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Research paper

## Role of redoximiRs in fibrogenesis

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### ABSTRACT

Fibrosis can be defined as an excessive accumulation of extracellular matrix (ECM) components, ultimately leading to stiffness, scarring and devitalized tissue. MicroRNAs (miRNAs) are short, 19–25 nucleotides (nt), non-coding RNAs involved in the post-transcriptional regulation of gene expression. Recently, miRNAs have also emerged as powerful regulators of fibrotic processes and have been termed “fibromiRs”. Oxidative stress represents a self-perpetuating mechanism in fibrogenesis. MiRNAs can also influence the expression of genes responsible for the generation of reactive oxygen species (ROS) and antioxidant defence and are termed “redoximiRs”. Here, we review the current knowledge of mechanisms by which “redoximiRs” regulate fibrogenesis. This new set of miRNAs may be called “redoxifibromiRs”.

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## 1. Fibrosis

Fibrosis is the final common pathological feature of most chronic inflammatory diseases. In some diseases, such as idiopathic pulmonary fibrosis (IPF), liver cirrhosis, cardiovascular fibrosis, systemic sclerosis and nephrosclerosis, extensive tissue remodeling and fibrosis can ultimately lead to permanent scarring organ failure and death [1]. Fibrosis results when a normal wound-healing response persists or becomes dysregulated, usually in response to a severe or repetitive injury [2]. Regardless of their distinct etiology and clinical features, chronic fibrotic disorders share the portfolio of growth factors, proteolytic enzymes, angiogenic factors and fibrogenic cytokines that stimulate the excessive production, deposition and contraction of ECM components, such as hyaluronic acid, fibronectin (FN), proteoglycans and interstitial collagens that progressively remodel and damage the normal tissue architecture [3]. Transforming growth factor- $\beta$  (TGF- $\beta$ ) is considered to be the most potent and ubiquitous pro-fibrogenic cytokine [4,5]. TGF- $\beta$  and local mechanical forces, produced by infiltrated macrophages or resident cells, together with the ectodomain A (ED-A) sequence of FN stimulate the activation of alpha-smooth muscle actin ( $\alpha$ -SMA)-expressing and ECM-producing myofibroblasts, which are the key mediators of fibrotic tissue remodeling and ECM assembly [6,7]. The persistent activation of myofibroblasts leads to an excessive accumulation of ECM components that progressively distorts normal tissue

architecture [2]. The origin of myofibroblasts has been a controversial subject in recent years because they differentiate from various precursor cells that differ according to the nature of the insult and the affected organ [8]. Thus, although activation of local tissue fibroblasts is thought to be the primary instigator of ECM production following injury in many organs [8,9], epithelial and endothelial cells [10–13], hepatic stellate cells [14,15], pericytes [16], smooth muscle cells [17,18] and bone marrow-derived cells [19–21] have been shown to differentiate into pro-fibrotic myofibroblasts during fibrosis development. Whilst myofibroblasts are the primary drivers of fibrosis, it is becoming increasingly clear that various monocyte-derived cell populations can control the fibrotic process by exerting direct effects on matrix remodeling and regulating activated myofibroblasts [22].

Although it is well established that TGF- $\beta$  and myofibroblasts play central roles in the development of fibrosis, no effective therapy is yet available to revert the evolution of this group of severe connective tissue disorders.

## 2. Oxidative stress and fibrosis

Oxidative stress as a concept in redox biology and medicine was formally defined in 1985 by Helmut Sies [23] and it is currently known as an unbalance between the generation of ROS or reactive nitrogen species (RNS) and the capacity of cells and tissues to detoxify or scavenge them [24]. Mitochondria electron-transport chain (ETC), membrane-bound NADPH oxidases (NOXs) and endoplasmic reticulum (ER) are the three major intracellular sources of ROS [25]. ROS-derived NOXs are particularly interesting

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in fibrogenesis, as described in the following sections. Glutathione (GSH) is considered the most abundant molecule among endogenous anti-oxidants [26]. GSH contributes to detoxify deleterious metabolites maintaining redox homeostasis by preserving redox potential and facilitating redox signaling [26]. The nuclear factor-erythroid 2 related factor 2 (Nrf2) pathway is the major regulator of cyto-protective responses to oxidative stress by regulating the expression of several enzymes involved in the antioxidant response such as NOX:quinoneoxidoreductase1 (NQO1), glutathione S-transferase (GSTs), cysteine–glutamate exchange transporter and multidrug resistance associated protein [27,28]. ROS, generally through the action of its quintessential signaling arm, hydrogen peroxide ( $H_2O_2$ ), have a role in various signaling cascades, such as in the response to growth factor stimulation and control of inflammatory responses [29]. They participate in the regulation of many cellular processes, including differentiation, proliferation, growth, apoptosis, cytoskeletal regulation, migration, and contraction [30]. ROS have crucial roles in critical physiological processes [31], including the intracellular killing of bacteria by neutrophil granulocytes [29], detoxification by the liver [32], thyroid hormone production [33] and crosslinking of ECM [34]. Despite its important role in physiological signaling, excessive amounts of ROS cause direct cellular injury by inducing lipid and protein peroxidation and damaging nucleic acids [35–37]. ROS contribute to a wide range of pathologies including cancer, cardiovascular diseases, neurological disorders, chronic inflammation and autoimmune diseases [33]. Although many of the important pathogenetic mechanisms that promote fibrosis have been identified in the last decades, the precise molecular mechanisms involved are not fully understood. There is a substantial and growing body of evidences indicating the involvement of oxidative stress in the development of fibrosis in multiple organs.

