts initially produce collagen type III, EDA-containing cellular fibronectin (EDA-FN), thrombospondin (TSP), and tenascin C to promote migration of more myofibroblasts and regulation of the healing response [56]. Myofibroblasts are supplied with oxygen and nutrients during the repair process via angiogenic signals stimulating microvasculature formation in the infarct area. As the collagen scar is established, most myofibroblasts undergo cell death possibly though apoptosis and the lack of growth factors required for their survival.

Finally, during the maturation phase, collagen type III is replaced with collagen type I. Crosslinking of the collagen fibers also occurs though lysyl oxidase (LOX) [52]. Unfortunately, cardiac myofibroblasts persist within the heart for many years after the initial infarct, and the reasoning behind this remains to be elucidated. The exact mechanisms and the regulation of the reactive phase of fibrosis are not clearly understood.

Inflammation

Inflammation plays an important role in the wound healing and repair in the heart. Fibroblasts have been shown to express toll-like receptors (TLRs) which are pattern recognition receptors, and the activation of these receptors can lead to the activation of fibroblasts and stimulate their conversion towards the myofibroblast phenotype [57]. Both the innate and adaptive immune responses play a role in fibrosis in the heart. In the innate system, platelets express a number of TLRs and have been implicated in the cardiac inflammatory response. Platelets are a source of TGF-β; however, macrophages are a more potent TGF-β producer. Macrophages are a main source of MMPs including MMP-1, -7, -8, and -9. MMP-9 is the most pro-fibrotic where the inhibition or deletion of the MMP-9 protein showed decreased fibrosis in dilated cardiomyopathy in infarcted hearts. Patients with heart failure have been shown to have decreased MMP-1 and elevated TIMP-1 (metalloproteinase inhibitors) expression which promotes collagen production within the heart [57]. The adaptive immune response involves the T cells and mice deficient of T cells have shown to develop fibrosis [57]. The T helper 2 cell release cytokines including interleukin (IL)-4, IL-5, and IL-13. IL-13 is a noted mediator of fibrosis and together with IL-4 is able to contribute to the conversion of fibroblast to myofibroblast phenotype in a c-Jun NH_2-terminal kinase-dependent manner [57]. Furthermore, IL-13 has been reported to inhibit fibroblast MMP activity to decrease ECM degradation and subsequently increase collagen accumulation. The T helper 1 cells secrete IFN-γ, which interestingly have been shown to have both pro-fibrotic and anti-fibrotic effects. IFN-γ has pro-fibrotic properties by producing both pro-inflammatory and pro-fibrotic molecules including TNF-β; however, the exact contribution of IFN-γ to the differentiation of cardiac fibroblast to myofibroblast is not known [57].

IFN-γ has anti-fibrotic effects and in combination with IFN-α have a potent ability to inhibit collagen production. In a mouse model of lung fibrosis, IFN-γ reduced the overexpression of TGF-β and thus decreasing collagen levels. The actions of TGF-β are reduced through the Jak/STAT1 pathway, which induces the expression of SMAD7 and the phosphorylation of SMAD3 to prevent TGF-β from signaling the nucleus [57]. IFN-γ also has the ability to inhibit IL-4 and IL-13 release to promote fibrocyte to myofibroblast conversion [57].

