Interplay between Selenium Levels, Selenoprotein Expression, and Replicative Senescence in WI-38 Human Fibroblasts*

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Background: Selenoproteins are important enzymes for antioxidant defense, redox homeostasis, and redox signaling. Our data indicate that the selenoproteome is regulated at the translational and mRNA levels in WI-38 cells. Selenium is an essential trace element, which is incorporated as selenocysteine into at least 25 selenoproteins using a unique translational UGA-recoding mechanism. Selenoproteins are important enzymes involved in antioxidant defense, redox homeostasis, and redox signaling pathways. Selenium levels decline during aging, and its deficiency is associated with a marked increase in mortality for people over 60 years of age. Here, we investigate the relationship between selenium levels in the culture medium, selenoprotein expression, and replicative life span of human embryonic lung fibroblast WI-38 cells. Selenium levels regulate the entry into replicative senescence and modify the cellular markers characteristic for senescent cells. Whereas selenium supplementation extends the number of population doublings, its deficiency impairs the proliferative capacity of WI-38 cells. We observe that the expression of several selenoproteins involved in antioxidant defense is specifically affected in response to cellular senescence. Their expression is selectively controlled by the modulation of mRNA levels and translational recoding efficiencies. Our data provide novel mechanistic insights into how selenium impacts the replicative life span of mammalian cells by identifying several selenoproteins as new targets of senescence.

Conclusion: Our data indicate that the selenoproteome is regulated at the translational and mRNA levels in WI-38 cells. Replicative senescence is controlled by selenium levels and selectively modulates selenoprotein expression.

Significance: We have characterized a strong interplay between selenium levels, selenoprotein expression, and replicative senescence.

Selenium is an essential trace element, which is incorporated as selenocysteine into at least 25 selenoproteins using a unique translational UGA-recoding mechanism. Selenoproteins are important enzymes involved in antioxidant defense, redox homeostasis, and redox signaling pathways. Selenium levels decline during aging, and its deficiency is associated with a marked increase in mortality for people over 60 years of age. Where selenium supplementation extends the number of population doublings, its deficiency impairs the proliferative capacity of WI-38 cells. We observe that the expression of several selenoproteins involved in antioxidant defense is specifically affected in response to cellular senescence. Their expression is selectively controlled by the modulation of mRNA levels and translational recoding efficiencies. Our data provide novel mechanistic insights into how selenium impacts the replicative life span of mammalian cells by identifying several selenoproteins as new targets of senescence.

Significance: We have characterized a strong interplay between selenium levels, selenoprotein expression, and replicative senescence.

Selenium is an essential element for human health, known for its antioxidant properties at trace levels. It is incorporated into 25 selenoproteins as selenocysteine, a rare amino acid implicated in redox reactions when present in the active site of enzymes (1). Many selenoproteins are involved in antioxidant defense, redox homeostasis, and redox signaling through the action of glutathione peroxidases (Gpx1–Gpx6), methionine sulfoxide reductase of (MrsB1 or SelX), thioredoxin reductases (TR1–TR3), and endoplasmic reticulum (ER) selenoproteins (Sel15, SelS, SelK, and SelM). However, about half of selenoproteins remains without specific function. Selenoprotein synthesis follows an unusual mechanism, which involves the translational recoding of a UGA codon, normally used as a stop signal. A selenocysteine insertion sequence (SECIS), present in the 3′-UTR of selenoprotein mRNAs, is the platform for an mRNP assembly to direct faithful UGA recoding as selenocysteine (2–6). Among the recoding factors involved in the mRNPs, SECIS-binding protein 2 (SBP2) has been proposed to dictate selenocysteine insertion efficiency (7–10). Acting as a platform, SBP2 recruits the selenocysteine-specific elongation factor (EFsec) complexed with the tRNA^Sec and delivers it to the ribosome, in which L30 is acting as an anchor (11).

Aging is a slow, complex, and multifactorial process, resulting in a gradual and irreversible decline of various functions of the body. The replicative senescence of cultured human diploid fibroblasts has been proposed to recapitulate several cellular aspects of organismal aging. Indeed, the number of senescent cells increases with age, and senescence-associated phenotypes are considered to be predictive features of age-related pathologies and longevity in humans (12, 13). Replicative senescence is associated with morphological changes, accumulation of oxidized proteins, telomere shortening, and increased positive cells for senescence-associated β-galactosidase (SABG) and for senescence-associated heteromochromatin foci (SAHF). These
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Changes are often associated with a decreased efficiency of antioxidant defense mechanisms and repair of oxidized molecules (14). The p53-p21 and p16-pRb signaling pathways mainly control the cellular senescence; however, upstream regulators and downstream effectors of the telomere-associated senescence remain unclear. The historical and well accepted model to study replicative senescence is the use of WI-38 cells that are diploid fibroblasts derived from human embryonic lung tissue. They have a finite lifetime of ~55 cumulative population doublings (CPDs), also known as the Hayflick limit. When senescent, WI-38 cells display all of the characteristics described above.

