MYOFIBROBLAST (T KISSELEVA, SECTION EDITOR)

Reversibility of Liver Fibrosis and Inactivation of Fibrogenic Myofibroblasts

Xiao Liu · Jun Xu · David A. Brenner · Tatiana Kisseleva

Abstract Many studies have demonstrated that hepatic fibrosis is reversible. Regression of liver fibrosis is associated with resorption of fibrous scar and the disappearance of collagen-producing myofibroblasts. The fate of these myofibroblasts has been recently revealed: some myofibroblasts undergo senescence and apoptosis during reversal of fibrosis, whereas other myofibroblasts revert to a quiescent-like phenotype. Inactivation of myofibroblasts is a newly described phenomenon (Kisseleva et al. in *Proc. Natl .Acad. Sci. USA* 109:9448–9453, 2012) which now requires mechanistic investigation. Understanding the mechanism of inactivation of hepatic stellate cells on cessation of fibrogenic stimuli may identify new approaches to cause already existing activated hepatic stellate cells/myofibroblasts to revert to a quiescent-like state. This review summarizes the research on the inactivation of hepatic myofibroblasts.

Keywords Reversibility of liver fibrosis · Hepatic fibrosis · Inactivation of myofibroblasts · Hepatic stellate cells · Collagen · Pathobiology

Abbreviations

aHSCs	Activated HSCs
α-SMA	α-Smooth muscle actin
Col-α2(I)	Type II collagen α_2
Col-α1(I)	Type I collagen α_1
ECM	Extracellular matrix
GFAP	Glial fibrillar acidic protein
HSCs	Hepatic stellate cells
iHSCs	Inactivated HSCs
PPARγ	Peroxisome-proliferator-activated receptor γ
qHSCs	Quiescent HSCs
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
YFP	Yellow fluorescent protein

X. Liu · J. Xu · D. A. Brenner
Department of Medicine, University of California,
San Diego, La Jolla, CA, USA

T. Kisseleva (✉)
Department of Surgery, University of California,
San Diego, 9500 Gilman Drive, Leichtag Biomedical Research
Building, H349, La Jolla, CA 92093-0602, USA
e-mail: tkisseleva@ucsd.edu

Introduction

Hepatic fibrosis is an outcome of many chronic liver diseases, including hepatitis B, hepatitis C, alcoholic liver disease, and nonalcoholic steatohepatitis [2]. Hepatic fibrosis is characterized by extensive deposition of extracellular matrix (ECM) proteins, mostly type I collagen, forming a scar. Chronic liver injury damages hepatocytes. Injured or apoptotic hepatocytes secrete factors that facilitate activation and recruitment of inflammatory cells to the injured liver. Activated macrophages secrete IL-6 and transforming growth factor (TGF) β_1, which in turn activate hepatic myofibroblasts. Myofibroblasts are not present in the normal liver, but in response to injury they transdifferentiate from hepatic stellate cells (HSCs), upregulate collagen, and produce the fibrous scar. Myofibroblasts are characterized by expression of α-smooth muscle actin (α-SMA) and type I collagen, and in all clinical and experimental liver fibroses serve as a major source of the ECM. Thus, activation and proliferation of hepatic myofibroblasts is a key mechanism in the development of liver cirrhosis.

Myofibroblasts Are the Primary Target of Antifibrotic Therapy

Hepatic myofibroblasts are the major source of type I collagen in fibrotic liver. Therefore, elimination of myofi-broblasts or their inactivation is a goal of therapy. Several sources of myofibroblasts have been identified [3–6]. It is believed that HSCs are the major source of fibrogenic myofibroblasts, and contribute more than 80 % of the collagen-producing cells [1••, 2, 7]. Under physiological conditions, HSCs reside in the space of Disse and exhibit a quiescent phenotype (quiescent HSCs, qHSCs). They express neural markers, such as glial fibrillar acidic protein (GFAP), synemin, synaptophysin, and nerve growth factor receptor p75, and store vitamin A in lipid droplets [3]. In response to injury, qHSCs decrease vitamin A storage and peroxisome-proliferator-activated receptor γ (PPARγ) expression, and activate into type I collagen and α-SMA expressing myofibroblasts [2]. Although the mechanism of HSC activation has been comprehensively studied, insights into the fate of HSCs during regression of liver fibrosis are new [8•]. In addition to HSCs, portal fibroblasts [9, 10] and bone-marrow-derived fibrocytes [11] can also contribute to hepatic myofibroblasts.