### 2.1. Oxidative stress in pulmonary fibrosis

Pulmonary fibrosis is a lung disease that includes a heterogeneous group of lung disorders characterized by the progressive and irreversible destruction of lung architecture caused by scar formation that ultimately leads to death from respiratory failure [1]. Among the fibrotic lung diseases, IPF, with unknown etiology, is the more common and severe form. Lung fibrosis can also develop after viral infections and after exposure to radiotherapy, chemotherapeutic drugs and environmental toxins [38–41]. There are some evidences showing that ROS play an important role in the development of pulmonary fibrotic diseases. Oxidized proteins and lipid products have been identified in exhaled air, bronchoalveolar lavage fluid and lung tissues from patients with fibrotic lung diseases. Importantly, increased ROS and oxidative stress markers are detected in patients with IPF [42,43] and levels of ROS negatively correlate with pulmonary function in IPF and may predict disease severity [44]. An increase in oxidative DNA damage has been also detected in patients with silicosis and asbestosis [45]. The mouse model of bleomycin-induced pulmonary fibrosis, the most commonly used experimental model, is associated with a marked increase in ROS levels and in oxidized proteins, DNA and lipids [46]. In this experimental model the treatment with anti-oxidants attenuates fibrosis [47–49], suggesting a possible key role of oxidative stress in the development of this pathology. TGF- $\beta$  isoforms are secreted in an inactive, latent form bound to a latency association protein (LAP). It has been proposed that ROS can increase the TGF- $\beta$ -induced fibrosis by activating latent TGF- $\beta$  in asbestos-induced fibrosis [50] and by increasing the gene expression and secretion of TGF- $\beta$  in many cell types including alveolar epithelial cells (AECs) and macrophages [51]. ROS can also trigger the TGF- $\beta$ 1-induced epithelial-to-mesenchymal transition (EMT), a process that results in a loss

of epithelial markers and an acquisition of a mesenchymal phenotype in many fibrotic diseases, including IPF [52–54]. During EMT, epithelial cells lose their cell to cell junctions and the apical-basal polarity. EMT also enables cells to acquire invasive properties, thus degrading ECM and synthesizing new ECM components [55]. Several recent studies indicate that ROS production by NOXs plays a central role in the pathogenesis of pulmonary fibrosis and inflammation [47,56–59]. A recent study has found that TGF- $\beta$ 1 regulated the expression of NOX4, one of the major cellular ROS sources, thus generating oxidative stress [59]. Importantly, these NOX4-produced ROS are required for TGF- $\beta$ 1-induced myofibroblast differentiation, ECM production and contractility and genetic knock-down or pharmacologic inhibition of NOX4 prevented bleomycin-induced lung fibrosis in mice [59]. Moreover, NOX4 is up-regulated in lungs of mice treated with bleomycin and in patients with IPF [59], further highlighting the active role of ROS in the establishment and progression of pulmonary fibrosis. NOX4 is strongly expressed in the hyperplastic alveolar epithelium of patients with IPF, particularly in type 2 AECs. Death of AECs in pulmonary fibrosis is thought to be mediated, at least in part, by TGF- $\beta$ 1 and it constitutes a key initiating and perpetuating event in pulmonary fibrosis [60,61]. Studies in a NOX4 knock-down mouse support that NOX4-dependent ROS are critical for the induction of AECs death by apoptosis and the subsequent development of lung fibrosis, thus confirming an important role of NOX4 induction in the pathogenesis of tissue fibrosis [60].

### 2.2. Oxidative stress in kidney fibrosis

Renal fibrosis, characterized by glomerulosclerosis and tubulointerstitial fibrosis, is the final common manifestation of a wide variety of chronic kidney diseases (CKD) [62]. The excessive and progressive accumulation of ECM in the kidney promotes a gradual destruction of renal tubules and functional nephrons. Oxidative stress plays a central role in the pathogenesis of diverse chronic inflammatory disorders including CKD. Patients with CKD have markedly increased levels of oxidative stress, including increases in lipid peroxidation, oxidation proteins products and changes in GSH content [63–66]. The increased oxidative stress and inflammation likely contribute to the high rates of morbidity and mortality in this patient population [67]. Angiotensin II (Ang II), the key contributor to the progression of renal diseases by activating pro-inflammatory and matrix synthesis pathways, induces the generation of ROS by NOXs [68]. Other studies have shown a close relationship between ROS production by NOXs and the development of renal fibrosis. For example, the production of FN in mesangial cells induced by Ang II involves the reaction of NOX4-derived superoxide ( $O_2^-$ ) with endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) resulting in ONOO $^-$  production and uncoupling of eNOS, further promoting  $O_2^-$  generation [69]. During hyperglycemia, NOX4 promotes podocyte apoptosis, an event that significantly contributes to the development of CKD [70]. Although the origin of myofibroblasts in the particular case of the kidney is strongly debated [71], the role of oxidative stress has been reported as a contributing factor in every cell type [72–79]. In the context of renal disease, inhibition of NOX4 expression in kidney fibroblasts significantly reduces  $\alpha$ -SMA and ECM production [73,80], thus confirming the role of the NOX4-mediated redox imbalance in the pro-fibrotic transformation of renal fibroblasts.

