1-1-2015

Role of cellular senescence and NOX4-mediated oxidative stress in systemic sclerosis pathogenesis.

Sonsoles Piera-Velazquez
Thomas Jefferson University

Sergio A. Jimenez
Thomas Jefferson University

Role of cellular senescence and NOX4-mediated oxidative stress in Systemic Sclerosis pathogenesis.

Sonsoles Piera-Velazquez, Ph.D. and Sergio A. Jimenez M.D.

The Scleroderma Center and The Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia PA, 19107, USA

Running Title: Cellular Senescence, Oxidative Stress, and NOX4 in Systemic Sclerosis Pathogenesis

Address all correspondence to:

Sergio A. Jimenez, MD

Jefferson Institute of Molecular Medicine

Thomas Jefferson University

233 South 10th Street, Suite 509 BLSB

Philadelphia, PA 19107

E-mail: Sergio.jimenez@jefferson.edu

Phone: (215) 503-5032

Fax: (215) 923-4649

Keywords: Systemic Sclerosis; fibrosis; cellular senescence; oxidative stress; ROS; NOX4.

ABSTRACT

Systemic Sclerosis (SSc) is a systemic autoimmune disease characterized by progressive fibrosis of skin and numerous internal organs and a severe fibroproliferative vasculopathy resulting frequently in severe disability and high mortality. Although the etiology of SSc is unknown and the detailed mechanisms responsible for the fibrotic process have not been fully elucidated one important observation from a large U.S. population study was the demonstration of a late onset of SSc with a peak incidence between 45 and 54 years of age in African-American females and between 65 and 74 years of age in white females. Although it is not appropriate to consider SSc as a disease of aging, the possibility that senescence changes in the cellular elements responsible for its development may play a role has not been thoroughly examined. The process of cellular senescence is extremely complex and the mechanisms, molecular events, and signaling pathways involved have not been fully elucidated, however, there is strong evidence to support the concept that oxidative stress caused by the excessive generation of reactive oxygen species may be one important mechanism involved. On the other hand, numerous studies have implicated oxidative stress in SSc pathogenesis, thus, suggesting a plausible mechanism in which excessive oxidative stress induces cellular senescence and that the molecular events associated with this complex process play an important role in the fibrotic and fibroproliferative vasculopathy characteristic of SSc. Here, recent studies examining the role of cellular senescence and of oxidative stress in SSc pathogenesis will be reviewed.

INTRODUCTION

Systemic Sclerosis (SSc) is a systemic autoimmune disease of unknown etiology characterized by progressive fibrosis of skin and numerous internal organs and a severe fibroproliferative vasculopathy resulting frequently in functional disability and high mortality [1-3]. The pathogenesis of SSc is complex and despite numerous studies that have examined its intricate picture, the exact mechanisms involved in the severe cutaneous and systemic fibrotic process have not been fully elucidated [4-7]. One important demographic feature identified in a large SSc population is that SSc disease onset and its peak incidence occur between the ages of 45 and 54 years for African American women and between the ages of 65 and 74 years for white women [8]. The reasons for the late onset and higher frequency of SSc in individuals older than 45 years of age are not known and have not been examined in detail. Although it is not appropriate to consider SSc as a disease of aging, the possible role of cellular senescence in SSc pathogenesis should be considered as an important factor. Among the reactions or pathways that may be involved in this process there is strong evidence that oxidative stress mediated by an excessive generation of reactive oxygen species (ROS) plays a crucial role. We will review here recent experimental evidence supporting the participation of cellular senescence and oxidative stress in SSc pathogenesis emphasizing the potential role of oxidative stress in the fibrotic process that is the hallmark of SSc. We will also discuss recent evidence indicating that the NADPH oxidase NOX4 may be one of the most important mediators of ROS generation in SSc, and the potentially beneficial effects of inhibition of NOX4 activity as a cogent therapeutic approach for SSc-associated tissue fibrosis and fibroproliferative vasculopathy.

CELLULAR SENESCENCE AND SSc PATHOGENESIS.

Cellular Senescence and Senescence Associated Secretory Phenotype.

Cellular senescence, a process first identified by Hayflick and Moorehead in their pioneering studies on human diploid fibroblasts cultured *in vitro* [9,10], is characterized by the permanent arrest of cell division associated with a variety of phenotypic changes including cellular enlargement, flattening, and vacuolization, as well as, numerous functional alterations [Reviewed in 11-16]. The most prominent of these alterations are the expression of novel specific gene products such as the senescence-associated β-galactosidase isoform (SA-β gal), and the acquisition of a unique complement of secreted molecules which includes inflammatory cytokines and chemokines, growth factors, and various proteases and other pro-inflammatory molecules collectively known as the senescence-associated secretory phenotype or SASP [17**-21]. The most important pro-inflammatory and pro-fibrotic SASP components are listed in Table 1. Although it was initially considered that cellular senescence may be solely a protective mechanism to prevent the uncontrolled cellular proliferative activity of malignant cells, extensive studies have demonstrated that it may participate in numerous physiological processes including embryonic development [22**,23**], as well as in pathologic conditions associated with aging [11-13, 24,25].

The mechanisms responsible for the irreversible arrest in cellular proliferative capacity are highly complex and although they have been studied extensively, they have not been fully elucidated. However, it has been shown that a variety of stimuli and numerous signaling pathways may be involved [11-13, 16,18]. The most important triggers of cellular senescence are the activation of the DNA-damage response (DDR) initiated by the occurrence of structural changes in DNA and complex molecular events involving oncogene effects, abnormalities in cell

cycle kinases, including various cyclin-dependent kinases, and telomere shortening or dysfunction [11-13, 26-28]. Of relevance to the topic of this review are the extensive observations demonstrating that two important mechanisms involved in cellular senescence are reactive oxygen species (ROS)-induced oxidative stress [29-34] and persistent stimulation by type I interferons [35-37].

Cellular Senescence and SSc.

There has been strong interest in the role of cellular senescence in malignancies and disorders of aging, however, there is very little information about its possible contribution to SSc pathogenesis. Among the few investigations that have explored the possible connection between senescence, aging, and SSc, a recent study conducted an evaluation of normal fibroblasts obtained from donors of various ages (up to 33 years of age) and of several dermal fibroblast cell strains obtained from patients with SSc employing a proteomic approach [38**]. The study identified numerous age-dependent differences including the accumulation of SA-βgal and showed that SSc fibroblasts displayed evidence indicative of cellular senescence and decreased autophagy. Another study [39] examined bone marrow derived mesenchymal stem cells from SSc patients and demonstrated that these cells displayed markers of early senescence and had an impairment in their ability to differentiate into endothelial cells suggesting that these alterations may be important for the pathogenesis of the vascular involvement in SSc.

Numerous studies have examined alterations in telomere length as playing a role in the development of various autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, SSc, and Sjögren syndrome, however, the available evidence is not conclusive and may be even contradictory in some studies [40,41]. There are very few studies of telomere length in SSc [42-44]. Although an early study described that telomere length was significantly

shortened in SSc patients and suggested that this observation may be either reflective of a genetic predisposition to chromosomal instability or the result of exposure to a noxious environmental agent [42], a more recent study described contradictory results demonstrating that telomere length was longer in peripheral blood mononuclear cells from SSc patients [44]. Therefore, the original description of shortened telomeres in cells from SSc patients requires further experimental validation. In this respect, a study of telomerase activity in peripheral blood mononuclear cells from patients with various autoimmune diseases demonstrated that in contrast with cells from patients with rheumatoid arthritis and systemic lupus erythematosus that displayed elevated activity of the enzyme, cells from SSc patients had remarkably low levels of telomerase activity [45]. However, the significance of this observation to SSc pathogenesis has not been elucidated.