ROS

In human cardiac fibroblast cells, an increase in ROS from NAD(P)H oxidase 4 (NOX4) increases TGF-β mediated activation of SMAD 2/3 to indicate a potentially important role for NOX4 in TGF-β stimulated conversion of fibroblast to myofibroblast phenotype. ROS can act as a secondary messenger molecule to regulate a variety of signaling cascades. A chronic increase in ROS has been linked to cardiac remodeling and heart failure. Bai and colleagues [58] reported that angiotensin II (Ang II) significantly increased the production of intracellular ROS, and it has been hypothesized in the past to be a possible contributing factor for fibroblast to myofibroblast differentiation. In the cardiovascular system, ROS is generated through Ang II activation of NADPH oxidase, although the exact mechanism of how this occurs in cardiac fibroblasts remains not well understood [58]. Past research have shown that Ang II acts to stimulate membrane-bound nicotinamide dinucleotide phosphate (NADPH) oxidase to generate ROS in cardiac fibroblast cells [59, 60]. In cultured cardiac rat fibroblast cells, AngII stimulated the production of soluble collagen, an effect that was attenuated by NADPH oxidase inhibitors (apocynin and diphenyleneiodinium (DPI)) [60]. There are five identified isoforms of the Nox catalytic subunit, Nox1, Nox2, Nox3, Nox4, and Nox5. NADPH oxidase is commonly composed of membrane-bound Nox, p22phox, and cytosolic subunits p40phox, p47phox, and p67phox with GTPase Rac [61]. It has been postulated that the Nox4 NADPH oxidase isoform is most likely to be involved in TGF-β-induced fibrosis [61] due to a previous study finding that TGF-β increased the gene expression of Nox4 in human cardiac fibroblast cells without an increase in the expression of the other Nox isoforms [61]. It was also reported that Nox4 may be involved in the conversion of fibroblast cells to myofibroblast cells when a siRNA (silencing RNA) was used to target Nox4, which suppressed the expression of TGF-β target genes such as α-smooth muscle actin and pro-collagen I [61]. Interestingly, NADPH oxidase may have a potential role in fibrotic effects mediated by TGF-β. In cardiac fibroblasts, it was seen that TGF-β-induced Smad 2/3 activation was reduced by anti-oxidant agents and through Nox4 gene silencing. Furthermore, there has been speculation that age may play an important role for Nox4. Nox4-induced wound healing has beneficial roles in the young; however, in the aged population, Nox4 can prompt fibrosis formation due to a persistent presence of myofibroblast cells [62].

Mechanical tension

Mechanical tension of the cardiac tissue has been widely accepted to be a contributor to the conversion of fibroblast to myofibroblast phenotype. The increased mechanical stress in the non-infarcted left ventricular wall can induce the activation of TGF-β in the myocardium [52]. The cardiac fibroblast interacts with the extracellular matrix via integrins [32]. Cardiac fibroblast cells have integrins αvβ3 and αvβ5 that activate latent TGF-β. During HF, a series of neurohormones are released to compensate the failing heart as follows; Ang II increases the production of aldosterone, which in turn causes fluid retention and pressure overload. AngII is also produced when mechanical tension occurs, which can also stimulate the TGF-β signaling pathway [48]. AngII binds to two types of receptors, angiotensin receptor 1 (AT_1) and angiotensin receptor 2 (AT_2). Stimulation of the AT_1 increases blood pressure and sodium water retention. However, the AT_2 has not been characterized fully but is believed to be counteractive of the effects caused by the AT_1 [63]. AngII binding to AT_1 causes the phosphorylation of Smad2 and Smad3 via the ERK/p38/MAPK pathway. Activated Smad2/3 forms a complex with Smad4 to translocate into the nucleus to allow transcription of target genes including TGF-β, procollagen I, and fibronectin [63]. In vivo studies have demonstrated a positive relationship, where an increase in mechanical stress resulted in an elevated expression of α-SMA and ED-A fibronectin, which are indicative of fibroblast to myofibroblast conversion [32]. α-SMA expression was observed in neonatal cardiac fibroblast cells that were grown in serum media and subjected to mechanical tension following the phosphorylation of extracellular signal-regulated kinases (ERK) and activation of the mitogen-activated protein kinases (MAPK) cascade [64]. Moreover, α-SMA expression in fibroblasts did not occur in serum-free media when mechanical force was also applied. It was also reported that cardiac fibroblasts cultured on agar coated with collagen resulted in no α-SMA expression; however, when cultured on plastic collagen-coated plates, α-SMA expression was observed, thus indicating conversion to the myofibroblast phenotype [64]. This research suggests that the environment of the cardiac fibroblast cells can influence the degree of mechanical tension on fibroblast cells and is an important factor in the conversion to the myofibroblast phenotype.

