Oxidative exposure impairs TGF- β pathway via reduction of type II receptor and SMAD3 in human skin fibroblasts

Tianyuan He · Taihao Quan · Yuan Shao ·
John J. Voorhees · Gary J. Fisher

Received: 31 July 2013 / Accepted: 20 January 2014 / Published online: 20 February 2014
© American Aging Association 2014

Abstract Exposure to oxidants results in cellular alterations that are implicated in aging and age-associated diseases. Here, we report that brief, low-level oxidative exposure leads to long-term elevation of cellular reactive oxygen species (ROS) levels and oxidative damage in human skin fibroblasts. Elevated ROS impairs the transforming growth factor- β (TGF- β) pathway, through reduction of type II TGF- β receptor (T β RII) and SMAD3 protein levels. This impairment results in reduced expression of connective tissue growth factor (CTGF/CCN2) and type I collagen, which are regulated by TGF- β . Restoration of T β RII and SMAD3 together, but not separately, reinstates TGF- β signaling and increases CTGF/CCN2 and type I collagen levels. Treatment with the anti-oxidant *N*-acetylcysteine reduces ROS elevation and normalizes TGF- β signaling and target gene expression. These data reveal a novel linkage between limited oxidant exposure and altered cellular redox homeostasis that results in impairment of TGF- β signaling. This linkage provides new insights regarding the mechanism by which aberrant redox homeostasis is coupled to decline of collagen production, a hallmark of human skin aging.

Electronic supplementary material The online version of this article (doi:10.1007/s11357-014-9623-6) contains supplementary material, which is available to authorized users.

T. He · T. Quan · Y. Shao · J. J. Voorhees · G. J. Fisher (✉)
Department of Dermatology, Medical School, University of Michigan,
1301 E Catherine, Rm 6447 Med Sci I, Ann Arbor, MI
48109-5609, USA
e-mail: gjfisher@umich.edu

Keywords Connective tissue · Oxidative stress · Signal transduction · Extracellular matrix · Skin

Introduction

Reactive oxygen species (ROS) are produced during aerobic respiration. Elevated levels of ROS, beyond cellular anti-oxidant capacity, can occur as a consequence of abnormal activities of cellular anti-oxidant enzymes, or external factors, such as toxins, pharmacological agents, or ultraviolet (UV) irradiation (Herrling et al. 2006). Excess ROS can oxidize cellular lipids, proteins, DNA, and carbohydrates. This oxidation is implicated in a number of human diseases as well as aging (Droge 2002; Cadenas and Davies 2000).

Type I collagen (COL1) is the most abundant structural protein in skin connective tissue. Collagen fibrils are responsible for mechanical strength and resiliency of skin. Dermal fibroblasts are the primary source of collagen synthesis. Regulation of collagen gene expression is an important biological event and closely associated with the pathophysiology of skin connective tissue diseases and aging. Numerous studies have investigated the mechanisms that regulate COL1 synthesis. Transforming growth factor- β (TGF- β) has been shown to play a central role through its downstream effectors, SMAD transcription factors and their target gene connective tissue growth factor (CTGF, recently named CCN2) (Quan et al. 2010).

TGF- β is a multiple functional cytokine that has profound cell type-specific effects on many biological

processes, including proliferation, differentiation, and migration. TGF- β initiates its actions by binding and activating specific cell surface receptor complex composed of type I (T β RI), type II (T β RII), and type III (T β RIII) receptors. The activated TGF- β receptor complex transiently interacts with and phosphorylates downstream receptor-regulated SMAD proteins, SMAD2 and SMAD3. Phosphorylated SMAD2 and SMAD3 combine with SMAD4 and then translocate to nucleus, where they bind to specific sequences in the promoter region of target genes and regulate transcription. The actions of TGF- β are antagonized by SMAD7, which interacts with T β RI to prevent activation of the receptor-regulated SMADs. Studies indicate that CCN2 is rapidly induced by TGF- β and cooperates with TGF- β to stimulate COL1 gene expression (Brigstock 1999; Duncan et al. 1999; Quan et al. 2010).

TGF- β has been shown to stimulate ROS levels in a variety of cells (Hertig et al. 1993; Thannickal et al. 1993; Ohba et al. 1994; Thannickal and Fanburg 1995), and that ROS may be an important mediator of many TGF- β -regulated responses (Jiang et al. 2003; Wu 2006; Kim et al. 2006; Rhyu et al. 2005). However, the impact of ROS on TGF- β /SMAD signal transduction pathway and collagen gene expression in human dermal fibroblasts has not been addressed. Here, we report that oxidative exposure causes elevation of cellular ROS levels, and this elevated ROS results in substantial reduction of type I α 1 procollagen protein (ProCOL1) and gene (*COL1A1*) expression through impairment of TGF- β signaling. These alterations can be mitigated by the anti-oxidant reagent *N*-acetylcysteine (NAC).

Results

Oxidative exposure induces cellular ROS levels in human dermal fibroblasts

To assess intracellular ROS levels, primary human dermal fibroblasts were labeled with the redox-sensitive dye RedoxSensor Red and monitored by fluorescence microscopy. A single short-term exposure (30–60 min) of fibroblasts to H₂O₂ (200 μ M) caused a rapid increase in intracellular ROS levels, followed by a return to baseline within 8 h. ROS levels remained unchanged for at least 7 days (data not shown). In contrast, exposure of fibroblasts to H₂O₂ (200 μ M) for 1 h on two

consecutive days resulted in biphasic, long-lasting elevation of intracellular ROS levels. Following the second exposure, ROS levels rapidly rose and returned to baseline, as observed after the first H₂O₂ exposure. Intracellular ROS levels then were slightly increased on day 3 and substantially increased on day 7 after the second exposure to H₂O₂ (Fig. 1a). Elevated intracellular ROS levels did not diminish for at least 28 days (the longest time point we have measured, data not shown). To quantify ROS levels, we performed flow cytometry with the redox-sensitive fluorescence probe carboxy-H2DCFDA. Analysis revealed significantly increased ROS levels 4 days after the second H₂O₂ exposure. ROS levels continued to rise for the next 3 days (Fig. 1b) and remained elevated at a level threefold to fourfold over baseline for at least seven additional days (data not shown).

