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Myofibroblasts and Fibrosis-Mitochondrial and metabolic control of cellular differentiation

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Abstract

Cardiac fibrosis is mediated by the activation of resident cardiac fibroblasts, which differentiate into myofibroblasts in response to injury or stress. While myofibroblast formation is a physiological response to acute injury, such as myocardial infarction, myofibroblast persistence, as occurs in heart failure, contributes to maladaptive remodeling and progressive functional decline. While traditional pathways of activation, such as transforming growth factor beta (TGFβ) and angiotensin II (AngII), have been well characterized, less understood are the alterations in mitochondrial function and cellular metabolism that are necessary to initiate and sustain myofibroblast formation and function. In this review, we highlight recent reports detailing the mitochondrial and metabolic mechanisms that contribute to myofibroblast differentiation, persistence and function with the hope of identifying novel therapeutic targets to treat, and potentially reverse, tissue organ fibrosis.

Keywords

Fibrosis; Myofibroblast; Metabolism; Mitochondria; Epigenetics; Myocardial

Introduction

Cardiac fibrosis occurs in ischemic and non-ischemic heart failure, genetic cardiomyopathies, diabetes, and aging[1, 2]. A hallmark of fibrosis is the excessive remodeling and accumulation of extracellular matrix (ECM), a three-dimensional network of macromolecules including collagens and glycoproteins that provide structural and biochemical support to neighboring cells[3–5]. Based on correlative expression of ECM proteins, the primary cell-type implicated in the myocardial fibrotic response is the cardiac fibroblast. Cardiac fibroblasts are abundant in the myocardium, comprising ~10–20% of the cell population in the heart[6]. In response to injury, cardiac fibroblasts differentiate into highly specialized synthetic and contractile myofibroblasts, increasing fibrillar collagen, matricellular protein production/secretion, as well as the expression of smooth muscle α-actin (αSMA, *ACTA2* gene)[7]. Myofibroblasts are essential for maintaining the structural

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integrity of the myocardium by contracting surrounding tissue and remodeling the ECM scaffold[8–12]. As proof, experimental elimination of activated myofibroblasts following myocardial infarction (MI) results in substantial mortality due to inefficient scar formation and ventricular rupture[13], highlighting their importance in cardiac injury repair. However, excessive fibrosis driven by sustained and unremitting myofibroblast activation results in a progressive decline in tissue compliance, reduced nutrient and oxygen delivery, and increased cardiomyocyte atrophy and cell death, resulting in progressive left ventricular dilation and dysfunction[14–16]. This is important as nearly 6M Americans suffer from heart failure, an epidemic projected to encumber the nation with a cost of care of ~$70 billion by 2030[17]. On the bright side, a recent report demonstrated that ablation of myofibroblasts under chronic stress was cardioprotective and that removal of this stress stimuli following myofibroblast activation permitted these cells to revert back to a quiescent state[13]. This suggests it's possible to target myofibroblasts to reduce fibrosis and lessen disease severity. Therefore, it is imperative we identify the molecular mechanisms initiating and sustaining myofibroblast activation to generate novel therapeutics for heart failure and other fibrotic diseases.

Numerous mitochondrial and metabolic mechanisms have recently been reported to be necessary for myofibroblast formation. For example, changes in mitochondrial ROS (mtROS) generation and oxidative phosphorylation appear essential for myofibroblast formation[18, 19]. Further, our lab recently identified that myofibroblast differentiation was dependent on a reduction in mitochondrial calcium (Ca^{2+}) uptake to initiate specific and highly coordinated metabolic changes to activate the differentiation gene program[20]. In this article, we review what defines a cardiac fibroblast and the evidence implicating mitochondrial signaling and cellular metabolism in myofibroblast differentiation, which may represent novel therapeutic targets for treating fibrosis.

The Cardiac Myofibroblast

Defining the myofibroblast—Fibroblasts mediate tissue repair of nearly every organ. In the heart, fibroblasts have been identified and characterized in the uninjured, infarcted, and pressure overloaded myocardium animal models and human patients[21–24]. During embryonic development, fibroblast progenitors infiltrate the myocardium at embryonic day (E) 13.5, developing into a molecularly distinct fibroblast population by E17.5–18.5[21, 25–28]. While the expression of numerous epicardial genes, such as transcription factor 21 (*TCF21*)[21], Wilms tumor protein (*WT1*), and T-box transcription factor 18 (*TBX18*) define this developmental fibroblast population, only TCF21 reliably labels most all adult cardiac fibroblasts[13, 21, 27, 29], denoting its utility as a lineage tracing marker (see review by Tallquist and Molkentin[28]). In the quiescent state, fibroblasts are small, spindle-shaped cells with a reduced amount of rough endoplasmic reticulum (ER). Cardiac fibroblasts contribute to myocardial homeostasis by synthesizing and maintaining the ECM network critical for structural and functional integrity (Fig. 1)[30]. Fibroblasts also provide insulation of the electrical conduction system, potentiate blood vessel formation, and secrete paracrine signals that control muscle growth[31, 32]. During activation, fibroblasts express cytoplasmic actin and adhesion complexes, permitting migration to sites of tissue injury[33]. Additionally, activated fibroblasts undergo rapid proliferation dependent on the activation of p38-MAPK[34] and

ERK1/2[35] signaling pathways and the upregulation of cell cycle proteins[13]. Following stress or injury, fibroblasts reach a maximum proliferation rate in 2 to 4 days[36]. Once localized to sites of injury fibroblasts secrete various ECM proteins, such as collagens and fibronectin (FN), to remodel the ECM and initiate wound healing[37]. In addition, the *de novo* expression of periostin (POSTN), a matricellular protein important in development and wound healing, is a robust and early marker of fibroblast activation (Fig. 1)[13, 38, 39].

Upon differentiation fibroblasts become a phenotypically distinct cell referred to as the myofibroblast. Transmission electron microscopy of myofibroblasts in the pressure-overloaded heart identified several morphological features including: multiple dendritic processes, elongated and serrated nuclei, extensive rough ER, and the formation of an extensive stress-fiber network[40]. Other defining features are copious production and secretion of ECM proteins such as POSTN, collagens (e.g. COL1 and COL3), and a specialized isoform (splice-variant) of fibronectin (FN-EDA)[41]. The most defining characteristic is the *de novo* expression of the globular microfilament protein, alpha-smooth muscle actin (αSMA, *ACTA2* gene)[8] (see Fig. 1). In addition, myofibroblasts increase the expression of cadherin-2 and −11 for the formation of cell-to-cell adhesions critical for force generation and transduction[42, 43]. Experimentally, the ability to contract a collagen gel-matrix is commonly used to demonstrate myofibroblast formation[44]. Upon the formation of a fully matured scar 7–10 days after MI, myofibroblasts were recently reported to morph into a new differentiated state recently referred to as a matrifibrocyte[36].

In addition to these classical myofibroblast characteristics, emerging evidence point to mitochondrial alterations and metabolic remodeling as initiators of differentiation. For instance, alterations in mitochondrial morphology[45] and mtROS production[46] are reported to occur early in myofibroblast formation. Remodeling of cellular metabolism is universally observed and is necessary to support the biomass required for cellular growth and differentiation, as well as the energetic demand of the newly acquired contractile phenotype. Compared to the quiescent state, myofibroblasts increase both aerobic glycolysis and oxidative metabolism[20]. Interestingly, recent work from our lab identified changes in mitochondrial Ca^{2+} uptake as a key mediator linking metabolism to the epigenetic modifications required for the differentiation program[20]. While the phenotypic characteristics of the myofibroblast are well defined, our understanding of the signaling and molecular events necessary to initiate the differentiation program is evolving.

Activation signaling pathways

Myofibroblast differentiation is initiated by various cytokines, hormones and ligands (e.g. TGFβ, AngII, ET-1)[47, 48], ions (e.g. Ca^{2+})[49], and environmental mechanical forces[8, 12, 49–51]. In addition, recent studies have brought to light the importance of mitochondrial signaling and function, as well as alterations in cellular metabolism, as key regulators of myofibroblast formation and persistence. Here we will briefly highlight well-established signaling pathways initiating myofibroblast differentiation, reviewed in more detail elsewhere[47, 48], with a special emphasis on the mitochondrial and metabolic underpinnings that instigate and promote myofibroblast differentiation.

TGFβ-mediated canonical and non-canonical signaling—The prototypical signaling effector of myofibroblast differentiation is the cytokine TGFβ. Secreted by macrophages, infiltrating immune cells, and fibroblasts themselves, TGFβ acts in both an autocrine and paracrine fashion. TGFβ binds to a heterodimeric transmembrane receptor composed of type I (TGFBR1) and type II (TGFBR2) proteins, for both canonical and non-canonical signaling[52]. The canonical TGFβ pathway involves TGFBR1-mediated phosphorylation of SMAD2/3, permitting its association with SMAD4 and subsequent nuclear translocation for transcriptional activation of the fibrotic gene program. Experimental evidence confirms the importance of canonical signaling in myofibroblast differentiation, as deletion of *Tgfβr1* prevented TGFβ-mediated differentiation[53, 54] and a constitutively-active TGFBR1 mutant promoted myofibroblast formation[53]. Furthermore, Verrecchia et al.[55] demonstrated *Col1a2*, *Col3a1*, *Col6a1*, *Col6a3*, and *Timp1* as TGFβ/SMAD3 gene targets, reporting that expression of a dominant negative SMAD3 construct, or overexpression of inhibitory SMAD7, prevented TGFβ-mediated increases in promoter activity. Similar results were obtained in *Smad3*-null cardiac fibroblasts, which were resistant to TGFβ-mediated induction of collagens and FN *in vitro*, and demonstrated reduced collagen deposition in response to MI [56]. SMAD3 influences αSMA expression[56], and not surprisingly, there are several SMAD transcription factor binding motifs within the αSMA (*ACTA2*) promoter(add Lombardi Ref)[57]. In contrast to SMAD3, recent genetic studies suggest SMAD2 is likely not a mediator of this process[58, 59].