Reversal of Liver Fibrosis

Mechanism of Regression of Liver Fibrosis

Sequential liver biopsies from patients with liver fibrosis have demonstrated that removing the underlying etiologi-cal agent may reverse hepatic fibrosis in patients with secondary biliary fibrosis [12], hepatitis C [13], hepatitis B [14], nonalcoholic steatohepatitis [15], and autoimmune hepatitis [16]. Withdrawal of the etiological source of the chronic injury (e.g., hepatitis B virus and hepatitis C virus) [2] results in a decrease in the levels of proinflammatory and fibrogenic (including TGF-β_1) cytokines, increased collagenase activity [2, 3], decreased ECM production, and the disappearance of activated myofibroblasts.

Studies of liver fibrosis in rodents have confirmed that established liver fibrosis can reverse on cessation of the action of the etiological agent. Reversal of liver fibrosis has been successfully studied in experimental models of CCl_4 [17, 18], alcohol, and bile duct ligation [19] induced liver fibrosis [2]. Several events have been identified to be critical for regression of liver fibrosis. These include the disappearance of hepatic myofibroblasts/activated HSCs (aHSCs), recruitment of collagenese-secreting monocytes/ macrophages, and the prevalence of matrix-degrading metalloproteinases over their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs).

Conditions for Reversal of Liver Fibrosis

Bone-marrow-derived monocytes and liver-resident mac-rophages play an important role in reversal of liver fibrosis. Selective ablation of CD11b$^+$ cells in mice (CD11b-DTR) during the phase of recovery from liver injury significantly attenuates fibrosis resolution, suggesting that macrophages mediate distinct functions at the onset of fibrosis and dur-ing recovery. Increased collagenase activity is a primary pathway of fibrosis resolution. At this stage, activated macrophages/Kupffer cells secrete matrix metalloprotein-ases, e.g., matrix metalloproteinase13, interstitial collage-nase, and other enzymes responsible for matrix degradation [6, 20]. Moreover, increased activity of collagen-degrading enzymes during fibrosis resolution correlates with decreased amounts of TIMPs [21•, 22]. Activated myofi-broblasts/aHSCs serve as a significant source of TIMPs, and the disappearance of myofibroblasts/HSCs during recovery is associated with reduced production of TIMPs.

An established mechanism for the elimination of acti-vated myofibroblasts is due to senescence [23] and apop-tosis of aHSCs [24]. Several mechanisms are implicated in the apoptosis of aHSCs: (1) activation of death-receptor-mediated pathways (Fas receptor or TNF receptor 1) and caspases 3 and 8; (2) upregulation of proapoptotic proteins (e.g., p53, Bax, and caspase 9); and (3) decrease of pro-survival genes (e.g., Bcl-2) [22]. A population of liver-associated natural killer cells and γδ T cells stimulatse apoptosis of aHSCs. Drugs that induce apoptosis in aHSCs (glyotoxin, sulfasalazine, IκB kinase inhibitors, and anti-TIMP antibodies) cause liver fibrosis to regress [2, 3].

Conditions for Irreversible Liver Fibrosis

Whether end-stage cirrhosis can revert to a normal liver architecture remains controversial [25, 26]. However, sig-nificant improvement in hepatic structure and function provides evidence of regression of liver fibrosis [21•, 27]. Perhaps ECM remodeling is limited in cirrhosis by the formation of nonreducible cross-linked collagen and an ECM rich in elastin fibers preventing its degradation. This pathophysiological state may lead to a "point of no return" for liver fibrosis [26, 27]. The characteristics of the myo-fibroblast environment play a critical role in myofibroblast survival. For example, stiffness of the ECM and increased contact between myofibroblasts and collagen scar promote survival of myofibroblasts and facilitate their activation [28]. In support of this notion, transgenic mice expressing an uncleavable form of type I collagen are more suscepti-ble to liver fibrosis, and demonstrate a defect in sponta-neous resolution of liver fibrosis on cessation of liver injury [29]. Similarly to what is found in these transgenic mice, prolongation of the duration of fibrogenic liver injury often

results in the formation of persistent and uncleavable scars caused by irreversible cross-linking of collagen fibers. Furthermore, the presence of elastin fibers distinguishes "biochemically mature scars," which are more likely to persist in recovering liver [18]. In addition, persistent scars are associated with the formation of areas of hypocellularity, suggesting that lack of biodegrading macrophages in these areas may contribute to poor desorption of fibrous scars [18].