Only a few analyses have investigated a link between selenium, age, and longevity. One study conducted in Italy on healthy subjects showed a decrease in serum selenium and in selenium-dependent Gpx activity with age (15). This drop is particularly noteworthy in people over 60 years of age. It has been subsequently shown in two independent studies on elderly people that selenium level in the blood was a predictor of longevity (16, 17). These studies suggest that low selenium levels lead to a weakening of the antioxidant defense and show that the concentration of blood selenium is an important predictor of longevity in elderly patients, although the molecular mechanisms and selenoproteins involved remain to be elucidated. At a cellular level, only studies performed in 1987 (18), before many selenoproteins were identified and characterized, report an extension of the replicative life span of bovine adrenocortical cells with 20 nM selenium supplementation of the culture medium.

The expression of selenoproteins is controlled by the bioavailability of selenium in the body or in the culture medium. Long term selenium deficiency is hypothesized to decrease antioxidant defenses and redox-regulatory pathways through a deregulation of selenoprotein expression. To investigate the potential role of selenium in replicative senescence, we follow the proliferative capacity of WI-38 cells in response to varying selenium concentrations of the culture media, defined as depleted (Dpl), control (Ctrl), and supplemented (Sup) conditions. These changes in selenium concentration that regulate the expression of selenoprotein hierarchically in several cell models are used to reproduce in vitro the deficient, adequate, and supplemented levels observed in the human cohorts (19–21). In the present work, we show that the change in selenium concentration in young cells triggers rapid changes in replicative senescence-associated markers and signaling pathways and regulates the entry into replicative senescence. A comprehensive analysis of selenoproteins involved in antioxidant defense identified novel targets of the replicative senescence. We find that the altered expression of selected selenoproteins occurs simultaneously at the level of mRNA abundance and translational GUC recoding efficiency. A change in SBP2 localization between the cytoplasm and nucleus in response to senescence is associated with the modulation of selenoprotein synthesis. Our data expand the repertoire of mechanistic controls of selenoprotein expression and open novel anti-aging strategies.

**EXPERIMENTAL PROCEDURES**

*Cell Line and Culture Conditions*—Human embryonic fibroblasts WI-38 were purchased from Sigma (passage 15, estimated CPD 30), grown, and maintained in DMEM supplemented with 10% fetal calf serum (FCS). Different culture media, referred to as Ctrl, Dpl, Dpl + Se, and Sup, were used according to Refs. 20–22. Selenium concentration was determined by inductively coupled plasma MS in a FCS lot number, which was kept throughout the experiments. Therefore, selenium concentration in the Ctrl medium was 15 nM. In the Dpl medium, we expected 3 nM selenium because 2% FCS was used instead of 10%. To cope with the decrease of growth factors in Dpl medium, 5 mg/liter transferrin, 10 mg/liter insulin, 100 μM 3,5,3'-triiodothyronine, and 50 nM hydrocortisone were added, as described and validated previously for selenoprotein expression studies (20–22). Supplemented medium was made by an addition of 30 nM sodium selenite to the Ctrl medium, which resulted in a total of 45 nM selenium. The Dpl + Se medium was obtained by adding 30 nM sodium selenite to the Dpl medium. Cells were cultured in 5% CO2 at 37 °C and humidified atmosphere. Cells were passaged in a new flask at 10,000 cells/cm2 when they reached confluence. WI-38 cells were counted at each passage to calculate the population doubling (PD) using the equation, \( \Delta PD = \frac{\text{log}(\text{final cell number}/\text{initial cell number})}{\log(2)} \). Cumulative PD was plotted as a function of time.

*Protein Extraction and Western Immunoblotting*—Whole protein extracts were obtained with cell lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM DTT, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). Equal protein amounts (50 μg) were separated in BisTris NuPAGE Novex Midi Gels (Invitrogen) and transferred onto nitrocellulose membranes using the iBlot dry blotting system (Invitrogen). Antibodies were purchased from Epitomics (Gpx1, Sel15, SelP, and SelM), Abfrontier (Gpx4 and TR1), Sigma-Aldrich (TR2, SelS, SelK, and actin), Cell Signaling (p16, p21, and pRb), Santa Cruz Biotechnology, Inc. (p53), and ProteinTech (SBP2). Membranes were probed with primary antibodies (as indicated) and HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Sigma). Detection of total carbonyl contents in cell extracts was performed with the Protein Oxidation Detection Kit purchased from Millipore. Protein carbonyls from cell extracts (5 μg) were derivatized using 2,4-dinitrophenylhydrazine, separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and revealed with an anti-2,4-dinitrophenol antibody according to the manufacturer’s instructions. The chemiluminescence signal was detected using the ECL Advance Western blotting detection kit (GE Healthcare) and revealed by the LAS3000 CCD camera (GE Healthcare).