Non-canonical signaling by TGFBR2 (SMAD-independent) also appears to be required for myofibroblast differentiation. Deletion of fibroblast *Tgfbr2* in mice resulted in reduced fibrosis in a bleomycin-induced lung injury[60] model and in dermal wound healing[61]. The most well-characterized non-canonical (SMAD-independent) pathway is the MAPK/JNK/p38 pathway. Genetic deletion of mitogen-activated protein kinase 2 (*Mapk2*) prevented TGFβ-induced expression of αSMA[62]. p38 has been implicated as a key effector of MAPK, as pharmacologic inhibition of p38 prevented myofibroblast formation, whereas gain-of-function experiments resulted in upregulation of phenotypic markers of myofibroblast activation in cardiac fibroblasts[54]. Furthermore, conditional deletion of *p38 in vivo* blocked myofibroblast formation and fibrosis following ischemic injury or chronic neurohormonal stimulation[63]. Although canonical and non-canonical pathways act through independent signaling cascades, they are also likely interconnected since p38 can also phosphorylate SMAD3[64]. Further, TGFβ specifically binds to TGFBR2 in the heterodimeric complex, which in turn catalyzes the phosphorylation and activation of TGFBR1[65], providing a mechanism to coordinate canonical and non-canonical signaling at the receptor level. Other non-canonical pathways, such as Rho-GTPase-Actin, PI3K/Akt, NF-kB, and TNF receptor-associated factors, are discussed elsewhere[47, 48, 66].

G protein-coupled receptor (GPCR) signaling—Cardiac fibroblasts transcriptionally express ~190 GPCRs[67], including putative receptors for angiotensin II (AngII) and endothelin 1 (ET-1). AngII stimulation is sufficient for fibroblast proliferation, collagen synthesis, and inhibition of collagen degradation[68, 69]. AngII induces fibroblast expression of TGFβ[70] and its receptors[71], suggesting AngII is a pioneering signal to initiate differentiation through a positive-feedback mechanism[50, 69]. To this point, cardiac

fibroblasts treated with anti-TGFβ1 antibody were resistant to AngII-mediated increases in collagen expression[72]. However, other studies have shown proximal activation of canonical and non-canonical TGFβ pathways, suggesting AngII signals independently of TGFβ. For example, AngII increases the phosphorylation of SMAD2/3 and the expression of SMAD4 [72], independent of TGFβR2 activation [73]. Furthermore, knockdown of *Tgfbr*1 in cardiac fibroblasts did not prevent the upregulation of αSMA and gel contraction in response to AngII stimulation[54], a response associated with p38 activation and binding of serum response factor (SRF) transcription factor[54] to the αSMA (*Acta2*) promoter[74]. Collectively, these studies suggest an important role for AngII in both canonical and non-canonical signaling in myofibroblast activation.

Endothelin-1 (ET-1) is an endothelium-derived vasoactive peptide secreted in response to mechanical and hemodynamic stress[75–79] and is associated with unfavorable outcomes post-MI[80]. *In vitro*, ET-1 increases fibroblast proliferation[81], collagen synthesis[82], and the ability to contract a collagen gel matrix[83]. Myofibroblast formation in response to ET-1 appears, at least in part, to be mediated through the activation of nuclear factor of activated T-cells (NFAT)[84]. In addition to endothelial-derived ET-1, AngII has been shown to induce the expression of ET-1 in cardiac fibroblasts[85], further intertwining these signaling effectors and pathways. To date, few studies have rigorously investigated the effects of ET-1 *in vivo*, likely because ET-1 is not exclusively produced by coronary endothelial cells, making genetic targeting of this pathway in cardiovascular disease difficult.

Mechanical and Ca^{2+} dependent signaling—Fibroblasts also differentiate in response to changes in ECM stiffness[86, 87]. Mechanical tension is sensed through the newly synthesized, rigid matrix proteins, such as COL1, FN, secreted during initial scar formation[47, 88]. Culturing fibroblasts on low tensile strength substrates maintains cellular quiescence while high tensile strength substrates promote myofibroblast formation[89]. Mechanical tension activates Ras homolog family member A (RhoA) and downstream effector Rho-associated, coiled-coil-containing protein kinase (ROCK) for actin filament stabilization[90]. Actin stabilization potentiates the nuclear translocation of myocardin related transcription factor (MRTF), where together with SRF, transcription of the myofibroblast gene program is initiated[91].

Plasma membrane mechanoreceptors likewise transduce mechanical tension into intracellular signals to activate or promote fibroblast formation[86, 92]. Among these mechanoreceptors are transient receptor potential (TRP) cation channels, a family of extracellular Ca^{2+} uptake channels implicated in myofibroblast differentiation[49], likely through calcineurin-mediated NFAT activation and nuclear translocation[93–96] (Fig. 2). Of these TRP channels, the most widely studied is TRP vanilloid 4 (TRPV4). TRPV4 demonstrates mechanosensitive properties in cardiac fibroblasts[97] and TRPV4-dependent increases in cytosolic Ca^{2+} appear to mediate differentiation through nuclear translocation of MRTF-A, an αSMA and SRF transcriptional coactivator[98].

Upregulation of TRP channel 6 (TRPC6), via TGFβ-mediated p38/SRF signaling, is necessary for both *in vitro* and *in vivo* ventricular myofibroblast formation and wound healing[54]. Similar to TRPV4, TRPC6-dependent increases in cytosolic Ca^{2+} activates

calcineurin-NFAT signaling to promote differentiation[54]. Additionally, TRP member 7 (TRPM7) and TRP channel 3 (TRPC3) expression and activity are increased in fibroblasts from human and animal models of atrial fibrillation-associated fibrosis[99, 100]. Interestingly, atrial cardiac fibroblasts appear to be partial to TRPM7, as they have no detectable activity of TRPC6[97], suggesting different populations of cardiac fibroblasts express different TRP channels. In summary, TRP channels increase and sustain cytosolic Ca^{2+} to activate and potentiate downstream signaling[49, 54, 100], suggesting a unified/conserved mechanism in myofibroblast differentiation.

Liberation of intracellular Ca^{2+} stores, such as ER Ca^{2+}, also contribute to differentiation as many pro-fibrotic agonists have been shown to enhance IP3R-mediated ER Ca^{2+} release[101, 102] (Fig. 2). Pro-fibrotic increases in COL1A1 and FN in lung fibroblasts were blocked by pharmacological inhibition of both IP3R[103] and RyR[104], demonstrating ER Ca^{2+} release is required for myofibroblast differentiation. Furthermore, deletion of the ER Ca^{2+}-binding protein calreticulin impairs the upregulation of COL1A1 and FN in mouse embryonic fibroblasts by reducing TGFβ-mediated increases in cytosolic Ca^{2+} and subsequent NFAT translocation[105].

In agreement with the above reports, recent work from our lab confirmed that pro-fibrotic agonists (i.e. TGFβ, AngII) increase cytosolic Ca^{2+} and NFAT nuclear translocation[20]. More importantly, our study highlights a novel mechanism by which decreases in mitochondrial Ca^{2+} uptake not only contribute to increases in local cytosolic Ca^{2+} (see Fig. 2), but also coordinate specific changes in cellular metabolism for activation of the fibrotic gene program[20], discussed in further detail below. While changes in mitochondrial Ca^{2+} dynamics appear to control myofibroblast differentiation, recent evidence also suggests that changes in mitochondrial-derived reactive oxygen species (ROS) coordinates intracellular signaling to promote and amplify fibrotic signaling. To these points, the next section explores the evidence for mitochondrial structural and functional changes and alterations in cellular metabolism that promote myofibroblast differentiation.

Mitochondrial Mechanisms of Myofibroblast Differentiation

Influence and mechanisms of ROS generation—Reactive oxygen species are byproducts of (patho)physiological processes with wide ranging cellular implications, from influencing intracellular signaling cascades to thiol-dependent posttranslational modifications. A major source of superoxide ($O_2{\cdot}^-$) in the cell is the ETC via electron slippage at Complex I and III[106, 107]. Mitochondrial-derived ROS (mtROS) may be a critical component of differentiation, as Complex III-derived $O_2{\cdot}^-$ is increased following TGFβ-treated lung fibroblasts and mitochondrial-targeted antioxidants attenuated fibrotic gene induction[46]. In rat cardiac fibroblasts, β-adrenergic stimulation increased ROS production and the activation/phosphorylation of p38-MAPK to increase proliferation and collagen production[108]. p38-dependent increases in proliferation, and collagen production were reversed with antioxidant treatment, suggesting ROS-dependent activation of the p38-MAPK pathway[108], likely through ROS-induced oxidation of a cysteine residue on MAPK phosphatase to reduce acitivty[109] (Fig. 3A).

Mitochondrial fission, the process of dynamin-related protein 1 (Drp1)-mediated mitochondrial fragmentation, provides one mechanism to increase mtROS[45, 110]. Genetic overexpression of mitochondrial fission factor (MFF) in immortalized human fibroblasts increased ROS production, which correlated with increased αSMA expression[110]. Conversely, decreasing fission through knock down of *Drp1* decreased the AngII-mediated production of αSMA and COL-1 in adventitial fibroblasts, which the authors attributed to the blockade of fission-induced mtROS production[45]. Additionally, fibroblasts increase the production of $O_2{\cdot}^-$ through the upregulation of NADPH oxidase 4 (NOX4)[46]. Members of the NOX family are typically located at the plasma membrane, however NOX4 is reportedly localized to mitochondria in various cell types[111, 112]. In lung fibroblasts, TGFβ-induced mitochondrial ROS generation transcriptionally upregulated NOX4, potentially as a means to sustain elevated intracellular ROS levels for activation of ROS-sensitive pathways (e.g. AKT and MAPK[46]). Indeed, siRNA against NOX4 inhibited the induction of αSMA and FN-EDA via TGFβ non-canonical (ERK1/2) signaling[113]. The expression of NOX4 appears to be downstream of SMAD3, suggesting that NOX4-generated ROS amplifies TGFβ-mediated gene transcription, rather than initiating it[59, 113]. A NOX4 splice variant has also been shown to localize to the nucleus where it may play a role in activating p38-MAPK and ERK1/2-dependent transcriptional signaling[109, 114].

Mitophagy, which is essential for the clearance of dysfunctional mitochondria, is downregulated in pathological conditions such as cancer and cardiovascular disease[115, 116]. Without proper autophagic clearance, dysfunctional mitochondria accumulate and contribute to excessive mtROS[117]. Interestingly, the expression of parkin, an autophagic effector encoded by the gene *PARK2*, is significantly reduced in lungs from idiopathic pulmonary fibrosis patients[118]. In fibroblasts, knockdown of *Park2* results in the upregulation of αSMA[118, 119] in a platelet-derived growth factor receptor (PDGFR)-mediated manner, which is attenuated by mitochondrial-targeted antioxidant supplementation[118]. In a mouse model of bleomycin-induced lung fibrosis, deletion of *Park2* enhanced lung fibrosis whereas treatment with a PDGFR inhibitor diminished this response[118], suggesting a role for mitophagy in tissue fibrotic remodeling.