Although not directly related to SSc pathogenesis, several studies of another fibrotic disease that displays a clear pattern of increased occurrence in older individuals; namely idiopathic pulmonary fibrosis (IPF), have provided evidence to support the notion that cellular senescence may play an important role in its pathogenesis [46-48]. Indeed, recent studies showed an unexpectedly high frequency of telomerase mutations and telomere shortening in a large population of patients affected with the familial form of IPF [49,50].

Caveolin-1 and PTEN in cellular senescence.

Strong experimental evidence has demonstrated that caveolin-1 (cav-1), the protein responsible for the remarkable functional properties of caveolae, is decreased in affected cells and tissues from SSc or IPF patients [51-54]. Numerous recent studies have shown that cav-1 plays and important role in the pathophysiology of tissue fibrosis [51,52,55**] most likely, owing to its ability to induce internalization of activated TGF-β receptors into cav-1 lipid rafts leading to

their rapid proteasomal degradation [52,55**]. Although an earlier study indicated that expression of cav-1 induced premature cellular senescence in primary cultures of murine fibroblasts [56], and a more recent investigation demonstrated that cav-1 deficiency protects from bleomycin-induced pulmonary fibrosis in mice [57], recent evidence indicates that cav-1 exerts antifibrotic effects by modulating the activity of the phosphatase and tensin homolog (PTEN). PTEN is a tumor suppressor protein that has been implicated in cellular senescence. A recent study found that PTEN was markedly decreased in lung fibroblasts from IPF patients as well as in lungs from mice with bleomycin-induced pulmonary fibrosis and that this reduction was mediated by decreased cav-1 [58]. In light of previous studies demonstrating that a reduction of PTEN exerted potent pro-fibrotic effects *in vitro* and *in vivo* [59], the newly uncovered interaction between cav-1, PTEN and cellular senescence represents a novel pro-fibrotic mechanism that may be of relevance to SSc pathogenesis.

ROS and OXIDATIVE STRESS IN SSc PATHOGENESIS.

Evidence of oxidative stress in SSc.

In 1993, Murrell proposed a unifying hypothesis to explain the pathogenesis of the cutaneous fibrotic process in a variety of systemic fibrotic disorders including SSc suggesting a crucial role for oxygen free radical induced oxidative stress [60]. Following this report numerous studies have provided experimental support for this hypothesis and explored the mechanisms involved [Reviewed in 61- 63]. It has been shown that oxidative stress in SSc is the result of an imbalance between the production of oxidative stress producing systems and their antagonist antioxidant mechanisms. Increased generation or overproduction of ROS appears to be the main mechanism whereas ROS inactivation plays a less important role. Although one recent study demonstrated increased levels of antioxidants in serum of about 25% of SSc patients as measured by a total

antioxidant power assay [64**], numerous other studies have shown increased ROS production by various cells in SSc, as well as elevated levels in plasma from SSc patients [65*-69] providing strong evidence for the important contribution of oxidative stress in SSc pathogenesis. A recent study compared the total oxidant status (TOS), total antioxidant status (TAS) and an oxidative stress index (OSI) between SSc patients and healthy volunteers. The results demonstrated that TOS and OSI levels were significantly higher in SSc patients than in controls, whereas there were no significant differences in TAS between the two groups [65**]. These results indicated a remarkable imbalance between the profoundly increased oxidative stress levels and the relatively insufficient antioxidant status in SSc. In agreement with these observations elevated levels of various oxidative stress-related products have been detected in various biological fluids from SSc patients including increased urinary 8-oxodG levels [70], and increased levels of isoprostanes in SSc serum [71,72], in the urine [73,74] or in exhaled breath [75]. Isoprostanes such as F2-isoprostanes are markers of lipid peroxidation [76], produced *in vivo* in humans by free radical-catalyzed peroxidation of arachidonic acid. Other markers of oxidative stress in SSc include increased serum levels of N(epsilon)-(hexanoyl)lysine [77] and elevated serum levels of heat shock protein 70 [78]. The functional relevance of the elevated oxidative stress components in the circulation of SSc patients was evidenced in one recent study showing that sera from patients with SSc pulmonary hypertension caused oxidative stress induced activation of collagen synthesis in human pulmonary smooth muscle cells [79].

Role of ROS in SSc fibrosis and fibroproliferative vasculopathy.

ROS are a group of oxygen-derived molecules characterized by high chemical reactivity. It has been recently recognized that under physiological conditions ROS play important functions in intracellular signaling by activating redox-sensitive pathways including the cellular responses to

growth factor stimulation and the establishment of inflammatory responses [Reviewed in 80-82]. However, in pathological states, higher ROS levels can induce oxidative stress causing damage to proteins, lipids, and DNA, as well as inducing cellular senescence as discussed above. Following Murrell's provocative hypothesis linking SSc pathogenesis to the deleterious effects of ROS [60] numerous studies have implicated excessive oxidative stress and the generation of deleterious ROS in the pathogenesis of SSc [Reviewed in 61-63]. The studies of Sambo et al. [69] provided elegant experimental evidence that SSc dermal fibroblasts produce increased levels of ROS compared with normal cells and that the elevated ROS may be involved in the increased expression of a profibrotic phenotype in these cells. The same group also demonstrated that monocytes from SSc patients spontaneously release increased amounts of superoxide anion *in vitro* [66]. Other studies supporting the role of ROS in the development of the fibrotic and vasculo-proliferative lesions in SSc include the induction of high production of ROS by endothelial cells and fibroblasts *in vitro* following exposure to sera from SSc patients [68] and in pulmonary artery smooth muscle cells exposed to serum from SSc patients with pulmonary hypertension [79], as well as the ROS mediated inhibition of the anti-Wnt protein, Wint inhibitory factor 1 (WIF-1) leading to activation of Wnt pathway-induced tissue fibrosis (83**). Another study described a dose dependent abrogation of the increased production and secretion of type I collagen and fibronectin characteristic of SSc fibroblasts following *in vitro* exposure to the potent antioxidant, epigallocatechin-3-gallate (EGCG). EGCG also reduced the expression of the fibrotic marker CTGF, inhibited collagen gel contraction, and suppressed intracellular ERK1/2 kinase signalling and NF-κB activity [84**].

Although the detailed mechanisms involved in ROS effects in induction and establishment of the fibrotic process in SSc have not been fully elucidated there is intense ongoing research exploring

various novel directions. Among these, one recent study described a novel mechanism by which ROS may promote a profibrotic phenotype in SSc fibroblasts [85**]. This mechanism involves the ROS-mediated oxidative inactivation of protein tyrosine phosphatase 1B (PTP1B) leading to pronounced platelet derived growth factor receptor (PDGFR) activation. In this study, PTP1B activity was significantly reduced in SSc fibroblasts, most likely as a result of increased cysteine oxidation caused by higher levels of ROS. Confirmation of the important role of PTP1B on the regulation of the fibrotic process was obtained from studies showing that decreased PTP1B expression in normal fibroblasts led to increased expression of the genes encoding type I collagen and to elevated production of the corresponding protein [85**].