*Microscopy Analyses*—WI-38 cells were washed in PBS and fixed for 5 min at room temperature in a 2% formaldehyde, 0.2% glutaradehyde solution. To detect SAHF, the cells were permeabilized by PBS, 0.1% Tween and stained with 0.13 μg/ml DAPI for 2 min at room temperature. To detect SABG, the cells were then washed and incubated 16 h at 37 °C (without CO2) with fresh senescence-associated β-gal stain solution composed of 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), 40 mM citric acid/sodium phosphate, pH 6, 5 mM.
potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM de MgCl₂. To detect lysosomal β-gal, staining solution was buffered at pH 4.

To perform cellular localization of SBP2, young and senescent cells were seeded and grown on coverslips in 6-well plates; washed with PBS; fixed in 4% paraformaldehyde, PBS; permeabilized with 0.5% Triton, PBS; and blocked in 1% BSA, PBS. Coverslips were incubated with anti-SBP2 antibody at 4 °C overnight, washed with PBS, and incubated with an Alexa Fluor 488 dye-conjugated anti-rabbit secondary antibody (Invitrogen). Nuclei were stained with DAPI. Epifluorescence images were captured in 10 Z-sections on an AxioObserver Z1 microscope (Zeiss) equipped with an Evolve EMCCD camera (Roper Scientific). Image analysis was performed with National Institutes of Health ImageJ software.

**Measurement of Telomere Length by qPCR**—Genomic DNA was extracted with the DNeasy blood and tissue kit (Qiagen). Concentration and purity of extracted genomic DNA were determined by A_{260}/A_{280} measured on a NanoVue spectrophotometer (GE Healthcare). The telomere length measurement assay consisted of a quantitative PCR performed in triplicate using LightCycler® 480 SYBR Green I Master (Roche Applied Science) according to Ref. 23. The amplification program for telomeric PCR was as follows: 30 cycles of (i) 1-s denaturation (96 °C) and (ii) 60-s annealing (54 °C) with fluorescence data collection. The amplification program for single gene copy PCR was as follows: eight cycles of (i) 15-s denaturation (95 °C), (ii) 1-s annealing (58 °C), (iii) 20-s extension (72 °C), followed by 35 cycles of (i) 1-s denaturation (96 °C), (ii) 20-s annealing (58 °C), (iii) 20-s extension (72 °C), and (iv) 5 s at 83 °C with data collection. The primers used are listed in Table 1.

**Enzymatic Activities**—Gpx activity was measured in an enzymatic coupled assay according to Ref. 21. The reaction mixture was composed of 50-µg protein extracts, 0.25 mM NADPH, 2 mM reduced L-glutathione, 1.5 IU of glutathione reductase adjusted to a total volume of 250 µl with 50 mM potassium phosphate buffer, pH 7.5. Reaction was started by the addition of 300 nm tert-butyl hydroperoxide, and consumption of NAPDH was followed at 340 nm with the Fluostar Optima reader (BMG Labtech). Enzymatic activities (units/mg) were expressed as nmol of glutathione·min⁻¹·mg⁻¹. TR activity was measured as described (24). Briefly, the catalytic reduction of oxidized 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by TR is coupled with the oxidation of one molecule of NAPDH. In the assay, the recycling of NAPD⁺ in NAPDH is associated with the conversion of DTNB into 5-thio-2-nitrobenzoic acid, which is visible at 410 nm. The reaction mixture was composed of 50 µg of protein extracts, 0.2 mM NADPH, 10 mM EDTA, 0.2 mg/ml BSA, adjusted to a total volume of 250 µl with 50 mM potassium phosphate buffer, pH 7.5. The reaction was started by the addition of 25 mM DTNB, and production of 5-thio-2-nitrobenzoic acid was followed at 340 nm. Enzymatic activities (units/mg) were expressed as nmol of NAPDH·min⁻¹·mg⁻¹. The enzymatic assays were performed in duplicates from three independent experiments.