A prominent consequence of excess ROS is oxidation of cellular proteins. Direct oxidation of amino acids results in formation of carbonyl groups. Protein carbonyls are a well-validated marker of cellular oxidative status. Consistent with the observed elevation of steady-state ROS levels, the level of protein carbonyls was significantly elevated (nearly sevenfold) 7 days following oxidative exposure of fibroblasts (Fig. 1c). In addition, oxidative exposure reduced fibroblast proliferation and increased expression of senescence-associated β -galactosidase activity (data not shown), indicative of premature senescence.

These observations demonstrate that short-term exposure to H₂O₂ on two consecutive days, hereafter termed oxidative exposure, causes increased endogenous steady-state levels of ROS and oxidative damage in human dermal fibroblasts.

Oxidative exposure causes long-term reduction of type I procollagen in human dermal fibroblasts

We next investigated the effects of oxidative exposure on ProCOL1, a major protein product in human dermal fibroblasts. Type I procollagen is composed of two α 2 protein chains, encoded by the *COL1A1* gene, and one α 1 protein chain, encoded by the *COL1A2* gene. We found substantial, long-lasting reduction of ProCOL1 mRNA (*COL1A1* gene) and protein (alpha1 chain) (Fig. 2a). Reduction of ProCOL1 mRNA at day 7 was nearly 75 % (Fig. 2a, left panel), compared to matched vehicle-treated control fibroblasts. Reduced ProCOL1 mRNA observed on day 7 remained unchanged for at

Fig. 1 Oxidative exposure increases steady-state cellular reactive oxygen species levels and oxidative damage in human dermal fibroblasts. Fibroblasts were exposed to vehicle control (*Ctrl*) or hydrogen peroxide (200 μ M) for 1 h on two successive days (*oxidative exposure*). Intracellular ROS levels were measured at the indicated time points, following second hydrogen peroxide

exposure, by **a** RedoxSensor Red fluorescence or **b** flow cytometry using the redox-sensitive dye carboxy-H2DCFDA. **c** Protein oxidation levels were determined by Western analysis of carbonyl residues. Results are means $\pm$ SEM, $N=4$, $*p<0.05$. *Insets* show representative Western blot

Fig. 2 Oxidative exposure represses COL1 expression in human dermal fibroblasts. Fibroblasts were exposed to vehicle control (*Ctrl*) or hydrogen peroxide (200 μ M) for 1 h on two successive days (*oxidative exposure*) and analyzed 7 days later. **a** COL1A1 mRNA (*left panel*) and intracellular ProCOL1 protein (*right panel*) were quantified by real-time RT-PCR and Western analysis, respectively. COL1A1 mRNA levels were normalized to

housekeeping gene *36B4* mRNA, *RPLP0* gene. Data are means $\pm$ SEM, $N=8$, $*p<0.01$. *Insets* show representative Western blots. Data are means $\pm$ SEM, $N=7$, $*p<0.01$. **b** Cellular expression of ProCOL1 protein was determined by immunohistochemistry. Representative images are shown, $N=6$. **c** Intracellular and **d** secreted extracellular ProCOL1 protein levels were quantified by ELISA. Data are means $\pm$ SEM, $N=6$, $*p<0.01$

least 28 days (data not shown). ProCOL1A2 (*COL1A2* gene) mRNA levels showed similar reductions by oxidative exposure (data not shown). ProCOL1 protein reduction, measured by Western analysis, was similar to mRNA reduction, with 80 % decrease at day 7

(Fig. 2a, right panel). Immunohistochemistry revealed that the vast majority of fibroblasts had substantially reduced ProCOL1 protein expression (Fig. 2b). ELISA analyses revealed that both intracellular (Fig. 2c) and secreted (Fig. 2d) ProCOL1 protein were significantly

reduced to 79 and 70 %, respectively, by oxidative exposure.

Interestingly, reduction of ProCOL1 protein was observed within 2 to 3 days after oxidative exposure (Supplemental Fig. 1). This time falls between the first and second phases of ROS elevation (Fig. 1a, b). These data suggest that the early phase of increased ROS initiates loss of ProCOL1 expression, and the second phase further enhances and maintains this loss.

ProCOL1 production is dependent on SMAD3 in human skin fibroblasts

TGF- β is known to strongly regulate ProCOL1 production in human skin fibroblasts (Quan et al. 2010). Blocking TGF- β signaling by T β RI kinase inhibitor SB431542 markedly reduced expression of ProCOL1

mRNA (Fig. 3a, left panel) and protein (Fig. 3a, right panel) by 76 and 75 %, respectively. Similar results were obtained with siRNA-mediated knockdown of SMAD3, an intracellular signal transducer of the TGF- β signaling pathway. As shown in Fig. 3, SMAD3 siRNA specifically and markedly reduced SMAD3 protein (Fig. 3b, left panel) and repressed ProCOL1 expression (Fig. 3b, right panel). These data indicate that the TGF- β /SMAD3 signal transduction pathway plays an essential role in the regulation of ProCOL1 expression in primary adult human dermal fibroblasts.

ROS impairs TGF- β signaling through reduction of T β RII receptor and SMAD3 protein

Given that oxidative exposure significantly reduces ProCOL1 expression and ProCOL1 expression is

Fig. 3 TGF- β signaling is required for COL1 expression in human skin fibroblasts. **a** Fibroblasts were treated with vehicle (Ctrl) or T β RI inhibitor SB431542 (10 μ M) and analyzed 48 h after treatment. COL1A1 mRNA (left panel) and protein (right panel) were quantified by real-time RT-PCR and Western analysis, respectively. COL1A1 mRNA levels were normalized to mRNA

for housekeeping gene *36B4*, *RPLP0* gene. **b** Fibroblasts were transfected with scrambled control (*siCtrl*) or SMAD3 siRNA (*siSMAD3*). Forty-eight hours after transfection, SMAD3 (left panel) and ProCOL1 proteins (right panel) were quantified as described above. Data are means $\pm$ SEM, $N=5$, $*p<0.01$. Insets show representative Western blots

dependent on TGF- β signaling, we hypothesized that reduction of procollagen expression by oxidative exposure may be mediated by impairment of the TGF- β signal transduction pathway. To address this hypothesis, we first determined levels of TGF- β 1, 2, and 3 mRNAs and TGF- β 1 protein levels by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) and ELISA, respectively. We found that oxidative exposure had no effect on the levels of any of these ligands (data not shown).

