Cigarette Smoke Induces Cellular Senescence via Werner's Syndrome Protein Down-regulation

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Rationale: Werner's syndrome is a genetic disorder that causes premature aging due to loss-of-function mutations in a gene encoding a member of the RecQ helicase family. Both Werner's syndrome and cigarette smoking accelerate aging. No studies have examined the effect of cigarette smoke on Werner's syndrome protein.

Objectives: To investigate the role of Werner's syndrome protein in cigarette smoke-induced cellular senescence.

Methods: Cellular senescence and amounts of Werner's syndrome protein were measured in fibroblasts isolated from patients with emphysema and compared with age-matched nonsmokers. The *in vitro* effects of cigarette smoke on amounts of Werner's syndrome protein, function, and senescence were also evaluated in primary human lung fibroblasts and epithelial cells.

Measurements and Main Results: Cultured lung fibroblasts isolated from patients with emphysema exhibited a senescent phenotype accompanied by a decrease in Werner's syndrome protein. Cigarette smoke extract decreased Werner's syndrome protein in cultured fibroblasts and epithelial cells. Werner's syndrome protein-deficient fibroblasts were more susceptible to cigarette smoke-induced cellular senescence and cell migration impairment. In contrast, exogenous overexpression of Werner's syndrome protein attenuated the cigarette smoke effects.

Conclusions: Cigarette smoke induces cellular senescence and cell migration impairment via Werner's syndrome protein down-regulation. Rescue of Werner's syndrome protein down-regulation may represent a potential therapeutic target for smoking-related diseases.

Keywords: aging; smoking; emphysema; oxidative stress; cell migration

Werner's syndrome (WS) is a genetic disease causing premature aging characterized by accelerated development of atherosclerosis, cancer, graying of the hair, type 2 diabetes mellitus, and osteoporosis (1–3). Most patients with WS prematurely die because of cancer or atherosclerosis (2, 3). The WS phenotype resembles accelerated normal aging (4, 5) and has been widely used to investigate the mechanisms of normal human aging (6). Various loss-of-function mutations in a gene encoding a member

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Both heavy smokers and patients with Werner's syndrome manifest similar clinical features consistent with accelerated aging. Despite this, no prior studies have investigated a link between Werner's syndrome protein (WRN protein) and smoking.

What This Study Adds to the Field

Lung fibroblasts isolated from smokers with emphysema exhibit a decrease in WRN protein. Cigarette smoke also induces cellular senescence via WRN down-regulation in cultured fibroblasts. WRN protein may play an important role in smoking-related diseases.

of the RecQ helicase family (WRN) result in genomic instability, contributing to accelerated aging (2, 7). Thus, the family of RecQ helicases is referred to as a guardian of the genome (8). The WRN gene product, WRN protein, consists of 1,432 amino acids and is ubiquitously expressed in all tissues (9). WRN protein plays a key role in DNA metabolism, including replication, recombination, and repair (10–12).

Cellular senescence is defined as complete and irreversible loss of replicative capacity occurring in primary somatic cells (13) and is characterized by an enlarged and flat morphologic change and an increase in senescence-associated β -galactosidase (SA β -Gal) activity. WRN protein loss due to the gene mutation or RNA interference promotes cellular senescence (14–16).

Some individuals who smoke cigarettes exhibit many of the features of Werner's syndrome, including cancer (17) and atherosclerosis (18, 19). It also is clear that cigarette smoke accelerates aging (20–22) and induces cellular senescence in the emphysematous lung (23, 24). However, nothing is known about the effect of cigarette smoke on WRN protein expression. Cigarette smoke, which contains numerous reactive oxygen species (ROS) and cytotoxic by-products of oxidation reactions (25), including acrolein and hydroperoxides, induces DNA damage (26), resulting in cell growth inhibition and cellular senescence (27). The role of redox regulation and balance in cigarette smoke extract (CSE)-induced cell death has been extensively studied by using *in vitro* models (28–32); however, little is known about the role of redox regulation in cellular senescence. In this study, we evaluated a possible link between CSE and WRN protein regulation.