NADPH OXIDASES (NOX).

The NOX family of membrane-associated enzymes catalyze the reduction of O_2 to form ROS. The crucial roles of NOX in normal cellular physiology is evidenced by the remarkable increase in the number of NOX isoforms during eukaryotic evolution and their striking conservation through multiple species [86,87]. Several biochemical processes and enzyme systems are capable of producing ROS *in vivo*, however, the NADPH oxidases are the primary enzymes responsible for inducible ROS formation [Reviewed in 88-90]. The role of NOX enzymes in a variety of human disorders is currently the focus of intense investigation and numerous studies have provided strong experimental evidence to support their participation in a variety of pathologic conditions. Currently seven distinct NOX isoforms have been identified in humans and there are substantial differences in their tissue distribution, however, several of them display prominent expression in tissues and cells of substantial relevance to the pathophysiology of SSc. The three most important NOX isoforms related to SSc are NOX1, NOX2, and NOX4. Although the highest expression levels of NOX1 are in colonic epithelium, this isoform is also abundantly

expressed in endothelial cells and vascular smooth muscle cells. NOX2 is the classic inflammatory isoform found in neutrophils and macrophages but is also expressed in B-lymphocytes and in endothelial cells. NOX4 is highly expressed in the kidneys, however, it displays very high expression in fibroblasts, smooth muscle cells, and endothelial cells as well as in the lung.

ROLE OF NOX4 IN TISSUE FIBROSIS

Numerous recent studies have shown that NOX4 is a crucial molecule involved in the initiation, establishment and development of tissue fibrosis. Indeed, multiple growth factors and related polypeptides which participate in SSc pathogenesis [4-7] including TGF-ß, the most potent cytokine implicated in the fibrotic process, as well as other profibrotic polypeptides including PDGF, angiotensin II, and endothelin-1 have been shown to modulate the expression of NOX, and in particular, that of NOX4. Stimulation of NOX4 expression by TGF-β has been demonstrated in numerous recent studies [91-93]. PDGF, angiotensin II, and endothelin-1 have also been shown to induce increased NOX4 expression [94-96]. NOX4 has been identified as a source of ROS responsible for the generation and activation of TGF-β induced pulmonary, cardiac and renal myofibroblasts *in vitro* [91-93, 97-102]. NOX4 is upregulated in lungs of patients with IPF and in kidney and liver fibrosis [99,103,104]. NOX4-dependent generation of ROS has been postulated to be involved in the fibrotic process in SSc [105,106], however, experimental evidence in support of this hypothesis is still lacking. Of great relevance to the role of NOX4 in the pathogenesis of the fibroproliferative vasculopathy in SSc is a recent study demonstrating that ROS were capable of inducing the phenotypic conversion of endothelial cells into activated myofibroblasts through a TGF-β dependent mechanism [107**].

<u>Regulation of NOX4 activity.</u>

The mechanisms involved in the regulation of NOX activity are quite complex and it is likely that they vary depending on a specific cellular and functional context. However, it is important to emphasize that, in contrast with all other NOX enzymes, NOX4 does not require other protein subunits for its activity and, therefore, the levels of its activity are dependent on the levels of expression of its corresponding gene [108]. Given the important functions of NOX4 in a variety of physiological processes and in the pathogenesis of numerous diseases, there has been intense interest in unveiling the intimate mechanisms of its regulation. The mechanisms of increased NOX4 expression by TGF-β are the focus of intense investigation [109,110]. A recent study identified a far upstream AP-1/Smad binding element in the human NOX4 promoter that was involved in the regulation of NOX4 expression by TGF-β [109].

Regarding the regulation of NOX4 activity one important mechanism has been recently recognized. This study demonstrated a novel and quite important interaction of NOX4 with the polymerase delta interacting protein 2 (PDIP2) that resulted in a three-fold stimulation of NOX4 activity by PDIP2 [111**]. The full significance of this important discovery is still not apparent but undoubtedly represents a promising novel area for further investigation. Although the exact mechanisms involved in the regulation of NOX4 levels in normal cells are becoming unveiled the possible alterations responsible for the constitutive elevation in NOX4 levels and activity in SSc cells have not been studied.

<u>NOX4 inhibitors as a potential antifibrotic therapeutic intervention.</u>

Following Murrell's provocative hypothesis [60], numerous studies have provided strong supporting evidence for a role of ROS in abnormal, exaggerated fibrogenesis, and for the therapeutic targeting of ROS in fibrotic disorders such as SSc [112]. Indeed, a plethora of clinical studies have examined a variety of pharmacological or naturally occurring antioxidant

compounds as potential therapeutic agents for various aspects of the complex clinical manifestations of SSc [113-122]. Most of the clinical studies examining the effects of antioxidant therapy for SSc including some large placebo controlled clinical trials [122] have not provided conclusive evidence of a therapeutical benefit. However, the recent demonstration of the crucial role of NOX4 in the generation of ROS and the development of highly selective small molecule inhibitors [123**,124**] and/or specific small synthetic peptide inhibitors targeting NOX4 [125**] offers substantial promise for the treatment of currently incurable fibrotic disorders, such SSc or IPF [Reviewed in 126-128]. Indeed, numerous recent studies have shown that a selective NOX1/NOX4 inhibitor exerted highly effective antifibrotic effect in various animal models of tissue fibrosis [129-132]. The extensive study of Hecker et al. (131**) demonstrated remarkable differences in the extent and severity of bleomycin-induced pulmonary fibrosis induced in young mice compared to aged mice. These differences were caused by a NOX4-induced senescence phenotype in fibroblasts from the aged animals that prevented fibrosis resolution by rendering these cells resistant to apoptosis. The results demonstrated that persistent lung fibrosis in aging was mediated by NOX4 induced oxidative stress that resulted in cellular senescence and the acquisition of an apoptosis-resistant fibroblast phenotype. A remarkable observation was the reversal of these phenotype following treatment with the NOX4 selective small molecule inhibitor, GKT137831.

Although the extensive investigational studies reviewed above have suggested that specific or selective NOX4 inhibitors may be of benefit for the therapy of SSc patients their beneficial effects need to be conclusively demonstrated in rigorously controlled clinical trials.

CELLULAR SENESCENCE AND OXIDATIVE STRESS PATHWAYS LEADING TO TISSUE FIBROSIS IN SSc.