Lastly, myofibroblasts appear to downregulate the expression of various antioxidant enzymes [e.g. superoxide dismutase (SOD) and catalase] to sustain elevated intracellular ROS levels. For example, pro-fibrotic stimulation with AngII decreased catalase expression[120] and mitochondrial SOD2 activity[121], resulting in increased ROS (Fig. 3A). Importantly, the profibrotic effects of AngII were prevented with pretreatment of SOD mimetics[121], suggesting a link between fibrotic signaling and the impaired ability to neutralize mtROS. Collectively, these studies suggest a critical role for mtROS in myofibroblast differentiation, however further study is required to elucidate the molecular mechanisms that instigate and promote mtROS and how mtROS directly influences the fibrotic gene program.

Resistance to Apoptosis—Resolution of wound healing is possible via the de-differentiation of myofibroblasts or loss of these cells by cell death signaling. Considering the latter, a characteristic feature of the myofibroblast is extreme resistance to cell death, which contributes to their persistence in the stressful environment associated with wound

healing and fibrosis. One way mitochondria regulate apoptosis is by the release of apoptogens, such as cytochrome c or Smac/DIABLO, to activate the proteolytic caspase cascade. Anti-apoptotic factors such as B-cell lymphoma 2 (BCL-2) and B-cell lymphoma-extra-large (BCL-XL) prevent the release of apoptogens and formation of the apoptosome in opposition to pro-cell death factors such as Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist killer (BAK)[122, 123]. The increased expression of anti-apoptotic factors is thought to be a primary mechanism by which myofibroblasts gain resistance to cellular demise (Fig. 3B). Fibrotic stress is reported to increase the expression of anti-apoptotic BCL-2 and BCL-XL resulting in decreased cell death in models of scleroderma[124], Crohn's disease[125], and cardiac fibrosis[126] while pharmacological inhibition of anti-apoptotic BCL-2 reduced ECM deposition and αSMA expression[125]. Conversely, TGFβ activation of dermal fibroblasts decreased the expression of pro-apoptotic factor BAX[127], suggesting a shift in the balance of BH3-only proteins. In cardiac fibroblasts, AngII stimulation promotes apoptotic resistance by activating MAPK-ERK1/2 signaling to inhibit capase-3 cleavage[128]. While myofibroblast activation is, in part, maintained by apoptotic resistance, targeting apoptosis pathways may provide novel therapeutic strategies to lessen the impact of persistent tissue fibrosis in disease progression.

Mitochondrial Ca^{2+} Exchange—As previously stated, sustained cytosolic Ca^{2+} is a driver of myofibroblast differentiation. In addition to this observation, we discovered that mitochondrial calcium uptake is reduced during fibroblast activation, which further augments cytosolic Ca^{2+} signaling and reduces matrix calcium levels[20]. Mitochondrial Ca^{2+} uptake is mediated by the mitochondrial Ca^{2+} uniporter complex (mtCU). The mtCU is a multimeric protein complex comprised of the pore forming component, mitochondrial calcium uniporter (MCU), and a number of regulatory subunits, such as mitochondrial calcium uptake 1 (MICU1) that serves as a channel gatekeeper by preventing opening at low cytosolic Ca^{2+} concentrations and enhancing MCU opening at higher concentrations via Ca^{2+} binding to EF-hand motifs on MICU1. While there are many additional components of the mtCU[129], we will focus on MICU1 due to its role in myofibroblast differentiation. Our recent work demonstrates that pro-fibrotic stimulation (TGFβ or AngII) of mouse embryonic or cardiac fibroblasts results in increased expression of MICU1. Given the large increase in mRNA level, we speculate that this is a transcriptional mechanism, a hypothesis supported by prominent transcription factor binding motifs for SRF, NFAT and SMADs in the MICU1 promoter. The increase in MICU1 expression was sufficient to enhance mtCU gating and reduce mitochondrial Ca^{2+} uptake during fibroblast activation[20] (refer to Fig. 2). In agreement, genetic deletion of *Mcu* results in increased myofibroblast differentiation *in vivo* following MI. The regulatory role of decreased mtCU activity in myofibroblasts is likely multifactorial. First, by decreasing the capacity of mitochondria to buffer local cytosolic Ca^{2+}, more free Ca^{2+} is available to enhance the myofibroblast gene program though Ca^{2+}-dependent transcription factor signaling (i.e. increase in microdomain Ca^{2+} for the activation of NFAT). Second, decreasing Ca^{2+} entry into the mitochondria could protect the myofibroblast against mitochondrial-dependent cell death in the stressful fibrotic environment. Third, altering mitochondrial matrix Ca^{2+} concentration is a direct mechanism to regulate cellular metabolism. For example, $_{mt}Ca^{2+}$ is a regulator of pyruvate dehydrogenase (PDH) via activation of pyruvate dehydrogenase phosphatase, and fibroblast

activation correlated with decreased PDH activity. Further, we found that fibrotic stimulation increased aerobic glycolytic flux, increased glutaminolysis, and elicited discrete changes in various metabolites that potentially regulate the myofibroblast differentiation program[20]. For example, activated fibroblasts increased the bioavailability of metabolites that are co-factors for enzymes that post-translationally modify histones and DNA, chromatin accessibility, and downstream gene expression[20]. Thus, mitochondria are a key regulatory hub for myofibroblast formation by participating in the modulation of signaling and metabolism that support the changes in genetic programming necessary for cell state transition. Next, we will summarize the literature, describing metabolism as a key driver of myofibroblast differentiation.

Cellular metabolism in myofibroblast differentiation

A prominent feature of fibroblast activation is an increase in aerobic glycolysis and lactate production in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation, a phenomenon referred to as The Warburg Effect[130, 131]. *It is important to note that the changes in metabolism during initial fibroblast activation are distinct from the metabolic programs supporting the function of fully differentiated myofibroblasts.* Metabolism impacts fibroblast differentiation by numerous mechanisms (Fig. 4). For example, increased ATP production is crucial for the contractile apparatus while the synthesis of building blocks (e.g. nucleotides and phospholipids) are necessary to support the initial proliferative burst, as well as the subsequent cellular growth of the fully differentiated, non-proliferative myofibroblast. Increased synthesis of various amino acids for *de novo* collagen production is essential for ECM remodeling. Metabolites themselves also serve as signaling molecules and are cofactors for epigenetic modifying enzymes that regulate chromatin structure and gene transcription. Here, we summarize the experimental evidence that changes in metabolism not only accompany the myofibroblast phenotype, but that these metabolic changes are in fact necessary for the activation and differentiation of fibroblasts themselves.

Glucose and lactate—Changes in glycolysis are critical not only for energy provision, but also for coordinating ancillary biosynthetic pathways to provide the elements essential for cellular growth and differentiation[132]. The first evidence of glycolytic remodeling in activated fibroblasts was discovered in cystic fibrosis patients where increased activity of hexokinase, phosphofructokinase-1 (PFK1), pyruvate kinase muscle isozyme (PKM), and lactate dehydrogenase (LDH) were observed[133]. A fibroblast glycolytic switch has also been confirmed in numerous fibroblast populations[20, 134, 135]. One mechanisms to increase glycolysis is the transcriptional upregulation of numerous glycolytic genes[134, 136–138], many of which are metabolic checkpoints (Fig. 5). In fact, simply increasing glucose concentrations in cardiac fibroblasts in the absence of TGFβ stimulation is sufficient to increase the expression of canonical signaling effectors TGFβ and SMAD2/3 and induce a myofibroblast phenotype[139]. As the hallmark of diabetes mellitus is an increase in blood glucose levels, and epidemiological and experimental studies demonstrate a strong association between diabetes and heart failure (reviewed elsewhere[140, 141]), the idea that extracellular glucose concentrations alone are sufficient to promote myofibroblast differentiation and tissue fibrosis is of clinical interest. Causal experimental evidence

suggests that altering glucose utilization is necessary for myofibroblast formation. For example, inhibition of glucose uptake and glycolysis using 2-deoxyglucose (2DG) prevents the expression of αSMA and Fn[134, 138]. PFK1, the rate-limiting and committed step of glycolysis, is allosterically activated by fructose-2,6-bisphosphate, the levels of which are regulated by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB). Genetic deletion or pharmacologic inhibition of PFKFB3, an isoform with a ~740-fold higher kinase than phosphatase activity[142], diminished myofibroblast formation[138], Similarly, overexpression of a kinase-deficient PFKFB1 mutant isoform prevented differentiation, while overexpression of the phosphatase-deficient PFKFB1 mutant isoform was sufficient to promote differentiation, independent of exogenous ligands/activators[20]. Indeed, the inhibition of glucose uptake or PFK1 significantly attenuated the fibrotic response in *in vivo* models of renal and pulmonary fibrosis[138]. Further, inhibition of glyceraldehyde dehydrogenase facilitated phenotypic reversal of activated hepatic myofibroblasts into a less mature form as evidenced by a reduction in αSMA expression and collagen secretion[143].

An additional site of regulation that appears critical for myofibroblast formation is pyruvate oxidation. Pyruvate kinase 2 (PKM2), the less catalytically active isoform, is upregulated in myofibroblasts[134] and TGFβ rapidly increases PKM2 phosphorylation at Tyr105[134], an inhibitory phosphorylation site that favors dimerization as opposed to the more active tetrameric state, further promoting the Warburg effect[144]. Intriguingly, PKM2 dimerization is associated with a redirection of glucose into the pentose phosphate pathway to augment nucleotide and serine biosynthesis, both of which are critical for *de novo* collagen formation[145]. In line with this regulatory mechanism, overexpression of pyruvate dehydrogenase kinase-1 (PDK), which inhibits PDH activity, is sufficient to promote myofibroblast formation[146]. We also reported that pro-fibrotic stimulation with either TGFβ or AngII rapidly upregulates MICU1, to limit $_{mt}Ca^{2+}$ .uptake, which reduces PDH phosphatase activity resulting in increased phospho-PDH (decreased PDH activity)[20] (see Fig. 2 & 5). *In vivo* activation of PDH by dichloroacetate prevented the induction of myofibroblast markers and interstitial fibrosis in a mouse model of kidney injury[147]. Collectively, these studies suggest that reducing glucose oxidation, in the face of enhanced glycolytic rates, is a key metabolic driver of myofibroblast differentiation with numerous checkpoints in place to ensure coordinated metabolic remodeling.