### 2.3. Oxidative stress in liver fibrosis

Liver fibrosis results from chronic damage to the liver in conjunction with the accumulation of ECM proteins, which is a characteristic of most types of chronic liver diseases [81]. The main

causes of liver fibrosis in industrialized countries include chronic hepatitis C virus (HCV) infection, alcohol abuse, and nonalcoholic steatohepatitis (NASH). Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation [14]. Most of the chronic liver diseases, regardless of the cause of the liver disorder, are characterized by increased oxidative stress which induces liver injury and loss of liver function [82]. Oxidative stress has been also identified as a feature of experimental models of fibrosis and cirrhosis, bile duct ligation (BDL) or carbon tetrachloride (CCl<sub>4</sub>) intoxication, suggesting their possible role in liver fibrosis. ROS contributes to the hepatic fibrosis from various kinds of liver injuries, including alcohol abuse, HCV infection, iron overload, and chronic cholestasis [83,84]. ROS can stimulate the production of collagen, type I, alpha 1 (Col1 $\alpha$ 1) and may act as intracellular signaling mediators of the fibrogenic action of TGF- $\beta$  [85–87] in hepatic stellate cells (HSCs). Accumulating evidence indicates that NOXs-mediated ROS play a critical role in HSCs activation, a key event in the initiation and progression of liver fibrogenesis due to the role of these cells in orchestrating the ECM deposition in normal and fibrotic liver. Direct evidence for the contribution of NOX1 and NOX2 to hepatic fibrogenesis was provided by attenuation of hepatic fibrosis in either NOX1 or NOX2-deficient mice after CCl<sub>4</sub> treatment or BDL [88–90]. Several studies have shown that NOX4-generated ROS participate in hepatic fibrogenesis by inducing TGF- $\beta$ 1-mediated HSCs activation [91] and triggering TGF- $\beta$ - or death ligand-induced hepatocyte apoptosis [91–93].

#### 2.4. Oxidative stress in cardiac fibrosis

Cardiac fibrosis is one of the detrimental factors that contributes to heart failure (HF) during increased cardiac workload under conditions such as hypertension or aortic stenosis. Increased accumulation of ECM within the myocardium, especially in the interstitium and in perivascular areas has been implicated in the progression of HF [2,12,94–96]. A large body of work indicates that ROS generated by NOXs and their interactions with NO are especially important in redox signaling during the development of HF [97–99]. Interstitial fibrosis induced either by Ang II infusion or chronic activation of the renin-angiotensin system was abrogated in NOX2 knock-out mice [100–102]. It has been also shown that NOX2-derived ROS contribute to contractile dysfunction, myocardial atrophy, increased cardiomyocyte apoptosis, interstitial fibrosis, and inflammatory cell infiltration in an *in vivo* model of doxorubicin cardiotoxicity in mice [103], further confirming the possible role of NOX2 in cardiac fibrogenesis. Some *in vitro* studies have suggested that NOX4 is involved in the proliferation of cultured human cardiac fibroblasts and their transformation into myofibroblasts in response to TGF- $\beta$ 1 [104]. However, the relevance of these results remains to be established *in vivo*.

#### 2.5. Oxidative stress in skin fibrosis

In dermatology, fibrosis is the hallmark component of many skin diseases, including systemic sclerosis (SSc), keloids, hypertrophic scars, and graft-versus-host disease [105]. Fibrosis develops in the dermis, the connective tissue layer between the basement membrane and the epidermis and is mainly composed by ECM [106]. Fibrotic skin disorders can greatly impact patient quality of life with organ dysfunction and psychological sequelae [107–109]. Oxidative stress is thought to play a significant role in cellular functions involved in skin fibrosis [110]. NOX-derived ROS are involved at various levels of skin fibrosis and interfere with redox-sensitive intracellular signaling pathways, including inhibition of protein tyrosine phosphatases, activation of certain transcription factors, and modulation of enzymes [111]. In the skin,

NOX enzymes play a key role in excess ECM protein synthesis via indirectly activating key protein tyrosine kinases [112] and may interfere with ECM degradation by activating matrix metalloproteinases (MMPs) [113]. In scleroderma, a complex connective tissue disorder characterized by the excessive deposition of ECM proteins in the skin and internal organs [114], the source of oxidative stress is attributed to ROS production by fibroblasts and activated leukocytes via NOX enzymes [115]. NOXs promote fibroblast proliferation and differentiation into myofibroblasts by increasing  $\alpha$ -SMA and collagen (Col) expression and induce the expression of TGF- $\beta$ 1, the chemokine ligand (CCL)-2 and PDGFR [115–121], thus highlighting the importance of NOX enzymes in SSc.

