

# Cardiac myofibroblast and fibrosis

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**Abstract:** Fibroblasts are differentiated to myofibroblasts and produce collagen and other extracellular matrix when the heart is exposed to stresses. Myocardial infarction and pressure overload-induced hypertrophy are major stresses to induce differentiation of fibroblasts. Since collagen can compensate the missing tissue due to injury, appropriate production of collagen is beneficial for the injured heart against rupture. However, excessive deposition of collagen is called fibrosis and causes cardiac dysfunction. After fibroblasts are differentiated to myofibroblasts, myofibroblasts can further change their phenotypes. In addition, myofibroblasts are found to have a new function other than collagen production. Myofibroblasts have macrophage-like functions that engulf dead cells and secrete anti-inflammatory cytokines. So far, research on fibroblasts has been delayed due to the lack of available markers for selective isolation of fibroblasts. In recent years, it has become possible to genetically label fibroblasts, sequence the cells at single cell levels, and manipulate function or the number of cells. Based on new technologies, the origin of fibroblasts and myofibroblasts, time-dependent changes of fibroblast states after injury, and heterogeneity have been demonstrated. Here, I will introduce recent advances in fibroblasts and myofibroblasts.

**Keywords:** myofibroblasts; fibrosis; heart failure

## Introduction

Cardiac fibrosis is defined as the state with excess extracellular deposition of collagens and extracellular matrix [1]. It occurs when the heart is exposed to stresses such as ischemic injury and chronic high blood pressure. Since fibrosis causes cardiac dysfunction, it is a target for treatment with drugs, medical devices or tissue transplantation. Collagen and extracellular matrix are produced by myofibroblasts that are differentiated mainly from resident fibroblasts. Manipulation of activity and number

of myofibroblasts is proposed to be important for inhibition of progression to more severe fibrotic states or recovery from fibrotic state [2]. Since myofibroblasts are an important player in inflammation and fibrosis after cardiac injury [3], it is an urgent need to understand the origin, function and fate of myofibroblasts. Recent technological advances reveal some of these issues. progress of research topics for fibroblasts, myofibroblasts and fibrosis will be reviewed.

## **1. Classification of fibroblasts**

There are several types of cells in the heart such as cardiomyocytes and immune cells. They interact with each other to regulate homeostasis in healthy and diseased conditions [4]. Among several types of cells, fibroblasts are unique, since they produce extracellular matrix that supports morphological integrity at resting state. Recent histology-based and flow cytometric methods have demonstrated that fibroblasts account for about 13% of cells in the mouse heart [5, 6]. When the heart is exposed to injury such as myocardial infarction and hypertrophy, fibroblasts differentiate into myofibroblasts and produce extracellular matrix. Myofibroblasts are only the cells that produce extracellular matrix. Excess deposition of extracellular matrix causes fibrosis leading to tissue dysfunction. It has been recognized that management of the number or function of myofibroblasts is important for treatment of fibrosis.

Origin of myofibroblasts is analyzed by labeling the various types of cells with reporter genes under cell-specific promoter (lineage-tracing experiment) [7]. Genetic labeling has advantage over immunological detection of marker proteins, since marker proteins sometimes disappear during development or differentiation. In addition, marker proteins are expressed in not only cells that are analyzed but also functionally irrelevant cells. Lineage tracking experiment is to express reporter gene under the control of cell-type specific promoter. It labels the cells permanently even after the promoter activity turns off and then allows to trace the cells that promoter is once activated.

Lineage-tracing experiments showed that about fibroblasts present in the left ventricle and ventricular septum are derived from endocardial cells via endothelial-mesenchymal transition (EndoMT) and epicardial cells through epithelial-mesenchymal transition (EMT) [8]. A small number of fibroblasts were generated by differentiation of neural crest cells. However, mature endothelial cells, epicardial cells, or bone marrow-derived cells

did not contribute to population of fibroblasts. After labeling fibroblasts using cell-type specific promoter-reporter gene, it was investigated whether fibroblasts exhibit different functions depending on their origin. After pressure overload, epicardial-derived fibroblasts (labeled with Tbx18 promoter-GFP) and endocardial-derived cells (labeled with Tie2 promoter-GFP) were isolated. Analysis of RNAs showed similar expression profiles between epicardial-derived and endocardial-derived cells [9-11]. In addition, these two groups of cells had similar proliferative activity [12]. Therefore, it was concluded that there is no significant relationship between function of fibroblasts and origin of fibroblasts.

Molkentin's group used genetic labeling technique to trace the changes in the characteristics of fibroblasts over time after myocardial infarction [13]. They found that fibroblasts change their properties four different states after myocardial infarction, that is, resting fibroblasts, active fibroblasts, myofibroblasts and matrifibrocytes (Figure 1).