TGF- β signaling requires T β RI and T β RII and intracellular mediators SMAD2, 3, and 4, which are counteracted by SMAD7. Oxidative exposure did not alter transcript levels of any of these genes. However, following oxidative exposure, the protein levels of T β RII and SMAD3, but not other components, were significantly reduced by 70 and 60 %, respectively (Fig. 4a, b). Similar to reduction of ProCOL1 protein, reduction of T β RII and SMAD3 proteins was detectable 2 days after the oxidative exposure and continued to decline for the next 4 to 5 days (Supplemental Fig. 1).

We used two well-characterized TGF- β response reporter constructs, 4X SBE-LUX, which contains four repetitions of GTCTAGAC SMAD3-binding elements, and pCOL1 α 2-CAT, which contains SMAD3-binding element from type I α 2 collagen gene promoter (-772/+58), to determine the impact of oxidative exposure on TGF- β signaling. Oxidative exposure significantly reduced SBE-LUX activity by 70 % (Fig. 4c, left panel), and collagen promoter reporter was reduced by 93 % (Fig. 4c, right panel). This reduction of SMAD3 activity is consistent with impaired TGF- β signaling due to T β RII and SMAD3 reduction, which resulted in 61 ± 7 % reduction of SMAD3 phosphorylation (Supplemental Fig. 2). These data indicate that downstream components of the TGF- β signal transduction pathway are functionally impaired by oxidative exposure.

To further confirm the functional impact of oxidative exposure on the TGF- β pathway, we determined expression of CCN2, a well-documented TGF- β /SMAD target gene in dermal fibroblasts (Quan et al. 2010). Consistent with reporter assays, both CCN2 mRNA

Fig. 4 Oxidative exposure impairs TGF- β signaling through repression of type II receptor and SMAD3 in human dermal fibroblasts. Fibroblasts were exposed to vehicle control (*Ctrl*) or hydrogen peroxide (200 μ M) for 1 h on two successive days (*oxidative exposure*) and analyzed 7 days later. **a** T β RI and T β RII and **b** SMAD2 and SMAD3 proteins were quantified by Western analysis. Data are means $\pm$ SEM, $N=5-8$, $*p<0.01$. *Insets* show representative Western blots. **c** Fibroblasts were co-transfected with β -galactosidase expression vector (internal control) and either 4X SBE-LUX (containing SMAD3/4-binding motif, *left*

panel) or COL1 α 2 promoter (-772/+58) CAT reporter (containing SMAD3/4-binding motif, *right panel*). Whole cell protein extracts were prepared 48 h after transfection. Aliquots containing identical β -galactosidase activity were used for promoter activity assays. Data are means $\pm$ SEM, $N=5$, $*p<0.01$. **d** CCN2 mRNA and protein levels were measured 7 days post oxidative exposure, by real-time RT-PCR and Western analysis, respectively. mRNA levels were normalized to mRNA for housekeeping gene *36B4*, *RPLP0* gene. Data are means $\pm$ SEM, $N=7$, $*p<0.01$. *Insets* show representative Western blots

and protein were significantly reduced by 70 and 62 %, respectively, (Fig. 4d) in dermal fibroblasts following oxidative exposure.

Restoration of T β RII and SMAD3 overcomes oxidative exposure reduction of ProCOL1 expression in human dermal fibroblasts

The above data indicate that impairment of the TGF- β signaling pathway by oxidative exposure is coupled to reduction of ProCOL1 expression. We next determined whether restoration of T β RII and/or SMAD3 is able to overcome oxidative exposure impairment of TGF- β signaling and reduction of ProCOL1 and CCN2 expression. Following oxidative exposure, fibroblasts were transfected with T β RII or SMAD3 expression vector alone or together. Expression of T β RII alone did not increase the levels of SMAD3 phosphorylation (Fig. 5 and Supplemental Fig. 2), ProCOL1, or CCN2 (Fig. 5). Although the expression of SMAD3 alone modestly increased SMAD3 phosphorylation (Fig. 5 and Supplemental Fig. 2), it did not alter the ProCOL1 or CCN2 levels (Fig. 5). However, restoration of both T β RII and SMAD3 together resulted in substantial increases of SMAD3 phosphorylation (Fig. 5 and Supplemental Fig. 2), ProCOL1, and CCN2 expression. These data provide further evidence that downregulation of ProCOL1 and CCN2 by oxidative exposure is mediated by reduction of T β RII and SMAD3.

Anti-oxidant treatment protects against impairment of TGF- β signaling by oxidative exposure in human dermal fibroblasts

We next investigated whether boosting cellular anti-oxidant capacity could protect TGF- β signaling from impairment by oxidative exposure. We chose *N*-acetylcysteine (NAC), which is an anti-oxidant and metabolic precursor of glutathione. Glutathione serves as a co-factor for the anti-oxidant enzyme glutathione peroxidase, which plays a critical role in protecting cells from oxidative damage by reducing lipid peroxides and converting hydrogen peroxide to water. Treatment of fibroblasts with NAC (10 mM) every other day for 7 days, beginning 1 day after oxidative exposure, a time when ROS levels are not yet elevated, markedly prevented elevation of endogenous ROS levels (Fig. 6a).

We next determined whether the addition of NAC could mollify the negative influence of oxidative exposure on levels of ProCOL1 or components of the TGF- β signaling pathway. NAC treatment, 1 day after oxidative

Fig. 5 Expression of T β RII plus SMAD3 overcomes oxidative exposure repression of COL1 and CCN2 production in human skin fibroblasts. Seven days following treatment with vehicle (*Ctrl*) or hydrogen peroxide (200 μ M) for 1 h on two successive days (*oxidative exposure*), human skin fibroblasts were transfected with pCDNA3.1 (control vector) and SMAD3 and/or T β RII, expression vectors. Whole cell protein extracts were prepared 48 h after transfection. Protein levels were determined by Western analysis. Data are means $\pm$ SEM, $N=4$, $*p<0.05$ vs. oxidative exposure control. Insets show representative Western blots

exposure, largely prevented the downregulation of ProCOL1 and CCN2 protein levels by oxidative exposure, as measured by Western analysis (Fig. 6b). In addition, NAC completely prevented reduction of T β RII and SMAD3 protein levels (Fig. 6c). Administration of NAC also effectively prevented inhibition of COL1 α 2 promoter activity (pCOL1 α 2-CAT reporter) by oxidative exposure (Fig. 6d). These results indicate that the deleterious effects of oxidative exposure are largely preventable by anti-oxidant treatment.