Unsurprisingly, enhanced glycolysis and reduced glucose oxidation results in increased lactate production in myofibroblasts[134–136, 148]. Recently, lactate has been implicated in myofibroblast formation as inhibition of LDH prevents the expression of αSMA, FN, *Col1* and *Col3*[135, 148], whereas overexpression of LDH induced myofibroblast formation[136]; however, it should be noted that others have shown no role for LDH in fibroblast to myofibroblast transition[147]. As glycolysis is necessary and sufficient for differentiation, the activity of LDH is essential for the regeneration of NAD+, a required cofactor for glycolysis. One study[134] had the surprising result that addition of exogenous lactate was sufficient to increase the expression of phenotypic myofibroblast markers in a dose-dependent manner. Lactate in the heart likely promotes myofibroblast differentiation through numerous mechanisms. For example, activation of latent TGFβ in the ECM is pH sensitive and regulated by HIF1α[136]. Since HIF1α is a master transcriptional regulator of the hypoxic

response, the idea that hypoxia-mediated tissue lactate accumulation may be a signal for fibroblast activation is noteworthy and warrants further investigation. Interestingly, HIF1α degradation is regulated by an αKG-dependent prolyl hydroxylase[149], further intertwining metabolic regulation during myofibroblast formation. Lactate is also a signaling molecule for infiltrating immune cells responding to cardiac injury, resulting in M2 polarization and the production of pro-fibrotic cytokines[150, 151]. Interestingly, monocyte-derived MHCII $^{+}$Ly6C^{lo} macrophages (i.e. M1-like phenotype) are associated with a reduction of myocardial fibrosis[152], indicating metabolic control of macrophage polarization may be a novel therapeutic target to treat cardiac fibrosis. Histone lactylation (addition of a lactyl group to lysine amino-acid residues) has been identified as an epigenetic modification regulating gene expression in macrophages and cancer cells[153], providing an additional means of lactate-dependent regulation of differentiation (see Fig. 5). The fact that M2 macrophages, myofibroblasts, and cardiomyocytes all increase glycolysis under conditions of cardiac stress, suggests lactate may drive a futile cycle of myofibroblast activation and tissue fibrosis. The notion that exogenous lactate can promote myofibroblast differentiation has numerous physiological implications. First, while exercise clearly has a beneficial effect on cardiovascular health, reports of cardiac pathology in ultra-athletes suggest deleterious functional and structural cardiac remodeling including cardiac fibrosis[154–156]. While it is plausible that the chronic vs. intermittent nature of this type of exercise training could result in sustained elevations in circulating blood lactate leading to fibroblast activation, this hypothesis has yet to be investigated. Secondly, numerous cardiac diseases (e.g. MI) induce a hypoxic environment, long implicated in the pathogenesis of fibrotic disease. Tissue hypoxia increases cellular glycolytic rates and lactate production in all cells (e.g. cardiomyocytes, fibroblasts, immune cells), which in turn promote myofibroblast differentiation and tissue fibrosis.

The universal finding that tissue hypoxia occurs in wound healing and fibrotic disease suggests a critical role in myofibroblast activation and differentiation (REF). Overexpression of HIF1α promoted myofibroblast formation and in support genetic knockdown or inhibition prevented differentiation[136, 146]. While the transcriptional upregulation of glycolytic genes is a primary target of HIF1α, it also appears to directly regulate the fibrotic gene program, as it can bind to the promoter region of αSMA (*Acta2*)[138]. Activation of Hedgehog signaling also appears to play a role in metabolic remodeling as pharmacologic inhibition or genetic interruption of Hedgehog in hepatic fibroblasts reduced the expression of glycolytic genes and myofibroblast formation[135]. Glycolytic enzymes are also transcriptionally upregulated by canonical signaling; the inhibition of TGFβR1 or siRNA targeting *Smad2/3* abolished the upregulation of PFKFB3 and myofibroblast formation[138] and FACS analysis of activated pulmonary fibroblasts showed high correlation between PFKFB3 and αSMA expression[138]. Canonical signaling has also been shown to modulate PI3K/AKT signaling in vascular smooth muscle cells[157], providing yet another mechanism to enhance aerobic glycolysis. In summary, there is copious causal experimental evidence linking glycolysis to myofibroblast activation and differentiation.

Acetyl-CoA—Acetyl-CoA is a versatile two-carbon metabolite utilized for many cellular processes. Conventionally, acetyl-CoA is utilization to generate reducing equivalents

through the Krebs cycle for cellular bioenergetics, however acetyl-CoA can exit the mitochondrial matrix as citrate where it is converted back to acetyl-CoA by ATP citrate lyase (ACLY) for anabolic purposes, such as *de novo* lipid synthesis and protein acetylation[158, 159] (Fig. 5). Cytosolic and nuclear acetyl-CoA can also be formed by Acyl-CoA Synthetase Short Chain Family Member 2 (ACSS2) dependent acetate metabolism[160]. Furthermore, histone acetyltransferases (HATs) utilize acetyl-CoA for histone acetylation[158, 160, 161] in cellular reprogramming and differentiation in many physiological contexts[162, 163]. For example, AKT activation of ACLY promotes myofibroblast gene expression in human kidney fibroblasts *in vitro* and pharmacological inhibition of ACLY suppressed fibrosis in mice subjected to ureteral obstruction[164]. In line with this idea, mesangial cells, which can differentiate into a fibroblast-like phenotype, increased the expression of *Tgfβ1*, *Ctgf*, and *Fn* when exposed to high glucose, in an ACLY/HAT-dependent manner[165]. However, others have shown TGFβ-induced myofibroblast differentiation results in decreased acetyl-CoA abundance and an overall decrease in total H3 acetylation[166]. Interestingly, αSMA expression was decreased in renal fibroblasts supplemented with citrate or acetate, which reversed an observed global decrease in H3 acetylation[166]. However, while global acetylation was decreased, TGFβ increased H3K18 and H3K27 acetylation, suggesting a level of specificity in fibrotic gene program activation/silencing[166]. These studies suggest that, even in the same cell type, it is unclear how, or even if, acetyl-CoA metabolism regulates myofibroblast differentiation, warranting further study.

Multiple classes or families of HATs and histone deacetylases (HDACs) add or remove acetyl-CoA from histone residues to alter chromatin accessibility[167, 168] (see Fig. 5). Inhibition of various HDACs have been shown to ameliorate myofibroblast differentiation and tissue organ fibrosis[169–171]. Similarly, evidence indicates that inhibiting HATs, the p300 family in particular, is an effective strategy to prevent cardiac myofibroblast differentiation and fibrosis[172]. This paradox of inhibiting either HATs or HDACs further implicates a level of specificity in regulating certain gene programs that likely coordinate cellular differentiation[173, 174].

Another interesting avenue to explore is the catabolic generation of acetyl-CoA in fibroblast activation. Many carbon sources fuel acetyl-CoA production including: glucose, fatty acids, glutamine, and ketone bodies[175–177]. Hepatic stellate cells (HSCs) in the liver transition from lipid- and vitamin A-storing pericytes to myofibroblasts upon injury[178]. Activated HSCs upregulate the expression of peroxisome-proliferator-activated receptor β (PPARβ), a nuclear hormone receptor that promotes fatty acid oxidation[179], potentially providing a supply of acetyl-CoA from lipids rather than glucose. Additionally, HSC activation and PPARβ expression was counteracted by ectopic expression of PPARγ and sterol regulatory element-binding protein 1 (SREBP1), transcription factors that promote lipid synthesis as opposed to catalysis[179]. These data suggest that fibroblast quiescence may be maintained by fatty acid synthesis with a switch to oxidation mediating activation. Interestingly, very few studies have investigated the effects of fatty acids on myofibroblast formation, providing opportunity for investigation.

Ancillary biosynthetic pathways—Numerous ancillary pathways of intermediary metabolism contribute to myofibroblast activation and the provision of biosynthetic

materials for cellular growth and ECM remodeling. Collagen is unique in its amino acid structure due to its high (33%) glycine composition (ref). As myofibroblasts increase collagen production and secretion, this requires an increase in *de novo* synthesis of glycine and its precursor serine. To fuel biosynthetic demand, a coordinated increase in aerobic glycolysis and decrease in glucose oxidation shunts glucose-derived carbons into the serine biosynthetic pathway for collagen synthesis[137, 180]. TGFβ induces the expression of serine biosynthetic genes (*PHGDH, PSAT1, PSPH, SHMT2*)[137, 180] (Fig. 5), via canonical (SMAD3) and noncanonical (mTOR) upregulation of activating transcription factor 4 (ATF4), a transcriptional master regulator of amino acid metabolism[137]. Importantly, genetic and pharmacologic attenuation of serine biosynthesis diminished collagen synthesis in pulmonary fibroblasts *in vitro*[180]. *In vivo*, pharmacological inhibition of phosphoglycerate dehydrogenase (PHGDH), the rate limiting step of serine biosynthesis, attenuated bleomycin-induced pulmonary fibrosis[181]. Proline, another amino acid making up ~17% of collagen, is also increased in abundance in TGFβ-stimulated fibroblasts and is necessary for the myofibroblast phenotype[182], providing further evidence to the importance of amino acids in fibroblast activation and function.

Increased serine biosynthetic pathway flux also provides s-adenosylmethionine, the methyl donor for cytosine and histone methyltransferases (Fig. 5). Specifically, histone lysine methyltransferase KMT7 has been implicated in tissue fibrosis as genetic silencing significantly attenuated TGFβ-induced ECM gene expression accompanied by a correlative decrease in promoter H3K4me levels[183]. Furthermore, inhibition of Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2) diminished H3K27me3 and increased collagen production[184]. While these data suggest that loss of methylation at H3K27 and H3K4 enhances myofibroblast formation, KMT2H (*ASH1L* gene which methylates H3K9) is reported to directly bind regulatory elements of pro-fibrotic genes, and knockdown of KMT2H suppresses fibrogenic gene expression in the liver[185]. Collectively, these studies demonstrate the importance of these pathways in both the biosynthetic and transcriptional requirements necessary for myofibroblast formation.