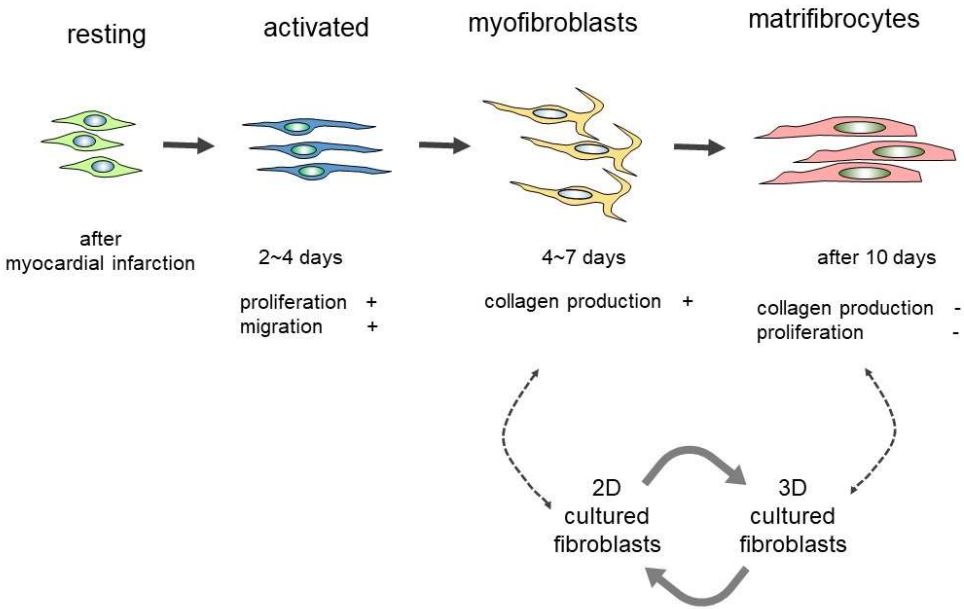


Figure 1: Differentiation of fibroblasts to myofibroblast and matrifibrocytes. Fibroblasts of each state have different proliferating activity and function. Dotted line is not established pathway.

Tcf21 promoter can label fibroblasts of resting conditions, which is tissue-resident fibroblasts. When proliferative activity of fibroblasts was measured by feeding mice with

5-ethynyl-2'-deoxyuridine (EdU) or immunodetection of Ki-67 after myocardial infarction, they could detect actively proliferating fibroblasts 2 to 4 days after myocardial infarction (active fibroblasts), and also found that fibroblasts convert to myofibroblasts 4 to 7 days after myocardial infarction. Myofibroblasts were derived from tissue-resident fibroblasts. Their findings are consistent with other studies. Myofibroblasts were transformed to new type of cells, which is called matrifibrocytes, 10 days after myocardial infarct. Analysis of the expressing mRNAs suggested that each cell has different properties. Fibroblasts of active state had high proliferative and migration activities. Myofibroblasts produced collagen and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). Matrifibrocytes are unique, since they localized at scar and express the genes detected in tendons, bones and cartilage. The physiological implications of these bone-related genes in the heart are not known. Since matrifibrocytes localize only at scar, it suggests that they play a special role in scar formation or maintenance. Matrifibroblasts have several unique properties. When diphtheria toxin was administrated to mice with the toxin receptor expressing in myofibroblasts, matrifibrocytes were resistant to killing by the toxin [13]. Susceptibility to diphtheria toxin treatment was different from resting and active fibroblasts. In addition, matrifibrocytes did not show proliferative activity when mice were treated with angiotensin II and phenylephrine. In this context, Kim's group reported an interesting finding. In diabetes, inter- $\alpha$ -trypsin inhibitor heavy chain 1 (ITIH1) secreted from liver was found to be responsible for systemic glucose intolerance. It blocked insulin action on adipose tissue and skeletal muscle (14). ITIH1 works as a glue to tighten the binding between extracellular matrix. Anti-ITIH1 neutralizing antibody releases the inhibition of ITIH1 and recovered insulin sensitivity. Inaccessibility of diphtheria toxin to periostin-expressing cells at the late stage of fibrosis may be caused by ITIH1 or ITIH1-like molecule. Future studies are waited to reveal the roles of matrifibrocytes in cardiac fibrosis.

## 2. Heterogeneity of fibroblasts

Single-cell RNA sequencing (scRNAseq) is a relatively new but rapidly developing technology [15]. It allows to comprehensively characterize gene expression and relationships between individual cells. Single-cell analysis of 11,492 cells revealed heterogeneity of fibroblasts and cardiomyocytes during pressure overload-induced cardiac hypertrophy [16]. In their report, fibroblasts were grouped into 6 clusters: FB1 to

FB6. FB1 corresponds to active fibroblasts in previous reports and FB6 is myofibroblast-like cells that highly express extracellular matrix and periostin. It is unknown whether the cells of each group differently contribute to fibrosis and which group corresponds to the cells of the previous classification. In contrast to fibroblasts, cardiomyocytes were divided into four groups (FC1~FC4) based on their expressing proteins. The cells of each group expressed different combination of proteins that are involved in muscle development, metabolism and contraction. Among them, FC3 and FC4 are interesting due to expression of endothelial or fibroblast markers such as cadherin 5, von Willebrand factor, vimentin and decorin. FC3 and FC4 were not fibroblast origin, since they did not express marker proteins that label fibroblasts such as transcription factor Tcf21 and PDGF $\alpha$  receptor. Correlation analysis suggested that the changes in FC3 and FC4 groups are highly correlated to cardiomyocyte pathology at late stage. However, it remains to be determined whether FC3 and FC4 have specific function in the progression of myocardial infarction-induced heart failure.

