Review Article

Therapeutic Targeting of Redox Signaling in Myofibroblast Differentiation and Age-Related Fibrotic Disease

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Myofibroblast activation plays a central role during normal wound healing. Whereas insufficient myofibroblast activation impairs wound healing, excessive myofibroblast activation promotes fibrosis in diverse tissues (including benign prostatic hyperplasia, BPH) leading to organ dysfunction and also promotes a stromal response that supports tumor progression. The incidence of impaired wound healing, tissue fibrosis, BPH, and certain cancers strongly increases with age. This paper summarizes findings from *in vitro* fibroblast-to-myofibroblast differentiation systems that serve as cellular models to study fibrogenesis of diverse tissues. Supported by substantial *in vivo* data, a large body of evidence indicates that myofibroblast differentiation induced by the profibrotic cytokine transforming growth factor beta is driven by a prooxidant shift in redox homeostasis due to elevated production of NADPH oxidase 4 (NOX4)-derived hydrogen peroxide and supported by concomitant decreases in nitric oxide/cGMP signaling and reactive oxygen species (ROS) scavenging enzymes. Fibroblast-to-myofibroblast differentiation can be inhibited and reversed by restoring redox homeostasis using antioxidants or NOX4 inactivation as well as enhancing nitric oxide/cGMP signaling via activation of soluble guanylyl cyclases or inhibition of phosphodiesterases. Current evidence indicates the therapeutic potential of targeting the prooxidant shift in redox homeostasis for the treatment of age-related diseases associated with myofibroblast dysregulation.

1. Myofibroblast Biology

The myofibroblast is a specialized cell type that combines the extracellular matrix (ECM)-producing characteristics of fibroblasts with the cytoskeletal and contractile properties of smooth muscle cells (SMCs) as reviewed recently [1]. Defined by (i) their *de novo* expression of alpha-smooth muscle cell actin (α-SMA, encoded by the gene *ACTA2*) in stress fibers and (ii) contractile force, myofibroblasts play a critical role during normal wound healing and thereby in maintaining tissue integrity [2]. In addition, myofibroblasts secrete growth factors that attract epithelial cells for subsequent wound closure (reepithelialization) [3–5]. Normal tissue function and architecture is restored upon completion of reepithelialization via massive apoptosis of myofibroblasts

and vascular cells followed by their subsequent clearance from the wound site [6]. Whilst relatively poorly understood, induction of cellular senescence via telomere-dependent and -independent mechanisms may also facilitate completion of wound healing [7–10]. For example, upon tissue injury telomerase activation is thought to enable cell proliferation for repair of local tissue damage [9]. However, its subsequent downregulation appears to be required for downstream induction of cellular senescence, cessation of wound healing, and cell clearance by the immune system [7, 10].

Dysregulation of the wound healing response has significant pathological consequences. On the one hand, a major clinical problem in the elderly is impaired wound healing, whereby wound repair is temporally delayed and all phases of wound healing exhibit characteristic changes

[11]. By contrast, excessive and/or persistent myofibroblast activity results in continued synthesis of ECM, dysregulation of growth factor signaling and consequently to tissue fibrosis and organ dysfunction [12]. This paper focuses on growing evidence indicating that redox signaling downstream of dysregulated transforming growth factor beta (TGFβ) promotes myofibroblast differentiation and the development/progression of several age-related fibrotic diseases. Findings from cellular fibrosis models are highlighted, which have improved our understanding of the molecular mechanisms underlying such disorders.

2. Myofibroblast Origin and Induction

Although cell types other than fibroblasts, (e.g., vascular SMCs, pericytes, bone marrow-derived fibrocytes, resident epithelial cells via epithelial-to-mesenchymal transition, and endothelial cells via endothelial-to-mesenchymal transition) have been reported to undergo differentiation into myofibroblasts *in vitro*, the extent of their contribution to the *in vivo* myofibroblast pool remains controversial [12, 13]. Rather, it is widely considered that myofibroblasts predominantly originate from the differentiation of local tissue fibroblasts [13].

Following injury or during chronic inflammation, fibroblast-to-myofibroblast differentiation occurs via a two-step process, initiated by changes in mechanical tension of the ECM that are transmitted to the fibroblast cytoskeleton via RhoA/ROCK signaling [14]. Consequently, fibroblasts adopt an "activated" phenotype (referred to as "proto-myofibroblast") and deposit new ECM components [15]. Soluble factors and cytokines produced by platelets and infiltrating leukocytes play a major role in the differentiation to the α-SMA-expressing myofibroblast phenotype. In particular, the combined action of the splice variant ED-A of cellular fibronectin and TGFβ, especially TGFβ1, that initially is secreted by platelets and phagocytic cells at the wound site [15]. However, proto-myofibroblasts and myofibroblasts also secrete and activate TGFβ1 thereby generating an autocrine feed-forward loop further driving myofibroblast differentiation [3, 16].

TGFβ1 is a key inducer of myofibroblast differentiation in cells of diverse histological origin, including breast, skin, prostate, kidney, heart, lung, and liver [17–23]. TGFβ1 exerts its effects via transcriptional events downstream of Smad2/3 activation and Smad-independent regulation of mitogen-activated protein kinase (MAPK) and PI3 kinase/Akt pathways [1, 3, 24]. Collectively, activation of these pathways results in the deposition of ECM and secretion of paracrine- and autocrine-acting growth factors [3, 25]. Importantly, the ECM can directly bind to and release growth factors, such as heparan sulfate binding to fibroblast growth factor 2 [26]. Such interactions can serve to sequester and protect growth factors from degradation and/or enhance their activity [27]. In addition, indirect interactions are required for signal transduction of some growth factors, for example, integrin binding is necessary for induction of angiogenesis by vascular endothelial cell growth factor [28]. Thus, maintaining ECM homeostasis is critical to regulate not only tissue architecture but also cellular signaling cascades.

3. Aging, Dysregulation of Myofibroblast Differentiation, and Fibrosis

Dysregulation of the wound healing response, particularly in association with chronic inflammation and injury (e.g., in the liver due to viral infection, in the lung from chronic obstructive pulmonary disease, in the heart following myocardial infarction) can result in excessive myofibroblast activation and organ fibrosis [29]. It is perhaps therefore not surprising that the incidence of many fibrosis-associated diseases increases sharply with advancing age, for example, cardiovascular disease, fibrosis of the liver, lung and kidney, and benign prostatic hyperplasia (BPH), a classic age-related fibrotic-like disease characterized by fibroblast to myofibroblast differentiation and ECM deposition [24, 30–35]. Fibrosis is also observed in the stromal response to many tumors, including liver and prostate cancer, both of whose incidence is strongly linked with aging and chronic inflammation [36–39]. Myofibroblasts in the tumor-adjacent stroma (termed "reactive stroma") not only actively promote tumorigenesis and angiogenesis via ECM deposition and mitogen secretion but also support cancer cell invasion and metastasis by producing ECM remodeling enzymes [40–44].