Changes in glycolytic flux can also redirect glucose into the hexosamine biosynthetic pathway[132] (Fig. 5). One critical function of this pathway is the post-translational addition of *O*-Linked β-*N*-acetylglucosamine (O-GlcNAc) to Ser/Thr residues to alter protein function[186]. Increased protein O-GlcNAcylation is observed in ischemic injury, heart failure and diabetic cardiomyopathy, all of which are associated with fibrosis[186–189]. In rat cardiac fibroblasts cultured under high-glucose conditions, SP1, a transcription factor associated with myofibroblast activation[190–193], demonstrated higher levels of O-GlcNAcylation[139]. This O-GlcNAcylation of SP1 enhanced transcription factor binding to the promoter region of *Col1a1* resulting in increased transcription and collagen production. This was abrogated with genetic overexpression of the O-GlcNAc removal enzyme, O-GlcNAcase[139]. In addition to GlcNAcylation post-translational modification, the hexosamine biosynthetic pathway is necessary for the synthesis of hyaluronic acid, a chief component of the ECM involved in cell-cell adhesions, migration, proliferation, and differentiation[194]. Genetic silencing of hyaluronan synthase prevented the expression of αSMA[194, 195] and was mechanistically linked to TGFβ1 signaling[196, 197]. Furthermore, depletion of cytoplasmic UDP-glucuronic acid, essential for hyaluronan-chain elongation, decreased the expression of

αSMA[195], an effect not reproduced by exogenous administration of hyaluronan[198], suggesting the macromolecular assembly of *de novo* hyaluronan is required. In addition, overexpression of glucosamine—fructose-6-phosphate aminotransferase isomerizing 1 (GFPT1) , a rate limiting step in the hexosamine pathway, is sufficient to increase TGFβ1 mRNA and protein levels in 3T3 fibroblasts, partly by enhancing *Tgfβ1* promoter activity[199], suggesting direct transcriptional regulation of the fibrotic gene program via the hexosamine pathway.

Glutamine—Glutamine is the most abundant amino acid in the body and serves an anaplerotic role by replenishing Krebs cycle intermediates, at the level of alpha-ketoglutarate (αKG), under conditions of increased aerobic glycolysis and reduced oxidative phosphorylation[176.] In TGFβ treated fibroblasts, glutamine utilization (i.e. glutaminolysis) is enhanced and contributes to increased levels of αKG[20, 182] (Fig. 5). Increased rates of glutaminolysis in fibroblasts is accomplished by the upregulation of the rate-limiting enzyme, glutaminase 1 (GLS1), via TGFβ[182, 200] and Hedgehog signaling[181, 201]. Glutaminolysis is a strict requirement for fibroblast activation *in vitro* as pharmacologic inhibition and genetic silencing of *Gls1* or removal of extracellular glutamine prevents myofibroblast formation [20, 182, 200, 201]. Myofibroblast persistence appears also dependent upon glutaminolysis as removal of extracellular glutamine or siRNA knockdown of GLS1 in a lung fibroblast cell line reverted myofibroblasts into a more quiescent state[200]. Importantly, protection from bleomycin-induced pulmonary fibrosis was observed with pharmacological inhibition of GLS1[202].

One potential mechanism by which glutamine controls myofibroblast formation may be at the level of epigenetic modifications that regulate chromatin remodeling. For example, αKG is a cofactor for numerous dioxygenases, including the epigenetic modifiers ten-eleven translocation enzymes (TETs) and jumonji-C (JmjC)-domain-containing demethylases (JmjC-KDMs), which demethylate DNA cytosine and histone lysine residues, respectively, to regulate chromatin structure (see Fig. 5). We recently discovered that JmjC-KDMs are necessary for demethylation of H3K27me2/3 residues for profibrotic gene expression in mouse embryonic fibroblasts[20]. In support, treatment with cell-permeable dimethyl-αKG was sufficient to promote differentiation and inhibition of JmjC-KDMs, with the drug JIB-04, during TGFβ stimulation was sufficient to block myofibroblast formation[20]. Additionally, removal of glutamine from lung fibroblast media decreased KDM6B activity resulting in increased H3K27me3 levels[203]. Chromatin immunoprecipitation confirmed that JMJD3 interacts with the promoter region of anti-apoptotic genes in a glutamine-dependent manner[203], suggesting one potential mechanism allowing for myofibroblast persistence in injured tissues. Collectively, these results implicate glutaminolysis as a prominent link with the epigenome to induce cellular differentiation. This warrants further investigation into chromatin remodeling mechanisms in myofibroblast activation and persistence in disease, which has been touched upon elsewhere[204].

Glutamine is also an essential substrate for *de novo* collagen synthesis. In lung fibroblasts, the glutamate-consuming enzymes phosphoserine aminotransferase 1 (PSAT1) and Δ^1-pyrroline-5-carboxylate synthase (P5CS) are necessary for glycine and proline biosynthesis and collagen production[205] (Fig. 5). Additionally, glutaminolysis not only enhances collagen

production but also its stability, as inhibition of GLS1 increased collagen degradation and exogenous αKG prevented degradation in a proline hydroxylation dependent manner[182]. Lastly, glutamine may play a role in myofibroblast differentiation through *de novo* synthesis of the antioxidant glutathione. A reduction in the GSH:GSSG ratio, suggesting an increase in the oxidative environment, occurs with TGFβ stimulation[20] and is implicated in regulating the fibrogenic effects of TGFβ through SMAD3 activation[206] (see above section on Influence and mechanisms of ROS generation).

Conclusions

Myofibroblasts are a specialized cell type that are responsible for the pathological remodeling observed in tissue organ fibrosis. Numerous pro-fibrotic signaling cascades contribute to mechanisms for myofibroblast differentiation, most of which integrate with mitochondrial and metabolic pathways for cellular reprogramming (Fig. 6). Of these mechanisms, the interactions between metabolism and the epigenome are a logical candidate for additional study. While identifying the epigenetic signatures that mediate myofibroblast differentiation and tissue fibrosis is an emerging field, and has been reviewed thoroughly elsewhere[173, 174], identifying the specific metabolic substrates and pathways that contribute to DNA and histone modifications for chromatin remodeling and fibrotic gene expression may provide novel therapeutic targets to treat tissue organ fibrosis in disease.

Encouragingly, metabolic targets highlighted throughout this review, such as inhibition of GLS1 via the small molecule CB-839 and dichloroacetate to inhibit PDK, are in phase 1 and 2 cancer clinical trials. If found safe, the repurposing of such drugs for the treatment of cardiac fibrosis may quickly follow. Additionally, increasing mtCU uptake through the use of the natural plant flavonoid kaempferol[207], may present a potential strategy to reverse the widespread alterations in metabolism contributing to myofibroblast differentiation/persistence. To avoid off target effects of pharmacological interventions, the use of nanoparticle drug delivery systems by loading drug inhibitors into lipid vesicles (e.g. exosomes) that bind specific surface markers of a cardiac fibroblasts could be developed. Gene targeting may provide another therapeutic avenue as the use of systemically injected adeno-associated viruses (AAVs) have been used to direct gene transfer to a variety of tissues. Recently, the use of AAV9 carrying a *Postn* promoter targeted a myofibroblast-like lineage in mouse hearts following myocardial infarction, potentially providing a new avenue for gene therapy to treat cardiac fibrosis[208]. The idea of periostin-directed gene therapy is made more appealing by the knowledge that periostin expression is largely absent from the non-diseased heart and is specifically upregulated in activated fibroblasts[13], suggesting gene therapy can be restricted to myofibroblasts and hence avoid on-target negative effects. Lastly, recent studies utilizing methodologies designed to combat cancer report that chimeric antigen receptor T-cell (CAR-T) therapy may be amendable to override myofibroblast resistance to apoptosis. Epstein and colleagues discovered that engineered T cells targeting activated cardiac fibroblasts via fibroblast activation protein (FAP), reduced pathological myofibroblast number and fibrosis in a model of chronic neurohormonal stimulation[209]. While an effective treatment is still elusive, the continued study of myofibroblast mitochondria and metabolism will likely yield novel therapeutic strategies to treat cardiac fibrosis and improve patient outcomes.

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Nonstandard Abbreviations and Acronyms:

TGFβ	transforming growth factor beta
AngII	angiotensin 2
ECM	extracellular matrix
αSMA	alpha smooth muscle actin (protein)
Acta2	alpha smooth muscle actin (gene)
MI	myocardial infarction
mtROS	mitochondrial-derived reactive oxygen species
Ca2+	calcium
TCF21	transcription factor 21
WT1	Wilms tumor protein
TBX18	T-box transcription factor 18
ER	endoplasmic reticulum
p38	p38 mitogen-activated protein kinases
MAPK1/2	mitogen-activated protein kinase 1/2
ERK1/2	extracellular signal related kinase 1/2
FN	fibronectin
POSTN	periostin
COL-1	collagen type 1
COL-3	collagen type 3
Fn-EDA	fibronectin containing extra domain A
TGFβR1/2	transforming growth factor beta receptor type 1 or 2
SMAD2/3/4/7	SMAD family member 2/3/4/7
AKT	phosphoinositide 3 kinase (PI3K)-protein kinase B
NF-kB	nuclear factor-kB

GPCR	G protein-coupled receptor
SRF	serum response factor
ET-1	endothelin 1
NFAT	nuclear factor of activated T-cells
RhoA	Ras homolog family member A
ROCK	Rho-associated, coiled-coil-containing protein kinase
MRTF	myocardin related transcription factor
TRP	transient receptor potential cation channels
TRPV4	transient receptor potential vanilloid 4
TRPC6	transient receptor potential channel 6
TRPM7	transient receptor potential member 7
TRPC3	transient receptor potential channel 3
IP3R	inositol triphosphate receptor
RyR	ryanodine receptor
$O2^{\cdot-}$	superoxide
Drp1	dynamin-related protein 1
MFF	mitochondrial fission factor
NOX4	NADPH oxidase 4
PARK2	Parkin RBR E3 Ubiquitin Protein Ligase (gene)
PDGFR	platelet-derived growth factor receptor
SOD2	mitochondrial superoxide dismutase
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large (BCL-XL)
BAX	Bcl-2-associated X protein
BAK	Bcl-2 homologous antagonist killer
mtCU	mitochondrial calcium uniporter
MCU	mitochondrial calcium uniporter
MICU1	mitochondrial calcium uptake 1
PDH	pyruvate dehydrogenase

PFK1	phosphofructokinase 1
PKM	pyruvate kinase muscle isozyme
LDH	lactate dehydrogenase
2-DG	2-deoxyglucose
PFKFB	phosphofructo-2-kinase/fructose-2,6-bisphosphate
PKM2	pyruvate kinase isozyme 2
PDK	pyruvate dehydrogenase kinase-1
HIF1α	hypoxia inducible factor 1 alpha
ACLY	ATP citrate lyase
ACSS2	Acyl-CoA Synthetase Short Chain Family Member 2 (ACSS2)
HAT	histone acetyltransferase
HDAC	histone deacetylase
HSC	Hepatic stellate cells
PPARβ/γ	peroxisome-proliferator-activated receptor β/γ
SREBP-1	sterol regulatory element-binding protein 1
PHGDH	phosphoglycerate dehydrogenase
PSAT1	phosphoserine aminotransferase 1
PSPH	phosphoserine phosphatase
SHMT2	serine hydroxymethyltransferase
mTOR	mammalian target of rapamycin
ATF-4	activating transcription factor 4
KMT7	lysine N-methyltransferase 7
KMT2H	lysine N-methyltransferase 2H
O-GlcNAc	O-Linked β-N-acetylglucosamine
SP1	transcription factor Sp1
GFPT1	glucosamine-fructose-6-phosphate aminotransferase isomerizing 1
αKG	alpha ketoglutarate
GLS	glutaminase