There are several studies that use scRNAseq for analyzing cellular states. Skelly et al. reported new cardiac fibroblast states with the cells isolated from healthy hearts [17]. A new fibrocyte population of cells was identified, expressing markers of both fibroblasts and immune cells. However, the functional role of these cells in the heart at baseline or injury was not investigated. Farbehi et al. used lineage tracing to isolate the cells expressing PDGF receptor  $\alpha$  and sequenced them [18]. They identified novel myofibroblast subtypes expressing both profibrotic and antifibrotic signatures. McLellan et al. studied fibroblast populations present after angiotensin II infusion by scRNAseq [19]. They found that myofibroblasts expressing  $\alpha$ SMA are not detected. Instead, they identified two fibroblast subpopulations expressing the matricellular proteins Cilp and thrombospondin 4.

These results demonstrate the heterogeneity of fibroblasts but the relationship and identity between the cells assigned by different groups are unknown. Furthermore, their function and contribution to cardiac fibrosis remain to be determined in future.

### 3. Differentiation of fibroblasts to other cells

The possibility that fibroblasts convert to other cells or vice versa after maturation has been investigated [20]. Prolonged culture of macrophages resulted in the cells that express various fibroblast markers such as type I collagen, prolyl-4-hydroxylase, fibroblast specific protein-1, and fibroblast activation protein [20]. Next, the animals that express yellow fluorescent protein (YFP) only in the cells of myeloid lineage were created. These marker fibroblast proteins were detected in infiltrating YFP-positive macrophages after myocardial infarction. Clodronate liposome treatment to deplete macrophages reduced the number of collagen positive fibroblast marker-expressing cells. These results suggested that fibroblasts are derived from macrophages. It is interesting to examine the contribution of macrophage-fibroblast transition to cardiac fibrosis. Inhibition of the transition to fibroblasts may help reduce fibrosis after myocardial infarction.

It was reported that endothelial cells are not a major source of fibroblasts in adult mouse heart [10]. However, there is a controversy on conversion of endothelial cells to myofibroblasts. There is a report that fibroblasts acquire the endothelial cell-like phenotype during ischemia-reperfusion [21, 22]. A series of experiments using mice with genetically labeled fibroblasts demonstrated that 20-40% of fibroblasts express various markers of endothelial cells, and the isolated cells can form a capillary network. Expression of p53 was essential for the process of conversion from fibroblasts to endothelial cells [21]. In addition, stimulation of p53 signaling improved cardiac dysfunction during ischemia-reperfusion. However, an opposite result was reported by different group, in which resident fibroblasts did not contribute to neovascularization after cardiac injury [22]. In the report, pulse chase labeling of fibroblasts after ischemia-reperfusion showed that resident fibroblasts do not express the genes involved in angiogenesis, which are characteristics of endothelial cells. Origin of almost all endothelial cells was resident endothelial cells. Different approaches resulted in distinct conclusion. Thus, it may be necessary to confirm the findings with other techniques such as scRNAseq and proteomic analysis of isolated cells.

### 4. Myofibroblasts as phagocytes

Myofibroblasts have been recognized only as the cells that produce extracellular matrix such as collagen and fibronectin. They also interact with the inflammatory cells through

secreted factors. New role of myofibroblasts in inflammation induced by myocardial infarction was reported. Myofibroblasts phagocytose apoptotic cells and secrete cytokines that suppress the inflammatory responses [23]. This activity is similar to that of macrophages which induce immuno-suppressive responses by engulfment of apoptotic cells.

Myocardial infarction induces necrosis of cardiomyocytes. Phosphatidylserine is presented on the cellular surface of the cells that have undergone necrosis as observed at apoptosis. Therefore, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining is conveniently used as a marker of dead cells. Apoptotic cells are thought to be engulfed by phagocytes such as macrophages. Nakaya et al. examined expression of various molecules involving in engulfment after myocardial infarction [23] (Figure 2). The expression of a factor called milk fat globule-EGF factor 8 (MFG-E8) was increased. MFG-E8 binds both phosphatidylserine expressing on the membrane of apoptotic cells and integrin being present at the surface of phagocytic cells [24]. Since integrin does not directly bind phosphatidylserine, MFG-E8 functions as a bridge between apoptotic cells and phagocytes. MFG-E8 was found to be produced by myofibroblasts and was used by myofibroblasts to phagocytose dead cells. By phagocytosing dead cells, myofibroblasts release anti-inflammatory cytokines and prevent excessive inflammation. Myofibroblasts are similar to macrophages in phagocytosis of dead cells and subsequent secretion of anti-inflammatory cytokines. It indicates that myofibroblasts behave like macrophages at myocardial infarction. The activity of myofibroblasts to engulf dead cells is weaker than that of macrophages. However, fibroblasts are easily differentiated to myofibroblasts at the injury sites and the number of myofibroblasts is supposed to be high at ischemic area during myocardial infarction. Thus, myofibroblasts compensate low ability of engulfment with the number of cells that engulf. However, there is a big difference between myofibroblasts and macrophages. Unlike macrophages, myofibroblasts do not have antigen presentation activity [23]. Myofibroblast-mediated phagocytosis of apoptotic cells is considered to be an efficient way to prevent excess inflammation at the injured site. It is interesting that not all myofibroblasts phagocytose dead cells in vitro. Thus, distinct group members of myofibroblasts may have different functions such as phagocytosis and differentiation to other types of cells.