The causative agent or events and the exact mechanisms responsible for SSc development remain unknown. However, extensive recent information about cellular senescence and oxidative stress suggest a cogent hypothesis that may explain numerous observations obtained from the study of SSc cells and tissues. This hypothesis postulates that the potent profibrogenic growth factor, TGF-β becomes activated following its release from the TGF-β latent binding protein and engages its cognate receptors in the surface of TGF-β-responsible cells. The sequence of events resulting from active TGF-β receptor engagement leads to the upregulation of expression of NOX4 mediated by the canonical Smad pathway although other non-canonical TGF-β signaling pathways may be involved as well. Owing to the fact that NOX4 does not require additional co-factors and that its activity is largely determined by the levels of protein expression, the TGF-β induced increase in NOX4 transcript levels results in increased NOX4-mediated ROS production. Elevated ROS production would then initiate a cascade of events leading to the establishment of an autocrine and paracrine self-stimulating pathway responsible for the progressive fibrotic process in SSc. The main components of the autocrine/paracrine loop induced by elevated ROS levels include the following:

1. Further release of TGF-β from the latent TGF-β complex.
2. Intracellular activation of TGF-β-dependent signaling pathways leading to the activation of quiescent fibroblasts and the induction of the myofibroblast phenotype in these cells, as well as in endothelial cells through the process of endothelial to mesenchymal transition. The activated myofibroblasts are the cells ultimately responsible for the fibrotic process in SSc. TGF-β also causes downregulation of genes encoding for antifibrotic proteins such as cav-1 and PTEN or MMPs and other ECM degrading

enzymes. Reduction of cav-1 and PTEN is also involved in stimulation of cellular senescence as well as in stimulation of the pro-fibrotic phenotype.

3. ROS-mediated initiation and maintenance of cellular senescence. This is one of the crucial components of the pathway initiated by ROS-mediated oxidative stress in the target cells that results in tissue fibrosis. Although other mechanisms that cause cellular senescence such as DDR or telomere shortening may also be involved, oxidative stress, an important initiating event of cellular senescence is induced by elevated ROS levels.
4. The establishment of cell senescence in the target cells is accompanied by profound structural and molecular changes including the expression of SASP and the secretion of multiple proinflammatory and profibrotic molecules (Table 1).
5. The multiple components of the SASP exert potent effects on a variety of cells that are involved in SSc pathogenesis including fibroblasts, endothelial cells and monocytes.
6. Cytokines and growth factors present in the SASP induce quiescent fibroblasts to become activated and to acquire the profibrotic phenotype of activated myofibroblasts. These cells produce exaggerated levels of various ECM macromolecular components and, also display reduced expression and secretion of relevant metalloproteinases and other proteases capable of degradation of ECM components.
7. The endothelial cells present in the microvasculature in proximity to the senescent cells are also induced by SASP components to change their phenotype and become activated myofibroblasts through endothelial to mesenchymal cellular transdifferentiation processes.
8. Monocytes are attracted from the circulation through the effects of various cytokines and chemokines present in the SASP and become activated. Following their activation

macrophages are induced to change their phenotype into profibrotic M_2 macrophages which then establish a chronic inflammatory infiltrate in the affected tissues.

9. The activated M_2 macrophages can directly contribute to the fibrotic process through the production and secretion of profibrotic cytokines and growth factors causing further stimulation of ECM production as well as, by enhancing the activation of quiescent fibroblasts into myofibroblasts and by inducing the endothelial to mesenchymal transition of endothelial cells.
10. The activated macrophages also contribute to the establishment of a vicious cycle mediated by the macrophage production of TGF-β, and interferons and related macromolecules. The macrophage production of TGF-β can induce further NOX4 production, and the interferon and related peptides can re-enforce the process of cellular senescence, thus allowing the establishment of an autocrine and paracrine self-sustaining mechanism that leads to tissue fibrosis.

CONCLUSION

There is strong experimental evidence supporting the concept that cellular senescence and ROS-mediated oxidative stress play a crucial role in the initiation, establishment and the progression of fibrosis in SSc and that these effects appear to involve NOX4. Therefore, the potential use of specific NOX4 inhibitors for the treatment of SSc would be expected to be an effective therapeutic approach. Although numerous clinical studies have examined the potentially beneficial effects of various therapeutic interventions aimed at the reduction of oxidative stress for SSc the results have not conclusively shown therapeutic effectiveness. Therefore, it will be necessary to perform *in vitro* studies with specific or selective small molecule or peptide NOX4

inhibitors followed by well designed placebo controlled clinical trials to document conclusively any beneficial effects. Furthermore, clinical trials should also consider evaluating the use of NOX4 inhibitors in combination with other drugs modulating different pathways of the complex pathogenesis of SSc as this multidrug approach may result in improved therapy for this disabling and frequently fatal disease.

ACKNOWLEDGMENTS: Supported by NIH grant 5 R01 AR019616-29 to SAJ. The expert assistance of Ruth Johnson in the preparation of this manuscript is gratefully acknowledged. We are grateful to Dr. Joel Rosenbloom for his critical reading of this manuscript and for his valuable and constructive comments. We sincerely apologize to the numerous investigators whose relevant publications have not been included in this review owing to space limitations.

REFERENCES

1. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med. 2009;360:1989-2003.

2. Matucci-Cerinic M, Kahaleh B, Wigley FM. Review: evidence that systemic sclerosis is a vascular disease. Arthritis Rheum. 2013;65:1953-62.

3. Elhai M, Meune C, Avouac J, Kahan A, Allanore Y. Trends in mortality in patients with systemic sclerosis over 40 years: a systematic review and meta-analysis of cohort studies. Rheumatology (Oxford). 2012;51:1017-26.

4. Eckes B, Moinzadeh P, Sengle G, Hunzelmann N, Krieg T. Molecular and cellular basis of scleroderma. J Mol Med (Berl). 2014;92:913-24.

5. Katsumoto TR, Whitfield ML, Connolly MK. The pathogenesis of systemic sclerosis. Annu Rev Pathol. 2011; 6:509-37.

6. Bhattacharyya S, Wei J, Varga J. Understanding fibrosis in systemic sclerosis: shifting paradigms, emerging opportunities. Nat Rev Rheumatol. 2011;25:42-54.

7. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest. 2007;117:557-67.

8. Mayes MD, Lacey JV Jr, Beebe-Dimmer J, Gillespie BW, Cooper B, Laing TJ, et al. Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. Arthritis Rheum. 2003;48:2246-55.

9. Hayflick L, Moorehead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961;25:585-621.

10. Hayflick L. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res. 1965;37:614-36.

11. van Deursen JM. The role of senescence cells in ageing. Nature. 2014;509:439-46.

12. Salama R, Sadaie M, Hoare M, Narita M. Cellular senescence and its effector programs. Genes Dev. 2014;28:99-114.

13. Muñoz-Espin D, Serrano M. Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol. 2014;15:482-96.

14. Sikora E, Arendt T, Bennett M, Narita M. Impact of cellular senescence signature on ageing research. Ageing Res Rev. 2011;10:146-52.

15. Erol A. Deciphering the intricate regulatory mechanisms for the cellular choice between cell repair, apoptosis or senescence in response to damaging signals. Cell Signal. 2011;23:1076-81.

16. Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. Int J Biochem Cell Biol. 2005;37:961-76.

17.** Campisi J, Anderson JK, Kapahi P, Melov S. Cellular senescence: a link between cancer and age-related degenerative disease? Semin Cancer Biol. 2011;21:354-9. *Review of the*

studies that lead to the identification of the "Senescence Associated Secretory Phenotype".

18. Tchkonia T, Zhu Y, van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest. 2013;123:966-72.

19. Freund A, Orjalo AV, Desprez PY, Campisi J. Inflammatory networks during cellular senescence: causes and consequences. Trends Mol Med. 2010;16:238-46.

20. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol. 2010;5:99-118.

21. Salminen A, Kauppinen A, Kaarniranta K. Emerging role of NF-kB signaling in the induction of senescence-associated secretory phenotype (SASP). Cell Signal. 2012;24:835-45.

22.** Muñoz-Espin D, Canamero M, Maraver A, Gomez-Lopez G, Contreras J, Murillo-Cuesta S, et al. Programmed cell senescence during mammalian embryonic development. Cell. 2013;155:1104-18.

23.** Storer M, Mas A, Robert-Moreno A, Pecoraro M, Ortells MC, Di Giacomo V, et al. Senescence is a developmental mechanism that contributes to embryonic growth and patterning. Cell. 2013;155:1119-30. *Refs. 22 and 23 describe elegant studies identifying the role of cellular senescence during embryonic growth and development.*

24. Chen JH, Hales CN, Ozanne SE. DNA damage, cellular senescence and organismal ageing: casual or correlative? Nucleic Acids Res. 2007;35:7417-28.

25. Collado M, Blasco MA, Serrano M. Cellular senescence in cancer and aging. Cell. 2007;130:223-233.

26. Gorgoulis VG, Halazonetis TD. Oncogene induced senescence: the bright and dark side of the response.Curr Opin Cell Biol. 2010;22:816-827.

27. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. Genes Dev. 2010;24:2463-79.

28. Harley CB. Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990;354:458-460.

29. Chen Q, Fischer A, Reagan JD, Yan LJ, Ames BN. Oxidative DNA damage and senescence of human diploid fibroblast cells. Proc Natl Acad Sci U S A. 1995;92:4337-41.

30. Chen QM. Replicative senescence and oxidant-induced premature senescence. Beyond the control of cell cycle checkpoints. Ann N Y Acad Sci. 2000;908:111-25.

31. Parrinello S, Samper E, Krtolica A, Goldstein J, Melov S, Campisi J. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. Nature Cell Biol. 2003;5:741-7.

32. Passos JF, Simillion C, Hallinan J, Wipat A, von Zglinicki T. Cellular senescence: unraveling complexity. Age (Dordr). 2009;31:353-63.

33. Muller M. Cellular senescence: molecular mechanisms, in vivo significance, and redox considerations. Antioxid Redox Signal. 2009;11:59-98.

34. Passos JF, Nelson G, Wang C, Richter T, Simillion C, Proctor CJ, et al. Feedback between p21 and reactive oxygen production is necessary for cell senescence. Mol Syst Biol. 2010;6:347.

35. Kim TK, Lee JS, Jung JE, Oh SY, Kwak S, Jin X, et al. Interferon regulatory factor 3 activates p53-dependent cell growth inhibition. Cancer Lett. 2006;242:215-21.

36. Song LL, Alimirah F, Panchanathan R, Xin H, Choubey D. Expression of an IFN-inducible cellular senescence gene, IFI16, is up-regulated by p53. Mol Cancer Res. 2008;6:1732-41.

37. Duan X, Ponomareva L. Veeranki S, Panchanathan R, Dickerson E, Choubey D. Differential roles for the interferon-inducible IFI16 and AIM2 innate immune sensors for cystosolic DNA in cellular senescence of human fibroblasts. Mol Cancer Res. 2011;9:589-602.

38.** Dumit VI, Kuttner V, Kappler J, Piera-Velazquez S, Jimenez SA, Bruckner-Tuderman L, et al. Altered MCM protein levels and autophagic flux in aged and systemic sclerosis dermal fibroblasts. J Invest Dermatol. 2014;134:2321-30. *Novel study demonstrating the occurrence of cellular senescence-associated changes in Systemic Sclerosis dermal fibroblast cell lines employing proteomics.*

39. Cipriani P, Guiducci S, Miniati I, Cinelli M, Urbani S, Marrelli A, et al. Impairment of endothelial cell differentiation from bone marrow-derived mesenchymal stem cells: new insight into the pathogenesis of systemic sclerosis. Arthritis Rheum. 2007;56:1994-2004.

40. Goronzy J, Fujii H, Weyand CM. Telomeres, immune aging, and autoimmunity. Exp Gerontol. 2006;41:246-51.

41. Debbi AZ, Radstake TRDJ, Broen JCA. Accelerated telomere shortening in rheumatic diseases: cause or consequence?. Expert Rev Clin Immunol. 2013;9:1193-1204.

42. Arlett CM, Black CM, Briggs DC, Stevens CO, Welsh KI. Telomere reduction in scleroderma patients: a possible cause for chromosomal instability. Br J Rheumatol. 1996;35:732-7.

43. Ohtsuka T. Life span of skin fibroblasts in patients with systemic sclerosis. Dermatology. 1998;196:204-7.

44. MacIntyre A, Brouilette SW, Lamb K, Radhakrishnan K, McGlynn L, Chee MM, et al. Association of increased telomere lengths in limited scleroderma, with a lack of age-related telomere erosion. Ann Rheum Dis. 2008;67:1780-2.

45. Tarhan F, Vural F, Kosova B, Aksu K, Cogulu O, Keser G. Telomerase activity in connective tissue diseases: elevated in rheumatoid arthritis, but markedly decreased in systemic sclerosis. Rheumatol Int. 2008;28:579-83.

46. Thannickal VJ. Mechanistic links between aging and lung fibrosis. Biogerontology. 2013;14:609-15.

47. Chilosi M, Carloni A, Rossi A, Poletti V. Premature lung aging and cellular senescence in the pathogenesis of idiopathic pulmonary fibrosis and COPD/emphysema. Transl Res. 2013;162:156-73.

48. Steele MP, Schwartz DA. Molecular mechanisms in progressive idiopathic pulmonary fibrosis. Annu Rev Med. 2013;64:265-76.

49. Armanios MY, Chen JJ, Cogan JD, Alder JK, Ingersoll RG, Markin C, et al. Telomerase mutations in families with idiopathic pulmonary fibrosis. N Engl J Med. 2007;356:1317-26.

50. Diaz de Leon A, Cronkhite JT, Katzenstein AL, Godwin JD, Raghu G, Glazer CS, et al. Telomere lengths, pulmonary fibrosis and telomerase (TERT) mutations. PLoS One. 2010; 5:e10680.doi:10.1371/journal.pone.0010680.

51. Del Galdo F, Sotgia F, de Almeida CJ, Jasmin JF, Musick M. Lisanti MP, et al. Decreased expression of caveolin 1 in patients with systemic sclerosis: crucial role in the pathogenesis of tissue fibrosis. Arthritis Rheum. 2008;58:2854-65.

52. Del Galdo F, Lisanti MP, Jimenez SA. Caveolin-1, transforming growth factor-beta receptor internalization, and the pathogenesis of systemic sclerosis. Curr Opin Rheumatol. 2008;20:713-9.

53. Wang XM, Zhang Y, Kim HP, Zhou Z, Feghali-Bostwick CA, Liu F, Ifedigbo E, et al. Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis. J Exp Med. 2006;203:2895-906.

54. Tourkina E, Richard M, Gööz P, Bonner M. Pannu J, Harley R, et al. Antifibrotic properties of caveolin-1 scaffolding domain in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol. 2008;294:L843-61.