Tissue and organ fibrosis are thought to arise from failure of the wave of myofibroblast apoptosis during wound healing, resulting in persistent myofibroblast activation, excessive ECM deposition, altered growth factor signaling and cellular proliferation [12, 45]. In addition, failure of the timely induction of cellular senescence and dysregulation of telomere biology may also lead to organ fibrosis [7–10]. Consistently, shortened telomeres and telomerase mutations have been observed in familial and idiopathic pulmonary fibrosis [46, 47]. However, cell senescence also ameliorates fibrosis via telomere-independent mechanisms [8] and may partially explain discrepancies observed in rodent fibrosis models, whereby telomere deficiency had no effect on chemical-induced fibrosis [48]. The contribution of cellular senescence and telomere biology to age-related fibrosis pathologies remains to be fully investigated.

Given its potent myofibroblast differentiating effects and ability to promote myofibroblast survival in an autocrine manner, TGFβ1 is considered a key molecule underlying the pathophysiology of fibrotic disease [29, 49, 50]. Interestingly, the TGFβ signaling intermediate Smad3 is a direct repressor of telomerase reverse transcriptase [51, 52], a key enzyme required for telomerase activity, suggesting that autocrine TGFβ signaling by myofibroblasts may override the protective mechanism of cellular senescence further exacerbating tissue fibrosis. Consistent with its central role in age-associated fibrogenic pathologies, elevated TGFβ1 levels and signaling are observed in BPH and preneoplastic prostatic lesions, tumor-associated reactive stroma, cardiovascular remodeling, renal interstitial fibrosis, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis (IPF), and in chronic liver disease [44–52].

4. Cellular versus Animal Model Systems for Studying Age-Associated Fibrosis

Several animal models for fibrotic diseases such as systemic sclerosis, pulmonary, liver, cardiac, and renal fibrosis have been established [53–58]. Whilst such models can be valuable tools to understand disease pathology and evaluate novel therapeutic strategies, fibrosis does not normally develop spontaneously in these animals and typically has to be artificially induced, for example by chemical means [53–58]. Moreover, there may be species-specific differences in organ anatomy, physiology, and disease susceptibility. A notable example is the prostate gland [32, 59, 60]. Significant anatomical differences between rodents and humans also have to be considered in other organs for example, lung and skin [61, 62]. In addition, whilst favorable for experimental reproducibility, the use of genetically identical inbred strains does not recapitulate the heterogeneity of human pathologies. Furthermore, animal models are costly and time-consuming and thus not suitable for high throughput drug screening.

Fibrotic diseases are often associated with inflammation, thus it is difficult to dissect if fibrosis suppression in animal models is due to direct antifibrotic effects of a drug or due to indirect anti-inflammatory effects. Cellular *in vitro* model systems to investigate specific cellular or molecular responses can be very useful to elucidate inflammation-independent antifibrotic targets. Moreover, cellular *in vitro* models offer the significant advantage that human-derived cells can be analyzed. In addition, they are less costly, deliver more rapid results than animal models, can be easily genetically manipulated and are amenable for high throughput screening.

Numerous *in vitro* models of fibrosis have been successfully devised. *In vitro* culture of fibroblasts in 3D collagen-gel matrices leads to progressive contraction of the gel over the course of several days mimicking wound contraction [63]. This model has been widely used to investigate the contractility of fibroblasts/myofibroblasts derived from various tissues affected by fibrotic disease including skin, mucosa, lung, cornea, and heart [64–71].

Overwhelming *in vitro* and *in vivo* data demonstrate the central role of TGFβ1 and myofibroblast differentiation in the etiology of diverse fibrotic disorders (see above). Thus, an *in vitro* approach successfully employed by us and others to model fibroblast-to-myofibroblast differentiation in BPH and prostate cancer reactive stroma applies TGFβ1 to primary prostatic fibroblasts, which induces their robust differentiation into myofibroblasts [3, 25, 39, 72, 73] (Figure 1). This approach has also been extensively employed to model myofibroblast differentiation of hepatic stellate cells and of fibroblasts from breast, skin, kidney, heart, and lung [20, 21, 23, 74–77]. Myofibroblast differentiation can be subsequently monitored at the molecular level by the induction of molecular markers such as α-SMA and collagens as well as at the morphological level whereby the thin and elongated phenotype characteristic of fibroblasts changes to the flattened, less light refractive myofibroblast phenotype, which is accompanied by the appearance of contractile actin bundles (Figure 1). The validity of this *in vitro* model system is highlighted by numerous molecular and cellular parallels with animal models and patient specimens that collectively reveal a central role of dysregulated redox signaling by NADPH oxidase 4 (NOX4) and nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) in the development of age-related fibrotic disease.

5. NADPH Oxidase-Derived Reactive Oxygen Species in the Regulation of Myofibroblast Differentiation

Accumulation of nonspecific oxidative damage by high levels of free radicals is thought to be a major contributor to organismal aging [78]. However, when produced in a regulated manner, reactive oxygen species (ROS), NO, and reactive nitrogen species (RNS) can act as biological second messengers in a variety of signal transduction pathways, including myofibroblast differentiation [79].

The NADPH oxidase (NOX) enzyme family, comprising seven members, catalyzes the transfer of electrons across biological membranes from NADPH to oxygen thereby generating superoxide ($O_2^{\bullet -}$) [80]. The NOX family is thereby unique as ROS production is their primary function and not a byproduct as is the case for other ROS-producing enzyme systems, such as xanthine oxidase, mitochondrial respiratory chain, lipid peroxidases, or uncoupled endothelial NO synthase [81].

NOX4 is unique among NOX enzymes in that it is constitutively active with primary regulation occurring at the transcriptional level [82, 83]. Moreover, whilst other NOX enzymes produce superoxide, NOX4 is associated with constitutive hydrogen peroxide (H_2O_2) production, however whether this occurs via superoxide dismutation or direct H_2O_2 production remains controversial [82, 84, 85]. The greater stability but lower reactivity of H_2O_2 compared to superoxide is consistent with a signaling function of NOX4-derived ROS as underscored by observations that sustained and elevated NOX4-derived ROS levels induced by cytokines and growth factors do not induce oxidative damage [3, 86, 87].