The above data indicate that NAC may prevent reduction of ProCOL1 and CCN2 expression by protecting against impairment of TGF- β signaling by oxidative exposure. To investigate this possibility, we treated fibroblasts with NAC alone or in combination with T β RI kinase inhibitor SB431542. SB431542 did not alter the ability of NAC to protect against reduction of T β RII (Fig. 7a) or SMAD3 (Fig. 7b). However, it

Fig. 6 Anti-oxidant NAC protects against impairment of the TGF- β pathway by oxidative exposure in human skin fibroblasts. Fibroblasts were exposed to vehicle control (*Ctrl*) or hydrogen peroxide (200 μ M) for 1 h on two successive days (*oxidative exposure*). One day later, cells were treated with vehicle (*Ctrl*) or NAC (10 mM) every other day for 7 days. **a** Intracellular ROS levels were measured by RedoxSensor Red fluorescence. Results show representative images from three separate experiments. **b** ProCOL1 (*left panel*) and CCN2 (*right panel*) proteins were measured by Western analysis. Data are means $\pm$ SEM, $N=5$, $*p<0.01$ vs. non-oxidative exposure control. *Insets* show representative Western blots. **c** Western blot analysis of T β RII (*left panel*) and SMAD3 (*right panel*) proteins. Data are means $\pm$ SEM, $N=4-5$, $*p<0.05$ vs. non-oxidative exposure control. *Insets* show representative Western blots. **d** Vehicle- (*Ctrl*) or NAC-treated fibroblasts were co-transfected with COL1A2 promoter (-772/+58) CAT reporter and β -galactosidase expression vector. Aliquots containing identical β -galactosidase activity were used for promoter activity assays, 48 h after transfection. Data are means $\pm$ SEM, $N=3$, $*p<0.05$ vs. non-oxidative exposure control

completely blocked the ability of NAC to prevent reduction of ProCOL1 (Fig. 7c) and CCN2 (Fig. 7d) protein levels by oxidative exposure. These data support the conclusion that ROS acts through impairment of the TGF- β /SMAD3 pathway to reduce ProCOL1 and CCN2 in human dermal fibroblasts.

Anti-oxidant treatment restores TGF- β signaling in human dermal fibroblasts

Having shown that addition of NAC 1 day after oxidative exposure prevents elevation of endogenous ROS levels and impairment of TGF- β signaling, we next examined whether addition of NAC 7 days after oxidative exposure is capable of reversing the deleterious effects of oxidative exposure. Beginning 7 days after oxidative exposure, when ROS levels are maximally elevated and TGF- β signaling is impaired, dermal fibroblasts were treated with NAC every other day for 7 days. This NAC treatment significantly reduced elevated intracellular ROS levels to near normal (Fig. 8a). This reduction of cellular ROS levels by NAC was accompanied by restoration of both T β RII and SMAD3 protein levels and type I α 2 collagen promoter reporter activity (Fig. 8b). Consistent with these observations, NAC largely restored ProCOL1 and CCN2 protein levels by 93 and 75 %, respectively (Fig. 8c). These data indicate that reduction of elevated endogenous ROS levels is coupled to restoration of TGF- β signaling and target gene expression, and the deleterious effects of oxidative exposure are largely reversible.

Discussion

We have previously reported that collagen, the most abundant structural protein in human skin, undergoes progressive loss and degradation during the aging process, and have proposed that these alterations are key drivers of age-related decline of human skin function (Fisher et al. 2002, 2009). TGF- β signaling, which regulates type I collagen production by human dermal fibroblasts (Quan et al. 2010), is impaired in aged human skin (Quan et al. 2004). Furthermore, elevated ROS levels have been implicated as a major driving force of the aging process. These observations led us to investigate the impact of oxidative exposure on the TGF- β signaling pathway and its target genes, ProCOL1 and CCN2, in human dermal fibroblasts. Our results demonstrate that limited exposure of cultured adult human dermal fibroblasts to low, non-toxic levels of hydrogen peroxide induces long-term stable elevation of endogenous ROS and reduces T β RII and SMAD3, thereby impairing TGF- β signaling. This impairment reduces ProCOL1 and CCN2 expression.

A single acute addition of hydrogen peroxide to cultured cells has been widely applied to examine the short-term effects of ROS on cellular functions (Liu et al. 2013; Meares et al. 2013). We sought to study the long-term effects of elevated ROS levels on human dermal fibroblast functions. To accomplish this objective, we investigated concentration, time, and frequency of exposure. We found that exposure of fibroblasts to low, non-toxic levels of hydrogen peroxide (200 μ M) for 1 h at 24-h intervals on two successive days resulted in long-term elevation of intracellular ROS. The mechanism by which this regimen of oxidative exposure leads to increased endogenous ROS levels remains to be determined. In general, intracellular redox homeostasis is maintained mostly by enzymatic anti-oxidant defenses, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). SODs are responsible for dismutation of superoxide radical, generated by NAD(P)H oxidases, to hydrogen peroxide. CAT thereafter converts hydrogen peroxide into water and oxygen. Impairment or imbalance of the enzymatic anti-oxidant defenses could contribute to elevated ROS generation. Interestingly, we found that oxidative exposure causes elevation of SOD-1, -2, and -3, and decline of CAT gene expression (unpublished observation). These findings suggest that imbalance of anti-oxidant enzyme levels may contribute to elevated ROS levels in dermal fibroblasts, in response to oxidative exposure.