55.** Gvaramia D, Blaauboer ME, Hanemaaijer R, Everts V. Role of caveolin-1 in fibrotic diseases. Matrix Biol. 2013;32:307-15. *Comprehensive review of the extensive published evidence demonstrating the crucial role of caveolin-1 in the pathogenesis of various fibrotic diseases including Systemic Sclerosis.*

56. Volonte D, Zhang K, Lisanti MP, Galbiati F. Expression of caveolin-1 induces premature cellular senescence in primary cultures of murine fibroblasts. Mol Biol Cell. 2002;13:2502-17.

57. Shivshankar P, Brampton C, Miyasato S, Kasper M, Thannickal VJ, Le Saux CJ. Caveolin-1 deficiency protects from pulmonary fibrosis by modulating epithelial cell senescence in mice. Am J Respir Cell Mol Biol. 2012;47:28-36.

58. Xia H, Khalil W, Kahm J, Jessurun J, Kleidon J, Henke CA. Pathologic caveolin-1 regulation of PTEN in idiopathic pulmonary fibrosis. Am J Pathol. 2010;176:2626-37.

59. Parapuram SK, Shi-wen X, Elliott C, Welch ID, Jones H, Baron M, et al. Loss of PTEN expression by dermal fibroblasts causes fibrosis. J Invest Dermatol. 2011;131:1996-2003.

60. Murrell DF. A radical proposal for the pathogenesis of scleroderma. J Am Acad Dermatol. 1993;28:78-85.

61. Gabrielli A, Svegliati S, Moroncini G, Pomponio G, Santillo M, Avvedimento EV. Oxidative stress and the pathogenesis of scleroderma: The Murrell's hypothesis revisited. Semin Immunopathol. 2008; 30:329-37.

62. Piera-Velazquez S, Jimenez SA. Role of oxidative stress and reactive oxygen radicals in the pathogenesis of systemic sclerosis. In: M.J. Alcaraz (eds.), Studies on Arthritis and Joint Disorders, Oxidative Stress in Applied Basic Research and Clinical Practice.doi 10.1007/978-1-4614-6166-1_10.pp.183-197.

63. Gabrielli A, Svegliati S, Moroncini G, Amico D. New insights into the role of oxidative stress in scleroderma fibrosis. Open Rheumatol J. 2012;6:87-95.

64.** Ogawa F, Shimizu K, Muroi E, Hara T, Sato S. Increasing levels of serum antioxidant status, total antioxidant power, in systemic sclerosis. Clin Rheumatol. 2011;30:921-25. *This study measured the total antioxidant power in serum from SSc patients and found that approximately one-quarter of these patients had elevated levels although there were no differences between patients with the diffuse or limited SSc subsets.*

65.** Savas E, Aksoy N, Pehlivan Y, Sayiner ZA, Oztürk ZA, Tabur S, et al. Evaluation of oxidant and antioxidant status and relation with prolidase in systemic sclerosis. Wien Klin Wochenschr. 2014;126:341-6. *This interesting study demonstrated that the "total oxidant status " and the "oxidative stress index" were higher in Systemic Sclerosis patients than in healthy controls whereas there were no differences in "total antioxidant status".*

66. Sambo P, Jannino L, Candela M, Salvi A, Donini M, Dusi S, et al. Monocytes of patients with systemic sclerosis (scleroderma) spontaneously release in vitro increased amounts of superoxide anion. J Invest Dermatol. 1999;112:78-84.

67. Allanore Y, Borderie D, Lemarechal H, Ekindjian OG, Kahan A. Acute and sustained effects of dihydropyridine-type calcium channel antagonists on oxidative stress in systemic sclerosis. Am J Med. 2004;116:595-600.

68. Servettaz A, Guilpain P, Goulvestre C, Chereau C, Hercend C, Nicco C, et al. Radical oxygen species production induced by advanced oxidation protein products predicts clinical evolution and response to treatment in systemic sclerosis. Ann Rheum Dis. 2007;66:1202-09.

69. Sambo P, Baroni SS, Luchetti M, Paroncini P, Dusi S, Orlandini S, et al. Oxidative stress in scleroderma: Maintenance of scleroderma fibroblast phenotype by the constitutive up-regulation of reactive oxygen species generation through the NADPH oxidase complex pathway. Arthritis Rheum. 2001;44:2653-64.

70. Avouac J, Borderie D, Ekindjian OG, Kahan A, Allanore Y. High DNA oxidative damage in systemic sclerosis. J Rheumatol. 2010;37:2540-47.

71. Stein CM, Tanner SB, Awad JA, Roberts LJ,2nd, Morrow JD. Evidence of free radical-mediated injury (isoprostane overproduction) in scleroderma. Arthritis Rheum. 1996; 39:1146-50.

72. Ogawa F, Shimizu K, Muroi E, Hara T, Hasegawa M, Takehara K, et al. Serum levels of 8-isoprostane, a marker of oxidative stress, are elevated in patients with systemic sclerosis. Rheumatology (Oxford). 2006;45:815-8.

73. Cracowski JL, Marpeau C, Carpentier PH, Imbert B, Hunt M, Stanke-Labesque F, et al. Enhanced in vivo lipid peroxidation in scleroderma spectrum disorders. Arthritis Rheum. 2001;44:1143-8.

74. Volpe A, Biasi D, Caramaschi P, Mantovani W, Bambara LM, Canestrini S, et al. Levels of F2-isoprostanes in systemic sclerosis: correlation with clinical features. Rheumatology (Oxford). 2006;45:314-20.

75. Tufvesson E, Bozovic G, Hesselstrand R, Bjermer L, Scheja A, Wuttge DM. Increased cysteinyl-leukotrienes and 8-isoprostane in exhaled breath condensate from systemic sclerosis patients. Rheumatology (Oxford). 2010;49:2322-6.76.

76. Morrow JD. The isoprostanes: Their quantification as an index of oxidant stress status in vivo. Drug Metab Rev. 2000;32:377-85.

77. Shimizu K, Ogawa F, Akiyama Y, Muroi E, Yoshizaki A, Iwata Y, et al. Increased serum levels of N(epsilon)-(hexanoyl)lysine, a new marker of oxidative stress, in systemic sclerosis. J Rheumatol. 2008;35:2214-19.

78. Ogawa F, Shimizu K, Hara T, Muroi E, Hasegawa M, Takehara K, et al. Serum levels of heat shock protein 70, a biomarker of cellular stress, are elevated in patients with

systemic sclerosis: Association with fibrosis and vascular damage. Clin Exp Rheumatol. 2008; 26:659-62.

79. Boin F, Erre GL, Posadino AM, Cossu A, Giordo R, Spinetti G, et al. Oxidative stress-dependent activation of collagen synthesis is induced in human pulmonary smooth muscle cells by sera from patients with scleroderma-associated pulmonary hypertension. Orphanet J Rare Dis. 2014;9:123.

80. Holmström KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol. 2014;15:411-21.

81. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol. 2014;24:R453-62.