Aging

Aging is an inevitable life process and is a major independent risk factor contributing to morbidity and mortality in

cardiovascular-related diseases. There are many factors that contribute to changes in the heart as an individual ages. It is well established age contributes to the progression towards cardiac fibrosis due to an increase in collagen and inflammation in the heart. As hearts age, changes include increased cell apoptosis, cell senescence (particularly in cardiomyocytes), an increase in cardiac fibroblast numbers, low inflammation, and accumulation of collagen. In the human heart, collagen content is said to increase by 50% in individuals < 65 years compared to individuals in their 20s [65], thus contributing to cardiac fibrosis. Cieslik and colleagues postulated that fibroblasts of different myeloid and mesenchymal origins crosstalk with each other in the development of fibrosis in aging hearts. The CD45+ fibroblast cells of myeloid origin were identified to be involved in the development of adverse fibrosis when caused by ischemia reperfusion induced cardiomyopathy, angiotensin II infusion, or aging [66]. MCP-1 expression on fibroblasts was also reported to be an important contributor to the development of cardiac fibrosis, whereby the genetic deletion of MCP-1 or receptor knockout resulted in no cardiac fibrosis. In mice, a progressive increase in the expression of MCP-1 along with greater numbers of myeloid fibroblasts were observed in aging hearts [66]. Interestingly, periostin is also highly expressed in aged rat [67] and human [68] hearts. Increased periostin expression in humans has been linked to greater levels of myocardial infarctions, myocardial hypertrophy, and dilated cardiomyopathy [68]. Aging also causes physiological changes in both human and rat hearts as there is increased afterload, impaired vasodilation, and increased left ventricular wall stress [69].

In vivo seeding fibroblasts at low density

In cell culture (in vivo), it was found that seeding fibroblasts at low density can cause them to convert into the myofibroblast cell phenotype [70], suggesting that the lack of cell-to-cell contact can contribute to fibroblast to myofibroblast conversion and TGF-β alone is not sufficient. In cells that were plated at low density, 70% of cells stained α-SMA positive while those stained at intermediate and high densities were 35 and 3%, respectively [70]. The myofibroblast phenotype of these cells, however, was found to be in a reversible state, whereby if the myofibroblast cells were trypsinized and seeded at a higher density, they reverted back towards the fibroblast phenotype.

Biomarkers for identifying cardiac fibroblast and myofibroblast cells

Specific biomarkers to delineate between cardiac fibroblasts and myofibroblasts have been difficult to determine. Recent research has created an interest in these cells they have been proven to be more than mere "structural cells" and so efforts have been to specifically and correctly identify these cells through various biomarkers. Cardiac fibroblasts have no known specific cell markers to distinguish it from other fibroblasts and as a result, are characterized as a uniform cell type. Recent studies compared the gene expression of 50 fibroblast cell lines from various origins to demonstrate the diversity among them [71]; however, it is challenging to define a cardiac fibroblast as there is still a lack of an identified definitive cell-specific marker. A summary of the current known biomarkers in determining the cardiac fibroblast and myofibroblasts can be seen in Table 1. A commonly used cell marker is vimentin, which labels fibroblasts with great sensitivity [8]. Vimentin was originally used as an endothelial cell marker. Although vimentin can positively identify fibroblasts, it also positively identifies macrophages and endothelial cells. As vimentin is not specific to cardiac fibroblast cells, other markers such as CD31 (endothelial cells) and CD45 (immune cells) are commonly used in combination with vimentin to distinguish fibroblasts from other cell types [72]. Vimentin has also been demonstrated in the cardiac myofibroblasts and therefore cannot be used to distinguish this cell type from fibroblast cells.

Another potential marker for cardiac fibroblast cells is the discoidin domain receptor 2 (DDR2), a collagen receptor that has been used to identify and distinguish cardiac fibroblasts [4]. The DDR2 receptor is present on the cell surface, and mediates a large range of cellular functions including growth, migration, and differentiation [8]. The DDR2 receptor is also present in myofibroblasts and other cells; therefore, again, it cannot be used to differentiate between other cells unless a combination of markers is used. Interestingly, the DDR2 marker is detected in both mice and rat hearts and is not expressed in myocytes or endothelial cells [16].