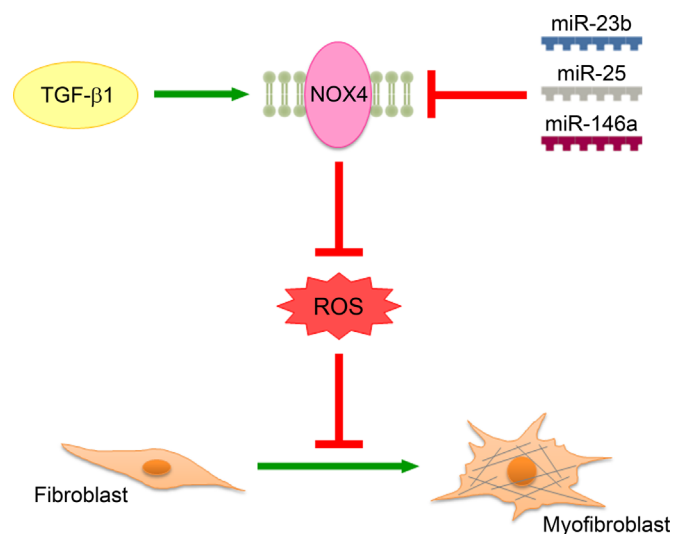
### 3. Redox-sensitive microRNAs

MiRNAs comprise a large family of conserved, small, non-coding RNAs of 19–25 nucleotides (nt) long that regulate gene expression at the post-transcriptional level by binding to the 3'-untranslated region (3'-UTR), coding sequences or 5'-untranslated region (5'-UTR) of target messenger RNAs and leading to the inhibition of translation or mRNA degradation [122,123]. Their discovery in 1993 by the laboratories of Ambros and Ruvkun, revolutionized the comprehension of the post-transcriptional regulation of gene expression [124]. In humans, miRNAs are predicted to control the activity of ~30% of the genome. At present there are more than 2500 miRNAs described. Each miRNAs is transcribed by RNA polymerase II as a long precursor RNA, called primary miRNA (pri-miRNA), which is then subjected to nuclear processing by the Drosha-DGCR8 'microprocessor' complex. The resulting intermediate, a hairpin shape precursor miRNA (pre-miRNA) of ~70 nt in length, is exported to the cytoplasm and then further shortened by Dicer, yielding a ~22-nt mature miRNA. In the cytoplasm, miRNAs associate with specific mRNAs within a multiprotein complex of Argonaute proteins, known as the RNA-induced silencing complex (RISC), providing sequence-specific silencing activity [124]. Considering hundreds of miRNA genes, each with many dozens of targets, it is expected that regulation by miRNAs may occur in any cellular function in health or disease [124–127]. In the last years, it has been found that miRNAs are able to "fine-tune" the regulation of redox signaling, by direct interaction with Nrf2, as mentioned above, the major transcriptional regulator of the defence against ROS [27,28]. MiRNAs may also interact with its co-regulators Kelch-like ECH-associated protein 1 (Keap1) and CNC homolog 1 (Bach1) or regulate the generation of ROS. These new subset of miRNAs that either regulate redox pathways or are themselves regulated by the cellular redox state have been termed "redoximiRs" [128].

### 4. RedoximiRs regulate fibrogenesis by diverse mechanisms

In the last decade, miRNAs have been shown to be regulators of pro- and anti-fibrotic processes in human diseases and this subset of miRNAs are called "fibromiRs" [129]. Aberrant expression of "fibromiRs" drives initiation and progression of the fibrotic process in response to persistent tissue injury [129]. Moreover, TGF- $\beta$  signaling through Smad proteins can regulate the transcription of miRNAs by DNA binding or the miRNA maturation by associating with the Drosha/DGCR8 complex [130], suggesting a possible link between miRNAs and fibrogenesis. Given the important role of oxidative stress in organ fibrosis, and the fact that miRNAs are implicated in fibrogenesis, the potential intersection between the sets of "redoximiRs" and "fibromiRs" team has gained increasing interest. We propose that this subset of miRNAs may be termed





**Fig. 1.** miRNAs regulating NOX4-mediated ROS production. TGF- $\beta$ -mediated induction of NOX4 contributes to ROS generation and transformation on fibroblasts into myofibroblasts. miRNAs inhibiting NOX4 expression abrogate this pro-fibrotic transformation.

“redoxifibromiRs” and in the following sections we provide examples of representative members as well as describe their redox-based mechanism of action and involvement in fibrogenesis.

#### 4.1. Regulation of ROS production and TGF- $\beta$ -dependent myofibroblast transformation

The best known example is constituted by those miRNAs regulating NOX4 expression. MiR-23b, miR-25 and miR-146a down-regulate the expression of NOX4 [131–133] and as mentioned earlier NOX4-dependent generation of H<sub>2</sub>O<sub>2</sub> was required for TGF- $\beta$ -induced myofibroblast differentiation, ECM production and contractility [59] (Fig. 1). Importantly, He et al. demonstrated that miR-146a inhibited the pro-fibrotic TGF- $\beta$  pathway by targeting SMAD4 and also reduced HSCs proliferation and induced HSCs apoptosis [134]. Other study showed that miR-146a modulated the TGF- $\beta$ -induced transformation of human dermal fibroblasts also by targeting SMAD4 [135], suggesting an anti-fibrotic role of miR-146a not only in liver but also in skin. Results from Fu et al. indicated that miR-25 contributed to the regulation of NOX4 expression in diabetic nephropathy (DN) [131], one of the major microvascular complications of diabetes and the most common cause of end-stage renal disease [136]. Thus, miRNAs can reduce ROS production by inhibiting NOX4 expression and modulating the levels of these miRNAs may represent a therapeutic strategy for the treatment of incurable diseases such as organ fibrosis.