Critically, several growth factors, in particular TGFβ1, and stimuli implicated in the pathogenesis of fibrotic disease induce NOX4 expression in diverse cell types as reviewed recently [88]. Consequently, NOX4-derived ROS have been implicated in the pathophysiology of fibrotic disorders, including BPH, IPF, cardiac remodeling, renal, and liver fibrosis, tumorigenesis and the stromal response to prostate, breast and liver cancers [3, 20, 74, 89, 90].

Using *in vitro* model systems, NOX4-derived ROS have been shown to be essential downstream inducers of TGFβ1-mediated myofibroblast differentiation in a myriad of cell types of diverse histological origin. For example, we demonstrated using NOX4 silencing and antioxidants that induction of NOX4-derived ROS in response to TGFβ1 drives myofibroblast differentiation of prostatic fibroblasts [3]. Similarly, TGFβ1-induction of NOX4-derived ROS was required for fibroblast-to-myofibroblast differentiation in *in vitro* models of cardiac, pulmonary, renal, and adventitial fibrosis [20, 21, 74, 75, 91]. Besides inducing differentiation
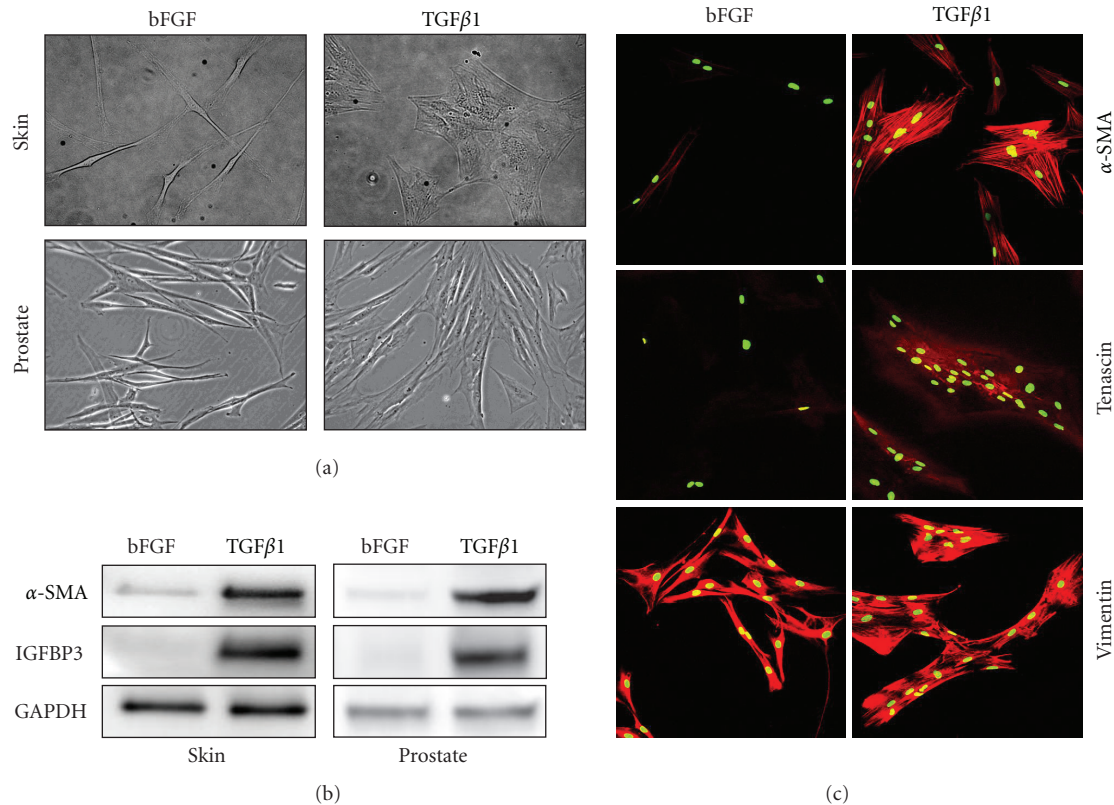
FIGURE 1: *In vitro* modeling of fibroblast-to-myofibroblast differentiation. Fibroblast-to-myofibroblast differentiation is induced following incubation of fibroblasts for at least 24 hours with 1 ng/mL transforming growth factor beta 1 (TGFβ1) in RPMI-1640 media supplemented with 1% steroid hormone-depleted charcoal-treated bovine calf serum (ctBCS). Steroid hormone depletion is particularly important for fibroblasts derived from endocrine tissues such as the prostate, since androgens attenuate TGFβ signaling and vice versa [32]. Similar to commercial formulations of fibroblast growth media, the fibroblast phenotype of mock control cells is maintained with 1 ng/mL basic fibroblast growth factor (bFGF) and serves to inhibit differentiation-inducing stimuli (e.g., TGFβ) in serum. Myofibroblast differentiation can be monitored at the morphological level by phase contrast microscopy (a) and molecular level by Western blotting (b) or immunofluorescence (c). (a) Primary human dermal fibroblasts *(top left)* and prostatic fibroblasts *(lower left)* exhibit a typical thin, elongated, and light refractive phenotype, whereas upon differentiation with TGFβ1 for 24 hours dermal *(top right)* and prostatic myofibroblasts *(lower right)* display a flattened, less light refractive phenotype. (b) TGFβ1 induces the expression of myofibroblasts markers α-smooth muscle cell actin (α-SMA) and insulin-like growth factor binding protein 3 (IGFBP3) [25] in primary human dermal *(left)* and prostatic *(right)* fibroblasts as determined by Western blotting. GAPDH served as loading control. (c) TGFβ1 treatment of primary human prostatic fibroblasts induces the expression of myofibroblast markers tenascin and filamentous α-SMA as determined by immunofluorescence and confocal laser scanning microscopy. Both myofibroblasts and fibroblasts express the mesenchymal marker vimentin. Nuclei were counterstained with SytoxGreen. (c) Adapted from [25], used with permission.

of fibroblasts, NOX4 was shown to play a role in TGFβ1-mediated cytoskeletal remodeling of vascular endothelial cells and in maintaining the differentiated phenotype of vascular SMCs [92, 93]. Moreover, induction of NOX4 by TGFβ1 is required for hepatic stellate cell activation and their subsequent transdifferentiation into myofibroblasts [22]. Interestingly, NOX4 induction was also required for insulin-induced adipocyte differentiation of preadipocytes [94]. Collectively, these data suggest a broader role of NOX4 in regulating differentiation in response to changes in the cellular environment.