TET	ten-eleven translocation enzymes
JmjC-KDMs	JmjC-domain-containing demethylases
JMJD3	jumonji domain-containing protein D3
P5CS	Δ1-pyrroline-5-carboxylate synthase
GSH	glutathione
GSSG	oxidized glutathione
AAV	adeno-associated viruses
CAR-T	chimeric antigen receptor T-cell
FAP	fibroblast activation protein

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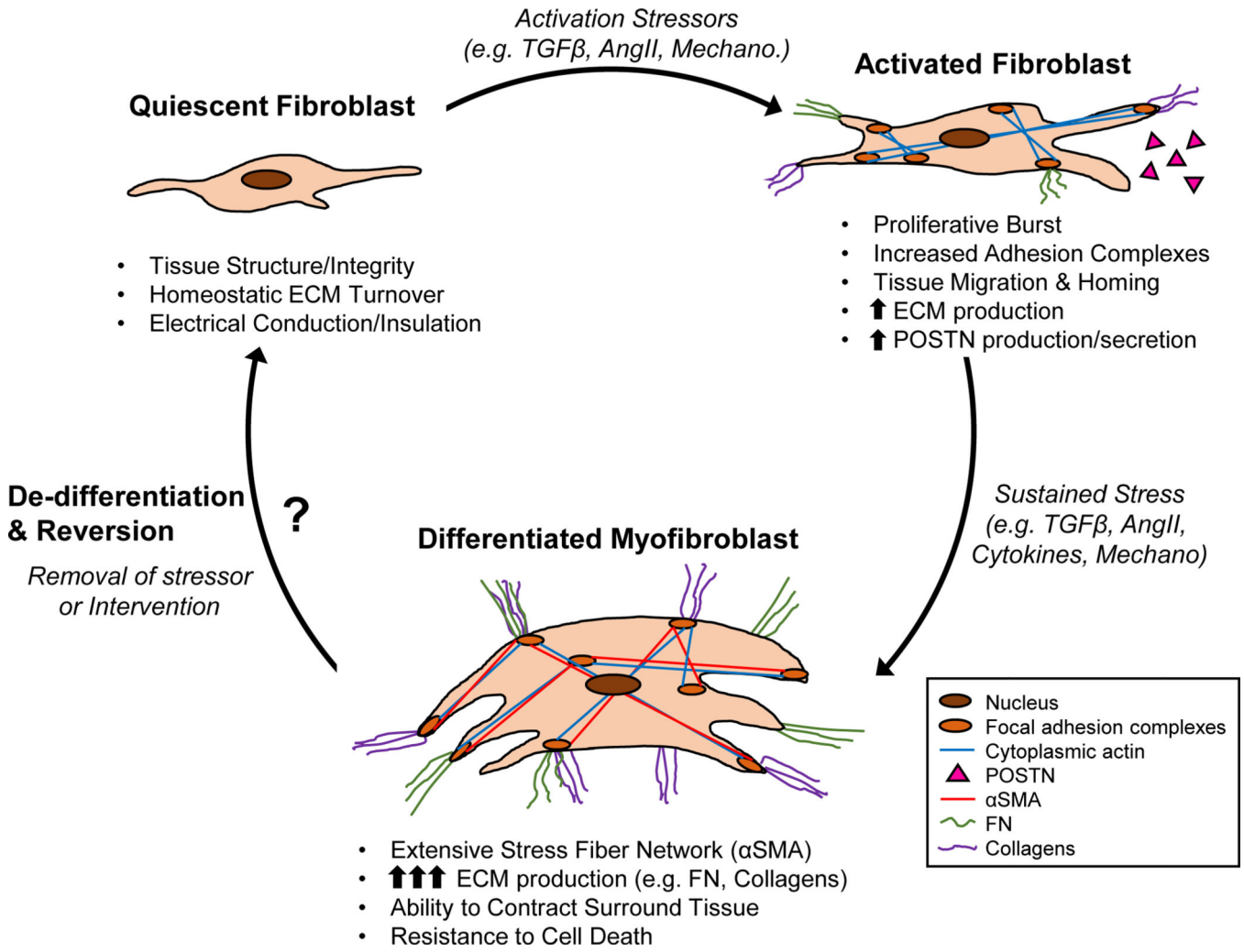

Figure 1. Progression and characterization of cardiac fibroblast to myofibroblast conversion.

Resident fibroblasts contribute to cardiac homeostasis under normal physiological conditions. Upon increased mechanical tension and pro-fibrotic mediators (e.g. TGFβ, AngII), these resident cardiac fibroblasts become activated, at which time they infiltrate and expand at the site of injury as well as begin to remodel the ECM. Upon sustained and unremitting activation signaling, these fibroblasts differentiate into myofibroblasts characterized by the *de novo* expression of αSMA and the excessive production of ECM proteins (FN, collagens). Myofibroblasts are also resistant to cell death, resulting in their persistence in the injured heart, eventually leading to maladaptive tissue remodeling. Recent evidence suggest that myofibroblasts are capable of de-differentiating upon removal of stress stimuli, however the mechanisms by which they do so, and the potential for targeted mechanistic interventions, require further investigation. ECM – extracellular matrix; TGFβ – transforming growth factor beta; AngII – angiotensin II; Mechano – mechanical stress; POSTN – periostin; αSMA – α-smooth muscle actin; FN – fibronectin.

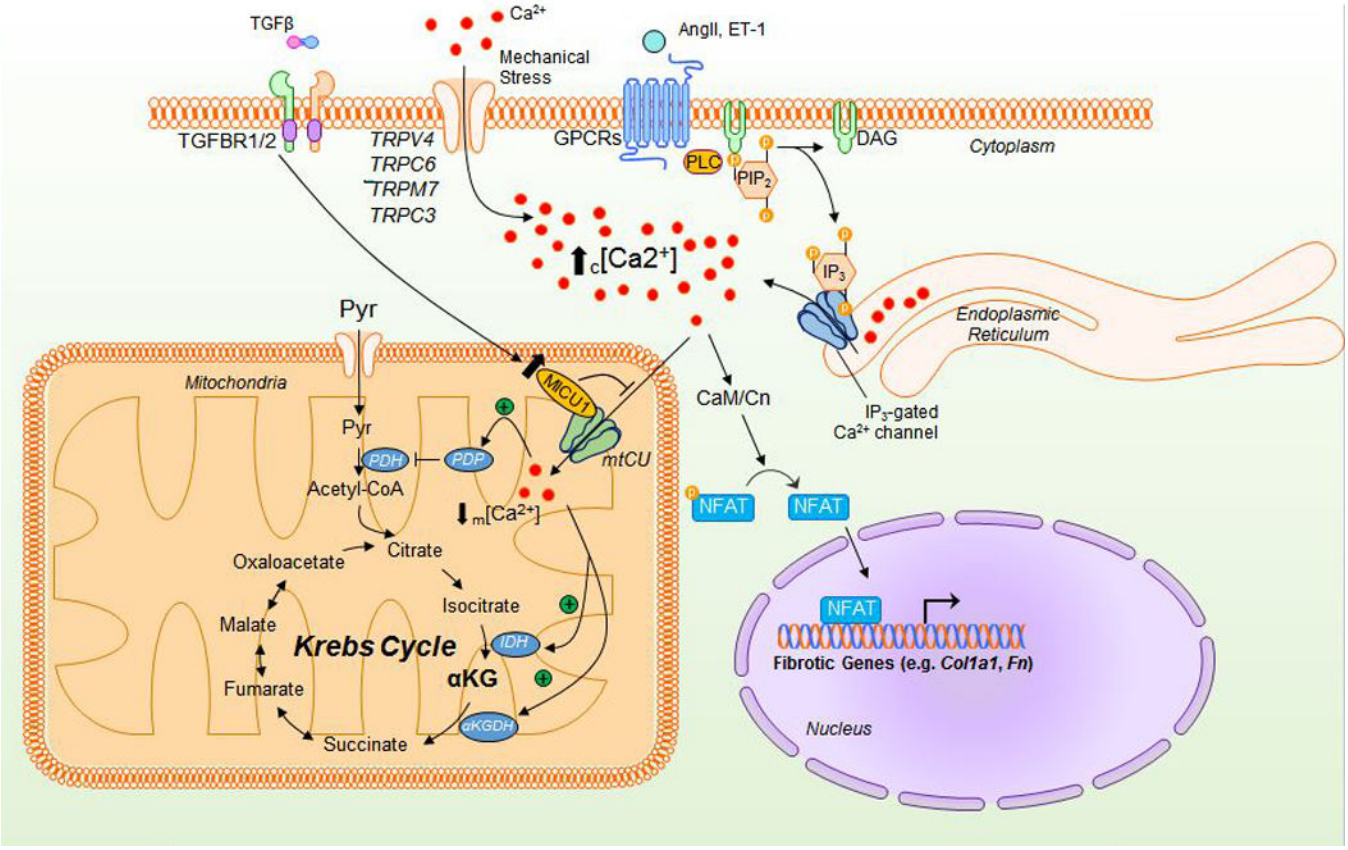

Figure 2. Mechanistic actions of Ca^{2+} during myofibroblast differentiation.

Mechanical activation of TRP channels permits Ca^{2+} entry from the extracellular space, increasing cytosolic [Ca^{2}+]. Additionally, activation of the G_q/PLC/IP_3 pathway allows for IP_3-mediated Ca^{2+} release from the ER, further increasing cytosolic [Ca^{2}+]. This increase in cytosolic [Ca^{2}+] activates calmodulin-dependent calcineurin phosphatase (CaM/CN) to dephosphorylate and permit nuclear translocation of NFAT and subsequent activation of the fibrotic gene program. While mitochondria can act to buffer increases in cytosolic [Ca^{2}+] through the mitochondrial calcium uniporter complex (mtCU), the transcriptional upregulation of MICU1 alters mtCU gating, reducing $_{m}Ca^{2+}$ uptake and reducing $_{m}[Ca^{2+}]$. As $_{m}[Ca^{2+}]$ is an activator of numerous dehydrogenases and phosphatases within the mitochondria, this significantly reduces pyruvate oxidation and overall Krebs cycle flux. TGFβ – transforming growth factor beta; AngII – angiotensin II; ET-1 – endothelin 1; TGFBR1/2 – TGFβ receptor 1/2; TRPs – transient receptor potential channels; PLC – protein lipase C; PIP_2 – phosphatidylinositol 4,5-bisphosphate; DAG – diacylglycerol; Pyr – pyruvate; PDH – pyruvate dehydrogenase; PDP – pyruvate dehydrogenase phosphatase; MICU1 – mitochondrial calcium uptake 1; NFAT – nuclear factor of activated T-cells; IDH – isocitrate dehydrogenase; αKGDH - α-ketoglutarate dehydrogenase; Col1a1 – collagen 1 type 1; Fn – fibronectin.