The fibroblast-specific protein 1 (FSP-1) was proposed to be a fibroblast marker; however, despite its name, this marker has been shown to be expressed on other cell types such as smooth muscle cells, endothelial cells, myocytes [73], cancer cells, leukocytes [8], immune cells, and endothelial cells [72]. The FSP-1 protein is only expressed in a subset of cardiac fibroblasts in the normal heart and is now considered a non-specific marker, those that use FSP-1 as a fibroblast marker with a combination of other markers to correctly distinguish it from other cells. A study by Souders and colleagues [8] has identified cadherin-11 as another potential fibroblast marker as it has so far been observed to be localized to fibroblasts and is thought to also have an important role in vascular remodeling. The expression of cadherin-11 in myofibroblasts is unclear, and therefore, it is unknown whether this marker can be used as to differentiate between cardiac fibroblasts and myofibroblasts.

Transcription factor 21 (Tcf21) has been found to be expressed in cardiac fibroblasts [73]. Immune cells positive for CD45 do not express Tcf21. Tcf21 cannot be used alone

Table 1 A summary of current known markers used to determine cardiac fibroblast and myofibroblast cells

Biomarker	Location	Role	Fibroblast	Myofibroblast	Expression in other cells	References
Alpha smooth muscle actin	Cytoskeletal	Cell contraction	No	Yes	Vascular smooth muscle cells and pericytes	[26, 75, 83, 84]
Angiotensin 1 (AT1) receptors	Surface	Initiates intracellular signaling pathways involved the renin angiotensin system	Yes	Yes, increased expression	Smooth muscle cells	[75, 85]
Cadherin-11	Surface	Mediate cell-to-cell interaction to regulate tissue morphogenesis, endothelial to mesenchymal transition, mesenchymal-epithelial transition, and cell rearrangement	Yes	Unknown	Mesenchymal cells and osteoblasts	[84, 86]
CD31	Surface	Adhesion molecule	No	No	Endothelial cells	[81]
CD45	Surface	Signal transduction in immune cells	No	No	Immune cells	[6]
Desmin	Cytoskeletal	Type III intermediate filament that integrates with the sarcolemma	No	No	Smooth muscle cells	[26]
Collagen1a1-GFP	Transgene	Targets collagen I matrix producing cells	Yes	Unknown/not tested	Endothelial and vascular smooth muscle cells	[86, 87]
DDR2	Surface	Growth, migration, and differentiation	Yes	Yes	Endothelial and vascular smooth muscle cells	[26, 74, 84, 86]
ED-A fibronectin	Secreted	Adhesive glycoprotein	No	Yes	Smooth muscle cells, endothelial cells, and macrophage	[26, 72, 75]
Fibroblast specific protein 1 (FSP-1/S100A-4)	Cytosolic	May function in motility and tubulin polymerization	Yes	Unknown/not tested	Vascular smooth muscle cells, endothelial cells, leukocytes, and cancer cells	[32, 84, 86]
Frizzled-2	Surface	Involved in tissue planar polarity during embryonic development	Yes	Yes, increased expression	Vascular smooth muscle cells and thoracic arterial system	[75, 84]
Osteopontin	ECM	Acidic phosphoprotein adhesion molecule: vascular smooth muscle remodeling and thought to contribute to fibroblast to myofibroblast differentiation	Yes	Yes, increased expression	Bone (osteoblasts and osteocytes), ovarian carcinomas, and dendritic cells	[76, 88]
Paxillin	Surface	Focal adhesion adaptor protein involved in recruiting signaling proteins	No	Yes, increased expression	Epithelial cells	[4, 75]
Periostin	ECM	Cardiac development, remodeling, cell trafficking, and ECM organization	No (not expressed in resting fibroblast cells, but is expressed in development)	Yes	Osteoblasts and cancer cells (glioblastomas)	[79, 89]
Platelet-derived growth factor receptor α (PDGF α)	Surface	Enhancing migratory and proliferative responses and extracellular matrix (ECM) synthesis [90]	Yes	Unknown/not tested	Platelets	[50]
Tenascin C	ECM	Upregulated in inflammation and tissue remodeling	No (not detectable in normal adult hearts but is expressed	Yes, increased expression after MI	Smooth muscle cells, neurons, glial cells, and breast carcinoma	[75]

Table 1 (continued)