These findings from *in vitro* fibroblast-to-myofibroblast differentiation model systems are supported by several *in vivo* data. We demonstrated that NOX4 mRNA levels specifically correlated with the myofibroblast phenotype in benign prostatic tissue [3]. NOX4 expression was higher in pulmonary

fibroblasts from patients with IPF compared with controls and correlated with mRNA levels of the myofibroblast markers α-SMA and procollagen I α1 [75]. Consistently, NOX4 is expressed *in situ* in fibroblastic foci in the lung of IPF patients and two mouse models of pulmonary fibrosis. Moreover, targeting NOX4 via siRNA or a nonspecific NOX inhibitor diphenylene iodonium attenuated lung fibrosis in two murine models of lung injury [74]. NOX4 may also be involved in vascular remodeling associated with IPF [95]. In animal models of diabetic nephropathy, treatment of diabetic rats with NOX4 siRNAs attenuated renal fibrosis strongly implicating a causative role of NOX4-derived ROS in the fibrogenic response to renal injury [96]. Recently, high levels of NOX4 were found in liver biopsy samples from patients with autoimmune hepatitis, which colocalized with α-SMA. Moreover, liver fibrosis could be attenuated in

mouse models via genetic deletion of NOX4 or application of a dual NOX1/NOX4 inhibitor GKT137831 [22], indicating a direct role of NOX4 in the pathogenesis of fibrosis.

NOX4 induction does not appear to contribute to fibrogenesis via direct oxidative stress but rather by chronic dysregulation of downstream signaling pathways. NOX-derived ROS mediate their signaling functions via reversible oxidation of thiol groups of low pKa cysteine residues in target proteins, including transcription factors, MAPKs, protein tyrosine phosphatases (PTPs), and protein tyrosine kinases (PTKs) [87]. Typically, thiol oxidation results in PTP inactivation but kinase activation thus promoting phosphorylation signaling cascades [87]. Only a small proportion of cysteines exhibit the necessary close proximity to basic amino acids to undergo transition to a sulfenic acid, thereby providing a basis for specificity of thiol redox signaling which reversibly regulates biological function by (i) chemically altering active site cysteines, (ii) altering macromolecular interactions, and (iii) modifying allosteric Cys [97].

The precise oxidative target(s) of NOX4-derived ROS that culminate in myofibroblast differentiation in response to TGFβ remain largely unknown. However, in lung fibroblasts TGFβ1 induced NOX4-derived ROS directly oxidatively inactivate MKP1, a nuclear dual-specificity MAPK phosphatase that targets JNK, and p38, leading to sustained activation of JNK and p38 MAPKs [98]. Similarly, in prostatic fibroblasts, NOX4-derived ROS were required for sustained phosphorylation of JNK whose activity in turn was essential for downstream α-SMA induction, and myofibroblast differentiation [3]. By contrast, NOX4 mediates TGFβ-induced myofibroblast differentiation of renal fibroblasts via ERK1/2 [21], whereas angiotensin II-induction of NOX4-derived ROS and fibronectin/ECM deposition in renal mesangial cells occurred via Src activation [99]. Thus, it is likely that the oxidative target(s) of NOX4-derived ROS are tissue-, cell-type, and/or context-specific.

In summary, whilst acute induction of NOX4 may be beneficial in inducing the myofibroblast phenotype for wound repair, the persistence of myofibroblasts together with autocrine TGFβ signaling may result in chronic NOX4 activation and dysregulation of signaling pathways culminating in myofibroblast differentiation, fibrosis, and organ dysfunction.

The cellular redox status and thus signaling potential of NOX4-derived ROS is regulated by antioxidant systems. Concomitant to TGFβ1-mediated induction of NOX4 during prostatic myofibroblast differentiation, a number of ROS-scavenging enzymes were downregulated, including the selenium transporter SEPP1 and selenium (Se)-containing ROS scavenging enzymes such as glutathione peroxidase 3 (GPX3) and thioredoxin reductase 1 (TXNRD1) [3]. The essential trace element Se is an integral component of GPX3 and TXNRD1 enzymes being incorporated as selenocysteine (Sec) at their active site and is critical for correct protein folding/function [100]. Consistent with the role of SEPP1 in delivering Se to peripheral tissues for selenoprotein biosynthesis [101, 102], supplementation of prostatic fibroblasts with exogenous Se restored expression of GPX3 and TXNRD1 as well as TXNRD1 enzyme activity, depleted

TGFβ1-induced ROS downstream of NOX4 induction and inhibited myofibroblast differentiation [3].

Given the central role of elevated TGFβ1 in fibrogenesis and that SEPP1 is a direct transcriptionally-suppressed target of TGFβ1 [103, 104], it is plausible that dysregulation of Se-dependent antioxidant systems occurs not only in BPH and the stromal response to prostate cancer but also in other fibrotic disorders. Indeed, the antifibrotic potential of Se is not restricted to prostatic stromal cells since exogenous Se also inhibited TGFβ-mediated myofibroblast transdifferentiation of hepatic stellate cells [105]. Moreover, in a rat model of thyroid fibrosis, Se deficiency promoted thyroid fibrosis in a TGFβ-dependent manner [106], whereas Se supplementation decreased hepatic fibrosis in mice [107]. Consistent with in vitro findings, SEPP1 was specifically lost in the periglandular tumor-associated stroma of prostate cancer patients [3]. These findings are consistent with a large body of animal and human clinical data that Se deficiency or supplementation increases or reduces tumor incidence, respectively [108–111]. Moreover, a recent dose-response meta-analysis revealed that overall prostate cancer risk was 15–25% (for advanced PCa: 40–50%) lower in men with plasma/serum Se levels between 135–170 ng/mL compared with 60 ng/mL [112].

Collectively, data from in vitro fibroblast-to-myofibroblast differentiation models together with in vivo findings indicate that myofibroblast differentiation in fibrotic disorders and tumor-reactive stroma is driven by a prooxidant shift in intracellular redox signaling caused by elevated ROS and/or reduced antioxidative potential. NOX4 appears to be the major source of elevated ROS and central mediator of TGFβ-induced myofibroblast differentiation in diverse tissues. Thus, restoring cellular redox homeostasis, for example by (i) targeting NOX4, (ii) Se supplementation, and/or (iii) application of antioxidants may represent a promising therapeutic strategy for fibrotic disease.

6. Nitric Oxide/cGMP Signaling in the Regulation of Myofibroblast Differentiation

The free radical NO is an important signaling molecule in a variety of biological processes. In vivo NO is biosynthesized from L-arginine by nitric oxide synthases (NOS), involving the oxidation of NADPH and the reduction of molecular oxygen. NO activates soluble guanylyl cyclase (sGC), which generates the second messenger cGMP. cGMP exerts multiple effects, for example it regulates cGMP-dependent protein kinases such as protein kinase G (PKG), cyclic nucleotide phosphodiesterases (PDEs), and cation channels and may have other unknown effects [113].