**RNA Extraction and Real-time PCR**—Total RNAs were extracted from cells using the Nucleospin RNA II kit (Macherey Nagel), and concentration and purity of extracted RNAs was determined by A_{260}/A_{280} measured on a NanoVue spectrophotometer (GE Healthcare). Synthesis of cDNA was carried out with 2.5 µg of RNA, using the Transcriptor High Fidelity cDNA Synthesis kit (Roche Applied Science) according to the manufacturer’s instructions. Real-time PCR experiments were performed in triplicate using LightCycler® 480 SYBR Green I Master (Roche Applied Science). The amplification program was as follows: 5-min denaturation (95 °C); 45 cycles of (i) 10-s denaturation (96 °C), (ii) 20-s annealing (60 °C), and (iii) 20-s elongation (72 °C). Data were analyzed using LightCycler® 480 software and normalized relative to the mRNA levels of Hprt. The primers used are listed in Table 1.

**Evaluation of UGA Recoding Efficiency Using Luciferase Activity**—Turbofect reagent (4 µl; Fermentas) was used to transfect WI-38 cells with 1 µg of luciferase and 1 µg of GFP plasmids per well in 6-well plates according to the manufacturer’s instructions. The luciferase constructs (Luc UGA/Gpx1, Gpx4, TR1, TR2, SelX or SelM, and Luc UGU/SelX) were described previously (20). Cells were harvested 24 h post-transfection in lysis buffer and analyzed for luciferase activity as described previously (20, 25) with the Lumistar reader (BMGLabtech). In all cases, the transfection efficiency was normalized over GFP fluorescence detected by the Fluostar Optima microplate reader (BMGLabtech).

**RESULTS**

**Selenium Levels Extend the Replicative Life Span of WI-38 Human Fibroblasts**—It has been reported that selenium levels in the blood are associated with the survival rate of human populations (16, 17), suggesting an interplay between selenium and longevity. Moreover, selenium supplementation has been reported to increase replicative life span in bovine adrenocortical cells (18). To examine this relationship in a human cellular model for replicative senescence, we have used previously validated growth media for WI-38 fibroblasts with various selenium concentrations mimicking adequate, deficient, and supplemented dietary intake (see “Experimental Procedures”). These conditions are referred to here as Ctrl, Dpl, and Sup, respectively. Another condition named Dpl+Se was used to verify that the effect observed in Dpl medium was essentially due to the deprivation of selenium. The proliferative capacity of the fibroblasts is measured by the population doubling at each passage. The CPD is then represented as a function of time to follow the life span of WI-38 from the mitotically active to the senescent state, as illustrated in Fig. 1. As described in the literature, the cells grown in Ctrl conditions maintained their proliferative capacity until CPD 51 and then became presenescent and reached the senescence phenotype at CPD 57 (Fig. 1A). When cells mitotically active (CPD 36) were separated and maintained in the different growth conditions, a significant effect of selenium concentration on population doubling was readily observed during the second passage (see the enlargement panel in Fig. 1A). It is noteworthy that the growth of WI-38 cells in Ctrl and in Dpl+Se conditions was very similar after two passages, indicating that the effect on population doublings in Dpl medium was essentially due to selenium deficiency. Additionally, in all cases, selenium supplementation led to an extended replicative lifetime. This alteration of cellular
proliferation was accompanied by morphological changes (Fig. 1B), especially in the Dpl conditions, where cells assumed a flattened senescence-associated morphology. Our data extend the observations from previous reports in showing that the selenium concentration in the culture medium affects the proliferative capacity of human diploid fibroblasts and regulates the entry into senescence.

The Selenium Levels Modify Senescence-associated Markers and Signaling Pathways—To monitor whether selenium concentration in the culture medium had an influence in the senescence-associated phenotype, we maintained young proliferating WI-38 fibroblasts in Dpl, Ctrl, and Sup media for two passages, which was sufficient to alter the population doubling, and analyzed different senescence-associated markers that have been validated for this cell line, including SAHF, SABG, and telomere length (Fig. 2, A–C). First, in the nucleus of WI-38 senescent cells, the chromatin underwent a dramatic remodeling, leading to the formation of so-called SAHF, that are thought to participate in the irreversible cell cycle arrest by repressing proliferation-promoting genes, such as E2F target genes. SAHF are characteristic of transcriptionally silent heterochromatin (26). Here we quantified the presence of compacted punctate DAPI-staining foci in the nucleus of young cells grown in the different media in comparison with senescent cells grown in the Ctrl medium. As shown in Fig. 2A, an almost 3-fold increase of SAHF-positive cells was observed between senescent and young cells grown in the Ctrl condition. Interestingly, in young cells grown in Dpl conditions, a similar increase was also observed in comparison with Ctrl counterparts, suggesting the emergence of transcriptionally silent heterochromatin.
matin in response to selenium deficiency (Fig. 2A). Another biomarker commonly used to characterize senescence status is the cytoplasmic SABG activity. As illustrated in Fig. 2B, an important increase in SABG-positive cells (reaching almost 100%) was observed in senescent WI-38 as compared with proliferating cells grown in the Ctrl medium. Interestingly, in young cells, we noted an inverse correlation between the level of selenium in the media and the percentage of SABG-positive cells, with an almost 3-fold difference between Sup and Dpl conditions. Another key determinant of the replicative life span of WI-38 cells is the gradual erosion of telomere length that results in cell cycle arrest. In agreement with the data in the literature (23), we observed a significant senescence-associated erosion in telomere length in Ctrl conditions (i.e. a loss of around 700 bp) (Fig. 2C). Interestingly, a similar alteration was obtained for young WI-38 cells grown in Dpl medium, which may explain the reduction of proliferating rate for these cells. In comparison, a significant maintenance of telomeric length appeared in Sup conditions, indicating an important role of selenium concentration in this feature. Taken together, our data indicate that the regulation of the life span of WI-38 cells by selenium levels of the culture media had many characteristics in common with the described phenotype of replicative senescence.