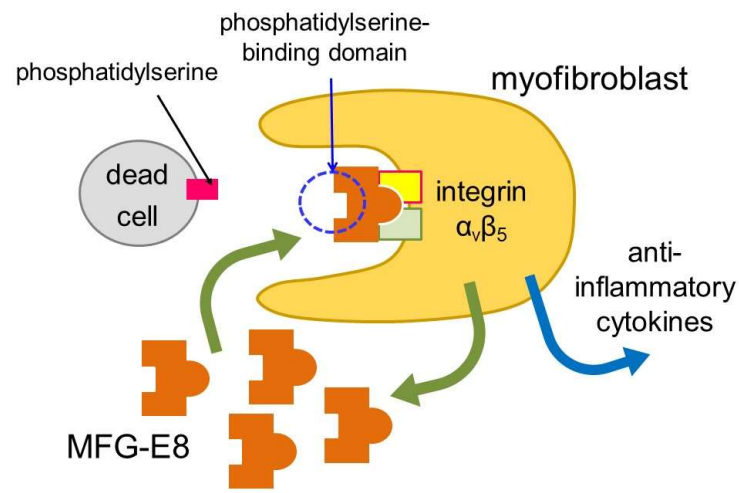


Figure 2: Engulfment of dead cells by myofibroblasts. Myofibroblasts engulf dead cells with the help of MFG-E8 secreted by themselves and then secrete anti-inflammatory cytokines.

## 5. Signaling that controls differentiation to myofibroblasts

TGF- $\beta$  is a strong inducer of differentiation of fibroblasts to myofibroblasts [25]. Inflammatory cells recruiting to the injury sites release cytokines including TGF- $\beta$ . Injury cells also release alarmins and damage-associated molecular patterns (DAMPs) [26]. These molecules cause inflammation leading to differentiation of fibroblasts to myofibroblasts. Thus, any inhibition of inflammatory responses will block appearance of myofibroblasts, which eventually suppresses fibrosis. At early stage of myocardial infarction, neutrophils are firstly recruited to the injury sites [27, 28]. Leukotriene B4 is a powerful attractant of neutrophils by binding to Leukotriene B4 receptor (BLT1). Inhibition of neutrophil recruitment by BLT1 gene knockout or BLT1 blocker decreased cardiac fibrosis by inhibition of inflammation [29, 30].

Interleukin (IL) is an important group of inflammatory cytokines. Multiple IL receptors are expressed in cardiac fibroblasts, which regulates fibroblast states and function [27]. The effects of proinflammatory ILs on cardiac fibroblasts are blocked by cardiac fibroblast-specific deletion of IL receptors. Knockout of IL11 receptor or IL17 receptor genes reduced injury-induced cardiac fibrosis and cardiac dysfunction [31, 32]. These results reveal that cardiac fibroblast-specific deletion of IL receptor genes decreases the

infiltration to or activity of immune cells in the injury area. It also suggests that cardiac fibroblasts play an important role in the regulation of injury-induced inflammation that is mediated by IL signaling.

After myocardial infarction, monocytes are mobilized from bone marrow and differentiated to macrophages at the injury sites [33]. Macrophages can be depleted by the treatment of mice with chlodronate-liposomes [34]. Macrophage-depleted mice showed decreased fibrosis and improved cardiac functions. These results show that single step of inflammation subsequent to myocardial infarction blocks fibrosis possibly through inhibition of differentiation to myofibroblasts.

TGF- $\beta$  stimulation activates both canonical Smad2/3 and non-canonical MAP kinase signaling pathways [25]. Fibrotic responses were inhibited by knockout of fibroblast-specific TGF- $\beta$  receptor 1/2 or knockout of transcription factor Smad3 that is activated downstream of TGF- $\beta$  receptor [35]. It demonstrated that TGF- $\beta$ -Smad2/3 signaling of fibroblasts is a major factor of cardiac fibrosis induced by pressure overload. It was interesting that knockout of TGF- $\beta$  receptor 1/2 in fibroblasts also inhibited cardiac hypertrophy by pressure overload [35]. It indicates that myofibroblasts interact with cardiac myocytes through direct cell-cell communication or indirect mediator-mediated interaction, which is secreted from myofibroblasts.