PDEs comprise a superfamily of phosphohydrolases that regulate cellular cGMP and cyclic adenosine monophosphate (cAMP) levels. PDE type 5 (PDE5) specifically hydrolyzes cGMP and is the major therapeutic target in erectile dysfunction (ED), whereby PDE5 inhibitors increase intracellular cGMP levels to enhance NO/cGMP signaling and thereby promote vasodilation [114].

Besides treatment of ED, PDE5 inhibitors are employed in the treatment of pulmonary arterial hypertension and

BPH [115, 116]. Noticeably, patients treated with PDE5 inhibitors for ED exhibited beneficial effects on lower urinary tract symptoms (LUTS) secondary to BPH [117, 118]. These effects were thought to be due to changes in prostatic smooth muscle tone [119–121]. However, our data implicate a direct role of PDE5, which is predominantly expressed in the stromal compartment of the prostate *in vivo*, and NO/cGMP signaling in myofibroblast differentiation [73]. Pharmacological or genetic inhibition of PDE5 significantly attenuated TGFβ1-induced myofibroblast differentiation of prostatic fibroblasts *in vitro*, indicating that enhancing intracellular cGMP levels inhibits myofibroblast differentiation [73]. Consistently, stimulating the generation of intracellular cGMP by the soluble NO donor sodium nitroprusside (SNP) dose-dependently inhibited TGFβ1-induced differentiation and additional blocking of cGMP hydrolysis by the PDE5 inhibitor tadalafil synergistically enhanced this effect [73].

These findings are consistent with numerous studies implicating an inhibitory role of the NO/cGMP pathway in fibroblast-to-myofibroblast differentiation in other tissues. On the one hand, treatment of human dermal fibroblasts with TGFβ1 significantly reduced NOS activity and NO levels, whereas restoring cGMP signaling downstream of NOS using SNP and the cell-permeable cGMP analog 8-bromo-cGMP significantly suppressed TGFβ1-induced collagen production [122]. Furthermore, the NOS inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME) synergistically potentiated TGFβ1-induced collagen production in dermal fibroblasts [122]. In addition, increasing cGMP levels using the PDE5 inhibitor sildenafil alone or in combination with the sGC activator BAY 58-2667 attenuated myofibroblast differentiation of fibroblasts from human Peyronie's disease plaques or lung, respectively [23, 123]. In a similar approach, 8-bromo-cGMP inhibited TGFβ1-induced myofibroblast differentiation of cardiac fibroblasts isolated from wild-type mice [76]. Likewise, the sGC stimulator BAY 41-2272 elevated intracellular cGMP levels and inhibited myofibroblast differentiation in cardiac fibroblasts [77] and dermal fibroblasts from healthy individuals and patients with systemic sclerosis [124].

In addition to the documented improvement of LUTS secondary to BPH by PDE5 inhibitors [117, 118], beneficial effects of enhancing NO/cGMP signaling have also been reported in several fibrosis models *in vivo*. For example, attenuating NO signaling by inhibition of inducible NOS (iNOS) activity in rats with TGFβ1-induced fibrotic plagues of tunica albuginea (a model of Peyronie's disease) resulted in increased myofibroblast abundance and collagen I synthesis in the plaques [125]. Similarly, fibrosis in the penile corpora cavernosa upon streptozotocin-induced diabetes was intensified in iNOS knockout mice compared with wild type [126]. Consistently, stimulating NOS activity in rats via oral administration of the NOS substrate L-arginine resulted in an 80–95% reduction in both plaque size and collagen: fibroblast ratio in PD-like plaques induced by TGFβ1 [123]. Similar effects were observed in parallel groups that received the competitive nonselective PDE inhibitor pentoxifylline or PDE5-selective inhibitor sildenafil [123]. Likewise, promoting NO synthesis via administration of the NOS substrate L-arginine, significantly elevated endothelial NOS expression but decreased TGF-β1 expression and ultimately ameliorated renal interstitial fibrosis, which was markedly aggravated by L-NAME administration in rats with unilateral ureteral obstruction [127].

Alternative approaches to increase cGMP production and NO/cGMP signaling by stimulating sGC activity exhibit similar antifibrotic effects. The sGC stimulator BAY 41-2272 significantly limited progression of anti-Thy-1-induced chronic renal fibrosis in rats [128]. Likewise, BAY 41-2272 reduced the number of myofibroblasts and decreased collagen accumulation in hypertension-induced cardiac fibrosis in rats [77]. Another sGC stimulator riociguat (BAY 63-2521) exerted similar inhibitory effects on cardiac and renal interstitial fibrosis in two rat models of hypertension [129] and attenuated fibrotic tissue remodeling in the myocardium and renal cortex of Dahl salt-sensitive rats [130]. Furthermore, BAY 41-2272 prevented the development of bleomycin-induced dermal fibrosis and skin fibrosis in Tsk-1 mice [124]. Interestingly, NO-independent activation of sGC by BAY 60-2770 also attenuated liver fibrosis in rats [131].

Many studies investigating NO/cGMP signaling in fibrotic disease exploit the specific hydrolytic activity of PDE5 for cGMP to indirectly enhance cGMP levels and thereby promote NO signaling. However, an active role of PDE5 in fibrogenesis via altered enzyme activity has also been reported. Glomerular PDE5 expression was increased during anti-Thy1-induced mesangial proliferative glomerulonephritis in the rat kidney *in vivo*, and PDE5 inhibition by vardenafil increased glomerular cGMP levels leading to subsequent inhibition of mesangial cell proliferation and ECM accumulation [132].

Collectively, findings from *in vitro* and *in vivo* model systems indicate that myofibroblast differentiation is associated with reduced NO/cGMP signaling, suggesting the potential therapeutic benefit of enhancing NO/cGMP signaling (by stimulating sGC activity, and/or preventing cGMP degradation via PDE5 inhibition) in fibrotic disease.

7. Potential Interplay of ROS and NO/cGMP Signaling in Regulating Myofibroblast Differentiation

NOX4-derived ROS play a key role in driving myofibroblast differentiation in response to TGFβ1, whereas increasing NO/cGMP signaling attenuates TGFβ1-induced fibroblast-to-myofibroblast differentiation. This raises an interesting possibility that crosstalk between NO/cGMP and NOX4-derived ROS signaling may coordinately regulate myofibroblast differentiation.