The Role of Myofibroblasts in Reversal of Liver Fibrosis

Although aHSCs undergo senescence [23] and apoptosis [17] during the regression of liver fibrosis, the quantitative contribution of aHSC apoptosis to the disappearance of activated myofibroblasts remains unknown [27]. Further, the cellular population of HSCs is restored in mice recovering from fibrosis, and the source of these quiescent-like HSCs is unknown. Recent studies have suggested that in addition to apoptosis, aHSCs/activated myofibroblasts can be eliminated by their undergoing inactivation and reverting to a quiescent-like phenotype. We [1••], and subsequently others [30••], have used genetic marking to demonstrate an alternative pathway in which myofibroblasts revert to a quiescent-like phenotype in CCl₄-induced liver injury and experimental alcoholic liver disease. The findings of these in vivo studies are in concordance with in vitro observations that suggested that cultured HSCs, at least in part, can revert to a quiescent-like phenotype. The quiescent phenotype of HSCs is associated with expression of lipogenic genes and storage of vitamin A in lipid droplets. Depletion of PPARγ is a key molecular event for HSC activation, and ectopic expression of this nuclear receptor results in the phenotypic reversal of aHSCs to quiescent cells in culture [31]. Treatment of aHSCs with an adipocyte differentiation cocktail or overexpression of SREBP-1c results in upregulation of adipogenic transcription factors and causes morphologic and biochemical reversal of aHSCs to quiescent-like cells [21•, 32, 33]. These in vitro and in vivo studies have provided new insights into the concept of reversibility of liver fibrosis, suggesting that the disappearance of activated myofibroblasts is attributed not only to their apoptosis but also to reversal of their phenotype to a quiescent-like phenotype.

Methods to Study Inactivation of HSCs

The Cre–lox system [34] provides a unique tool to monitor specific cellular populations and their progeny in mice [35]. This system is based on the ability of the small 38-kDa bacteriophage protein Cre recombinase to recognize and excise inverted 13 base pair loxP sequences and the flanking DNA [34, 36]. Genetic labeling of a specific cellular population is achieved by crossing mice expressing Cre under control of a cell-specific promoter with reporter Rosa26^f/f-YFP mice ubiquitously expressing the yfp gene in which transcription is blocked by a floxed Stop cassette [36]. Genetic Cre–loxP recombination causes excision of the floxed–Stop–floxed sequence from genomic DNA, with activation of yellow fluorescent protein (YFP) transcription in the resulting offspring. The cells of interest now irreversibly express YFP, so their phenotypic changes can be monitored in response to injury or stress [37].

Using Cre–LoxP-based genetic labeling of myofibroblasts, we [1••] and others [30••] elucidated the fate of aHSCs/activated myofibroblasts during recovery from CCl₄-induced liver fibrosis (Fig. 1a) [8•]. Genetic labeling of aHSC/myofibroblasts resulted from crossing mice expressing Cre under control of the type I collagen α₁ [Col-α1(I)] enhancer/promoter [Col-α1(I)^Cre mice] with reporter mice
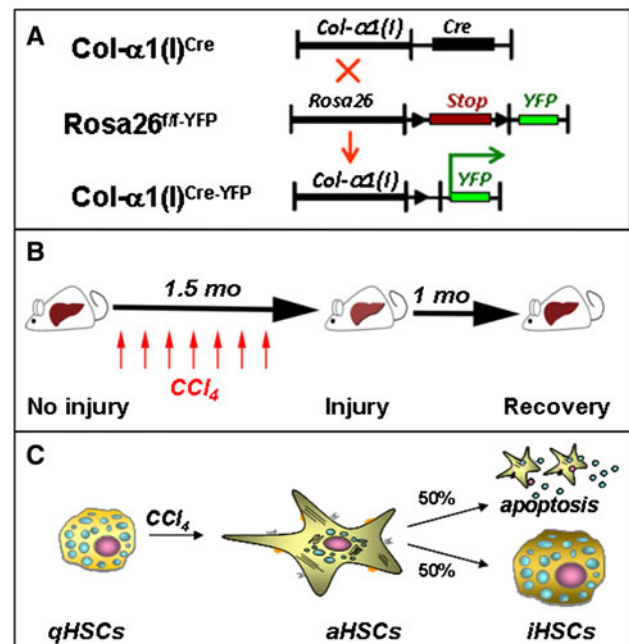