82. Finkel T. Signal transduction by reactive oxygen species. J Cell Biol. 2011;194:7-15.

83.** Svegliati S, Marrone G, Pezone A, Spadoni T, Grieco A, Moroncini G, et al. Oxidative DNA damage induces the ATM-mediated transcriptional suppression of the Wnt inhibitor WIF-1 in systemic sclerosis and fibrosis. Sci Signal. 2014;7(341):ra84.doi:10.1126/scisignal.2004592. *Extensive study that ties together ROS-mediated oxidative stress, DNA damage and activation of the Wnt fibrotic pathway caused by ROS-induced loss of the anti-Wnt protein, Wnt inhibitory factor 1 (WIF-1).*

84.** Dooley A, Shi-Wen X, Aden N, Tranah T, Desai N, Denton CP. Modulation of collagen type I, fibronectin and dermal fibroblast function and activity, in systemic sclerosis by the antioxidant epigallocatechin-3-gallate. Rheumatology (Oxford). 2010;49:2024-36. *Highly*

provocative study demonstrating modulaton of the profibrotic phenotype of Systemic Sclerosis dermal fibroblasts by the antioxidant epigallocatechin-3-gallate.

85.** Tsou PS, Talia NN, Pinney AJ, Kendzicky A, Piera-Velazquez S, Jimenez SA, et al. Effect of oxidative stress on protein tyrosine phosphatase 1B in scleroderma dermal fibroblasts. Arthritis Rheum. 2012;64:1978-89. *Pioneering study exploring the role of PTP1B in the regulation of the fibrotic process in SSc demonstrating that PTP1B activity was substantially reduced in SSc dermal fibroblasts as a result of increased ROS-induced cysteine residue oxidation in the protein.*

86. Kawahara T, Quinn MT, Lambeth JD. Molecular evolution of the reactive oxygen-generating NADPH oxidase (Nox/Duox) family of enzymes. BMC Evol Biol. 2007;7:109.

87. Sumimoto H. Structure, regulation and evolution of nox-family NADPH oxidases that produce reactive oxygen species. FEBS J. 2008;275:3249-77.

88. Lambeth JD, Neish AS. Nox enzymes and new thinking on reactive oxygen: a double-edged sword revisited. Annu Rev Pathol. 2014;9:119-45.

89. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev. 2007;87:245-313.

90. Geiszt M. NADPH oxidases: new kids on the block. Cardiovasc Res. 2006;71:289-99.

91. Cucoranu I, Clempus R, Dikalova A, et al. NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. Circ Res. 2005; 97:900-7.

92. Sturrock A, Cahill B, Norman K, Huecksteadt TP, Hill K, Sanders K, et al. Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Moll Physiol. 2006;290:L661-L673.

93. Hecker L, Vittal R, Jones T, et al. NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. Nat Med. 2009; 15:1077-81.

94. Datla SR, Peshavariya H, Dusting GJ, Mahadev K, Goldstein BJ, Jiang F. Important role of Nox4 type NADPH oxidase in angiogenic responses in human microvascular endothelial cells in vitro. Arterioscler Thromb Vasc Biol. 2007; 27:2319-24.

95. An SJ, Boyd R, Zhu M, Chapman A, Pimentel DR, Wang HD. NADPH oxidase mediates angiotensin II-induced endothelin-1 expression in vascular adventitial fibroblasts. Cardiovasc Res. 2007; 75:702-9.

96. Duerrschmidt N, Wippich N, Goettsch W, Broemme HJ, Morawietz H. Endothelin-1 induces NAD(P)H oxidase in human endothelial cells. Biochem Biophys Res Commun. 2000;269:713-17.

97. Manickam N, Patel M, Griendling KK, Gorin Y, Barnes JL. RhoA/Rho kinase mediate TGF-β1-induced kidney myofibroblast activation through Poldip2/NOX4-derived reactive oxygen species. Am J Physiol Renal Physiol. 2014;307:F159-71.

98. Bondi CD, Manickham N, Lee DY, Block K, Gorin Y, Abboud HE, et al. NAD(P)H oxidase mediates TGF-beta1-induced activation of kidney myofibroblasts. J Am Soc Nephrol. 2010;21:93-102.

99. Barnes JL, Gorin Y. Myofibroblast differentiation during fibrosis: role of NAD(P)H oxidases. Kidney Int. 2011;79:944-56.

100. Alili L, Sack M, Puschmann K, Brenneisen P. Fibroblast-to-myofibroblast switch is mediated by NAD(P)H oxidase generated reactive oxygen species. Biosci Rep. 2013.

101. Siani A, Tirelli N. Myofibroblast differentiation: main features, biomedical relevance, and the role of reactive oxygen species. Antioxid Redox Signal. 2014;21:768-85.

102. Shen WL, Gao PJ, Che ZQ, Ji KD, Yin M, Yan C, et al. NAD(P)H oxidase-derived reactive oxygen species regulate angiotensin-II induced adventitial fibroblast phenotype differentiation. Biochem Biophys Res Commun. 2006;339:337-43.

103. Amara N, Goven D, Prost F, Muloway R, Crestani B, Boczkowski J. NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFbeta-1-induced fibroblast differentiation into myofibroblasts. Thorax. 2010;65:733-8.

104. Paik YH, Kim J, Aoyama T, De Minicis S, Bataller R, Brenner DA. Role of NADPH oxidase in liver fibrosis. Antioxid Redox Signal. 2014;20:2854-72.

105. Babalola O, Mamalis A, Lev-Tov H, Jagdeo J. NADPH oxidase enzymes in skin fibrosis: molecular targets and therapeutic agents. Arch Dermatol Res. 2014;306:313-30.

106. Böhm M, Dosoki H, Kerkhoff C. Is Nox4 a key regulator of the activated state of fibroblasts in systemic sclerosis? Exp Dermatol. 2014;doi:10.1111/exd.12497.

107.** Montorfano I, Becerra A, Cerro R, Echeverria C, Saez E, Morales MG, et al. Oxidative stress mediates the conversion of endothelial cells into myofibroblasts via a TGF-β1 and TGF-β2-dependent pathway. Lab Invest. 2014. Jul 28. doi:10.1038/labinvest.2014.100.[Epub ahead of print]. *Important study showing that oxidative stress was capable of inducing a phenotypic change of endothelial cells into myofibroblasts through a TGF-β dependent mechanism.*

108. Serrander L, Cartier L, Bedard K, Bandi B, Lardy B, Plastre O. NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. Biochem J. 2007;406:105-14.

109. Jiang F, Liu GS, Dusting GJ, Chan EC. NADPH oxidase-dependent redox signaling in TGF-β-mediated fibrotic responses. Redox Biol. 2014;2:267-72.

110. Bai G, Hock TD, Logsdon N, Zhou Y, Thannickal VJ. A far-upstream AP-1/Smad binding box regulates human NOX4 promoter activation by transforming growth factor-β. Gene. 2014;540:62-7.

111.** Lyle AN, Deshpande NN, Taniyama Y, Seidel-Rogol B, Pounkova L, Du P, et al. Poldip2, a novel regulator of Nox4 and cytoskeletal integrity in vascular smooth muscle cells. Circ Res. 2009;105:249-59. *This paper describes the exciting new findings that polymerase delta interacting protein 2 (Poldip2) interacts with NOX-4 and is capable of increasing NOX-4 activity by three-fold.*

112. Simonini G, Pignone A, Generini S, Falcini F, Cerinic MM. Emerging potentials for an antioxidant therapy as a new approach to the treatment of systemic sclerosis. Toxicology. 2000; 155:1-15.