We show that lung fibroblasts isolated from patients with severe emphysema exhibit a senescent phenotype accompanied by a decrease in WRN protein. *In vitro*, we showed that exposure

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Figure 1. Lung fibroblasts isolated from patients with severe emphysema are deficient in Werner's syndrome protein. (A) Primary lung fibroblasts isolated from three patients with severe emphysema and from three age-matched non-smokers were cultured for 2 days. Immunoblot analysis was performed for WRN protein. Equal loading was determined by stripping the blot and reprobing with antibodies to β -actin. Densitometry is expressed as arbitrary units for each experimental band (see IMMUNOBLOTTING). Data are expressed as means $\pm$ SEM for each group ($n = 3$). (B and C) Primary lung fibroblasts were cultured at the starting density of $100 \times 10^3/\text{mL}$, and cell density and senescence-associated β -galactosidase (SA β -Gal) activity were measured on Day 4. Data are expressed as means $\pm$ SEM for each group ($n = 3$).

to CSE also induces WRN protein down-regulation, leading to cellular senescence in cultured fibroblasts and epithelial cells. WRN protein down-regulation appears to be dependent on a ubiquitin-proteasome degradation pathway. The CSE effects on cell growth and migration occurred spontaneously in fibroblasts isolated from patients with WS. WRN protein overexpression reversed the CSE-induced cellular senescence and migration impairment. Furthermore, *N*-acetylcysteine (NAC) attenuated WRN protein down-regulation and growth inhibition in CSE-exposed cells. These data suggest that cigarette smoke induces cellular senescence via oxidant-mediated WRN protein down-regulation.

METHODS

Reagents and Antibodies

See the online supplement for a list of sources of reagents and antibodies.

Cigarette Smoke Extract Preparation

Research cigarettes (2R4F, 100 mm) were purchased from the University of Kentucky (Lexington, KY). CSE solutions were prepared as previously described (27).

Cell Culture and Cell Count

See the online supplement for a description of methods. Experiments were performed in 12-well (20-mm) Costar tissue culture plates (Corning, Inc., Corning, NY) at a starting cell density of $0.10 \times 10^6/\text{mL}$. The cells were incubated with or without 1.2% CSE for various periods up to 14 days. In some instances, the cells were incubated with 3 mM NAC 30 minutes before exposure to CSE. Cell counts were performed with an electric particle counter.

Ex Vivo Human Lung Fibroblasts

Human lung fibroblasts were obtained under a protocol approved by the University of Iowa (Iowa City, IA) Institutional Review Board. See the online supplement for a description of these methods.

Human Tracheobronchial Epithelial Cells

Human tracheobronchial epithelial (hTBE) cells were obtained under a protocol approved by the University of Iowa Institutional Review Board. Epithelial cells were isolated from nonsmokers and cultured as previously described (33).

Establishment of Cell Lines with WRN Overexpression

Normal fetal lung fibroblasts (HFL-1 cells) were infected with retroviral vectors (LXSN) to overexpress WRN protein as previously described (34). Cells were selected with G418 (1.1 mg/mL for HFL-1) for 5–7 days and all the surviving colonies were collected as a pool.

Senescence-associated β -Galactosidase Activity

SA β -Gal staining was performed according to a previously described method (27).

Immunoblotting

Immunoblot analysis was performed as previously described (25). Protein levels were quantified with a Fluor S scanner and Quantity One software (Bio-Rad). When appropriate, data were statistically analyzed.

Real-time RT-PCR

RT-PCR and quantitative analysis for WRN mRNA were performed as previously described (35). The primers for WRN mRNA were ATG CAGCCACTGATGCTTATGCTG (forward) and TGGGCCTCAG TTCAGTCTCAATGT (reverse).

Immunoprecipitation of WRN Protein

Cells were treated with 20 μM lactacystin for 3 hours after a 24-hour incubation with CSE and whole cell lysates were obtained. Immunoblot analysis was performed for WRN protein. Immunoprecipitation of WRN protein was then performed as previously described (36). Immunoblot analysis for higher molecular weight forms of WRN protein was performed.

Scratch-induced Cell Migration/Proliferation Assay

A scratch-induced cell migration/proliferation assay was performed with some modifications of methods previously described (37, 38).