Crosstalk between superoxide and NO signaling has been extensively documented, in particular the ability of superoxide to reduce NO levels by direct chemical scavenging or by NOS uncoupling. For example, superoxide can react with NO generating peroxynitrite ($ONOO^-$), thereby depleting NO levels [133]. In addition, superoxide can oxidize the critical nitric oxide synthase (NOS) cofactor tetrahydrobiopterin (BH_4) leading to NOS uncoupling, which

results in superoxide generation rather than NO production [134]. However, TGFβ-induced fibroblast-to-myofibroblast differentiation is associated with induction of NOX4 and thus presumably generation of H_2O_2 [84, 85], which unlike superoxide does not appear to react directly with NO. Thus, any opposing regulation of myofibroblast differentiation by NO/cGMP signaling and NOX4-derived H_2O_2 presumably occurs via distinct mechanism(s).

Several examples of opposing interaction between H_2O_2 and NO/cGMP signaling have been reported. For example, incubation of isolated rat hepatocytes with an NO donor prevented H_2O_2-induced cell death, presumably via cGMP-activated downstream signaling since the prosurvival effect of NO was ablated by cycloheximide indicating the requirement of *de novo* protein synthesis [135]. Conversely, H_2O_2 impaired NO production in porcine aortic endothelial cells, possibly via direct oxidative inactivation of eNOS cofactors [136]. Moreover, H_2O_2 eliminates the endothelium-dependent vasodilatory response to acetylcholine, a potent inducer of NO synthesis [137].

There are several potential mechanisms by which NOX4-derived H_2O_2 and NO/cGMP signaling may interact to elicit opposing functions during myofibroblast differentiation. For example, both NOS and NOX require NADPH as an electron donor for enzyme activity. NOX4 induction is an early event during TGFβ1-mediated differentiation [3, 20], whereas enhancing cGMP levels inhibits/reverses prostatic fibroblast-to-myofibroblast differentiation without impairing NOX4 mRNA induction by TGFβ1 (our unpublished observations) [138], indicating that NO/cGMP signaling acts downstream of NOX4-derived H_2O_2 production. Thus, NADPH consumption/depletion due to elevated NOX4 activity may attenuate NO-dependent cGMP production and thereby impair NO/cGMP-mediated inhibition of differentiation.

A further potential mode of opposing interaction may occur via mutually exclusive modification of NOX/NO target proteins. For example, NO upregulates the activity of sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) via *S*-glutathiolation on cysteine 674 [139]. Interestingly, TGFβ1-mediated induction of NOX4 in aortic SMCs resulted in SERCA oxidation of the same thiol group, inhibited NO-mediated *S*-glutathiolation, and attenuated NO inhibition of SMC migration [140, 141]. Similar effects were observed upon exposure of vascular SMCs to H_2O_2 or high glucose, which induced NOX4 levels leading to SERCA oxidation, inhibition of NO-induced *S*-glutathiolation and migration. Moreover, NOX4 knockdown decreased SERCA oxidation and restored the inhibition of SMC migration by NO [142].

NOX4-derived H_2O_2 may also attenuate NO signaling by downregulating sGC expression/activity and consequently NO-dependent cGMP generation. H_2O_2 decreased sGC expression and NO-dependent cGMP generation in pulmonary arterial SMCs from lambs with persistent pulmonary hypertension of the newborn [143]. Moreover, incubation of rat aortic SMCs or freshly isolated vessels with ROS-generating agents (including H_2O_2) significantly decreased sGC expression and reduced SNP-induced cGMP formation in the SMCs [144]. In addition, PTP inhibitors or H_2O_2 promoted tyrosine phosphorylation of the beta 1 subunit of sGC, most likely via Src-like kinases [145]. Subsequent studies revealed that cGMP levels are cross-regulated via a mechanism that involves c-Src-dependent phosphorylation of sGC, which attenuates sGC activity and cGMP formation [146]. These studies suggest that elevated NOX4-derived H_2O_2 during myofibroblast differentiation may oxidatively inactivate protein tyrosine phosphatases and/or activate Src kinase, which in turn promote inhibitory phosphorylation of sGC leading to reduced cGMP signaling.

Interactions between TGFβ and NO signaling have also been reported. For example, the NO donor *S*-nitroso-*N*-acetyl-penicillamine or treatment with 8-bromo-cGMP decreased TGFβ3 mRNA levels in neonatal rat cardiac fibroblasts, whereas TGFβ1 mRNA levels were modestly increased [147]. Furthermore, *S*-nitroso-*N*-acetyl-penicillamine and ROS, most likely H_2O_2, both increased TGFβ1 release from human epithelial alveolar cells [148]. On the other hand, TGFβ1 decreased sGC and PKGI expression in pulmonary artery and aortic SMCs from adult rats and mice and a TGFβ-neutralizing antibody prevented the reduction of sGC and PKGI protein expression in chronic oxygen-induced lung injury in mouse pups [149].

Thus, similar to reciprocal inhibition of superoxide and NO, H_2O_2, and NO also appear to interact in a functionally opposing manner during myofibroblast differentiation. However, rather than direct radical quenching as observed for superoxide and NO, the basis of reciprocal inhibition between H_2O_2 and NO apparently occurs at multiple indirect levels. Cumulatively, such interplay would be expected to lead to downregulation of NO/cGMP signaling upon TGFβ1-mediated induction of NOX4-derived H_2O_2 and thereby promote fibroblast-to-myofibroblast differentiation. Consistent with this hypothesis, TGFβ1 significantly decreased NO production in dermal fibroblasts, whereas increasing NO signaling by stimulating sGC generation and/or inhibiting cGMP degradation counteracted ROS-mediated inactivation of NO signaling and prevented myofibroblast differentiation [122]. Inhibition of differentiation can also be achieved by treatment with 8-bromo-cGMP [122], indicating that suppression of myofibroblast differentiation by enhanced NO signaling is mediated by downstream cGMP-dependent mechanisms and not via the NO radical *per se*.

8. Reversal of Myofibroblast Differentiation and Clinical Implications

Tissue fibrosis is thought to arise from failure of the myofibroblast apoptotic wave during wound healing [12, 45]. Thus, whilst inhibition of myofibroblast differentiation may be suitable to prevent disease progression, curative treatments would also require targeting of preexisting myofibroblasts. Although some promising data for such approaches exist [150], extensive fibroblast heterogeneity and the lack of a consensus "myofibroblast-specific" surface marker mean that tissue- and even disease-specific targeting strategies will be required.