In addition to TGF- $\beta$ , lysophosphatidic acid (LPA) stimulation induces differentiation of fibroblasts to myofibroblasts [36, 37]. LPA binds their own GPCRs to activate cellular responses [38]. In vitro, LPA stimulation induced fibrosis by activation of myocardin-related transcription factor-serum responsive factor (MRTF-SRF) pathway. Rho regulated actin oligomerization that is regulated by phosphorylation of actin by Rho kinase (ROCK). ROCK-mediated phosphorylation of actin increased monomeric form of actin that results in release of SRF inhibition. SRF together with MRTF activated transcription of various genes including profibrotic genes [39]. Compound CCG-203971 is a small-molecule inhibitor of the Rho-mediated MRTF-SRF pathway [40]. Administration of CCG-203971 inhibited bleomycin-induced lung fibrosis. Rho is signaling molecule that is activated downstream of various receptors including GPCRs and TGF- $\beta$  receptors. Inhibitors of Rho-Rock pathway may suppress fibroblast activation and fibrosis more efficiently than receptor inhibition.

G protein-coupled Receptor Kinase 2 (GRK2) is known as a regulator of G protein-coupled receptors (GPCRs) by phosphorylating agonist-bound GPCRs [41]. Cardiomyocyte-specific knockout of GRK2 demonstrated that GRK2 ablation protects the heart against cardiac dysfunction and fibrosis by myocardial infarction [42]. These mice also showed the reduction of the development of heart failure by myocardial infarction. Fibroblast-specific knockout of GRK2 using the collagen 1 $\alpha$ 2 promoter reduced the secretion of TNF $\alpha$  and suppressed gene expression of profibrotic factors after ischemia-reperfusion [43]. Inhibition of fibroblast function improved cardiac dysfunction. These results show that inhibition of GRK2 protects the heart against cardiac stresses in cardiomyocytes as well as in fibroblasts.

Transient receptor potential channel canonical 6 (TRPC6) is a voltage-independent cation channel and mediates angiotensin II-stimulated hypertrophic responses [44]. The increased intracellular Ca<sup>2+</sup> also plays an important role in conversion of fibroblasts to myofibroblasts. It activates cellular signaling that is sufficient for promoting conversion to myofibroblast and resulting fibrosis [45]. TGF- $\beta$ -induced upregulation of TRPC6 was inhibited by blockade of p38-MAPK-mediated signaling [46]. It is interesting that TRPC6 knockout fibroblasts did not show changes in Ca<sup>2+</sup> signaling and did not promote conversion of fibroblasts to myofibroblasts when the cells are treated with angiotensin II and TGF- $\beta$ . These results demonstrate that TRPC6-Ca<sup>2+</sup> signaling is essential for induction of myofibroblasts in cardiac fibroblasts.

There are other signaling molecules involved in induction of fibrosis. Deletion of  $\beta$ -catenin gene in cardiac fibroblasts improves cardiac function and reduces fibrosis, which is due to decreased production of extracellular matrix proteins by cardiac fibroblasts [47]. It was recently found that functional primary cilia in cardiac fibroblasts is required for canonical TGF $\beta$  signaling-induced differentiation of cardiac fibroblasts to myofibroblasts [48]. Primary cilia express polycystin-1 that is known as regulator of cell proliferation, cell migration, and interactions with other cells. Polycystin-1 is required for maintaining cellular structures of primary cilia. When primary cilia of cardiac fibroblasts were disrupted specifically by deletion of polycystin-1 gene, TGF $\beta$ -Smad3 signaling-induced extracellular matrix protein production and fibroblast differentiation were impaired. Deletion of polycystin-1 gene enhanced pathological cardiac remodeling after myocardial infarction [49], suggesting the important role of primary cilia in hypertrophy and fibrosis.

It also suggested that primary cilia are functional and participate in TGF $\beta$ -induced fibrosis and myofibroblast differentiation. Heat shock protein (Hsp) is chaperon molecule for conversion of fibroblasts to myofibroblasts and resulting fibrosis. Among various Hsp proteins, Hsp47 is known as collagen-specific chaperone. Cardiac fibroblast-specific deletion of Hsp47 significantly reduced cardiac fibrosis and improved cardiac diastolic dysfunction after pressure overload [50]. However, the reduced collagen production in these mice increased lethality after myocardial infarction due to the insufficient scar formation.

Collectively, these results suggest that manipulation of expression and activity of various signaling molecules involved in fibrotic pathway modulates fibroblast states leading to alteration of fibrosis.