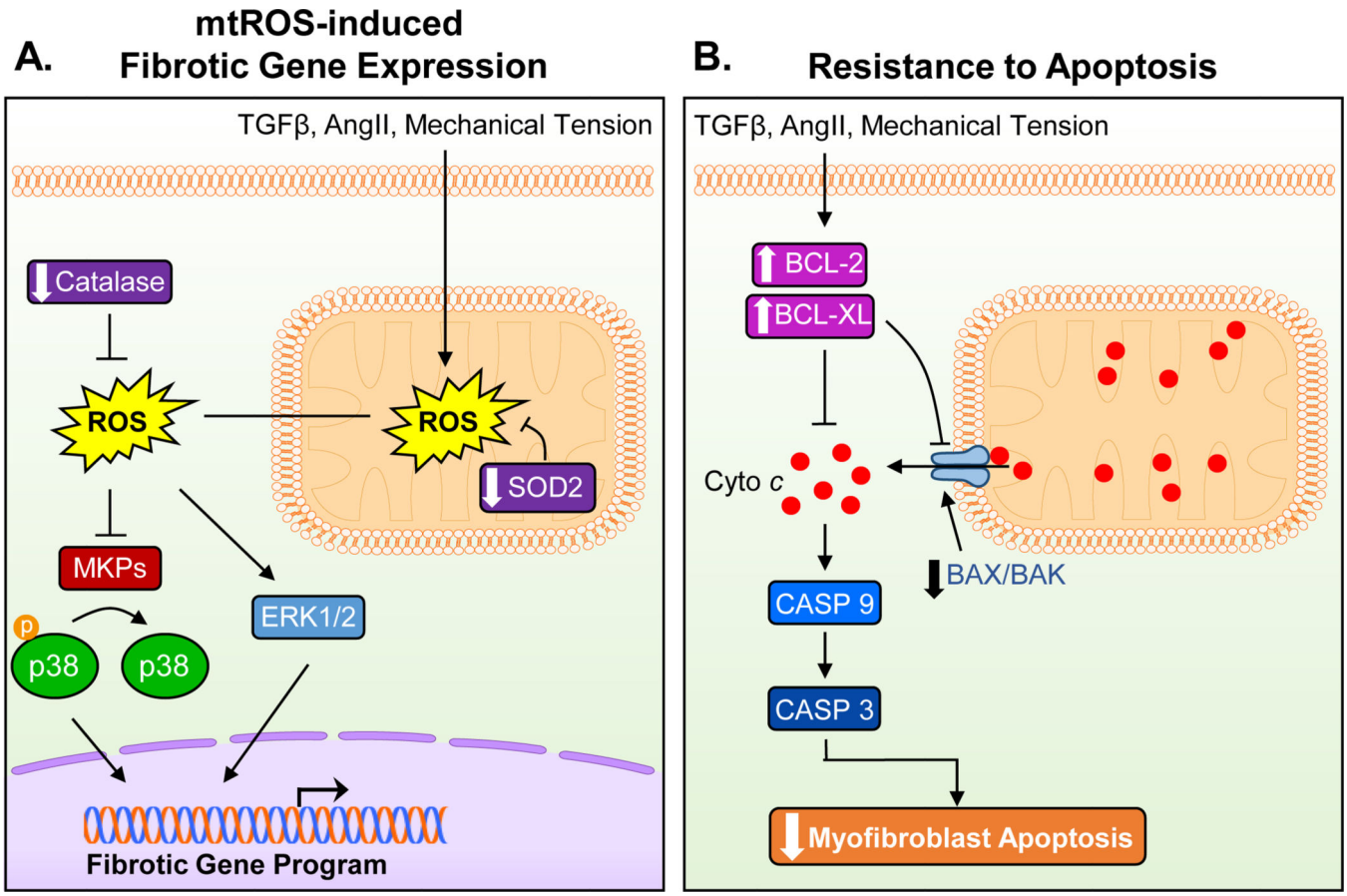

Figure 3. Mitochondrial mechanisms of myofibroblast differentiation and persistence.

(**A**) Pro-fibrotic stressors increase ROS production in the mitochondria, which then results in an increased and sustained intracellular ROS load, in part, through the downregulation of SOD2 and catalase. Increases in intracellular ROS in turn activates p38 and ERK1/2 signaling pathways, which are known to increase the transcription of the fibrotic gene program. (**B**) A key feature of myofibroblasts is their resistance to apoptosis. Mitochondrial cytochrome *c* (Cyto *c*) release is prevented through the upregulation of anti-apoptotic factors (BLC-2, BCL-XL) while pro-apoptotic factors (BAX, BAK) are downregulated. Reduced activation of the proteolytic caspase cascade due to decreased cytochrome *c* release from the mitochondria contributes to the persistence of myofibroblasts in the injured heart by imparting a resistance to cell death. TGFβ – transforming growth factor beta; AngII – angiotensin II; ROS – reactive oxygen species; SOD2 - superoxide dismutase 2; MKPs – mitogen-activated protein kinases; BCL-2 – B-cell lymphoma 2; BCL-XL - B-cell lymphoma-extra large; BAX – Bcl-2-associated X protein; BAK – Bcl-2 homologous antagonist killer; Casp9/3 – caspase 9/3.

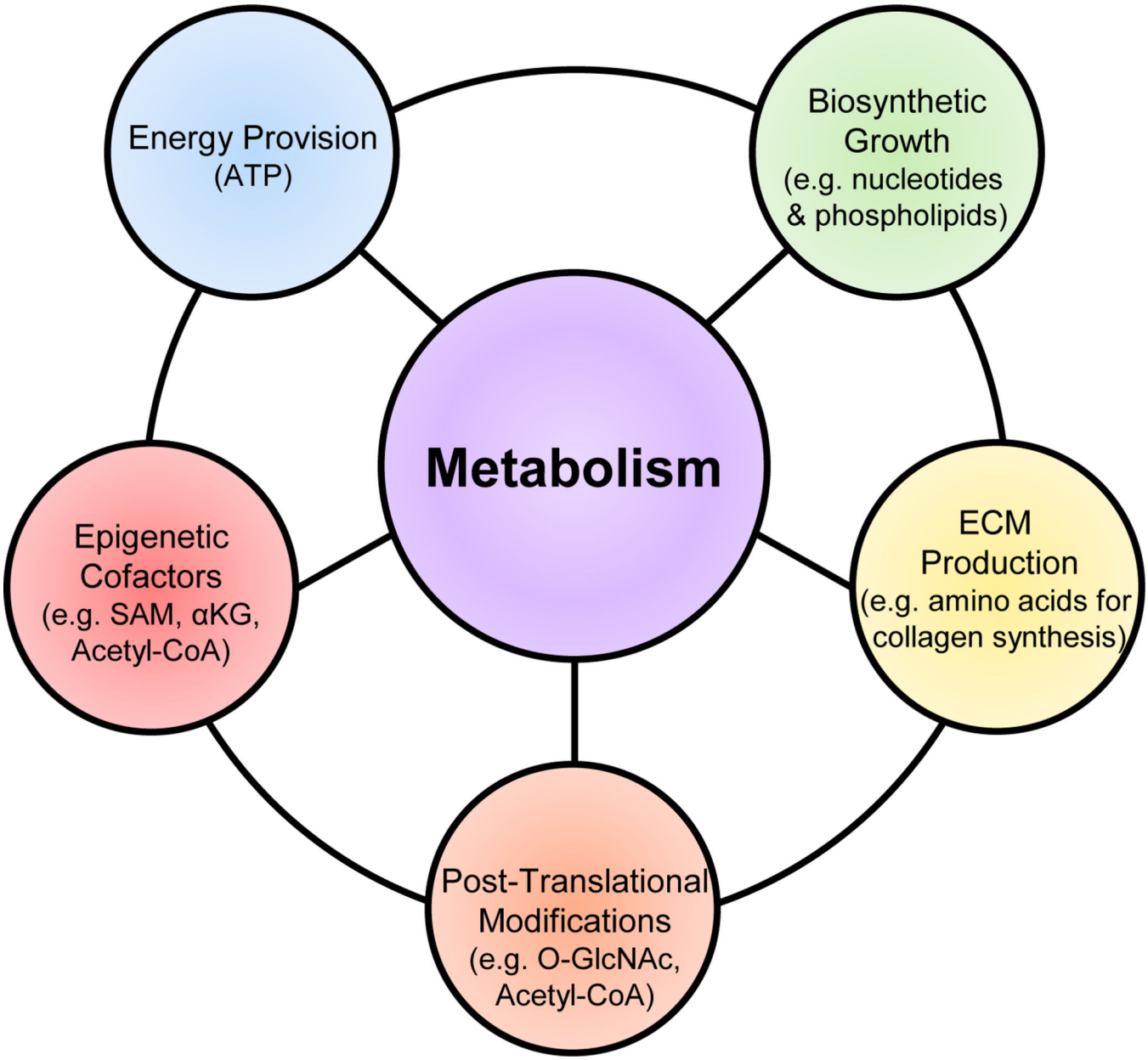

Figure 4. Metabolic components of cellular differentiation.

Overview of the metabolic influence regulating fibroblast differentiation. In addition to the energetic demands of a proliferating/differentiating cell, it is necessary to increase the biosynthetic intermediates to support such events. An increase in production and secretion of ECM components (e.g. collagens) requires increased amino acid synthesis. Post-translational modifications of proteins (e.g. transcription factors) can greatly alter their activities while regulation of the epigenetic landscape influences gene program activation & silencing. While each can work independently, the integration of these metabolic actions drive and coordinate cellular differentiation. ECM – extracellular matrix; SAM – S-adenosylmethionine; αKG - α-ketoglutarate.