An alternative approach to clear the fibrotic myofibroblast pool envisages inducing their dedifferentiation to the
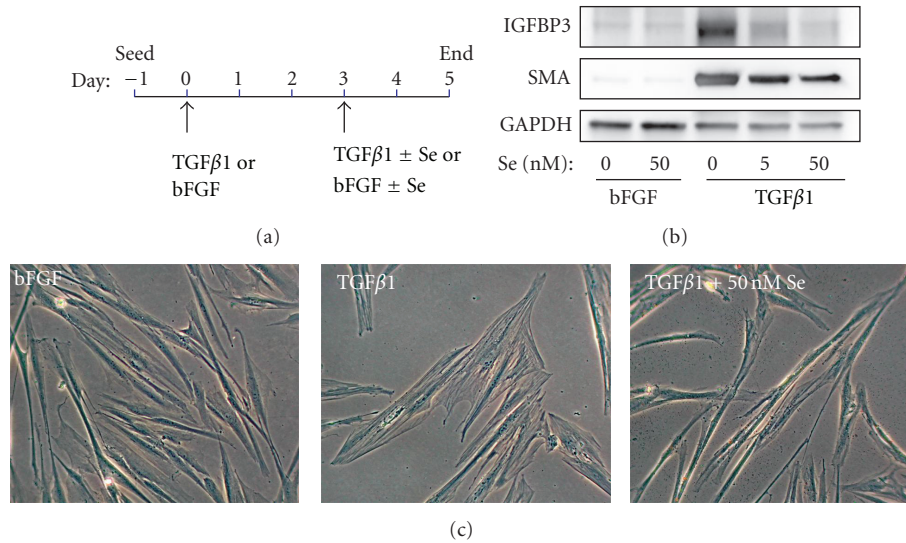
(a)

(b)

(c)

FIGURE 2: Selenium reverses myofibroblast differentiation of prostatic fibroblasts. Therapeutic targeting of myofibroblast dysregulation in fibrotic disease may be accomplished by promoting myofibroblast dedifferentiation to the nonactivated fibroblast/progenitor phenotype. (a) Methodology outline of selenium (Se)-mediated reversal of fibroblast-to-myofibroblast differentiation in primary human prostatic fibroblasts. Myofibroblast differentiation was induced with 1 ng/mL TGFβ1 or bFGF as mock control. After 72 hours, fresh media was added containing bFGF or TGFβ1 as before but supplemented with selenium (Se) as sodium selenite or vehicle equivalent. Cells were incubated for a further 48 hours before harvesting. Reversal of myofibroblast differentiation of primary human prostatic fibroblasts treated as outlined in (a) was verified by (b) Western blotting for the myofibroblast markers, α-SMA, and IGFBP3 and (c) morphological analysis using phase contrast microscopy. (b) Induction of myofibroblast differentiation by TGFβ1 in the absence of Se (0 nM) is indicated by increased production of myofibroblast markers. However, both α-SMA and IGFBP3 levels are reduced in the presence of Se in a dose-dependent manner. (c) At the morphological level, Se restores the thin, elongated and light refractive phenotype to cells predifferentiated with TGFβ1 *(far right)*, whereas cells treated with TGFβ1 alone *(center panel)* exhibit the typical enlarged and flattened myofibroblast phenotype with visible actin-like filaments. (b-c) Images are representative of three independent experiments using primary cells isolated from different donors.

nonactivated fibroblast/progenitor phenotype. It has long been considered that fibrosis and fibroblast-to-myofibroblast differentiation are irreversible processes. However, recent data from *in vitro* and *in vivo* models indicate that tissue fibrosis and fibroblast-to-myofibroblast differentiation can indeed be reversed. For example, we observe that exogenous Se or PDE5 inhibition restore morphological and molecular characteristics typical of the fibroblast phenotype to *in vitro* differentiated prostatic myofibroblasts even in the continued presence of the TGFβ differentiation-inducing stimulus (Figure 2) [138]. Our data are supported by studies employing myofibroblasts from IPF patients and a three-dimensional coculture model of porcine skin fibrosis that similarly demonstrate the potential utility of ROS scavenging in promoting myofibroblast dedifferentiation [151, 152]. Moreover, treatment of *in vitro* differentiated corneal myofibroblasts with fibroblast growth factor in combination with heparin decreased expression of α-SMA, TGFβ receptors, and cadherins, indicating reversal of myofibroblast differentiation to a fibroblast-like phenotype [153]. Recently, pharmacological inhibition of NOX4 after induction of liver fibrosis in mice was shown to reduce ROS levels and significantly attenuate fibrosis [22].

The principle of enhancing NO/cGMP signaling to induce myofibroblast to fibroblast reversal has also been successfully demonstrated in animal models. Treatment with the PDE5 inhibitor vardenafil reduced myofibroblast numbers and total size of preformed TGFβ1-induced Peyronie's disease plaques in rats [154]. Moreover, BAY 41-2272 reduced established fibrosis in a modified mouse model of bleomycin-induced skin fibrosis and in Tsk-1 mice [124].

Collectively, these findings and those discussed above suggest that signaling via ROS and local growth factors (such as TGFβ and fibroblast growth factor) play key roles not only in driving fibroblast-to-myofibroblast differentiation but also in subsequently maintaining the myofibroblast phenotype. Thus, we hypothesize that high NO/cGMP signaling and low NOX4-derived ROS production coordinately maintain the fibroblast phenotype, whereas differentiation into the myofibroblast phenotype may proceed upon elevated NOX4-derived ROS signaling and/or concomitant inactivation of NO/cGMP signaling (Figure 3).