## 6. Control of differentiation of fibroblasts by extracellular environment

Fibroblasts exist in interstitial spaces between cardiomyocytes in healthy condition. When the heart is exposed to stresses such as myocardial infarction and hypertrophy, fibroblasts differentiate to myofibroblasts and produce extracellular matrix such as collagen. Fibroblasts generated at the injury site actively proliferate and form aggregate. An in vitro three-dimensional culture system that mimics several states of fibroblasts was developed [51]. When fibroblasts were isolated and cultured in two or three dimensions, the properties of fibroblasts were changed. Standard polystyrene-coated culture plates (not coated with collagen or other extracellular matrix) were used for two-dimensional (2D) cultures, and ultra-low adhesion plates (coated with any extracellular matrix) for 3D cultures (Figure 1). Fibroblasts cultured with 3D structure plates formed spheres (spherical masses) within 24 hours. The morphology of fibroblasts formed by 2D and 3D cultures was reversible but did not depend on the tension or rigidity of the extracellular environment. Interestingly, a correlation of the expressing genes was found between 3D culture fibroblasts and the remodeling heart (treatment with isoprenaline for 3 weeks or cryo-injury treatment). However, the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) was decreased in the aggregates. Since the expression of  $\alpha$ -SMA, a marker of myofibroblasts, is decreased, it cannot be said that fibroblasts obtained by 3D culture is conventional myofibroblasts. However, expression pattern of mRNA of 3D culture fibroblasts is similar to that of matrifibrocytes reported by Fu et al. [52]. Analysis of fibroblasts with 3D culture may elucidate function and fate of matrifibrocytes. Interestingly, 3D cultured fibroblasts

reversibly changed their morphology and expressing genes by transferring the cells to 2D culture. Thus, 3D culture but not normal 2D culture provides a sufficient signal to trigger remodeling. Since in vitro system is essential for analyzing mechanism of differentiation and function of fibroblasts and myofibroblasts, the exchange of culture conditions may be a promising technique to analyze complex behavior of fibroblasts.

Fibroblast fate is also regulated by stiffness of extracellular matrix [53]. The stiffness around fibroblasts increases during progression of fibrosis. Increased stiffness was sensed by integrin receptors and actin cytoskeleton that promote translocation of p38-MAPK to the nucleus and stimulates remodeling [54]. Integrin-actin cytoskeleton signaling complex also activates tyrosine kinases such as focal adhesion kinase (FAK), Src and Fyn. These kinases stimulate GDP-GTP exchange of Rho through guanine nucleotide exchange factors (RhoGEFs). It leads to activation of Rho-ROCK-MRTF-A pathway that increases gene transcription in concerted action with SRF [55]. This pathway will be described in more detail in YAP-TAZ signaling.

## **7. YAP-TAZ signal in fibroblasts**

Hippo pathway is known for its inhibitory activity on cardiomyocyte proliferation [56]. Yes-associated protein (YAP) is a transcriptional coactivator in Hippo pathway that is negatively regulated by large tumor suppressor kinase 1 (Lats1) and Lats 2 (Figure 3).

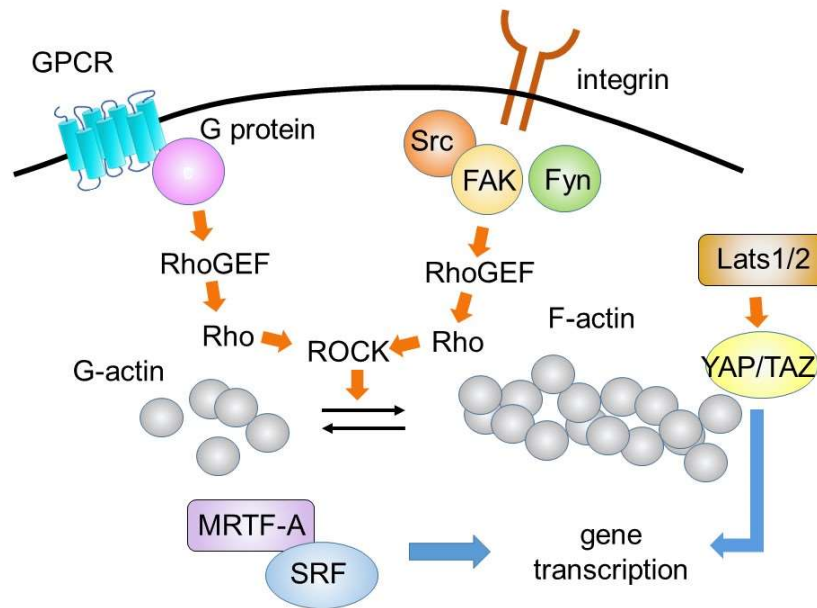


Figure 3: Rho- and YAP/TAZ-mediated signaling pathways.

Deletion of YAP from transcription factor 21 (Tcf21) and *Colla1*-expressing fibroblasts decreased their collagen deposition, proliferation, and activation after myocardial infarction [57]. Similar decreases in angiotensin II/phenylephrine-treated fibrosis were seen when YAP was knocked down from fibroblasts [58]. Myocardin-related transcription factor A (MRTF-A) levels were decreased in YAP knockout mice, suggesting that MRTF-A expression was regulated by YAP function. These results demonstrate the importance of YAP-MRTF-A signaling for determination of myofibroblast states in response to ischemic and chronic stresses. Interestingly, cardiomyocyte specific deletion of YAP decreased hypertrophy and significantly increased fibrosis [59]. Thus, YAP protects the heart against ischemic stress or pressure overload.