Fig. 7 Protection against reduction of COL1 and CCN2 expression by NAC is dependent on the TGF- β pathway. Human skin fibroblasts were exposed to vehicle control (*Ctrl*) or hydrogen peroxide (200 μ M) for 1 h on two successive days (*oxidative exposure*). One day later, cells were treated with vehicle (*Ctrl*) or NAC (10 mM) alone or together with T β RII kinase inhibitor

SB431542 (10 μ M), every other day for 7 days. Whole cell protein extracts were prepared and analyzed by Western blot for **a** T β RII, **b** SMAD3, **c** ProCOL1, and **d** CCN2 proteins. Data are means $\pm$ SEM, $N=3-5$, $*p<0.05$ vs. non-oxidative exposure control. Insets show representative Western blots

The family of NADPH oxidases (NOX) is an important source of intracellular ROS in most cell types (Bedard and Krause 2007; Jiang et al. 2011). Therefore, we considered NOX as a potential contributor to elevated ROS levels observed in dermal fibroblasts following oxidative exposure. However, oxidative exposure did not alter NOX family members' (NOX1, 3, 4) gene expression, determined by cDNA microarray analysis (data not shown). In addition, the NOX inhibitor diphenyliodonium did not reduce elevated ROS levels (data not shown). It appears that NOX may not be a major contributor to oxidative exposure-induced ROS levels in dermal fibroblasts.

Both T β RI and T β RII are indispensable for the TGF- β signal transduction pathway (Boyd and Massague 1989; Laiho et al. 1990; Quan et al. 2001, 2004). We found that oxidative exposure specifically reduces T β RII while not affecting T β RI. We also observed specific downregulation of T β RII, with sparing of T β RI, following UV irradiation of human dermal fibroblasts (Quan et al. 2004). These data indicate that T β RII and T β RI are differentially regulated and that T β RII, which is primarily responsible for ligand binding, is more responsive to cellular stress.

Fig. 8 NAC reverses impairment of TGF- β signaling by oxidative exposure in human skin fibroblasts. Fibroblasts were exposed to vehicle control (*Ctrl*) or hydrogen peroxide (200 μ M) for 1 h on two successive days (*oxidative exposure*). Seven days after oxidative exposure, cells were treated with vehicle (*Ctrl*) or NAC (10 mM) every other day for 7 days. **a** Intracellular ROS levels were measured by RedoxSensor Red fluorescence. Results show representative images from three separate experiments. **b** Western

blot analysis of T β RII (*left panel*), SMAD3 (*middle panel*), and COL1A2 promoter (-772/+58) CAT reporter activity (*right panel*). Promoter activity was measured 48 h after transfection and normalized to β -galactosidase activity. Data are means $\pm$ SEM, $N=3-5$, * $p<0.01$ vs. non-oxidative exposure control. **c** Western analysis of ProCOL1 (*left panel*) and CCN2 (*right panel*) proteins. Data are means $\pm$ SEM, $N=4$, * $p<0.01$ vs. non-oxidative exposure control. Insets show representative Western blots

The mechanism by which oxidative exposure selectively reduces T β RII and SMAD3 protein levels remains to be determined. Elevated ROS increases protein oxidation, which may alter protein susceptibility to proteolytic

degradation (Davies 1987; Davies and Delsignore 1987; Davies et al. 1987a, b; Davies and Goldberg 1987a, b). TGF- β receptors and SMADs have been reported to be degraded via ubiquitin-proteasome and sumoylation

pathway turnover (Bonni et al. 2001; Fukuchi et al. 2001; Izzi and Attisano 2004; Kavsak et al. 2000; Lin et al. 2000, 2003a, b; Long et al. 2004; Moustakas et al. 2001). Both protein degradation systems are regulated by ROS (Manza et al. 2004; Saitoh and Hinchey 2000; Zhou et al. 2004; Bossis and Melchior 2006). However, we find that oxidative exposure does not alter the half-life of either T β RII or SMAD3, arguing against accelerated protein degradation (unpublished observation). It appears more likely that reduced protein synthesis, which has been observed to occur in response to elevated ROS, may account for the observed reduction of T β RII and SMAD3 protein levels.

In the present study, addition of high concentration of anti-oxidant NAC (10 mM) reduced elevated levels of endogenous ROS that are generated following oxidative exposure of human dermal fibroblasts. When added 1 day after oxidative exposure, NAC prevented impairment of the TGF- β signaling pathway and reduction of ProCOL1 expression. When added 7 days after oxidative exposure, NAC largely restored T β RII, SMAD3, and ProCOL1 expression. NAC is an anti-oxidant and precursor of reduced glutathione (GSH), which is a co-factor for glutathione peroxidase (GPx). GPx decomposes H₂O₂ and lipid peroxides (Mukherjee et al. 2007). It is likely that addition of high concentration of NAC provides a reduced environment to counter increased ROS. These data suggest that T β RII and SMAD3 protein levels are regulated by a reversible, redox-dependent mechanism. Interestingly, several studies have reported increased ratio of oxidized to reduced glutathione content with aging (Favilli et al. 1994; Sohal et al. 1990; Teramoto et al. 1992). This alteration of GSSG/GSH ratio in aging represents reduction of anti-oxidant capacity and potential accumulation of ROS.

In summary, this study provides direct evidence that oxidative exposure in human dermal fibroblasts induces a stable state that is characterized by elevated endogenous ROS levels, impaired TGF- β signal transduction, and reduced levels of ProCOL1 and CCN2. Impaired TGF- β signal transduction results from reduction of T β RII and SMAD3/phospho-SMAD3 proteins, which downregulates ProCOL1 and CCN2 expression. Anti-oxidant treatment prior to elevation of endogenous ROS maintains normal ROS levels and protects against impairment of TGF- β signaling and loss of ProCOL1 and CCN2 expression. Moreover, long-term elevated ROS levels and impairment of the TGF- β /SMAD3 pathway that occur following oxidative exposure are largely normalized with anti-oxidant treatment. The reversible nature of these processes raises

the possibility that delivery of an effective anti-oxidant to dermal fibroblasts could restore ProCOL1 production and thereby improve function in aged human skin.

Materials and methods

Cell culture and treatments

Human skin dermal fibroblasts were established from healthy adult human buttock skin, as previously described (Quan et al. 2002). Cells were maintained in Dulbeccos' Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and were used at passages 3 to 10. For oxidative exposure, cultured dermal fibroblasts were grown to confluency and exposed to hydrogen peroxide (200 μ M) in serum-free DMEM for 1 h on two successive days. Cells were extensively washed after each exposure to remove the added hydrogen peroxide and placed in fresh DMEM containing 10 % FBS. Cells were analyzed at indicated times following last H₂O₂ treatment. This protocol is referred to as oxidative exposure throughout this article. Independent experiments, as indicated by *N* number in figure legends, were conducted with fibroblasts from different donors.