Figure 2. Cigarette smoke down-regulates Werner's syndrome protein. (A) Primary human lung fibroblasts and HFL-1 cells were cultured with or without 1.2% cigarette smoke extract (CSE) for various periods (1, 2, and 4 d). Immunoblot analysis was performed for WRN protein. Equal loading was determined by stripping the blot and reprobing with antibodies to β -actin. Densitometry is expressed as arbitrary units for each experimental band. Immunoblotting data are representative of three experiments. (B) Primary human tracheobronchial epithelial (hTBE) cells were cultured with or without 2% CSE for various periods (1, 2, and 4 d). Immunoblot analysis was performed for WRN protein. Equal loading was determined by stripping the blot and reprobing with antibodies to β -actin. Densitometry is expressed as arbitrary units for each experimental band. Immunoblotting data are representative of three experiments.

Fibroblasts were cultured until confluence in 12-well tissue culture plates and were further incubated with or without 1.5% CSE. After creating a scratch wound with a sterile 10- μ l pipette tip, complete medium was refreshed with or without 1.5% CSE. Closure of the open wound area at 24 hours is indicated as a percentage compared with the area of the initial wound.

Dihydroethidium Oxidation Assay

Intracellular superoxide production was measured by using a method previously described (39).

Statistical Analysis

Results are expressed as means $\pm$ SEM. The number of cells in each condition with or without CSE at various time points was compared using an unpaired *t* test.

RESULTS

Lung Fibroblasts Isolated from Patients with Severe Emphysema Are Deficient in Werner's Syndrome Protein

Various types of cells in the lungs of patients with chronic obstructive pulmonary disease exhibit a phenotype of cellular senescence characterized by a decreased proliferative capacity and increased SA β -Gal activity (23, 40). It is known that loss of function of WRN protein due to mutations or knockdown by RNA interference results in accelerated cellular senescence (2, 7, 16). However, no study has examined the effect of cigarette

smoke on WRN protein expression. We hypothesized that cigarette smoke exposure down-regulates WRN protein. To evaluate this, lung fibroblasts were isolated from three patients with a significant smoking history and severe emphysema, and from three age-matched nonsmokers. As shown in Figure 1, WRN protein, growth rate, and SA β -Gal activity were measured. Lung fibroblasts isolated from patients with emphysema exhibited a decreased growth rate and increased SA β -Gal activity, consistent with a senescent phenotype, accompanied by a decrease in WRN protein. These data suggest a role of cigarette smoke in WRN protein down-regulation in lung fibroblasts of patients with emphysema.

Cigarette Smoke Down-regulates Werner's Syndrome Protein

We next examined whether exposure to CSE decreases WRN protein expression in cultured fibroblasts. To evaluate this, primary lung fibroblasts and normal fetal lung fibroblasts (HFL-1) were cultured in the absence or presence of 1.2% CSE for up to 4 days. The concentration was chosen for prolonged exposure to maintain more than 90% fibroblast viability for up to 14 days (data not shown). Whole cell lysates obtained from each group were analyzed for WRN protein by Western blot. Exposure to CSE decreased steady state levels of WRN protein in cultured lung fibroblasts (Figure 2A). In the absence of CSE, as the cell density increased up to confluence at 4 days, there appeared to be a slight decrease in steady state levels of WRN protein in the control fibroblasts (Figure 2A), although there were some

A WRN mRNA **B** **C**

Figure 3. Cigarette smoke decreases Werner's syndrome protein in a ubiquitin-proteasome-dependent manner. (A) HFL-1 cells were cultured with or without 1.2% CSE. WRN mRNA was measured at various time points (1, 2, and 4 d). Data are expressed as means $\pm$ SEM for three independent experiments. Con = control; HPRT = hypoxanthine-guanine phosphoribosyltransferase. (B) HFL-1 cells were cultured with or without 1.5% cigarette smoke extract for 24 hours and then were incubated with 20 μ M lactacystin for 3 hours. Immunoblot analysis was performed for WRN protein. Equal loading was determined by stripping the blot and reprobing with antibodies to β -actin. Densitometry is expressed as arbitrary units for each experimental band. Immunoblotting data are representative of three experiments. (C) HFL-1 cells were treated as in (B). After immunoprecipitation of WRN protein, immunoblot analysis was performed for higher molecular weight (HMW) forms of WRN protein. Immunoblotting data are representative of three experiments. NS = nonspecific band.

variations among the cell strains or isolates (data not shown). To address whether or not the effects of CSE on WRN protein are cell type specific, primary hTBE cells were cultured in the presence or absence of 2% CSE. This concentration was determined to have a significant growth-inhibitory effect without cytotoxicity up to 4 days (data not shown). As shown in Figure 2B, exposure to CSE markedly decreased WRN protein in cultured hTBE cells. These results suggest a potential role of WRN protein in cigarette smoke-induced growth inhibition in various types of lung cells.