Lats1 and Lats2 phosphorylate YAP and inhibit YAP-mediated transcriptional activation of Hippo pathway [60]. Deletion of Lats1 and Lats2 increased YAP activity. Cardiac fibroblast-specific deletion of Lats1 and Lats2 induced spontaneous myofibroblast differentiation [61]. Lats1 and Lats2 knockout mice showed increased fibrosis both at baseline and after myocardial infarction. Mechanistic analysis revealed that YAP directly activates transcriptional machinery of myofibroblasts leading to fibrosis. These results

suggest that Lats1/2-dependent YAP inhibition plays an essential role for maintaining the resting state of fibroblasts.

Lats1 and Lats2 activities are regulated by actin cytoskeleton that are regulated by Rho. Rho are also involved in MRTF-A- and MRTF-B-mediated fibrotic pathway. MRTF-A and -MRTF-B help serum response factor (SRF) to bind a promotor sequence known as the serum response element (also known as the CArG box). Rho activates transcriptional machinery leading to fibrotic responses. Thus, Rho, Lats1/2, YAP and MRTF-A/B form complex network of induction of fibrotic responses.

## 8. Development of treatment of fibrosis and heart failure

GPCR stimulation dissociates heterotrimeric G protein in  $G\alpha$  and  $G\beta\gamma$ . Gallein is a small molecule that inhibits  $G\beta\gamma$ -dependent GRK2 activation [62, 63]. When administered in vivo, gallein suppressed cardiac injury after ischemia-reperfusion, activation of fibroblasts, and the onset of heart failure possibly by GRK2 inhibition [62]. Gallein could attenuate the activation responses of fibroblasts isolated from heart failure patients. However, the affinity of gallein for  $G\beta\gamma$  is low ( $K_i$  is about  $0.2\mu\text{M}$ ), non-specific effects of gallein should be examined by the experiments that gallein-mediated protective responses disappear in GRK2 knockout mice. From high throughput screen, paroxetine was identified as a GRK2 inhibitor that attenuated the development of heart failure by myocardial infarction [64, 65]. Although paroxetine is a selective serotonin reuptake inhibitor (SSRI), another SSRI fluvoxamine did not inhibit GRK2 and did not attenuate heart failure [65]. These results shows that new GRK2-selective inhibitor is a therapeutic option for treatment of heart failure.

Cyclic nucleotides (cAMP and cGMP) play important roles in cellular signaling. Cyclic AMP and cGMP are generated by adenylyl cyclase or guanylyl cyclase, respectively. Various drugs that increase cAMP or cGMP are already used in clinic for treatment of cardiovascular diseases. These nucleotides are degraded by phosphodiesterases (PDEs). PDE comprises superfamily and is categorized into 11 isozymes (PDE1~PDE11) [66]. Each PDE has different selectivity for cAMP or cGMP, although selectivity depends on cellular environment. It should be noted that cGMP selective PDE degrades cAMP when the concentration of cAMP is very high. There are PDEs that degrade both cAMP and cGMP. PDE2 is a PDE that is activated by cGMP and degrades cGMP and cAMP [67].

Inhibition of PDE2 suppressed the onset of heart failure by inhibiting cGMP degradation [68]. The elevated cGMP activated protein kinase G leading to activation of transcription factors (Nuclear Factor of Activated T cells, NFAT) and inhibition of TRPCs. PDE2 inhibitor such as BAY 60-7550 preferentially promoted NO-guanylyl cyclase-cGMP signaling and suppressed the onset of heart failure [69]. However, it should be careful to use PDE inhibitor for the treatment of heart failure, since cAMP causes harmful effects on heart failure.

Bcl2 family is involved in apoptosis and is categorized into three groups based on the homology of amino acid sequence, that is Bcl2-like, Bax-like and BH3-only. Bcl2-like protein has anti-apoptotic activity [70]. In contrast to Bcl2-like protein, Bax-like and BH3-only proteins promote apoptosis. BH3 domains of these apoptosis-promoting proteins bind hydrophobic BH3-binding pocket of anti-apoptotic Bcl2 to form heterodimer and inhibit anti-apoptotic activity by Bcl-2 [71]. Navitoclax (also known as ABT-263) is a BH3-mimetic antagonist of the Bcl2-anti-apoptotic protein [72]. It promoted apoptosis of myofibroblasts isolated from the patients of scleroderma [73]. Since enhanced apoptosis of myofibroblasts is effective in recovery from fibrosis, navitoclax will be a promising drug for treatment of scleroderma. Venetoclax that has excellent selectivity for BCL-2 is currently in clinical trials as an anticancer drug [74]. In the future, venetoclax may be also applied to treatment for cardiac fibrosis.