The entry into replicative senescence of cultured human diploid fibroblasts is due to an irreversible cell cycle arrest mainly controlled by the p53-p21 and p16-pRb signaling pathways (12, 13). Here, given the differences in proliferative capacity in response to selenium levels, we investigated the expression of these cell cycle proteins in young WI-38 cells cultured for two passages in Dpl, Ctrl, and Sup conditions. As shown in Fig. 2D, we observed that selenium supplementation significantly reduced (more than 3 times) the expression levels of p16, p21, p53, and pRb compared with Ctrl conditions. Inversely, selenium depletion raised the expression of p16, p53, and pRb, but not p21. Our results strongly suggest that the selenium concentration regulates the proliferation rate of WI-38 cells by regulating the expression levels of cell cycle proteins involved in replicative senescence.

The Levels of Oxidized Proteins Are Affected in Response to Selenium Variation—Oxidized proteins accumulate in senescent cells, due to an increased production of ROS, an impairment of proteasomal degradation, and a decrease in protein repair systems. To evaluate how much the concentration of selenium modified the levels of oxidized proteins in young cells, we determined the relative amount of carbonylated proteins using oxiblot assays (see “Experimental Procedures”). Several studies have reported an increase of carbonylated proteins dur-
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FIGURE 2. Senescence-associated characteristics and signaling pathways are modified in young WI-38 cells by the selenium levels. Young cells (gray bars) were cultivated for two passages in Dpl, Ctrl, and Sup medium and evaluated for senescence-associated markers in comparison with senescent cells grown in Ctrl conditions (black bars). A, the percentage of SAHF-positive cells is represented for the different growth conditions. B, the proportion of cells positive for SABG was measured in the different growth media. C, the length of telomeric DNA was quantified by qPCR on genomic DNA and normalized relative to a single copy gene (β-globin). The data are represented as the mean of three independent experiments ± S.D. (error bars). The significant -fold change differences between the different growth conditions and/or proliferative state are indicated above the respective brackets (p < 0.05). D, protein extracts from young WI-38 cells were evaluated for p16, p21, pRb, and p53 expression levels by immunoblotting, relative to actin (Ctrl condition being set at 1). E, these protein extracts were also analyzed by oxiblot in triplicates. The mean of the total signal from each lane is indicated below the immunoblot. Apparent molecular mass markers are indicated in the right side in kDa.

As illustrated in Fig. 2E, we found that the selenium levels of the culture media dramatically controlled the levels of carbonylated proteins. A 15-fold increase in the levels of oxidized proteins was detected in Dpl as compared with Sup conditions, indicating a dramatic impairment of antioxidant defense in selenium deficiency, presumably via a marked decrease of antioxidant selenoproteins. Our data suggest that the change in selenium concentration of the culture media rapidly alters the balance between ROS production and scavenging, which could subsequently lead to the activation of several senescence-associated characteristics.