Fig. 1 Study design to determine the fate of activated hepatic stellate cells (*aHSCs*) during regression of CCl₄-induced liver fibrosis. **a** Cre–loxP-based genetic labeling marks the fate of type I collagen α₁ [Col-α1(I)] expressing aHSCs/myofibroblasts in Col-α1(I)^Cre-YFP mice generated by crossing Col-α1(I)^Cre and Rosa26^f/f-YFP mice. **b** Col-α1(I)^Cre-YFP mice were subjected to CCl₄-induced liver injury (1.5 months), then recuperated on cessation of exposure to the injuring agent (for 1 month). Mice were killed and livers were analyzed for the presence of vitamin A positive, yellow fluorescent protein (*YFP*) positive and vitamin A positive, YFP-negative hepatic stellate cells. **c** CCl₄ induces activation of quiescent hepatic stellate cells (*qHSCs*) into aHSCs/myofibroblasts in Col-α1(I)^Cre-YFP mice. After CCl₄ withdrawal, some aHSCs undergo apoptosis and some become inactivated; the number of YFP-positive inactivated hepatic stellate cells (*iHSCs*) is less than 100 % of the number of aHSCs [6]. *mo* month. (Adapted from Kisseleva and Brenner [8•])

(Rosa26[f/f-YFP] mice). In the offspring [Col-α1(I)[Cre-YFP] mice], all type I collagen expressing cells expressed YFP. In uninjured mice, type I collagen is not expressed in the liver, and this correlates with minimal expression of YFP in livers of Col-α1(I)[Cre-YFP] mice. Following induction of liver injury in these mice, aHSCs and their progeny express collagen-driven YFP and are permanently labeled by YFP expression [1••]. Phenotypical changes of aHSCs and the mechanism of their inactivation can now be studied during regression of liver fibrosis (Fig. 1b) [8•]. Using two models of hepato-toxicity-induced liver fibrosis (CCl4 and intragastric alcohol feeding), we demonstrated that half of the myofibroblasts escape apoptosis during regression of liver fibrosis, down-regulate fibrogenic genes (Col1a1, Col1a2, α-SMA, TIMP1,

TGF-β receptor 1) and acquire a phenotype similar to, but distinct from, that of qHSCs [1••]. Similar results were obtained using inducible Cre-based systems, in which genetic labeling of aHSCs was achieved in Col-α1(I)–estrogen receptor–Cre and vimentin–estrogen receptor–Cre mice on tamoxifen administration.