Cigarette Smoke Decreases Werner's Syndrome Protein in a Ubiquitin-Proteasome-dependent Manner

We next evaluated the effects of CSE on WRN mRNA levels in HFL-1 cells. Exposure to CSE did not appear to decrease steady state levels of WRN mRNA except for a slight decrease on Day 1 ($P < 0.05$) (Figure 3A). We then examined the WRN protein stability using cycloheximide (50 μ g/ml) in the presence or absence of 1.5% CSE. Exposure to CSE accelerated WRN protein degradation (data not shown). A proteasome inhibitor, lactacystin attenuated the effect of CSE (Figure 3B). Furthermore, the CSE-exposed WRN protein appeared to be polyubiquitinated (Figure 3C). These data suggest that exposure to CSE increases WRN protein degradation via a ubiquitin-proteasome-dependent manner.

Modulation of Werner's Syndrome Protein Affects Cigarette Smoke-induced Cell Growth Inhibition

It is known that primary dermal fibroblasts isolated from patients with WS have a limited proliferative capacity (14, 15). We hypothesized that exposure to CSE further promotes cellular senescence in cultured fibroblasts deficient in WRN protein. We

first confirmed that primary dermal fibroblasts isolated from patients with WS (WSF1; AG03141) have no detectable expression of WRN protein and that CSE-exposed normal dermal fibroblasts decreased WRN protein expression at 48 hours (Figure 4A). To evaluate CSE effects on cell growth and SA β -Gal activity, WSF1 cells and normal fibroblasts were cultured in the absence or presence of 1.2% CSE for up to 14 days and their cell densities were monitored. WSF1 cells grew slowly compared with age-matched control fibroblasts even in the absence of CSE, and exposure to CSE almost completely inhibited cell growth (Figure 4A) without significant cell death (data not shown). There was a significant increase in SA β -Gal activity of WS fibroblasts (both WSF1 cells and WSF2 cells) in the absence of CSE and exposure to CSE markedly increased SA β -Gal activity in WS fibroblasts compared with normal fibroblasts at 7 days (Figure 4A). These data suggest that WRN protein expression is required for cell growth and that WRN protein defects further sensitize fibroblasts to cigarette smoke-induced cellular senescence.

To address whether WRN protein restoration prevents cigarette smoke-induced growth inhibition, we established an *in vitro* model of WRN overexpression, using HFL-1 cells and a retroviral vector (LXSN) encoding the complete WRN cDNA. In a control cell line that expressed only the empty retroviral vector, we confirmed that exposure to CSE decreases WRN protein at 48 hours (Figure 4B). In addition, the retroviral vector, itself, did not alter the effect of CSE on WRN protein expression (data not shown). In the cells overexpressing WRN protein, exposure to CSE slightly decreased levels of the protein; however, these cells were able to maintain significant amounts of the protein, compared with CSE-exposed control cells (Figure 4A).