Glutathione Peroxidase and Thioredoxin Reductase Activities Respond Differently to Selenium Variation and Senescence—Among the selenoproteins expressed in mammals, the Gpxs are well characterized in preventing ROS production by the reduction of hydrogen peroxides. Only Gpx1 and Gpx4, which are ubiquitous, are abundant in WI-38 cells, Gpx2, Gpx3, and Gpx6 being expressed essentially in intestinal, kidney, and olfactory tissues, respectively (29). For their part, TRs are essential oxidoreductases that use NADPH to reduce oxidized thioredoxin or other small molecules containing sulfur, selenium, or oxidized semiquinone (30) and therefore are involved in numerous cellular processes. TR1 and TR2 are expressed ubiquitously, in cytoplasm and mitochondria, respectively, whereas TR3 is only detected in testis (nucleus or ER) (30). To investigate how much selenium levels and replicative senescence control the antioxidant activities of these selenoproteins, we have analyzed their expression using enzymatic assays. As illustrated in Fig. 3, after two passages of young WI-38 cells in the different media, Gpx and TR activities were highly dependent on selenium concentration. An almost 30-fold increase was detected between extracts from Dpl and Sup conditions for Gpx activity (Fig. 3A). In comparison, TR activity was only stimulated ~5-fold by selenium supplementation (Fig. 3B). Then, when comparing the enzymatic activities between young and senescent cells grown in Ctrl medium, we surprisingly noted opposite changes between TR and Gpx. An almost 50% decrease in Gpx activity was detected in senescent cells, whereas an approximately 40% stimulation was measured for TR activity. Thereafter, to investigate whether these selenoproteins were still responsive to selenium variation in old cells, we analyzed Gpx and TR activities for senescent cells grown in other selenium conditions.
Unfortunately, we were unable to measure enzymatic activities in Dpl extracts due to a limited yield of proteins in this growth condition. However, we noted a strong stimulation of both TR and Gpx activities by long term selenium supplementation in senescent cells, indicating that these selenoproteins were still sensitive to this stimulus. Taken together, our data indicate that members of TR and Gpx selenoproteins, which are essential antioxidant enzymes, are up- and down-regulated, respectively, in response to replicative senescence.

Analysis of Selenoprotein Expression in Response to Selenium and Aging—In order to further our analysis on antioxidant selenoproteins, we have investigated the influence of selenium and replicative senescence on the expression of proteins located either in the cytoplasm (TR1, Gpx1, and SelP), the mitochondria (TR2 and Gpx4), or the ER (SelS, SelM, Sel15, and SelK) (Fig. 4). Selenoproteins that are present in the ER were quite recently shown to participate in protein folding with the assistance of ER chaperones and/or ER-associated degradation of misfolded proteins (31). The regulation of the selenoproteome is hierarchically tuned according to selenium intake. A differential sensitivity to selenium fluctuation is thought to maintain essential selenoproteins at the expense of the others, with a regulatory mechanism that is cell line- and tissue-dependent. This has been particularly described for Gpx1 and Gpx4, two members of the Gpx family that are ubiquitously expressed. It appeared from experiments performed in animals and in cultured cells that Gpx1 expression was more affected than Gpx4 by selenium availability (19–21). In young WI-38 cells, we confirmed this differential regulation, with Gpx1 being ~5-fold more responsive to selenium variation than Gpx4 (Fig. 4). Additionally, we could classify the selenoproteins studied as highly (SelM, SelS, Gpx1, and Gpx4), moderately (SelK, Sel15, TR1, and TR2), and weakly regulated (SelP) by selenium levels. This result extended previous descriptions of differential control of selenoprotein expression by selenium concentration in a novel cell line. However, when comparing the expression levels of these selenoproteins between young and senescent cells grown in Ctrl medium (Fig. 4), we observed a totally different hierarchy of expression, with proteins either down-regulated (Gpx4, SelS, SelK, and SelP), poorly affected (Gpx1, SelM, and Sel15), or up-regulated (TR1 and TR2) in response to replicative senescence. It confirmed that the change in enzymatic activities observed for Gpxs and TRs resulted from a modulated expression of the respective selenoproteins.

Selenoproteins Are Regulated by mRNA Levels in Response to Selenium Variation and Senescence—Selenoprotein expression is controlled at various levels in cells. First, like many other proteins, a change in mRNA can be responsible for variation of expression. However, due to the unique translational mechanism involved for selenocysteine insertion, a control of UGA recoding efficiency can also be implicated in this selenocysteine-regulated pattern of expression. To begin, a comprehensive analysis of the steady state levels of the 25 selenoprotein mRNAs was performed by RT-qPCR. As shown in Fig. 5, only 19 transcripts were detectable in WI-38 cells, with TR1, Gpx4, SelM, SelN, Sel15, and Gpx1 being the most abundant. Interestingly, in young WI-38 cells, we observed that in response to selenium supplementation, 12 selenoprotein mRNAs were significantly up-regulated (from 1.6 to 9.6 times), and only Sel15 mRNA was decreased (Fig. 5A). Subsequently, in response to senescence (in Ctrl medium), a strong reduction was detected for seven selenoprotein mRNAs (including SelN, SelT, SelH, Sps2, SelX, SelP, Dio2, and Gpx2); only Sel15 and Gpx6 mRNAs were increased (Fig. 5C). The comparison between selenium- and senescence-associated regulation resulted in a group of nine mRNAs, which were affected upon selenium deficiency and replicative senescence (Fig. 5C). Our data indicated that changes in mRNA levels account for the regulation of more than half of the selenoproteins detected in this cell line. Nevertheless, in several cases, no change was detected at the mRNA level, whereas a dramatic change was seen at the protein level, suggesting an additional translational control. This is particularly true for SelS, SelK, SelP, TR1, and TR2.