Generation of these novel transgenic mice provided a unique tool to study the fate of hepatic myofibroblasts in fibrotic liver. In addition, several studies have used GFAP–Cre mice to successfully target HSCs in the liver. Although these GFAP–Cre mice do not discriminate between qHSC, aHSC, and iHSC (iHSC) phenotypes, they were success-fully used to label HSCs for quantification purposes or for HSC-specific gene deletion. Since HSCs and astrocytes
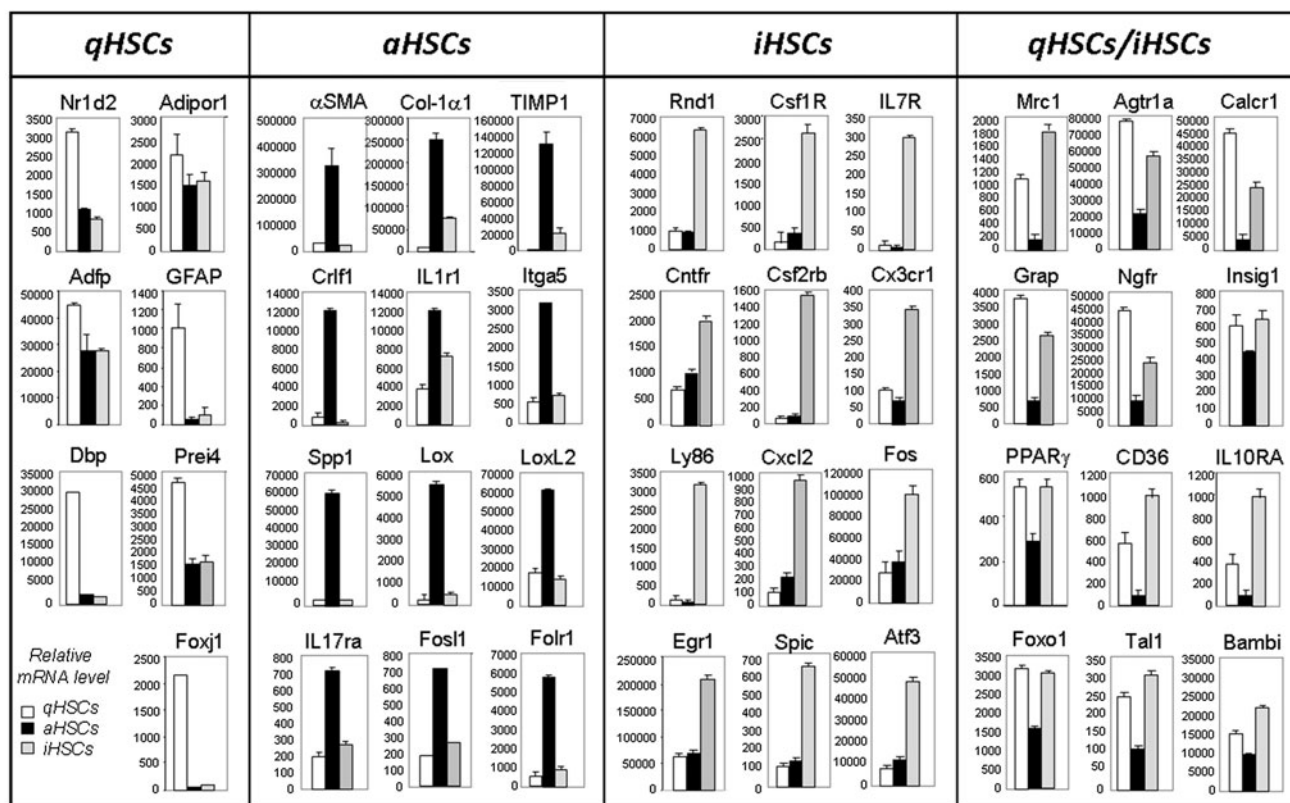

Fig. 2 Differential expression of cell surface antigens by different hepatic stellate cell types. On the basis of the whole genome microarray, we have identified messenger RNAs (mRNAs) that are specifically upregulated in qHSCs: Nr1d2, adiponectin receptor 1 (Adipor1), adipose-differentiation-related protein (Adfp), glial fibril-lary acidic protein (GFAP), D site of albumin promoter (albumin D-box) binding protein (Dbp), Prei4, and forkhead box protein J1 (Foxj1). The following mRNAs were specifically upregulated in aHSCs: α-smooth muscle actin (αSMA), type I collagen α1 (Col-1α1), tissue inhibitor of metalloproteinase 1 (TIMP1), cytokine-receptor-like factor 1 (Crlf1), interleukin-1 receptor type 1 (IL1r1), integrin α5 (Itga5), secreted phosphoprotein 1 (Spp1), lysyl oxidase (Lox), lysyl oxidase like 1 (LoxL2), interleukin-17 receptor A (IL17ra), fos-like antigen 1 (Fosl1), andfolate receptor 1 (Folr1). The following mRNAs were upregulated in iHSCs: Rho family GTPase 1 (Rnd1), colony stimulating factor 1 receptor (Csf1R), interleukin-7 receptor (IL7R),

ciliary neurotrophic factor receptor (Cntfr), colony stimulating factor 2 receptor, beta, low-affinity (granulocyte–macrophage) (Csf2rb), CX3C chemokine receptor 1 (Cx3cr1), lymphocyte antigen 86 (Ly86), chemokine (C-X-C motif) ligand 2 (Cxcl2), FBJ osteosarcoma oncogene (Fos), epidermal growth factor receptor (Egr1), Spi-C transcription factor (Spi-1/PU.1 related) (Spic), and activating tran-scription factor 3 (Atf3). Several mRNAs were upregulated in both qHSCs and iHSCs: mannose receptor 1 (Mrc1), angiotensin II receptor, type 1 (Agtr1a), calcitonin-receptor-like (Calcrl), GRB2-related adapter protein (Grap), the low-affinity nerve growth factor receptor (Ngfr), insulin-induced gene 1 (Insig1), peroxisome-prolif-erator-activated receptor γ (PPARγ), CD36, interleukin-10 receptor α (IL10RA), forkhead box O1 (Foxo1), helix–loop–helix protein (Tal1), and bone morphogenetic protein and activin membrane-bound inhibitor homolog (Xenopus laevis) (Bambi)