Biomarker	Location	Role	Fibroblast	Myofibroblast	Expression in other cells	References
			during embryonic development)			
Tensin	Intracellular	Expressed during wound healing	No (not completely confirmed)	Yes, increased expression	Mesangial cells (kidney), myocytes, and most cells in the body	[75]
Thymus antigen 1 (Thy 1/CD 90)	Surface	Cell matrix and cell-to-cell adhesion	Yes	No	Endothelial cells and leukocytes	[86]
Transcription factor (Tcf) 21	Nuclear	Embryonic development	Yes	Yes, Tcf21-positive fibroblasts can convert to myofibroblasts	Smooth muscle cell lineages	[86]
Transforming growth factor-βeta type II receptor	Surface	Regulates transcription of TGF-β responsive genes	Yes	Yes, increased expression	Myocyte cells and endothelial cells	[75]
Vimentin	Cytoskeletal	Motility and cell shape	Yes	Yes, increased expression	Endothelial and vascular smooth muscle cells	[47, 55, 74, 75]
Wilms tumor (WT) 1	Nuclear	Cardiac development	Yes	Unknown	Endothelial cells and epicardial cells	[72, 74]

and requires the use of other markers as it will only identify a subset of cardiac fibroblasts [73]. Using Tcf21 as an identification marker is also problematic as it is a transcription factor that acts in the nucleus [74].

Angiotensin 1 receptor (AT 1), transforming growth factor-βeta type II receptor, paxillin, tensin, and fibronectin extra dominant A splice variant are other markers whose expression has been reported to be increased in cardiac myofibroblast cells and may be used in combination with other markers to successfully confirm the myofibroblast phenotype [75]. Frizzled-2 is also another potential marker for cardiac myofibroblasts as expression levels increase in damaged areas of the heart. However, they are also expressed in cardiac fibroblasts and again, must be used with other markers for proper identification.

Collagen1a1-GFP is a reporter line that can be used to identify cardiac fibroblasts and is co-expressed along with platelet-derived growth factor receptor α (PDGF α) [72]. In combination, these markers identify most cardiac fibroblasts. The advantage of using PDGF α as a biomarker is that it is capable of being expressed in fibroblasts regardless of its development and disease state. Important to note, PDGF α is also broadly expressed during embryonic development in mesenchymal tissue [72]. The expressions of collagen1a1-GFP and PDGFα in cardiac myofibroblasts remain unclear and are yet to be determined.

Osteopontin was originally thought to be only involved in bone mineralization; however, it has now been accepted that osteopontin to have a role in post-infarcted hearts by orchestrating the signaling of myofibroblast proliferation, migration, and ECM deposition. In a study conducted in mice, it was reported that cardiac fibroblasts from osteopontin null expressing mice were unable to convert to myofibroblasts even when stimulated with TGF-β [76]. Osteopontin is expressed in both fibroblasts and myofibroblasts, though it is highly expressed in the myofibroblasts and may be useful as another potential marker to differentiate between the two [76].

Tenascin C is a molecule expressed in the wound-healing process of various tissues and only expressed during pathological conditions. Previous research in rat models showed the expression of α-smooth muscle actin (myofibroblasts) co-located with tenascin C-positive areas of the heart after a myocardial infarction [77]. Tenascin C is also observed to be highly expressed in myofibroblasts and may be a useful marker in confirming the myofibroblast phenotype. Unfortunately, tenascin C is also expressed in other cell lines such as smooth muscle cells, and therefore, another marker such as desmin is required to check for smooth muscle cell populations.

Periostin is a relatively new potential marker that may be used to identify cardiac myofibroblasts and has recently generated a large amount of interest. Periostin promotes the recruitment of myofibroblasts and increases in collagen production. Mice that lacked periostin demonstrated an increased

prevalence of cardiac rupture following a myocardial infarction [78]. A study has reported very little periostin expression in normal hearts and interestingly, increased mRNA expressed periostin in failing hearts [79]. Periostin is expressed during development in cardiac fibroblasts; however, it is not seen in resting fibroblasts [74]. Periostin can reappear after cardiac injury [80] exclusively in myofibroblasts as shown through genetic studies (refer to Kanisicak et al. [81] for more detail). Due to this, periostin has been noted as a marker of cardiac myofibroblasts in conjunction with other markers.