#### 4.2. Regulation of anti-oxidant responses and EMT process

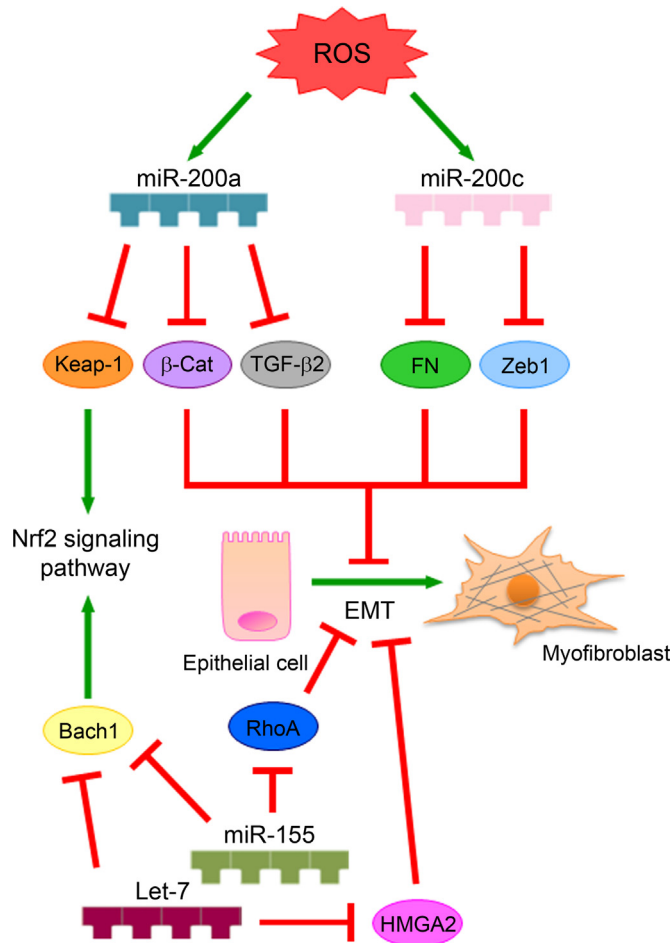
Regarding the miRNAs regulating cellular anti-oxidant defenses [137], those monitoring the Nrf2 signaling pathway are some of the most studied. Nrf2 signaling appears to be important for the pathogenesis of fibrosis. Several studies have demonstrated that Nrf2-deficient mice are more susceptible to chemically induced oxidative stress than wild-type mice, particularly in the liver [45,138].

MiR-34a has been shown to become up-regulated in the ageing liver acting as a post-transcriptional repressor for key genes related to oxidative defense in the rat liver during the ageing process [139]. The target genes repressed by miR-34a do not only include

two key genes, Sirtuin 1 (Sirt1) and microsomal glutathione S-transferase 1 (Mgst1), which have an important role in cellular redox balance and resistance to oxidative stress in the liver [140,141], but also their transcriptional activator factors, Sp1 and Nrf2 [139]. MiR-34a has been demonstrated to exert a pro-fibrotic role in a dimethylnitrosamine (DMN)-induced hepatic fibrosis in rats by targeting the acyl-CoA synthetase long-chain family member 1 (ACSL1) [142], thus suggesting the implication of miRNAs in the regulation of lipid/fatty acid metabolism involved in the development of liver fibrogenesis. In mice with preexisting pathological cardiac remodeling and dysfunction due to myocardial infarction (MI) or pressure overload via transverse aortic constriction (TAC), administration of locked nucleic acid (LNA)-based anti-miRs induced a decrease in miR-34a expression, improved systolic function and reduced cardiac fibrosis [143], providing evidence that silencing of the entire miR-34 family can protect the heart against pathological cardiac remodeling and improve cardiac function. Another study demonstrated that miR-34a is induced after acute MI and, importantly, the inhibition of miR-34a enhanced cardiac contractile recovery after acute MI [144]. These results strongly indicate that the inhibition of miR-34a is a potential therapeutic strategy to improve cardiac contractile function after acute myocardial infarction. In keeping with the growing interest on the regulation of antioxidant defenses by miRNAs, our group has recently described one miRNA, miR-433, which targets GSH biosynthetic enzymes in an Nrf2-independent manner, resulting in decreased GSH levels and antioxidant capacity with important implications for endothelial function as well as for kidney and liver fibrosis [145].

Of notice, Yang et al. showed an increase in miR-27a and miR-27b expression induced by c-Myc in mouse models of liver fibrosis relating it to a reduction in both the expression of GSH synthetic enzymes and Nrf2 [146], both major constituents of the anti-oxidant cellular pathways. It has been shown that the down-regulation of over-expressed miR-27a and miR-27b in culture-activated rat HSCs induce a switch towards a more quiescent HSC phenotype by restoring their ability to accumulate cytoplasmic lipid droplets and decreasing HSCs proliferation [147]. The retinoid X receptor alpha (RXR $\alpha$ ) was confirmed to be the target of both miRNAs [147], highlighting the importance of regulating fatty acid metabolism and cell proliferation during HSCs activation in an attempt to counteract hepatic fibrosis.