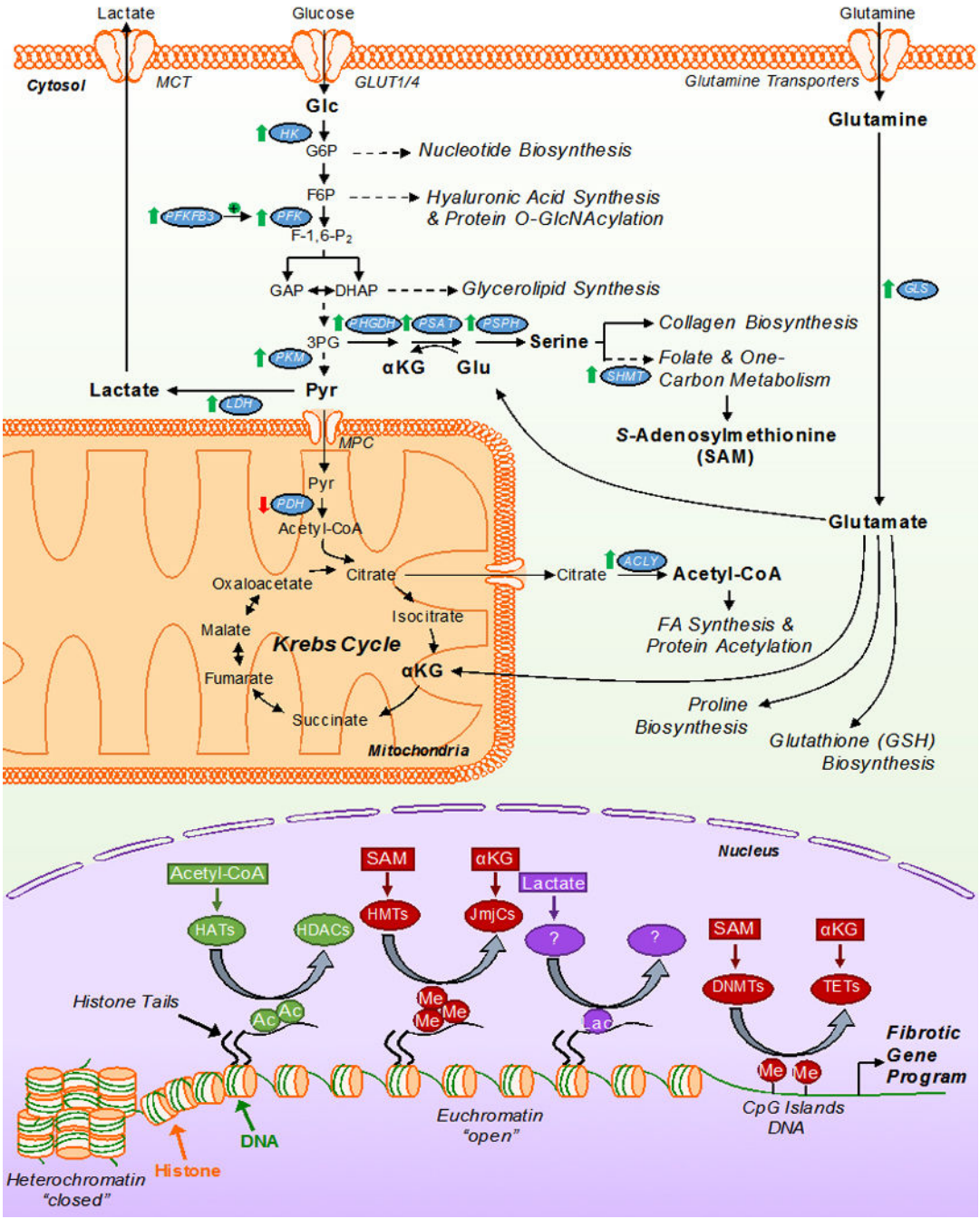

Figure 5. Metabolic inputs regulating the biosynthetic and transcriptional changes coordinating myofibroblast differentiation.

General schematic of myofibroblast metabolism on the differentiation program. Myofibroblasts are characterized by an increase in glutaminolysis and aerobic glycolysis, accompanied by decreased glucose oxidation. These key metabolic re-programming events permit the use of carbon intermediates by ancillary biosynthetic pathways to coordinate the production of ECM constituents, protein modifications, and epigenetic cofactors that initiate and sustain the myofibroblast differentiation program. Changes in the expression/activity of noted enzymes along with metabolites in bold are key to the differentiation process. Of these

processes, the ability of these key metabolites to act as cofactors for epigenetic modifying enzymes is critical for the coordinated activation and silencing of transcriptional events which promote the fibrotic gene program and myofibroblast differentiation/persistence. αKG – α-ketoglutarate; Pyr – pyruvate; HK – hexokinase; PFK – phosphofructokinase; PFKFB3 – 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PHGDH – phosphoglycerate dehydrogenase; PSAT – phosphoserine amino transferase; PSPH – phosphoserine phosphatase; PKM – pyruvate kinase muscle isozyme; LDH – lactate dehydrogenase; SHMT – serine hydroxymethyltransferase; PDH – pyruvate dehydrogenase; ACLY – ATP citrate lyase; HATs – histone acetyltransferases; HDACs – histone deacetylases; HMT – histone methyltransferases; JmJCs – Jumonji C-domain-containing histone demethylases; DNMTs – DNA methyltransferases; TETs – ten-eleven translocases; Ac – acetylation mark; Me – methylation mark; Lac – lactylation mark.

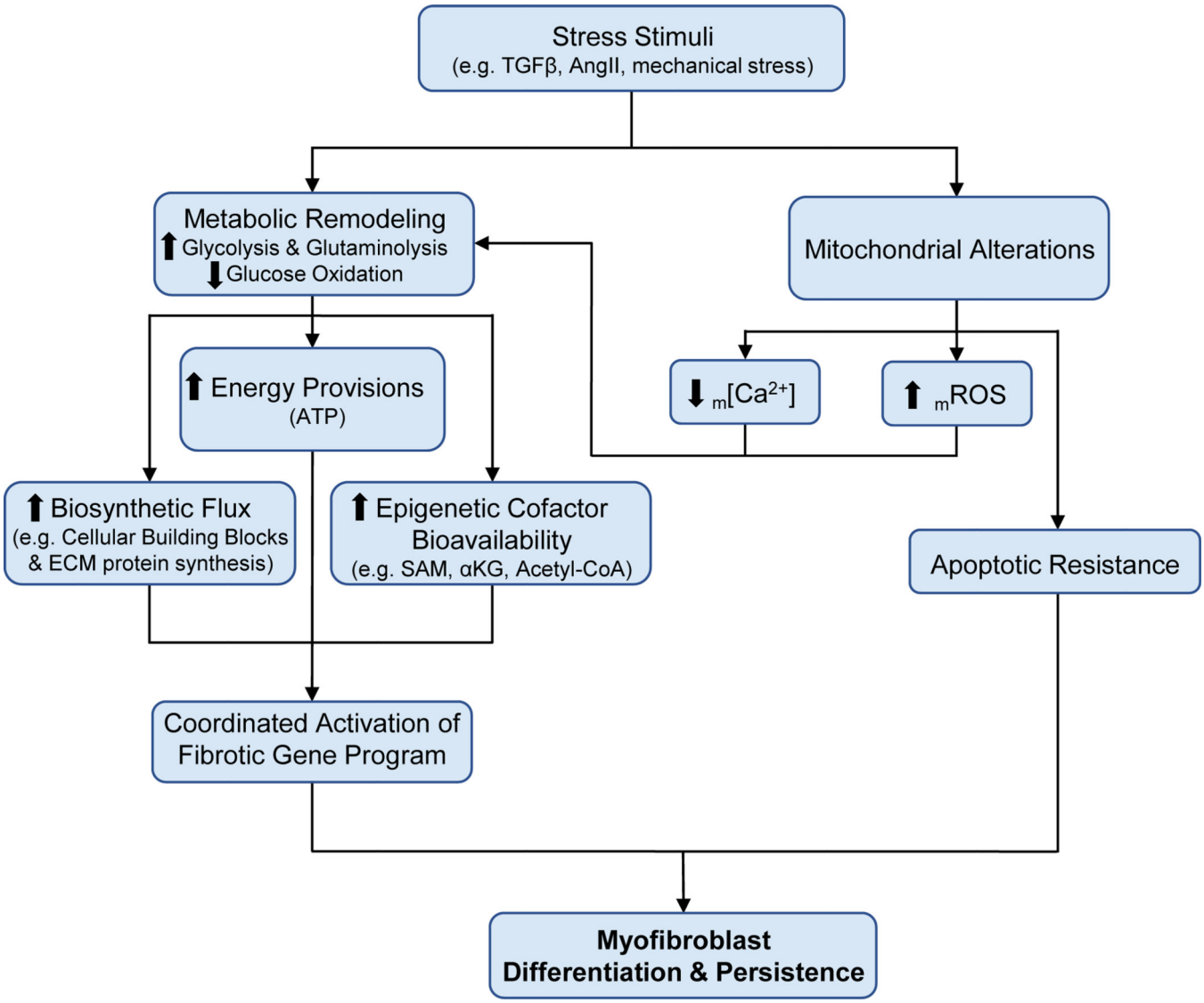

Figure 6. Working model of the influence of mitochondria and metabolism on myofibroblast differentiation and persistence.

Pro-fibrotic stress stimuli are critical for the activation and differentiation of resident cardiac fibroblasts to myofibroblasts. Pro-fibrotic stimulation results in significant metabolic remodeling that is critical for the energy provisions and synthesis of cellular building blocks required for proliferation, growth, and differentiation. Furthermore, these changes in cellular metabolism also regulate the bioavailability of metabolites which serve as cofactors for numerous epigenetic-modifying enzymes to coordinate the activation of the fibrotic gene program to promote differentiation. Recent work from our lab has shed new light on the mitochondria as a key regulator to this process by reducing mitochondrial Ca^{2+} uptake to promote the metabolic remodeling required for the differentiation process. The mitochondria also increase the generation of $_m$ROS to promote the necessary metabolic remodeling. Additionally, mitochondria under pro-fibrotic stimulation and in the terminally differentiated state downregulate the apoptotic pathways, promoting myofibroblast persistence which contributes to the progressive nature of cardiovascular disease. Collectively, the coordinated

efforts of mitochondrial and metabolic remodeling are critical for the activation, differentiation, and persistence of myofibroblasts in cardiac disease, providing novel therapeutic targets to mitigate and potential reverse tissue fibrosis in disease. TGFβ – transforming growth factor beta; AngII – angiotensin II; $_{m[}Ca^{2+}]$ - mitochondrial calcium concentration; $_{m}$ROS – mitochondrial-derived reactive oxygen species; ECM – extracellular matrix; SAM – S-adenosylmethionine; αKG - α-ketoglutarate.