The Efficiencies of UGA Recoding as Selenocysteine Govern the Selenoprotein Expression in Response to Selenium Variation and Senescence—As mentioned earlier, selenoprotein synthesis follows an intricate mechanism that is tightly controlled at the level of translation during the UGA-selenocysteine recoding process. To precisely evaluate the influence of selenium supply and replicative senescence at this step in WI-38 fibroblasts, we used a previously described set of validated luciferase-based reporter constructs (Fig. 6A) (20, 25). As reported for the
HEK293 cell line (20), we found that in WI-38 cells, UGA-re-coding activities were also highly dependent on selenium concentration for all luciferase-SECIS tested (Fig. 6B). However, only a subset of reporter constructs could be analyzed due to low transfection efficiencies and expression levels of the plasmids inherent to WI-38 cells. We confirmed that the SECIS element was a key determinant in modulating selenocysteine insertion in WI-38 in response to selenium variation (from 2.9-
FIGURE 6. The response of UGA/Sec recoding efficiency as a function of selenium levels and replicative senescence depends on the nature of the SECIS element. A, representation of the luciferase reporter assay used for the analysis of UGA recoding efficiency. The open reading frame of firefly luciferase with a UGA/Sec codon at position 258 is linked to different SECIS elements. A control construct of the Luc UGA/SelX was made, where the UGA was replaced by a UGU/cysteine. B, young cells were cultivated for two passages in either Dpl, Ctrl, or Sup medium and transfected with luciferase plasmid. Luciferase activities obtained with UGA construct were normalized relative to the signal measured from LucUGU/Gpx4 transfection. Data from three independent experiments were arbitrarily expressed relative to the activity obtained in young Ctrl extracts (set at 100%) ± S.D. (error bars). C, comparison of the UGA/Sec recoding efficiencies between young and senescent fibroblasts grown in Ctrl medium. D, cellular localization of SBP2 in young and senescent cells.
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to 16.5-fold change between Sup and Dpl). Then the luciferase activities were compared between young and senescent cells grown in Ctrl medium for the various constructs (Fig. 6C). Strikingly, a different response was observed, but it was also dependent on the nature of the SECIS element. Thus, whereas selenocysteine insertion was reduced for Gpx4 (−46%), SelX (−62%), and SelM (−40%) in old cells, it was increased for TR1 (+71%) and TR2 (+520%) and unchanged for Gpx1 reporter constructs. These data demonstrated that several selenoproteins and potentially others were regulated by a novel translational control mechanism in response to replicative senescence. To grasp a possible insight into this process, we investigated the cellular localization of SBP2, a key determinant in controlling selenoprotein synthesis, which has been shown to shuttle between cytoplasm and nucleus in response to oxidative stress conditions (8). In young WI-38 cells grown in Dpl conditions, SBP2 was predominantly detected in the cytoplasm. However, in almost all senescent cells, we observed a translocation of SBP2 in the nucleus. Given the central action of SBP2 in the nucleus, we hypothesized that the change in the pattern of UGA recoding efficiencies could result in a differential assembly of SBP2 to selenoprotein mRNAs. Taken together, our results demonstrated that a complex network between translational UGA/Sec recoding efficiencies and the levels of mRNAs controlled the expression of the selenoproteome in response to replicative senescence.

DISCUSSION

In the present work, we delineate how the selenium concentration in the culture medium significantly and rapidly influences the replicative life span of WI-38 cells by regulating the entry into senescence. The proliferation of WI-38 fibroblasts is a model of replicative senescence that shares several similarities with organismal aging, such as morphological changes, telomere shortening, increased oxidative damages, and activation of the pRb signaling pathway. We find that whereas selenium supplementation extends the number of population doubling, its deficiency dramatically impairs the proliferative capacity of WI-38 cells. Indeed, after two passages of young cells in the Dpl medium, the fibroblasts present several characteristics very similar to those of senescent cells, which include morphological changes, increased number of positive cells for SAHF and SABG, and telomere length shortening. Our data strongly suggest that the down-regulation of antioxidant selenoproteins during selenium deficiency raises the production of ROS, which thereby create oxidative damage and activate replicative senescence-associated signaling pathways, including p53, p16, p21, and pRb. On the other hand, selenium supplementation of the culture media, by increasing antioxidant defense capacity and limiting the production of ROS, has a chemopreventive action on replicative senescence.