Figure 4. Modulation of Werner's syndrome protein affects cigarette smoke-induced growth inhibition. (A) WSF1 cells (AG03141) and normal fibroblasts were cultured in the presence or absence of 1.5% cigarette smoke extract (CSE). Immunoblot analysis was performed for WRN protein at 48 hours. Equal loading was determined by stripping the blot and reprobing with antibodies to β -actin. Immunoblotting data are representative of three experiments. The cell counts were also monitored in the presence or absence of 1.2% CSE at various time points (2, 4, 8, and 14 d). SA β -Gal staining was performed for WSF1 cells (AG03141), WSF2 cells (AG12799), and normal fibroblasts in the presence or absence of 1.2% CSE and digital photographs were obtained at 7 days. The percentage of SA β -Gal-positive cells per total cell number is shown. Data are expressed as means $\pm$ SEM for three independent experiments ($*P < 0.01$). (B) HFL-1 cells were transduced with LXSN retroviral vectors encoding complete WRN cDNA. Transduced cells were selected with G418. HFL-1 cells transduced with LXSN-WRN were cultured in the presence or absence of 1.5% CSE. Immunoblot analysis was performed for WRN protein at 48 hours. Equal loading was determined by stripping the blot and reprobing with antibodies to β -actin. Immunoblotting data are representative of three experiments. The cell counts were monitored as in (A). SA β -Gal staining was also performed and digital photographs were obtained at 14 days. The percentage of SA β -Gal-positive cells per total cell number is shown. Data are expressed as means $\pm$ SEM for three independent experiments ($**P < 0.05$). (C) HFL-1 cells transduced with LXSN-WRN were cultured in the presence or absence of 1.5% CSE for 1, 3, and 6 hours. Immunoblot analysis was performed for phosphorylation of histone H2AX. Equal loading was determined by stripping the blot and reprobing with antibodies to β -actin. Densitometry is expressed as arbitrary units for each experimental band. Immunoblotting data are representative of three experiments. WRN OE = WRN protein overexpression.

Figure 5. Modulation of Werner's syndrome protein affects cigarette smoke-induced cell migration impairment. (A) WSF1 cells (AG03141) or normal fibroblasts were cultured until confluence in 12-well tissue culture plates and were further incubated with or without 1.5% cigarette smoke extract (CSE). After creating a scratch wound, the medium was refreshed with or without 1.5% CSE. Closure of the open wound area at 24 hours is shown as a percentage compared with the area of the initial wound. (B) HFL-1 cells transduced with LXSN-WRN were treated as in (A). Data are expressed as means $\pm$ SEM for three independent experiments (* $P < 0.01$). Digital photographs were obtained at 0 and 24 hours.

To evaluate the biological effect of WRN protein overexpression, WRN protein-overexpressing cells (WRN OE) and control cells were cultured with or without 1.2% CSE and cell density was monitored at various time points up to 14 days. As shown in Figure 4A, WRN protein overexpression blocked CSE-induced growth inhibition for 2 days (LXSN, $P < 0.01$; WRN OE, not significant; control group cell density compared with CSE-exposed cell density). Even after 4 days of exposure to CSE, WRN protein overexpression significantly improved CSE-induced growth inhibition (LXSN, 45% vs. WRN OE, 65% [% control group cell density/CSE-exposed group cell density at 14 d]; $P < 0.05$) (Figure 4B). Exogenous WRN protein overexpression also significantly attenuated an increase in SA β -Gal activity by CSE (LXSN, 76% vs. WRN OE, 40%;

$P < 0.01$) (Figure 4B). These data suggest that WRN protein overexpression significantly prevents cigarette smoke-induced cellular senescence in cultured fibroblasts.

Phosphorylation of histone H2A variant (H2AX), induced at DNA double-strand break sites, has been recognized as a measure of DNA damage (41). We hypothesized that WRN-overexpressing cells are resistant to cigarette smoke-induced DNA damage. To evaluate this, WRN-overexpressing cells were cultured in the presence of CSE for various time periods. As shown in Figure 4C, WRN overexpression attenuated phosphorylation of H2AX compared with the control in the presence of CSE. These data suggest a protective role of WRN protein in CSE-induced DNA damage.

Modulation of Werner's Syndrome Protein Affects Cigarette Smoke-induced Cell Migration Impairment

It is known that exposure to CSE impairs cell migration/proliferation in cultured fibroblasts (42, 43). However, the effects of WRN protein defects on CSE-induced cell migration/proliferation remain to be elucidated. To evaluate this, WSF1 fibroblasts (WSF1 cells) and normal fibroblasts were cultured until confluence and were further incubated with or without 1.5% CSE for 24 hours. After creating a scratch wound, the serum-containing medium was refreshed with or without 1.5% CSE. As seen in Figure 5A, wound closure was delayed in WSF1 cells in the absence of CSE and exposure to CSE markedly impaired cell migration/proliferation in WSF1 cells compared with normal fibroblasts. In contrast, WRN protein overexpression significantly improved the rate of cell migration/proliferation in CSE-exposed fibroblasts (Figure 5A). These data indicate that WRN protein modulation significantly affects cell migration/proliferation in cigarette smoke-exposed fibroblasts.