Members of the miR-200 family are not only the object of regulation by changes in the redox state [128] but are also up-regulated by oxidative stress themselves [148] (Fig. 2). Of interest, one specific member of the miR-200 family, miR-200a, is down-regulated in the TGF- $\beta$ 1-mediated activation of HSCs but during liver fibrosis progression in humans and mice it has been reported to increase [149]. This is in line with the induction in the expression of miR-200a in oxidative stress conditions in order to target Keap1, which negatively modulates the stability of the master antioxidant regulator Nrf2 [150] (Fig. 2). Noteworthy, miR-200 family members have also been closely related to EMT (Fig. 2). This process has been proposed to be an important mechanism responsible for the accumulation of interstitial myofibroblasts and Col production during kidney fibrosis [151], although this concept is currently still controversial [152,153]. Targets of miR-200a also include pro-fibrogenic factors such as TGF- $\beta$ 2 and  $\beta$ -catenin, both EMT-related down-stream genes [154], further confirming the involvement of miR-200a in the anti-oxidant response of HSCs during liver injury. Of interest, ROS induce the expression of miR-200c, which in turn represses the Zinc finger E-box binding homeobox 1 (Zeb1). This transcription factor is one of the master EMT-inducing genes associated with the mesenchymal phenotype and negatively modulates E-cadherin [155], whose down-regulation is a hallmark of EMT. FN is another annotated target gene for



**Fig. 2.** Different mechanisms of action of “redoximiRs” contribute to the regulation of the EMT process. Several miRNAs, regulating antioxidant responses and/or being regulated by ROS, can cooperate to control the expression of different genes related to EMT, thus having an important role in the development of fibrogenesis.

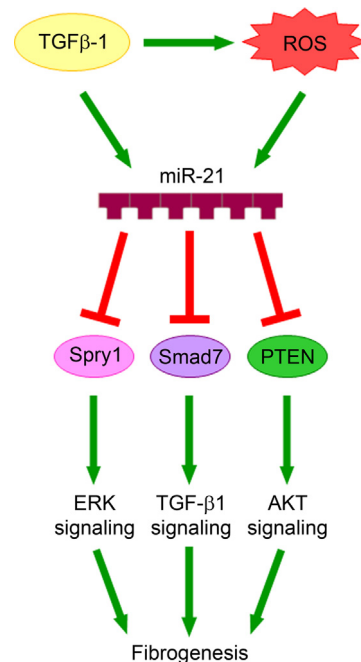
miR-200c [156], thus highlighting the role of miR-200c in organ fibrogenesis.

The Let-7 family of miRNAs has been implicated in the pathogenesis of fibrosis in several organs such as lung, skin or kidney [157–160]. Let-7d is transcriptionally inhibited by TGF- $\beta$  and is significantly decreased in the alveolar epithelium in lungs from patients with IPF [159]. Let-7d target genes include high mobility group A2 (HMGA2) [161] (Fig. 2), which is a key factor in the TGF- $\beta$ -EMT-induced process associated to activation of the zinc finger Snail and the basic helix–loop–helix (bHLH) Twist transcription factors and E-cadherin suppression [162]. Let-7a is down-regulated in skin tissue and fibroblasts from SSc patients and negatively regulates the expression of Col [158]. Moreover, over-expression of let-7a improved the skin fibrosis induced by bleomycin in mice [158]. A study comparing miRNA profiles of patients with SSc and healthy control individuals identified Let-7g as involved in SSc pathogenesis [157]. Finally, Let-7b expression was reduced in models of both diabetic and non-diabetic renal fibrosis. Over-expression of Let-7b prevented the pro-fibrotic effects of TGF- $\beta$  by repressing type I TGF- $\beta$  receptor (TGFB1) in rat proximal tubular epithelial cells [160]. Of note, some Let-7 family members have been shown to decrease Bach1 expression, a transcriptional repressor of Nrf2 [163] (Fig. 2), in a human hepatoma cell line, thereby attenuating oxidative injury via enhancement of Nrf2-mediated heme oxygenase-1 (HO-1) gene expression [164].

Similarly, miR-155 has been reported to inhibit Bach1 expression [128] (Fig. 2). Up-regulation of miR-155 correlated with the degree of lung fibrosis in mice and was also found to be over-expressed in patients with IPF [159,165]. The expression of miR-155 increased during TGF- $\beta$ -induced EMT in mammary epithelial cells through Smad4-mediated transcriptional up-regulation and facilitated loss of cell polarity and tight junctions by targeting the Ras homolog family member A (RhoA) [166], implicating miR-155 in EMT (Fig. 2).