Many studies report that fibrosis is inhibited by knockout of gene in myofibroblasts or cardiomyocytes. However, inhibition of ongoing fibrosis by compounds does not always recover the heart from fibrotic states. Metformin activates AMP-activated protein kinase (AMPK). It was reported that metformin recovers the lung from bleomycin-induced fibrosis [75]. The effects of metformin were proposed to promote apoptosis of myofibroblasts or inactivate myofibroblasts. Activation of AMPK with metformin or compound A769662 also suppressed hypertrophic responses of neonatal ventricular myocytes by phenylephrine stimulation [76]. These AMPK activators inhibited ERK activation and NFAT nuclear translocation. Mechanistic analysis showed that inhibition of cardiac hypertrophy by AMPK was associated with increase of protein O-linked acetylglucosamine (O-GlcNAcylation) and decrease of O-GlcNAcylation by inhibition of glutamine: fructose-6-phosphate aminotransferase (GFAT). AMPK activators decreased O-GlcNAcylation of troponin T that is associated with development of adverse

cardiac remodeling. Since the effects of AMPK activators were not observed in AMPK $\alpha$ 2-deficient mice, the effects of AMPK activators are mediated by AMPK $\alpha$ 2. Cardiac fibrosis is always accompanied by hypertrophy. Inhibition of hypertrophy will indirectly suppress fibrosis.

In the heart, aquaporin transfers hydrogen peroxide ( $H_2O_2$ ) from extracellular space to cytosol [77].  $H_2O_2$  entering into the cells modifies proteins and changes protein function that cause detrimental effects. Several isoforms of aquaporins are expressed in the mouse heart. Since  $H_2O_2$  are generated from superoxide anion ( $O_2^-$ ) at extracellular space produced by NADPH oxidases [78, 79], aquaporin-mediated transfer of  $H_2O_2$  to cytosol is critical for induction of hypertrophy and other responses. Treatment of mice with a clinically approved aquaporin 1 inhibitor, Bacopaside, attenuated cardiac hypertrophy [80]. These results suggest that aquaporin is a promising target to treat hypertrophy. However, aquaporins are expressed in whole body, and inhibition of water transfer activity of aquaporins by drug or antibody may cause side effects in other tissues. It has been reported that anti-aquaporin-4 antibody is main pathogen of human neuromyelitis optica spectrum disorders and level of the antibody in the plasma links to poor visual prognosis in human [81].

## 9. Immunotherapy

Immunological strategy to target cardiac fibrosis was recently reported [82]. Chimeric antigen receptor (CAR) T cells were engineered to specifically recognize myofibroblasts and induced ablation of myofibroblasts leading to improved cardiac function. CAR consists of antigen-recognizing regions of single chain Fv fragment, transmembrane domain, intracellular domains of T cell activation receptor (CD3 $\zeta$ ) and co-stimulation receptor (CD28). After binding of CAR to myofibroblast-specific protein, in this case mouse fibroblast activation protein, CAR T cells causes cytotoxic killing to decrease the number of myofibroblasts. CAR T cells are already used for cancer therapy and are reported to have beneficial effects on the treatment. Strategy to eliminate myofibroblasts is supported by the following experiments. When diphtheria toxin receptor specifically expressing in myofibroblasts binds diphtheria toxin, the toxin decreases the number of myofibroblasts and reduces cardiac fibrosis by myocardial infarction [83]. However, several concerns are raised before application of CAR T cells to heart failure patients who are seriously suffered from fibrosis [84]. CAR T cells release cytokines for attacking

myofibroblasts and heart failure patients are in advanced inflammatory state. Released cytokines will complicate the conditions of heart failure. It may cause detrimental effects on heart failure patients. Authenticity of elimination of myofibroblasts is also questioned. Myofibroblasts produce extracellular matrix such as collagen and fibronectin that are in part protective against cardiac rupture. Another point is the lack of information of myofibroblast-specific marker proteins in human. Specific antigen is essential for development of CAR T cells-dependent treatment. Although there are several concerns, proper management of number and appearance of myofibroblasts is expected to lead to the treatment of cardiac fibrosis with low side effects.

CAR T cell strategy utilizes myofibroblast-specific antigen and antibody-mediated cytotoxic actions. CAR T cell strategy requires dual recognition protein. A protein with dual inhibitory activities of programmed death ligand-1 (PD-L1) and TGF- $\beta$  (M7824) was successively used for the treatment of tumor growth and metastasis [85]. Dual inhibitor was more effective than treatment with TGF- $\beta$  inhibitor alone. It is expected to increase the specificity of TGF- $\beta$  inhibitor. Thus, to combine TGF- $\beta$  inhibitor and antibody that recognizes myofibroblast-specific protein into single molecule may be another strategy to treat cardiac fibrosis. Identification of myofibroblast-specific antigen helps increase specificity of TGF- $\beta$  inhibitor against cardiac fibrosis and restricts the action of TGF- $\beta$  inhibitor at the localized area.

## 10. Conclusion

Myofibroblasts are the cells that mainly differentiate from fibroblasts and are responsible for production of extracellular matrix in injury. Relationship between fibroblasts and myofibroblasts is more complex than previously thought. Single cell RNA sequencing and lineage tracing techniques demonstrate heterogeneity of fibroblasts, although functional differences are remained to be determined. Treatment of cardiac fibrosis is eagerly waited, signaling analysis of various fibroblast states will provide a therapeutic target that is suitable for drug development. Although direct reprogramming of cardiac fibroblasts to cardiomyocytes with chemical compounds is not mentioned in this review, technologies of direct reprogramming are currently in progress [86] and may be another option for the treatment of cardiac fibrosis in future.

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