The observation that fibroblasts and myofibroblasts are interconvertible phenotypes rather than terminally differentiated cell types has significant clinical implications for potential curative therapy of advanced fibrotic disease. In this respect, pharmacological regulation of redox signaling via NOX4 inhibitors, antioxidants, and/or enhancement of NO/cGMP signaling provides a promising option to modulate the fibroblast/myofibroblast ratio at multiple levels in several pathological conditions (Figure 3). For example, local activation of myofibroblast differentiation may provide a means to overcome impaired wound healing in the elderly,
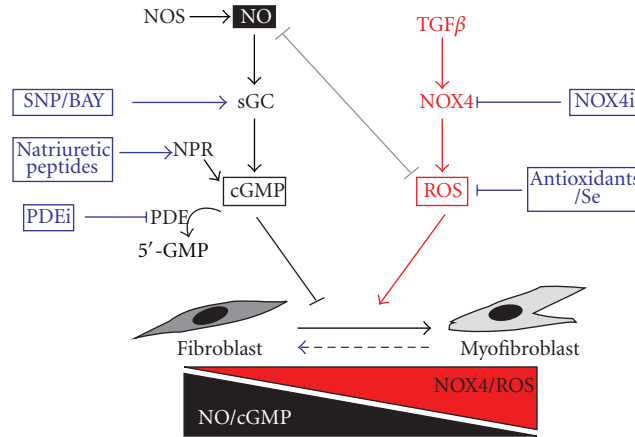
FIGURE 3: Potential therapeutic targeting of myofibroblast differentiation in the treatment of age-associated fibrotic disease. Myofibroblast differentiation induced by TGFβ1 plays a central role in the etiology of numerous age-related fibrotic disorders. In particular, TGFβ induces a prooxidant shift in intracellular redox homeostasis via the induction of NOX4-derived ROS (in particular H_2O_2), which modulates downstream phosphorylation signaling cascades and transcriptional events that culminate in myofibroblast differentiation. The concomitant downregulation of selenium-dependent ROS scavenging enzymes by TGFβ further potentiates NOX4-derived ROS signaling, which also downregulates NO/cGMP signaling and thereby relieves inhibition of myofibroblast differentiation by NO. Fibroblast-to-myofibroblast differentiation and subsequent tissue fibrosis are reversible processes *(broken horizontal arrow)*. Thus, pharmacological interference of these redox signaling processes to redress redox homeostasis and thereby restore the physiological fibroblast:myofibroblast ratio offers a promising therapeutic strategy for the treatment of age-related pathologies associated with myofibroblast dysregulation. Such pharmacological targeting may succeed at multiple levels. For example, targeting NOX4 directly via NOX4 inhibitors (NOX4i) or indirectly by ROS scavenging with Se or antioxidants would attenuate NOX4-derived ROS signaling and restore the inhibitory effects on differentiation by NO/cGMP signaling. Enhancing NO/cGMP signaling is also sufficient to prevent and reverse fibroblast-to-myofibroblast differentiation and may be achieved by (i) sGC stimulation using the NO donor sodium nitroprusside (SNP) and/or direct sGC stimulators/activators (BAY), (ii) promoting cGMP synthesis via administration of natriuretic peptides that bind to and activate transmembrane guanylyl cyclase natriuretic peptide receptors (NPRs), or (iii) by inhibiting phosphodiesterase (PDE)-mediated cGMP hydrolysis using PDE inhibitors (PDEi).

whereas inhibiting fibroblast-to-myofibroblast differentiation and restoring the fibroblast phenotype may represent a therapeutic strategy for fibrotic disease and also serve as a stromal-targeted chemotherapy approach for solid tumors, such as breast, liver, and prostate cancer.

Despite its critical role in the pathogenesis of fibrosis, TGFβ is not considered a direct clinical target due to its critical function in diverse biological processes and homeostatic maintenance [155]. As central downstream components of TGFβ signaling during fibroblast-to-myofibroblast differentiation, targeting NOX4-derived ROS and NO/cGMP might also be expected to elicit broad and undesirable effects. However, PDE5 inhibitors have a successful history in the treatment of ED, pulmonary arterial hypertension, and BPH [114–116]. Moreover, it should be noted that (i) none of the published Nox4 knockout animals display an obvious basal phenotype, (ii) a dual NOX1/NOX4 inhibitor (at doses that reduced liver fibrosis in mice) does not exert toxic effects in animal models, and (iii) the same inhibitor was also well tolerated in phase I clinical trials [22, 81]. This likely reflects observations that NOX4 does not mediate all of the signaling functions of TGFβ, as illustrated by the delayed temporal induction of NOX4 expression and ROS production (~2 hours following TGFβ1 treatment) compared to the rapid induction of phosphorylation cascades, such as phosphorylation of Smad2/3 and ERK1/2 that occurs within 5 minutes

of TGFβ treatment [3, 20, 83]. Thus, selective modulation of NOX4, PDE5, and/or sGC activities may permit continued physiological ROS/NO signaling due to the presence of multiple NOX, PDE, and GC isoforms. Moreover, the fact that these enzymes belong to multimembered families may be clinically exploited to selectively target those isoforms underlying pathology in a given tissue or disease state. For example in the heart, PDE1A appears to play a critical role in cardiac fibrosis and its selective targeting in rats and mice led to regression of cardiac remodeling that is associated with various cardiac diseases [156].

9. Conclusions

Myofibroblast activation and differentiation are central processes of normal wound healing. However, these beneficial effects of myofibroblasts are dysregulated in fibrotic disorders and in the reactive stromal response that promotes tumorigenesis and metastasis. Whilst TGFβ plays a key role in initiating myofibroblast differentiation and ECM deposition during normal wound healing, failure of myofibroblast clearance together with their autocrine production of TGFβ leads to ECM accumulation, fibrosis, and ultimately organ dysfunction. A large body of data from *in vitro* fibroblast-to-myofibroblast differentiation models indicates that TGFβ1-induced myofibroblast differentiation is mediated via induction of NOX4-derived H_2O_2, which

modulates downstream phosphorylation signaling cascades and transcriptional events that culminate in cytoskeletal remodeling and myofibroblast differentiation. In addition, NOX4-derived H_2O_2 appears to downregulate NO/cGMP signaling via multiple mechanisms and thereby relieves inhibition of myofibroblast differentiation by NO. Findings from these *in vitro* cellular models of fibrosis are supported by extensive *in vivo* data, underscoring the value of *in vitro* models in defining molecular mechanisms underlying fibrogenic disease and serving as screening platforms for the discovery of novel therapeutics. Targeting NOX4 directly via NOX4 inhibitors or indirectly by ROS scavenging with Se, antioxidants or enhancing NO/cGMP signaling by sGC stimulation, and/or inhibition of cGMP degradation attenuates TGFβ1-induced differentiation and inhibits myofibroblast activation. Moreover, these agents induce the dedifferentiation/reversal of preexisting myofibroblasts to a quiescent fibroblast phenotype. Thus, pharmacological interference of these redox signaling processes to restore the physiological fibroblast : myofibroblast ratio offers a promising therapeutic strategy not only for the treatment of fibrotic diseases but also for managing tumor invasion and metastasis at the level of stromal remodeling.

Abbreviations

α-SMA:	Alpha smooth muscle cell actin
bFGF:	Basic fibroblast growth factor
BPH:	Benign prostatic hyperplasia
cAMP:	Cyclic adenosine monophosphate
cGMP:	Cyclic guanosine monophosphate
ECM:	Extracellular matrix
ED:	Erectile dysfunction
IPF:	Idiopathic pulmonary fibrosis
L-NAME:	N_ω-nitro-L-arginine methyl ester
LUTS:	Lower urinary tract symptoms
MAPK:	Mitogen-associated protein kinase
NO:	Nitric oxide
NOS:	Nitric oxide synthase
NOX:	NADPH oxidase
PDE:	Phosphodiesterase
PKG:	Protein kinase G
PTK:	Protein tyrosine kinase
PTP:	Protein tyrosine phosphatase
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
Se:	Selenium
sGC:	Soluble guanylyl cyclase
SMC:	Smooth muscle cell
SNP:	Sodium nitroprusside
TGFβ:	Transforming growth factor beta.

Acknowledgments

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