For some studies, cultured dermal fibroblasts were treated with either NAC (10 mM, Sigma, St. Louis, MO) alone or NAC together with T β RI kinase inhibitor SB431542 (10 μ M; Tocris Bioscience, Ellisville, MO) every other day, starting 1 or 7 days after oxidative exposure. The cells were collected and subjected to mRNA and protein analysis.

Intracellular ROS measurements

Intracellular ROS was measured by either redox-sensitive fluorescent dye (RedoxSensor Red CC-1, Life Technologies, Grand Island, NY) or carboxy-H2DCFDA (C400, Life Technologies, Grand Island, NY). For RedoxSensor Red measurement, fibroblasts were seeded into 8-well chamber slides at 10³ cells per well for 24 h and incubated with RedoxSensor Red CC-1 (1 μ M) at 37 °C for 20 min in the dark. Cells were washed twice in PBS, fixed with 2 % paraformaldehyde for 20 min. The slides were stained with DAPI to visualize nuclei, and cells were observed and photographed using Zeiss HBO 100 fluorescence microscope through a $\times$ 10 objective. For ROS quantitation, fibroblasts were incubated with carboxy-H2DCFDA (5 μ M) at 37 °C for

30 min in the dark. Cells were then fixed, washed, and collected. Flow cytometric analysis was carried out using Beckman Coulter Elite ESP cell sorter in the flow cytometric core facility at the University of Michigan. For each analysis, cells without carboxy-H2DCFDA or cells incubated with 200 μ M H₂O₂ for 30 min served as negative and positive controls, respectively.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Quan et al. 2001) using reagents from BioGenex (San Ramon, CA) unless specifically stated. Briefly, fibroblasts (1×10^4) were seeded on 8-well chamber slides for 24 h. After fixation with cold acetone (Fisher Scientific, Pittsburgh, PA) for 10 min, the slides were blocked successively with Peroxide Block for 10 min and Protein Block for 1 h. Following blocking, the slides were incubated with monoclonal antibody against ProCOL1 (MAB1912, Millipore, Billerica, MA) for 1 h at room temperature. Slides were then incubated with Super Sensitive Multilink and Super Sensitive Label for 10 min. One Step AEC solution was used as a chromogen. Between steps, the slides were rinsed for 10 min in Tris-buffered saline with 0.1 % Triton X-100 (Sigma). All sections were lightly counterstained with CAT hematoxylin (Biocare Medical, Concord, CA).

RNA isolation and quantitative real-time RT-PCR

Total RNA from cultured dermal fibroblasts was prepared using a commercial kit (RNeasy Mini Kit, Qiagen, Chatsworth, CA) according to the manufacturer's protocol. mRNA levels were quantified by real-time RT-PCR as previously described (Quan et al. 2002). PCR primers and probes were purchased from Applied Biosystems custom oligonucleotide synthesis service. Primers and fluorescein amidite (FAM)-labeled probes for SMAD3 are as follows: sense primer, 5'-TGCGTC TCCAGTCATCTGTAAGAG-3'; antisense primer, 5'-TGAATGCAACTGACTACATAAACCAA-3'; and probe, 5'-TTGCTCCAGATTCTGATGCATACGGCT-3'. The primers and FAM-labeled probes for all other target genes have been previously described: ProCOL1 (Quan et al. 2004), CCN2, and VIC-labeled probe for *36B4* (*RPLP0* gene) (Quan et al. 2002). Target gene levels were normalized to the housekeeping gene *36B4*.

siRNA silencing of SMAD3 expression

siRNA was designed and purchased from Qiagen (Chatsworth, CA). The target sequence to SMAD3 was 5'-AAACCTATCCCCGAATCCGAT-3'. Human skin fibroblasts were transfected by electroporation using Amaxa Nucleofector (Koeln, Germany) with control-scrambled or SMAD3 siRNAs. Total RNA and whole cell protein extracts were prepared 48 h after transfection.

Western blot analysis, procollagen protein quantitation, and protein carbonyl detection

Following H₂O₂ exposure, whole cell protein extracts were prepared as previously described (Quan et al. 2010). Western blot analysis was performed as described (Quan et al. 2001). Briefly, cell extract proteins were resolved on 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA), which was then blocked in 5 % non-fat dry milk in TBST with 0.1 % Tween 20. Membranes were probed with antibodies against COL1 α 1, CCN2, SMAD2/SMAD3, T β RI and T β RII (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-SMAD3 (Cell Signaling Technology, Danvers, MA). Immunoreactive bands were visualized by enhanced chemifluorescence (ECF) and quantified by Storm Molecular Imager (GE Healthcare, Piscataway, NJ).

Intracellular and secreted ProCOL1 protein from cultured skin fibroblasts and culture media, respectively, were measured by procollagen type I C-peptide (PIP) EIA kit following manufacturer's instructions (Takara Bio Inc., Japan).

Protein carbonyl detection (OxyBlot) was performed using OxyBlot Protein Oxidation Detection Kit (Chemicon International Inc., Temecula, CA), according to manufacturer's instruction with slight modification. Briefly, cellular protein extracts (10 μ g) were denatured in 6 % SDS. Samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH), or sham for negative control, for 15 min. Reactions were neutralized and the samples were resolved on 10 % SDS-PAGE. After gel transfer and membrane block, as described in Western blot analysis, the membrane was probed by anti-DNP antibody for 1 h followed by secondary antibody conjugated with IgG-AP for 1 h. Oxidized proteins were detected and quantified as described in Western blot analysis. For control of equal protein loading, the

membrane was reprobed with antibody against β -actin (Sigma Chemical Co., St. Louis, MO).

Transient transfection and reporter assay

Primary human skin fibroblasts were transiently transfected by electroporation using Amaxa Nucleofector (Amaxa Biosystems). T β RII (pCMV-T β RII) and SMAD3 (pCMV-SMAD3) expression vectors were transfected separately or together. Forty-eight hours following transfection, total RNA and cellular proteins were extracted, and expression levels of target genes were determined by quantitative real-time RT-PCR and Western analysis, as described above.

