Chapter 5

Differential protein expression in senescent HUVECs

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Abstract

Senescence is a cellular process that is manifested by the loss of replicative ability and loss of cellular function. It is thought to be involved in the degenerative process of aging and in several diseases that are associated with aging. Senescence can be induced by various mechanisms, such as telomere erosion and oxidative stress, but the precise mechanisms and more importantly, the occurrence and significance of this phenomenon *in vivo* still remains to be fully elucidated. To resolve these questions, identification of the changes that occur prior to and during induction of senescence are essential. Here we describe preliminary results on the differential expression of several ER-resident proteins, involved in proper folding of proteins that enter the secretory pathway, and of the transmembrane protein endoglin, involved in the regulation of endothelial cell proliferation, upon induction of replicative senescence in human umbilical vein endothelial cells. The possible implications and consequences of the observed expression differences and the challenges of cellular senescence research will be discussed.

Introduction

Aging is accompanied by an increase in the prevalence of age-associated diseases. Of these diseases, atherosclerosis has one of the highest incidences in the aged population in western society and consequently causes a high stress on the medical system. The pathology is initiated by the accumulation and oxidation of LDL particles in the vessel wall and the subsequent intrusion of monocytes (Glass and Witztum, 2001). The disturbed integrity and functioning of the endothelial monolayer in blood vessels upon aging is thought to play a key role in the initiation of this disease. The endothelial cells (ECs) have an important regulatory barrier function on the interface of the blood and the rest of the vasculature. It is involved in the transport of macromolecules to and from the surrounding tissues, it regulates coagulant activity and it regulates the intrusion of white blood cells from the circulation. Endothelial integrity is normally maintained by several repair mechanisms. Damaged endothelial cells are either replaced by division of surrounding endothelial cells or by bone marrow-derived endothelial progenitor cells (EPCs) that circulate in the blood. Thus, both mechanisms are involved in maintaining endothelial monolayer integrity (Op den Buijs et al., 2004). As many other cell types, endothelial cells cannot divide indefinitely and eventually they will enter a state of irreversible growth arrest called senescence (Hayflick and Moorhead, 1961). This state can be caused by several mechanisms, like telomere shortening, induction of oncogenic proteins and intracellular oxidative stress (Campisi, 2000; Chiu and Harley, 1997; Serrano and Blasco, 2001), and is manifested by an enlarged and flattened cell morphology, increased granularity and vacuolization. Furthermore, proteins involved in cell cycle inhibition, such as p21, are expressed (Antropova et al., 2002; Chen et al., 2006; Vaziri and Benchimol, 1996). Although these cells are viable and metabolically active, they have altered gene expression (Shelton et al., 1999), which is reflected by altered protein expression (Eman et al., 2006). Although senescence is a process that is thought to have a protective function against the development of diseases, such as cancer, early in life, accumulation of these cells at specific sites could lead to loss of endothelial function and consequently a disturbed barrier function where cells and molecules can enter the rest of the vessel wall freely. Under normal circumstances, endothelial cells have a low average turnover rate of approximately once every 3 years (Foreman and Tang, 2003). However, at sites of injury, bifurcations and

branching points, there is a dramatic increase in cellular replication (Caplan and Schwartz, 1973). This can ultimately lead to induction of senescence and consequently to an increased risk in endothelial dysfunction.

Indications that senescent ECs are relevant *in vivo* and could be important in the development of pathological processes are the identification of cells with increased shortened telomeres at sites susceptible to atherosclerosis (Chang and Harley, 1995; Okuda et al., 2000) and the accumulation of senescent ECs on the surface of atherosclerotic plaques (Minamino et al., 2004; Vasile et al., 2001). Despite these findings, the occurrence and significance of this phenomenon *in vivo* still remain a matter of debate (Cristofalo et al., 2004).

Although several potential biomarkers for senescence have been described (Eman et al., 2006; Shelton et al., 1999), identification of additional changes that occur is needed to further unravel the mechanisms and consequences of EC senescence.