In young cells, one can consider the culture condition of selenium deficiency (Dpl) as a prolonged impairment of essential antioxidant selenoproteins that generates a constant oxidative stress and accumulation of ROS-induced cellular damage. First, Gpx1 and Gpx4, which are highly sensitive to selenium concentration (Figs. 3 and 4), act as a major defense against ROS by detoxifying hydrogen hydroperoxides and lipid peroxides. The Gpxs use GSH as a co-factor, which is subsequently recycled by glutathione reductases. The reduction of Gpx in cytosol (Gpx1) and in mitochondria (Gpx4) results in an increase in ROS production and subsequently in oxidized molecules and DNA damage (29, 32). Then TR1 (cytosol) and TR2 (mitochondria), which are less sensitive to selenium concentration than the Gpxs (Fig. 3 and 4), catalyze the NADPH-dependent reduction of oxidized thioredoxin and therefore participate in defense against oxidative stress, DNA synthesis, and redox signaling. Thereby, any imbalance of the TR/thioredoxin system dramatically impairs cellular homeostasis and cell cycle progression (30). In parallel, the ER-resident selenoproteins that include SelS, SelK, Sel15, and SelM, which have important functions in protein folding and ER stress response (31), could also participate in the senescence phenotype. Taken together, the lack of these selenium-sensitive selenoproteins in young cells in Dpl medium is expected to decrease antioxidant defense in different cellular compartments and thereafter activate the entry into senescence.

In young WI-38 cells, the accelerated senescence phenotype observed during selenium deficiency is accompanied by a decreased expression of almost all selenoproteins, including the Gpxs, TRs, and ER members. It is conceivable that only subset members are causing this phenotype. Indeed, in cells reaching senescence in Ctrl medium, only several selenoproteins, which include SelS, SelK, SelP, and, to a lesser extent, Gpx4, Sel15, and SelM, are down-regulated in comparison with young cells. Interestingly, the expression of TR1 and TR2 is up-regulated in senescent cells. Glutathione and thioredoxin systems are believed to work in parallel with several overlapping functions, but recently, accumulating evidence has shown a cross-talk between the two systems and possible mutual back-ups (33). Our results suggest that the senescent fibroblasts compensate for the decrease of the Gpxs/glutathione system by enhancing the TR/thioredoxin system, although insufficiently to avoid cell cycle arrest. On the other hand, an imbalance between the two systems could also signal cell cycle arrest and increasing redox damage.

In senescent cells, we characterize a novel selective regulation of selenoprotein expression, referred to as hierarchy and only described for selenium fluctuation. We establish that this regulation results from different control mechanisms at the stage of mRNA transcription/stability and translation. A dramatic change of selenoprotein mRNAs is observed for 11 cases of the 19 detected in WI-38 (Fig. 5); SelN, SelT, SelH, Sps2, SelX, SelP, Dio2, SelV, and Gpx6 are decreased (minus 50–97%), and Sel15 and Gpx2 are increased (plus 62–490%). This variation is unlikely to be due to activation of NRF-2 transcription factor because TR1 mRNA levels are not affected by variation of selenium levels or by replicative senescence in WI-38. Additionally, we find that selenocysteine insertion efficiency is also selectively modulated in response to replicative senescence by the nature of the SECIS element. We propose that this translational control results from the change in subcellular localization of SBP2. Indeed, many studies indicate that the SECIS-binding protein SBP2 is a key regulator of selenoprotein expression (4, 7–10). Its nucleocytoplasmic shuttling, which allows effective assembly of selenoprotein mRNP com-
complexes (34, 35), is stimulated upon $\text{H}_2\text{O}_2$-induced oxidative stress (8). One can speculate that the increase in oxidative stress during replicative senescence induces the nucleocytoplasmic shuttling of SBP2, by the reversible oxidation of redox-sensitive cysteine residues as described previously (8). In the nucleus, selenoprotein mRNP assembly is controlled by the Hsp90 chaperone machinery (Hsp90, R2TP, and Nufip) (36). In parallel, another nuclear protein, elf4A3, has been recently characterized as a selective regulator of selenocysteine insertion by preventing SBP2-SECIS complex formation for a subset of selenoprotein mRNAs (37). In any case, relocalization of SBP2 in the nucleus during senescence would certainly modify its stoichiometry versus auxiliary (Hsp90 and co-chaperones) and competing (elf4A3) factors to affect transcript-selective mRNP formation and therefore mRNA translation efficiency in the cytoplasm.

In conclusion, although the participation of the selenoproteins in organismal aging remains to be clarified, the changes in Gpx4, SelS, SelK, SelP, TR1, and TR2 expression can now be considered as novel biomarkers of replicative senescence in WI-38 cells. In summary, we provide evidence for an interplay between selenium concentration, selenoprotein expression, and cellular senescence in this human diploid fibroblast.

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