share expression of several neural markers, including GFAP, deletion of this gene in the brain might affect the phenotype in the liver.

Characterization of the Novel iHSC Phenotype

Inactivated HSCs (iHSCs) acquire a novel phenotype which has not been previously described (Fig. 1c) [8•]. In particular, iHSCs more rapidly reactivate into myofibroblasts in response to fibrogenic stimuli and more effectively contribute to liver fibrosis. Inactivated HSCs (iHSCs) down-regulate α-SMA, *Col1a1*, *Col1a2*, TIMP1, and TGF-β receptor 1 and obtain features of quiescent-like HSCs owing to upregulation of PPARγ and Bambi [1••]. Meanwhile, other quiescent-associated genes such as GFAP, *Adipor1*, *Adpf*, and *Dbp* are not reexpressed in iHSCs, suggesting that despite the similarities between qHSCs and iHSCs and their lack of fibrogenic gene expression, iHSCs possess properties distinct from those of qHSCs.

Inactivation of HSCs is associated with reexpression of the lipogenic genes PPARγ, *Insig1*, and cyclic AMP response element binding protein [31]. Our findings in mice in vivo support previous in vitro studies demonstrating the importance of PPARγ for maintaining and reestablishing the quiescent phenotype [31, 33]. On the basis of comparison of the global gene expression in qHSCs, aHSCs, and iHSCs, we have identified several genes that are differentially expressed in HSCs depending on their stage of activation, and can be used to distinguish iHSCs from qHSCs and aHSCs [1••]. This is of particular importance for identifying inactivation of human HSCs. Our strategy is based on identification of genes similarly and differentially expressed in qHSCs, aHSCs, and iHSCs, and that can be easily detected in a pool of HSCs by flow cytometry. Differential expression of cell surface antigens by different HSC types is illustrated in Fig. 2. Since qHSCs and iHSCs share many features, detection of surface markers may be compared in correlation with induction of phenotype-specific transcription factors, or other proteins.

Comparative analysis of phenotype-specific HSC signature genes may provide further insight into inactivation of HSCs in vivo (Fig. 2). Specifically, we identified seven qHSC-specific genes (that are expressed only in qHSCs), 12 aHSC-specific genes (expressed only in aHSCs), 12 iHSC-specific genes (upregulated in iHSCs), and 12 genes which are upregulated in both qHSCs and I HSCs (and therefore are associated with nonfibrogenic properties of HSCs). Furthermore, the genes *Nr1d2*, *Adipor1*, *Il17ra*, *Itga5*, *Egfr*, *Crlf1*, *Il1r1*, *Rnd1*, *Csf1r*, *Il7r*, *Cntfr*, *Csf2rb*, *Cx3cr1*, *Il10ra*, *Ly86*, *Cd36*, *Mrc1*, *Agtr1a*, *Calcr1*, *Grap*, and *Ngfr* are cell surface receptors, so their expression can be detected using flow cytometry (which can simultaneously detect expression of up to 11 to 12 surface antigens

in a single sample), allowing us to confidently identify and isolate live iHSCs. Furthermore, we identified several phenotype-specific transcription factors expressed in HSCs dependent on the stage of activation/inactivation.

Conclusions

Inactivation of myofibroblasts during reversal of fibrosis opens up new prospects for therapy. Hepatic fibrosis is reversible in patients and in experimental models with decreased fibrous scar and disappearance of the myofibroblast population. However, the fate of the myofibroblasts in patients with liver fibrosis is unknown. Although some myofibroblasts undergo death [17], an alternative untested hypothesis is that the myofibroblasts revert to their original quiescent phenotype or obtain a new phenotype. Understanding the origin and biology of fibrogenic myofibroblasts will provide a new target for antifibrotic therapy [21•].

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