NAC Alleviates Werner's Syndrome Protein Down-regulation and Growth Inhibition in Cigarette Smoke-exposed Fibroblasts

Previous *in vitro* studies demonstrated that exposure to CSE induces ROS production and oxidative stress (30, 44). However, little is known about ROS production in WRN protein-deficient cells. To evaluate this, WSF1 cells (AG03141) and normal dermal fibroblasts were cultured for 48 hours and a dihydroethidium oxidation assay was performed to estimate steady state levels of intracellular superoxide. CSE-exposed normal fibroblasts were used as a positive control for increased intracellular superoxide. Compared with normal fibroblasts, both WSF1 cells and CSE-exposed fibroblasts significantly increased intracellular ROS production (Figure 6A). These data suggest a potential link between cigarette smoke-induced WRN protein down-regulation and oxidative stress. We then tested whether pro-oxidants (i.e., H_2O_2) or an aldehydic by-product of oxidation reactions (i.e., acrolein), which are known components in cigarette smoke, decrease WRN protein in cultured fibroblasts. Both acrolein and H_2O_2 decreased steady state levels of WRN protein at 24 hours (Figure 6B) and inhibited cell growth without significant cell death at 48 hours (data not shown).

Because cigarette smoke-exposed cells accumulate intracellular ROS and reactive aldehydes (Figure 6A), we asked whether CSE-induced WRN down-regulation is blocked by a thiol antioxidant, *N*-acetylcysteine (NAC). To evaluate this, human pulmonary fibroblasts (HPFs) were cultured with or without 1.5% CSE in the presence or absence of 3 mM NAC for 48 hours. As shown in Figure 5C, NAC attenuated CSE-induced WRN protein down-regulation. In addition, NAC itself appears to increase WRN protein expression (Figure 6C). To confirm

actin. Immunoblotting data are representative of three experiments. (D) HPFs were treated as in (A). Cell density was measured at 48 hours. Data are expressed as means $\pm$ SEM for three independent experiments.

the biological relevance, cell density in the same groups was measured at 48 hours. NAC significantly reduced CSE-induced growth inhibition; however, NAC itself did not increase the growth rate in the absence of CSE (Figure 6D). These data suggest a significant role of redox regulation in WRN protein expression. In addition, an adequate level of WRN protein appears to be required, but not sufficient, for cell growth. These data again suggest a potential relationship between redox-mediated regulation of WRN protein expression and cell growth.

DISCUSSION

Both WS and an extensive smoking history result in clinical symptoms that resemble accelerated aging. There are no studies that have evaluated a possible connection between the protein responsible for WS and smoking-induced changes. In this study, we demonstrate that lung fibroblasts isolated from patients with a significant smoking history and severe emphysema exhibit a decrease in WRN protein expression. We confirm that exposure to CSE also down-regulates WRN protein expression. This induces the typical features of cellular senescence, growth inhibition, and defective cell migration. WRN protein over-expression partially rescues CSE-induced cellular senescence and abnormal cell migration/proliferation. In contrast, WRN protein defects sensitized CSE-exposed cells to develop a senescent-like phenotype. CSE-induced WRN protein down-regulation appears to be dependent on a ubiquitin-proteasome pathway. These data suggest a novel role of WRN protein in cigarette smoke-induced cellular senescence.

There are other cellular studies that link cigarette exposure to cellular senescence; however, none have addressed the mechanisms for this effect. Several *in vitro* studies have demonstrated that exposure to CSE significantly inhibits fibroblast proliferation (27, 45). In addition, some studies have shown that fibroblasts isolated from smokers with emphysema have a decreased proliferative capacity and a senescent phenotype (23, 40). Although the causative role of senescence in emphysema pathogenesis is not clear, growing evidence suggests that accumulation of senescent cells may contribute to age-related