113. Erre GL, De Muro P, Dellaca P, et al. Iloprost therapy acutely decreases oxidative stress in patients affected by systemic sclerosis. Clin Exp Rheumatol. 2008; 26:1095-98.

114. Volpe A, Biasi D, Caramaschi P, et al. Iloprost infusion does not reduce oxidative stress in systemic sclerosis. Rheumatol Int. 2008; 28:335-7.

115. Erre GL, Passiu G. Antioxidant effect of iloprost: Current knowledge and therapeutic implications for systemic sclerosis. Reumatismo. 2009; 61:90-7.

116. Yoshizaki A, Yanaba K, Ogawa A, et al. The specific free radical scavenger edaravone suppresses fibrosis in the bleomycin-induced and tight skin mouse models of systemic sclerosis. Arthritis Rheum. 2011; 63:3086-97.

117. Marut WK, Kavian N, Servettaz A, et al. The organotelluride catalyst (PHTE)NQ prevents HOCl-induced systemic sclerosis in mouse. J Invest Dermatol. 2012; 132:1125-32.

118. Sambo P, Amico D, Giacomelli R, et al. Intravenous N-acetylcysteine for treatment of raynaud's phenomenon secondary to systemic sclerosis: A pilot study. J Rheumatol. 2001; 28:2257-62.

119. Cracowski JL, Girolet S, Imbert B, et al. Effects of short-term treatment with vitamin E in systemic sclerosis: A double blind, randomized, controlled clinical trial of efficacy based on urinary isoprostane measurement. Free Radic Biol Med. 2005; 38:98-103.

120. Rosato E, Rossi C, Molinaro I, Giovannetti A, Pisarri S, Salsano F. Long-term N-acetylcysteine therapy in systemic sclerosis interstitial lung disease: A retrospective study. Int J Immunopathol Pharmacol. 2011; 24:727-33.

121. Allanore Y, Borderie D, Perianin A, Lemarechal H, Ekindjian OG, Kahan A. Nifedipine protects against overproduction of superoxide anion by monocytes from patients with systemic sclerosis. Arthritis Res Ther. 2005; 7:R93-100.

122. Furst DE, Clements PJ, Harris R, Ross M, Levy J, Paulus HE. Measurement of clinical change in progressive systemic sclerosis: A 1 year double-blind placebo-controlled trial of N-acetylcysteine. Ann Rheum Dis. 1979; 38:356-61.

123.** Laleu B, Gaggini F, Orchard M, Fioraso-Cartier L, Cagnon L, Houngninou-Molango S, et al. First in class, potent, and orally bioavailable NADPH oxidase isoform 4 (Nox4) inhibitors for the treatment of idiopathic fibrosis. J Med Chem. 2010;53:7715-30.

124.** Gaggini F, Laleu B, Orchard M, Fioraso-Cartier L, Cagnon L, Houngninou-Molango S, et al. Design, synthesis and biological activity of original pyrazolo-pyrido-diazepine,-

pyrazine and -oxazine dione derivatives as a novel dual Nox4/Nox1 inhibitors. Bioorg Med Chem. 2011;19:6989-99. *References 123 and 124 describe the design and synthesis of a novel class of potent and highly selective NOX4 small molecule inhibitors.*

125.** El-Benna J, Dang PM, Perianin A. Towards specific NADPH oxidase inhibition by small synthetic peptides. Cell Mol Life Sci. 2012;69:2307-14. *Description of novel and specific small synthetic peptides targeting NOX enzymes.*

126. Altenhöfer S, Radermacher KA, Kleikers PW, Wingler K, Schmidt HH. Evolution of NADPH oxidase inhibitors: selectivity and mechanisms for target engagement. Antioxid Redox Signal. 2014.

127. Altenhöfer S, Kleikers PW, Radermacher KA, Scheurer P, Rob Hermans JJ, Schiffers P, et al. The NOX toolbox: validating the role of NADPH oxidases in physiology and disease. Cell Mol Life Sci. 2012;69:2327-43.

128. Kim JA, Neupane GP, Lee ES, Jeong BS, Park BC, Thapa . NADPH oxidase inhibitors: a patent review. Expert Opin Ther Pat. 2011;21:1147-58.

129. Aoyama T, Paik YH, Watanabe S, Laleu B, Gaggini F, Fioraso-Cartier L, et al. Nicotinamide adenine dinucleotide phosphate oxidase in experimental liver fibrosis: GKT137831 as a novel potential therapeutic agent. Hepatology. 2012;56:2316-27.

130.** Jarman ER, Khambata VS, Cope C, Jones P, Roger J, Ye LY, et al. An inhibitor of NADPH oxidase-4 attenuates established pulmonary fibrosis in a rodent disease model. Am J Respir Cell Mol Biol. 2014;50:158-69. *Highly relevant study demonstrating that*

inhibition of NOX4 activity employing a small molecule antagonist decreased TGF-β1-induced upregulation of profibrotic genes in normal human lung fibroblasts in vitro and attenuated established lung fibrosis in vivo in bleomycin-induced animal model of pulmonary fibrosis.

131.** Hecker L, Logsdon NJ, Kurundkar D, Kurundkar A, Bernard K, Hock T, et al. Reversal of persistent fibrosis in aging by targeting Nox4-Nrf2 redox imbalance. Sci Transl Med. 2014;6:231ra47.doi:10.1126/scitranslmed.3008182. *This interesting study demonstrates that aged mice display persistent lung fibrosis following intratracheal bleomycin administration whereas young mice resolve the fibrotic process. The mechanisms involve NOX4/Nrf2 redox imbalance-induced fibroblast senescence coupled with apoptosis resistance causing a failure to resolve fibrosis.*

132. Sampson N, Berger P, Zenzmaier C. Redox signaling as a therapeutic target to inhibit myofibroblast activation in degenerative fibrotic disease. Biomed Res Int. 2014;2014:131737.doi:10.1155/2014/131737.

Legend to Figures.

Figure 1. Schematic diagram depicting the mechanisms involved in the induction of SSc-associated tissue fibrosis by cellular senescence and ROS-mediated oxidative stress and in the establishment of an autocrine/paracrine vicious cycle responsible for the progression and persistence of the fibrotic process. **1.** Release of TGF-β from the latent TGF-β binding protein, followed by its activation and binding to cognate TGF-β receptors. **2.** Stimulation of transcription of the NOX4 gene leading to increased NOX4 and to increased ROS production. **3.** Elevated ROS levels induce oxidative stress in the target cells as well as phenotypic changes in fibroblasts and endothelial cells causing their conversion into activated myofibroblasts. **4.** Oxidative stress induces morphological and functional changes of cellular senescence. **5.** Senescent cells express the senescence-associated secretory phenotype (SASP). **6-9.**The components of the SASP induce phenotypic changes in fibroblasts (6), endothelial cells (7) and monocytes (8). The monocytes become activated M_2 profibrotic macrophages that further increase the fibrotic process (9). **10.** The activated macrophages produce and secrete TGF-β and interferons and various interferon-related molecules. The secreted TGF-β and interferon related products close the vicious cycle and allow establishment of an autocrine/paracrine pathway that is responsible for the initiation, and progression of tissue fibrosis in SSc.