#### 4.3. Regulation of ERK, AKT and TGF- $\beta$ signaling pathways

MiR-21, one of the most extensively studied miRNAs, is also associated with oxidative stress [167]. Inhibition of miR-21 attenuated fibrosis in the heart, [168,169], kidney [170], lung [171] and skeletal muscle [172], evoking miR-21 inhibitors as a potential anti-fibrotic therapy. It has been proposed that the transcription factor NF- $\kappa$ B positively regulated miR-21 expression under H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in cardiomyocytes [167] and the up-regulation of this miRNA has been suggested to lie downstream of NOX activation [173]. Moreover, H<sub>2</sub>O<sub>2</sub>-induced ROS activity and cardiomyocyte apoptosis were partly protected by overexpression of miR-21 and displayed an important role in ROS-mediated cardiomyocyte injury [167]. MiR-21 has been implicated in cardiac fibrosis development as the expression of this miRNA was enhanced in the failing heart [169]. By targeting sprout homolog 1 (Spry1), an extracellular regulated kinase (ERK) inhibitor, miR-21 induces an up-regulation of ERK signaling. *In vivo* silencing of miR-21 was shown to reduce cardiac ERK-mitogen-activated protein (MAP) kinase activity, to inhibit interstitial fibrosis and to attenuate cardiac dysfunction in a mouse pressure-overload-induced disease model [169]. Results of another study shown that miR-21 played an important role in TGF- $\beta$ -induced endothelial-to-mesenchymal transition (EndMT), a process that contributes to fibroblast formation in fibrotic diseases of the heart [12,174], kidney [175–177], lung [178], liver [13] and carcinoma-associated fibrosis



**Fig. 3.** miR-21 as an archetypal “redoxifibromiR”. TGF- $\beta$ 1 and ROS induce the up-regulation of miR-21. This pro-fibrotic “redoximR” regulates key signaling pathways with specific relevance in the context of organ fibrosis.

[179], by targeting the protein phosphatase and tensin homologue (PTEN) thus regulating the AKT signaling pathway [180]. Liu et al. [171] confirmed the pro-fibrotic activity of miR-21 in human IPF and in a mouse model of bleomycin-induced lung fibrosis. The expression of miR-21 is increased in experimental fibrosis and importantly in patients with IPF [171] and miR-21 up-regulation was proportional to the severity of lung fibrosis in the bleomycin mouse model. The enhanced expression of miR-21 led to the suppression of Smad7 expression and favoured the assembly of Smad2/3-Smad4 heterodimers, a crucial event in the pro-fibrogenic TGF- $\beta$  signaling pathway [173]. Administration of miR-21 antisense oligonucleotides attenuated bleomycin induced-lung fibrosis [171]. Mir-21 has been reported to be increased in SSc skin tissues and fibroblasts and the regulation of Smad7 expression was also described in this context [181]. Studies on the role of miR-21 in renal fibrosis further confirmed the interaction of this miRNA with the TGF- $\beta$  signaling [170]. TGF- $\beta$  increased the expression of miR-21 in tubular epithelial cells. Smad3 knock-down mice were protected from miR-21 up-regulation and fibrosis after unilateral uretral obstruction (UUO) kidney injury, a useful and widely used model to examine mechanisms of tubulointerstitial fibrosis *in vivo* [182], indicating that the TGF- $\beta$ -induced up-regulation of miR-21 is mediated by Smad3 [170]. In addition to the induction of miR-21 in the mouse fibrotic kidney, this miRNA was also found to be up-regulated in human fibrotic kidney samples, especially in areas with abundant presence of myofibroblasts [183]. Importantly, serum levels of miR-21 were significantly elevated in patients with kidney fibrosis, showing good correlation with the severity of fibrogenesis [183], suggesting miR-21 as a potential biomarker of kidney fibrosis. It has been proposed that miR-21 is also involved in the development of skeletal muscle fibrosis by targeting PTEN and SMAD7 [172]. Importantly, the same authors described the increased expression of miR-21 in muscle biopsies of Duchenne muscular dystrophy (DMD) patients, correlating with extensive tissue fibrosis [172], thus confirming the role of this miRNA in skeletal muscle disease. Targets and signaling pathways regulated by miR-21 are summarized in Fig. 3.

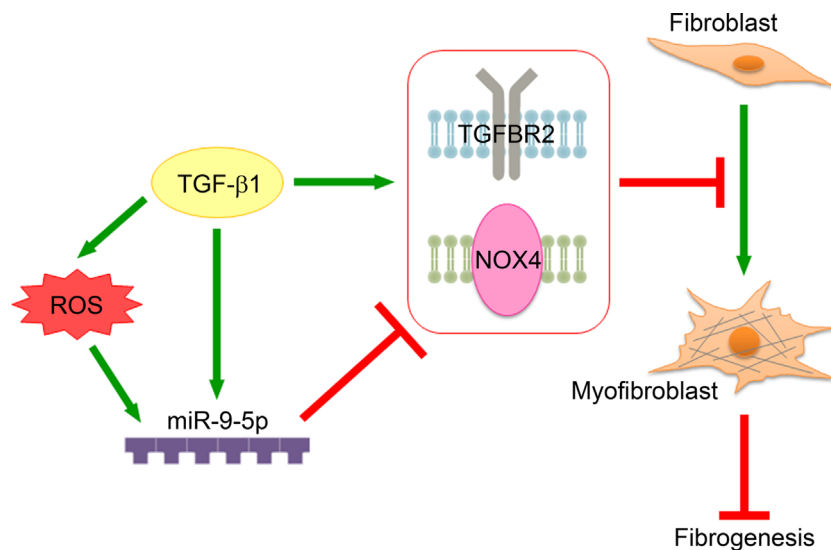
#### 4.4. Regulation of ROS production and TGF- $\beta$ signaling pathway