For reporter analysis, two TGF- β -regulated reporter constructs were utilized: (1) 4X SBE-LUX (Zawel et al. 1998), luciferase reporter containing four repetitions of the GTCTAGAC SMAD3-binding motif (provided by Dr. Bert Vogelstein of the Johns Hopkins Oncology Center, Baltimore, MD) and (2) pCol1 α 2-CAT (Ihn et al. 1997), a CAT reporter containing SMAD3-binding element from COL1A2 promoter (-772/+58) (provided by Dr. Maria Trojanowski, Boston University). β -Galactosidase expression vector (pCMV β Gal, Clontech Laboratories Inc., Palo Alto, CA) was co-transfected to provide an internal standard for transfection efficiency. Forty-eight hours after transfection, cells were collected, lysed, and assayed for luciferase activity using an enhanced luciferase assay kit (PharMingen International, San Diego, CA) according to the manufacturer's protocol. CAT assay was performed as previously described (Quan and Fisher 1999).

Statistical analysis

Comparisons among treatment groups were made with the paired *t* test (two groups) or the repeated measures of analysis of variance (more than two groups). Multiple pairwise comparisons were made with the Tukey's studentized range test. All *p* values are two-tailed and considered significant when <0.05 .

Acknowledgments We would like to thank Diane Fiolek for graphic material and administrative assistance; Dr. Maria Trojanowski (Boston University) for providing pCOL1 α 2-CAT; and Dr. Bert Vogelstein (Johns Hopkins) for providing 4X SBE-LUX. This work was supported, in part, by the National Institutes of Health, AG019364 (GF) and AG031452 (GF).

References

- Bedard K, Krause KH (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87(1):245–313. doi:10.1152/physrev.00044.2005
- Bonni S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Luo K, Wrana JL (2001) TGF- β induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation. *Nat Cell Biol* 3(6):587–595. doi:10.1038/35078562
- Bossis G, Melchior F (2006) Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Mol Cell* 21(3):349–357. doi:10.1016/j.molcel.2005.12.019
- Boyd FT, Massague J (1989) Transforming growth factor- β inhibition of epithelial cell proliferation linked to the expression of a 53-kDa membrane receptor. *J Biol Chem* 264(4):2272–2278
- Brigstock DR (1999) The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr Rev* 20(2):189–206
- Cadenas E, Davies KJ (2000) Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 29(3–4):222–230
- Davies KJ (1987) Protein damage and degradation by oxygen radicals. I. General aspects. *J Biol Chem* 262(20):9895–9901
- Davies KJ, Delsignore ME (1987) Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure. *J Biol Chem* 262(20):9908–9913
- Davies KJ, Goldberg AL (1987a) Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J Biol Chem* 262(17):8220–8226
- Davies KJ, Goldberg AL (1987b) Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells. *J Biol Chem* 262(17):8227–8234
- Davies KJ, Delsignore ME, Lin SW (1987a) Protein damage and degradation by oxygen radicals. II. Modification of amino acids. *J Biol Chem* 262(20):9902–9907
- Davies KJ, Lin SW, Pacifici RE (1987b) Protein damage and degradation by oxygen radicals. IV. Degradation of denatured protein. *J Biol Chem* 262(20):9914–9920
- Droge W (2002) Free radicals in the physiological control of cell function. *Physiol Rev* 82(1):47–95. doi:10.1152/physrev.00018.2001
- Duncan MR, Frazier KS, Abramson S, Williams S, Klapper H, Huang X, Grotendorst GR (1999) Connective tissue growth factor mediates transforming growth factor β -induced collagen synthesis: down-regulation by cAMP. *FASEB J* 13(13):1774–1786
- Favilli F, Iantomasi T, Marraccini P, Stio M, Lunghi B, Treves C, Vincenzini MT (1994) Relationship between age and GSH metabolism in synaptosomes of rat cerebral cortex. *Neurobiol Aging* 15(4):429–433
- Fisher GJ, Kang S, Varani J, Bata-Csorgo Z, Wan Y, Datta S, Voorhees JJ (2002) Mechanisms of photoaging and chronological skin aging. *Arch Dermatol* 138(11):1462–1470
- Fisher GJ, Quan T, Purohit T, Shao Y, Cho MK, He T, Varani J, Kang S, Voorhees JJ (2009) Collagen fragmentation promotes oxidative stress and elevates matrix metalloproteinase-1 in fibroblasts in aged human skin. *Am J Pathol* 174(1):101–114. doi:10.2353/ajpath.2009.080599
- Fukuchi M, Imamura T, Chiba T, Ebisawa T, Kawabata M, Tanaka K, Miyazono K (2001) Ligand-dependent degradation of

- Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. *Mol Biol Cell* 12(5):1431–1443
- Herrling T, Jung K, Fuchs J (2006) Measurements of UV-generated free radicals/reactive oxygen species (ROS) in skin. *Spectrochim Acta A Mol Biomol Spectrosc* 63(4): 840–845. doi:10.1016/j.saa.2005.10.013
- Hertig IA, Hassoun PM, Zulueta JJ, Thannickal VJ, Fanburg BL (1993) Mechanism of basal and transforming growth factor beta 1 stimulated H₂O₂ release by endothelial cells. *Trans Assoc Am Phys* 106:179–186
- Ihn H, LeRoy EC, Trojanowska M (1997) Oncostatin M stimulates transcription of the human alpha2(I) collagen gene via the Sp1/Sp3-binding site. *J Biol Chem* 272(39):24666–24672
- Izzi L, Attisano L (2004) Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. *Oncogene* 23(11):2071–2078. doi:10.1038/sj.onc.1207412
- Jiang Z, Seo JY, Ha H, Lee EA, Kim YS, Han DC, Uh ST, Park CS, Lee HB (2003) Reactive oxygen species mediate TGF-beta1-induced plasminogen activator inhibitor-1 upregulation in mesangial cells. *Biochem Biophys Res Commun* 309(4):961–966
- Jiang F, Zhang Y, Disting GJ (2011) NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. *Pharmacol Rev* 63(1):218–242. doi:10.1124/pr.110.002980
- Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL (2000) Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* 6(6):1365–1375
- Kim YK, Bae GU, Kang JK, Park JW, Lee EK, Lee HY, Choi WS, Lee HW, Han JW (2006) Cooperation of H₂O₂-mediated ERK activation with Smad pathway in TGF-beta1 induction of p21WAF1/Cip1. *Cell Signal* 18(2):236–243. doi:10.1016/j.cellsig.2005.04.008
- Laiho M, Weis MB, Massague J (1990) Concomitant loss of transforming growth factor (TGF)-beta receptor types I and II in TGF-beta-resistant cell mutants implicates both receptor types in signal transduction. *J Biol Chem* 265(30):18518–18524
- Lin X, Liang M, Feng XH (2000) Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor-beta signaling. *J Biol Chem* 275(47):36818–36822. doi:10.1074/jbc.C000580200
- Lin X, Liang M, Liang YY, Brunnicardi FC, Feng XH (2003a) SUMO-1/Ubc9 promotes nuclear accumulation and metabolic stability of tumor suppressor Smad4. *J Biol Chem* 278(33): 31043–31048. doi:10.1074/jbc.C300112200
- Lin X, Liang M, Liang YY, Brunnicardi FC, Melchior F, Feng XH (2003b) Activation of transforming growth factor-beta signaling by SUMO-1 modification of tumor suppressor Smad4/DPC4. *J Biol Chem* 278(21):18714–18719. doi:10.1074/jbc.M302243200
- Liu L, Gu L, Ma Q, Zhu D, Huang X (2013) Resveratrol attenuates hydrogen peroxide-induced apoptosis in human umbilical vein endothelial cells. *Eur Rev Med Pharmacol Sci* 17(1):88–94
- Long H, Shi T, Borm PJ, Maatta J, Husgafvel-Pursiainen K, Savolainen K, Krombach F (2004) ROS-mediated TNF-alpha and MIP-2 gene expression in alveolar macrophages exposed to pine dust. *Part Fibre Toxicol* 1(1):3. doi:10.1186/1743-8977-1-3
- Manza LL, Codreanu SG, Stamer SL, Smith DL, Wells KS, Roberts RL, Liebler DC (2004) Global shifts in protein sumoylation in response to electrophile and oxidative stress. *Chem Res Toxicol* 17(12):1706–1715. doi:10.1021/tx0497671
- Meares GP, Fontanilla D, Broniowska KA, Andreone T, Lancaster JR Jr, Corbett JA (2013) Differential responses of pancreatic beta-cells to ROS and RNS. *Am J Physiol Endocrinol Metab* 304(6):E614–E622. doi:10.1152/ajpendo.00424.2012
- Moustakas A, Souchelnytskyi S, Heldin CH (2001) Smad regulation in TGF-beta signal transduction. *J Cell Sci* 114(Pt 24): 4359–4369
- Mukherjee TK, Mishra AK, Mukhopadhyay S, Hoidal JR (2007) High concentration of antioxidants N-acetylcysteine and mitoquinone-Q induces intercellular adhesion molecule 1 and oxidative stress by increasing intracellular glutathione. *J Immunol* 178(3):1835–1844
- Ohba M, Shibamura M, Kuroki T, Nose K (1994) Production of hydrogen peroxide by transforming growth factor-beta 1 and its involvement in induction of egr-1 in mouse osteoblastic cells. *J Cell Biol* 126(4):1079–1088
- Quan T, Fisher GJ (1999) Cloning and characterization of the human protein kinase C-eta promoter. *J Biol Chem* 274(40):28566–28574
- Quan T, He T, Voorhees JJ, Fisher GJ (2001) Ultraviolet irradiation blocks cellular responses to transforming growth factor-beta by down-regulating its type-II receptor and inducing Smad7. *J Biol Chem* 276(28):26349–26356. doi:10.1074/jbc.M010835200
- Quan T, He T, Kang S, Voorhees JJ, Fisher GJ (2002) Connective tissue growth factor: expression in human skin in vivo and inhibition by ultraviolet irradiation. *J Invest Dermatol* 118(3): 402–408. doi:10.1046/j.0022-202x.2001.01678.x
- Quan T, He T, Kang S, Voorhees JJ, Fisher GJ (2004) Solar ultraviolet irradiation reduces collagen in photoaged human skin by blocking transforming growth factor-beta type II receptor/Smad signaling. *Am J Pathol* 165(3):741–751
- Quan T, Shao Y, He T, Voorhees JJ, Fisher GJ (2010) Reduced expression of connective tissue growth factor (CTGF/CCN2) mediates collagen loss in chronologically aged human skin. *J Invest Dermatol* 130(2):415–424. doi:10.1038/jid.2009.224
- Rhyu DY, Yang Y, Ha H, Lee GT, Song JS, Uh ST, Lee HB (2005) Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol* 16(3):667–675. doi:10.1681/ASN.2004050425
- Saitoh H, Hinchey J (2000) Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol Chem* 275(9):6252–6258
- Sohal RS, Arnold L, Orr WC (1990) Effect of age on superoxide dismutase, catalase, glutathione reductase, inorganic peroxides, TBA-reactive material, GSH/GSSG, NADPH/NADP+ and NADH/NAD+ in *Drosophila melanogaster*. *Mech Ageing Dev* 56(3):223–235
- Teramoto S, Fukuchi Y, Uejima Y, Ito H, Orimo H (1992) Age-related changes in GSH content of eyes in mice—a comparison of senescence-accelerated mouse (SAM) and C57BL/J mice. *Comp Biochem Physiol Comp Physiol* 102(4):693–696
- Thannickal VJ, Fanburg BL (1995) Activation of an H₂O₂-generating NADH oxidase in human lung fibroblasts by transforming growth factor beta 1. *J Biol Chem* 270(51): 30334–30338
- Thannickal VJ, Hassoun PM, White AC, Fanburg BL (1993) Enhanced rate of H₂O₂ release from bovine pulmonary

- artery endothelial cells induced by TGF-beta 1. *Am J Physiol* 265(6 Pt 1):L622–L626
- Wu WS (2006) The signaling mechanism of ROS in tumor progression. *Cancer Metastasis Rev* 25(4):695–705. doi:[10.1007/s10555-006-9037-8](https://doi.org/10.1007/s10555-006-9037-8)
- Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE (1998) Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell* 1(4):611–617
- Zhou W, Ryan JJ, Zhou H (2004) Global analyses of sumoylated proteins in *Saccharomyces cerevisiae*. Induction of protein sumoylation by cellular stresses. *J Biol Chem* 279(31):32262–32268. doi:[10.1074/jbc.M404173200](https://doi.org/10.1074/jbc.M404173200)