Figure 6. N-Acetylcysteine (NAC) alleviates Werner's syndrome protein down-regulation and growth inhibition in cigarette smoke-exposed fibroblasts. (A) WSF1 cells (AG03141) in the absence of cigarette smoke extract (CSE) and normal dermal fibroblasts in the absence or presence of CSE were cultured for 48 hours, and then incubated at 37°C for 40 minutes with dihydroethidium (DHE, 10 μ M/L) in 2 ml of phosphate-buffered saline containing pyruvate (5 mmol/L). Cells were trypsinized on ice and analyzed by flow cytometry. The mean fluorescence intensity (MFI) of each sample (10,000 cells) was measured. Values indicate the ratio of MFI relative to control MFI. Data are expressed as means $\pm$ SEM for three independent experiments. (B) HFL-1 cells were cultured in the presence or absence of 40 μ M acrolein, or 200 μ M hydrogen peroxide, for 24 hours. Western blot analysis was performed for WRN protein. Equal loading was determined by stripping the blot and reprobing with antibodies to β -actin. Immunoblotting data are representative of three experiments. (C) human pulmonary fibroblasts (HPFs) were cultured with or without 1.5% CSE in the presence or absence of 3 mM NAC for 48 hours. Immunoblot analysis was performed for WRN protein. Equal loading was determined by stripping the blot and reprobing with antibodies to β -

pathology and tissue dysfunction in bone marrow (46), brain (47), and pancreas (48). These defects appear to be caused by impaired regeneration of cells in these tissues (49).

WRN protein is constitutively expressed and is known to be transcriptionally regulated by some tumor suppressor proteins (50). However, in our study cigarette smoke-induced WRN protein down-regulation appears to be modulated posttranscriptionally, although exposure to CSE certainly activate both Rb and p53 pathways in cultured fibroblasts (27). These data may indicate a novel role of posttranscriptional regulation in WRN protein modulation (e.g., posttranslational modifications such as phosphorylation or oxidation).

One intriguing culprit for the posttranslational modification is oxidative stress. An *in vitro* study demonstrated that WRN protein oxidation contributes to loss of enzymatic functions (51). However, no study has demonstrated effects of pro-oxidants or cytotoxic by-products of oxidation reactions on WRN protein expression. In this study, we showed that treatment with cigarette smoke and an aldehydic by-product of oxidation reactions (acrolein) as well as H₂O₂ leads to decreases in WRN protein. Harrigan and colleagues suggest an oxidative stress-induced effect on activity of WRN protein without altering levels of the protein (51). However, they measured WRN protein levels for only 1 hour in the presence of H₂O₂. We observed that H₂O₂ exposure decreases WRN protein in cultured fibroblasts at 24 hours (Figure 6). The duration time of oxidant exposure of at least 24 hours may be necessary to decrease WRN protein. Moreover, a thiol antioxidant, NAC, partially alleviates WRN protein down-regulation and growth inhibition in cigarette smoke-exposed fibroblasts, suggesting the possible existence of a causal relationship between these phenomena. In nontransformed cells, WRN protein is required for cell proliferation. *In vitro* studies demonstrated that WRN protein defects from loss-of-function mutations or RNA interference result in an accelerated senescence phenotype (14, 15). In transformed cells, WRN protein may play a dual role in cell proliferation. Epigenetic silencing of WRN gene expression occurs in many tumor types and WRN restoration suppresses tumor growth, which suggests a role of

WRN in tumor suppression (52). In contrast, most transformed cell lines expressing WRN protein require WRN protein for cell proliferation, which suggests a role of WRN protein in tumorigenesis (53). In our study, WRN protein restoration by retroviral transduction attenuated CSE-induced growth inhibition. WRN protein may promote cell proliferation in the presence of oxidative stress and subsequent DNA damage by maintaining sufficient DNA repair and transcription activity.

This study demonstrates that exposure to CSE induces cell growth inhibition that is mediated, at least in part, via a ubiquitin-proteasome-mediated decrease in WRN protein expression (Figure 3). These data suggest a novel role of WRN protein in prevention of cellular senescence and defective cell migration associated with cigarette smoking. Future studies will be required to determine what oxidative stress-associated modifications of WRN protein are important for degradation of WRN protein and how this decrease in WRN protein expression is linked to disease processes associated with cigarette smoke such as premature aging, atherosclerosis, cancer, or emphysema.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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