Recently, we have described a clear example of a novel

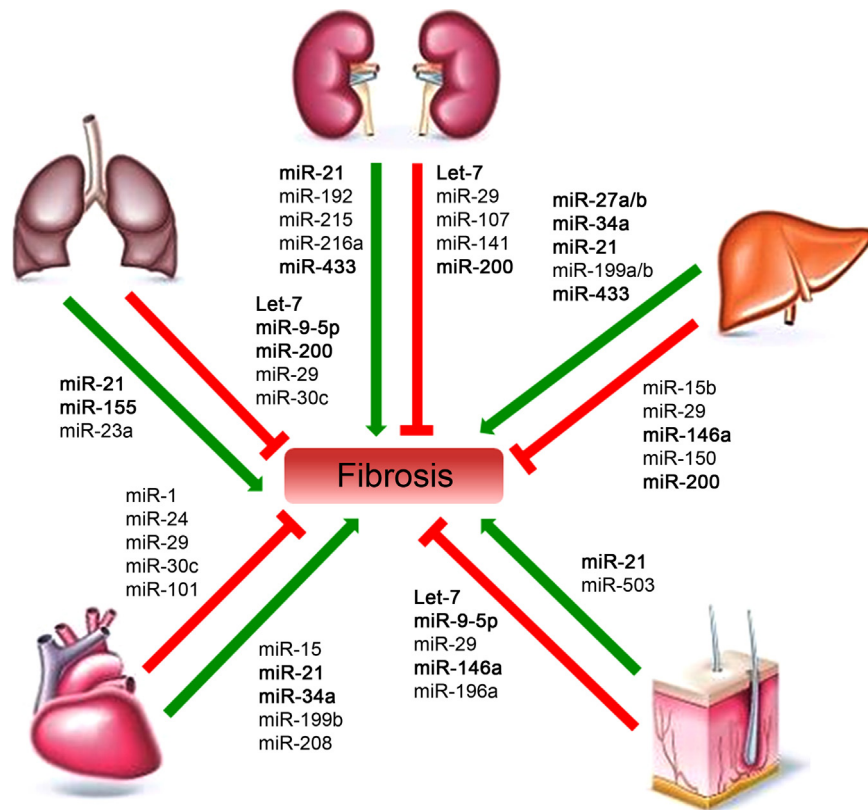
“redoximiR” regulating organ fibrogenesis. MiR-9-5p negatively regulates NOX4 expression, thus controlling ROS-NOX4 production, but it is also up-regulated in the presence of H<sub>2</sub>O<sub>2</sub>. By inhibiting NOX4 and type II TGF- $\beta$  receptor (TGFBR2) expression, miR-9-5p abrogated the TGF- $\beta$ -dependent pro-fibrotic transformation of lung fibroblasts, mesothelial cells (MCs) [184] and dermal fibroblasts (unpublished data) into myofibroblasts. Consistently, increased miR-9-5p levels reduced the pro-fibrogenic activities of TGF- $\beta$ 1 by inhibiting TGF- $\beta$ 1-mediated signaling. Increased expression of miR-9-5p results in a dramatic attenuation of bleomycin-induced lung [184] and skin fibrosis (unpublished data). On the contrary, inhibition of miR-9-5p both *in vitro* and *in vivo* resulted in a significant reduction of its anti-fibrotic effect [184]. Importantly, in lung specimens from patients with IPF and in omentum-derived MCs from patients subjected to peritoneal dialysis (PD) high levels of miR-9-5p were encountered. We propose that miR-9-5p may have a protective role in the initiation and progression of human fibrosis. It is conceivable that, for homeostatic purposes, the crucial pro-fibrogenic cytokine TGF- $\beta$  triggers counteracting responses that self-limit its deleterious action, such as miR-9-5p (Fig. 4). In addition, miR-9-5p could also represent a biological marker and a novel therapeutic target in lung fibrosis, still largely a devastating and incurable condition.

## 5. In summary

It has been demonstrated that oxidative stress and the antioxidant defences are crucial for fibrosis development and perdurability. The finding that miRNAs play an important role in the pathogenesis of organ fibrosis (Fig. 5) and even more, the recent discovery of the existence of miRNAs regulating and being regulated by ROS have uncovered the potential use of miRNAs as biomarkers in the diagnosis, prognosis and response to treatment. The novel set of miRNAs regulating fibrogenesis through redox-based mechanisms may be termed “redoxifibromiRs”. Therapeutic approaches based on “redoxifibromiRs” modulation may represent a novel alternative for many challenging human diseases, including fibrosis.



**Fig. 4.** Targets and anti-fibrotic actions of miR-9-5p. TGF- $\beta$  and ROS induce miR-9-5p expression. TGF- $\beta$ 1 may induce the expression of regulators, including miR-9-5p, to self-limit its pro-fibrogenic action. miR-9-5p inhibits the transformation of fibroblasts into myofibroblasts by targeting TGFBR2 and NOX4, thus counteracting fibrogenesis in different organs.



**Fig. 5.** Overview of miRNAs implicated in the pathogenesis of fibrosis. Selected “fibromiRs” inducing (green arrows) or blocking (red blunt arrows) organ fibrosis are shown. Those being part of the “redoximiRs” set of miRNAs are represented in bold, thus generating a novel set of miRNAs called “redoxifibromiRs”. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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