By far, the most commonly used standard marker for the identification of the cardiac myofibroblasts still remains to be the expression of the α-smooth muscle actin [26, 27, 82]. This protein is not found in cardiac fibroblasts; it appears following stressful cardiac conditions and marks the transdifferentiation of fibroblasts to the myofibroblasts phenotype. At this current stage, the use of the α-smooth muscle actin marker and other markers such as CD45 and CD31 are used in combination to correctly identify myofibroblasts.

It is important to note that the thymus cell antigen-1 (Thy-1/CD90), DDR2, Tcf21, periostin, cadherin-11, and fibroblast-specific protein-1 (FSP-1/S100A4) have all been used in research as markers to study cardiac fibroblasts, though they are only able to label a subset of fibroblasts from different origins. Wilms tumor 1 (WT1) is seen as a fibroblast lineage marker. WT1 alone is not sufficient to confirm fibroblast identity, although together with other cardiac fibroblast markers, this can also assist in their identification.

Table 2 Currently known and future potential targets of heart failure

Targets	Drug name	Mechanism of action	Observed effect	Ref
Known targets clinically trialed in humans				
ACE inhibitors	Lisinopril	Inhibits angiotensin-converting enzyme	Significant reduction in collagen volume fraction and hydroxyproline concentration (collagen protein) implying regression in fibrosis	[48]
		Inhibits angiotensin-converting enzyme	In biopsies taken at baseline and after 6 months, treatment with lisinopril (ACE inhibitor drug) in hypertensive patients showed a decrease of collagen volume group	[95]
Angiotensin II	Lorsartan	Selective competitive inhibition of angiotensin II	Patients who were administered losartan for a period of 12 months showed a decrease in collagen volume fraction and left ventricular stiffness in hypertensive patients with severe fibrosis	[96]
		Selective competitive inhibition of angiotensin II	Patients with losartan twice daily for 1 year and cardiac magnetic resonance imaging at baseline and 1 year after. Results showed that losartan effectively mitigated cardiac fibrosis progression and cardiac hypertrophy in patients with non-obstructive hypertrophic cardiomyopathy	[97]
Smad 2/3	Follistatin therapy	The follistatin therapy is being investigated through gene therapy techniques	Follistatin that is locally expressed was seen to be sufficient to reduce fibrosis in improve muscle function in cardiac myopathy. Unfortunately, there are other effects seen in the heart, and therefore, clinical trial with follistatin is currently on hold	[98]
Potential targets that have not yet been trialed in human clinical trials				
TGF beta	Tranilast	Suppresses the action of the TGF-β pathway by inhibiting TGF-β	Tranilast was seen to attenuate collagen production in cell culture models. In diabetic rat models, the administration of tranilast was able to reduce pathological fibrosis	[99]
			In a renovascular hypertension rat model, the long-term treatment of rats with tranilast resulted in a significantly attenuated left ventricular fibrosis	[100]
MicroRNA-130a	miR-130a	Not well understood; however, it may downregulate Smad-4 in the TGF-β pathway	Inhibition of miR-130a decreased cardiac fibrosis, collagen expression, and myofibroblast differentiation in mice models	[93, 101]
MicroRNA-15	miR-15	Potentially inhibits the TGF-β pathway	miR-15 is upregulated in human end-stage hypertrophic cardiomyopathy and may potentially be used to inhibit the TGF-β pathway for a more favorable outcome	[102]
Anti-oxidant	N-acetylcysteine	N-acetylcysteine is a precursor to glutathione, which is seen to be decreased in heart failure	Blunted cardiac fibrosis and remodeling by reducing oxidative stress in a mouse model	[103]

Clinical relevance

Identifying cardiac fibroblast and myofibroblasts cells and understanding their contribution to the development of heart failure may provide better treatment options. There have been many promising results in experimental models for potential heart failure treatments; however, in the clinical domain, clinical trials to reduce cardiac fibrosis have had mixed responses. Table 2 summarizes a list of the known and potential targets that may be useful for altering fibroblast and collagen deposition in heart failure treatment. Currently, ACE inhibitors remain the most commonly prescribed drug of choice for the treatment of heart failure. The use of ACE inhibitors is effective in heart failure treatment; however, there are unwanted adverse reactions such as coughing, reduced renal function, and gastrointestinal discomfort [91]. As a result, more specific heart failure targets involved in cardiac fibrosis are under investigation and include TGF-β_1 and microRNA-130a. TGF-β_1 blockade in animal ischemic heart models have shown an improvement in cardiac function and recovery [92] . Interestingly, the timing of TGF-β_1 inhibition is important. In previous research, inhibition of TGF-β_1 mediated actions before or immediately after a myocardial infarction resulted in increased mortality and cardiac dysfunction, whereas inhibition 24 h after the myocardial infarction resulted in better function and decreased cardiac remodeling [92]. Another potential target for heart failure treatment is reducing microRNA-130a levels which have only recently been described. MicroRNA-130a in the past has been shown to participate in angiogenesis and cardiac arrhythmia; however, it has now been implicated in cardiac fibrosis [39]. Angiotensin II was infused into mice to induce cardiac fibrosis, and an increased expression of microRNA-130a was observed [92]. Interestingly, upregulation of microRNA-130a is also observed in failing human hearts [93]. In this study, it was ascertained that an overexpression of microRNA-130a resulted in increased pro-fibrotic gene and myofibroblast phenotype expression. Moreover, when microRNA-130a was inhibited, a decrease in pro-fibrotic gene expression and transformation to myofibroblast was observed [21]. If these targets show promise, they have the potential to change the current treatment of heart failure and more specifically the development of cardiac fibrosis. Unfortunately, not all targets that are successful in experimental models are effective in clinical trials. An example of this occurred with TNF-α which is known to play a vital role in the development of cardiac fibrosis. In a study by Mann and colleagues [94], the effects of a TNF-α antagonist (infliximab) were examined in patients with HF. It was found to negatively affect patients with chronic HF with the authors suggesting that a loss of the beneficial traits of cytokine signaling was the cause.

Conclusions

The lack of definitive markers for cardiac fibroblast and myofibroblast cells makes it difficult to study and understand the exact mechanisms of these cells, how they function, and contribute to the development of heart failure. It is clear that fibroblasts have proven to be more than just structural cells and myofibroblasts have a pivotal role in cardiac fibrosis. We also reviewed the potential markers available for characterizing these cells and found that there is no single cell-specific marker that delineates fibroblast or myofibroblast cells. To characterize the cells of fibroblast origin, vimentin is commonly used. Cardiac fibroblasts can be identified using discoidin domain receptor 2 (DDR2) while α-smooth muscle actin is used to distinguish myofibroblasts. Markers such as angiotensin receptor 1 (AT 1), transforming growth factor-β type II receptor, paxillin, or tensin expression increase in cardiac myofibroblast cells. Finally, periostin is emerging as a potential marker for myofibroblasts. In order to study fibroblasts and myofibroblasts as accurately as possible, it is recommended that a combination of currently available markers to be used and a global recommended standard to be placed to ensure that research is being conducted in the same cells. In the future, implementing proteomics research may aid the identification of cardiac biomarkers for HF would allow for a more tailored approach for treatment. It is well established that the activation of fibroblast cells and their transformation to myofibroblasts is mediated by TGF-β_1 and that both cell types contribute to fibrosis and impaired heart function. Given that multiple factors contribute to the release and actions of TGF-β_1 in the pathogenesis of heart failure, recognition of these contributors provides opportunities to moderate the actions of TGF-β_1.

Box 1: Key summary points

- Cardiac fibroblast cells have no cell-specific marker for identification, and as a consequence, research within its field is often challenging.
- Cardiac fibroblasts can be triggered to transform into myofibroblast cells under numerous circumstances.
- There is a major need for identifying potential cell-specific markers for cardiac fibroblast and myofibroblast cells which will allow for new potential targets for the treatment of heart failure.
- Most current treatment targets available for heart failure/cardiac fibrosis are broad acting and not recommended for long-term use as they can affect the patient's quality of life due to unwanted side effects.
- TGF-β and microRNA-130a have potential as new targets in cardiac fibrosis and heart failure treatments in the future; however, further research must be conducted.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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