As there is an increase of oxidative stress with increasing age, several cellular processes are affected. An organelle that is likely to be more sensitive for this increased oxidative stress is the endoplasmic reticulum (ER), as glutathione (GSH), the major redox buffer of cells, is in a more oxidized state in the ER as compared to the cytoplasm (Hwang et al., 1992). Indeed it has been shown that upon severe oxidative stress primarily several ER-resident proteins are oxidized, which are responsible for proper folding of proteins that enter the secretory pathway (van der Vlies et al., 2002). Furthermore, carbonylation of these ER resident proteins has been shown to occur upon aging, which is thought to affect proper functioning of these proteins (Rabek et al., 2003). As the expression of several of these proteins is affected by various forms of ER stress via activation of the unfolded protein response (UPR) (Pakula et al., 2003; Schroder and Kaufman, 2005), we hypothesized that changes in expression of these proteins might occur during replicative senescence.

In addition, we hypothesized that as senescent cells lose the ability to replicate, the expression of proteins involved in the control of endothelial cell proliferation might change. A clear candidate is endoglin (CD105), a transmembrane protein involved in transforming growth factor (TGF)-β signaling, which is needed for cell proliferation (Lebrin et al., 2004). Endoglin modulates the balance between ALK1 and ALK5 signalling pathways, which are activated by binding of the TGF-β receptor to its ligand, but they have opposite effects. TGF-β/ALK5 signaling is a negative regulator of EC migration

and angiogenesis via induction of plasminogen activator inhibitor type 1 (PAI-1) expression (Stefansson and Lawrence, 1996; Stefansson et al., 2001), while TGF-β/ALK1 signaling promotes EC proliferation and migration. Endoglin is a positive regulator of the ALK1 pathway and a negative regulator of the ALK5 pathway. Consequently, endoglin protein levels determine the growth capacity of ECs (Lebrin et al., 2005). Here we describe a decrease in cellular endoglin expression and differential expression of several ER resident proteins in replicative senescent human umbilical vein endothelial cells (HUVEC). The decreased expression of endoglin was found in only part of the senescent population. Furthermore, the differential expression of the ER resident proteins differed slightly between different HUVEC isolations. The differences and possible consequences of differential expression of these proteins upon replicative senescence will be discussed. Additionally, the observed differences are used to reveal the challenges of aging research.

Materials and methods

HUVEC isolation and culturing

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from umbilical veins according to the method of Jaffe (Jaffe et al., 1973). Culturing of HUVECs was performed in fibronectin coated culture flasks in EGM-2 (Cambrex, New Jersey, U.S.A.) at 37ºC in 95% rel. humidity and 5% CO2. Medium was changed every two or three days and cells were passaged at 90% confluency. Cells were counted at the moment of seeding and passaging, to allow calculation of population doubling after each passage. When the cells ceased proliferation they were kept in culture for two weeks with continued medium replenishment. Prior to every experiment, a senescence-associated β-galactosidase assay (Cell Signalling Technology, Beverly, MA, U.S.A.) was performed according to the instructions of the manufacturer to determine the presence of senescent cells, together with microscopical inspection of cell morphology.

Western blot

For Western blot analysis, cells were grown in 10cm∅ dishes till 90% confluency, washed twice in PBS and lysed in 700μl lysis buffer [50mM Tris/HCl pH7.4; 100mM NaCl; 5mM EDTA; 1% (v/v) Triton X-100; 0.1% SDS; protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany)]. Non-soluble material was spun down (10.000g/ 5minutes/ 4°C) and the protein content of the supernatant was determined with a BCA protein quantification assay (Pierce, Rockford, U.S.A.). Equal amounts of protein were size-separated on a 10% poly-acrylamide gel and transferred to PVDF membrane. Before incubation with blockbuffer [2.5% (w/v) protifar plus (Nutricia, Zoetermeer, The Netherlands) in PBS], membranes were stained with ponceau red [0.1% (w/v) ponceau red; 0.5% (v/v) HAc] or coomassie brilliant blue (CBB) [0.1% (w/v) coomassie; 40% (v/v) MeOH; 10% (v/v) HAc] to confirm equal transfer of protein. Next, the membranes were incubated with the appropriate antibodies in block buffer. After every incubation with antibody, membranes were washed 5 times in PBST (0.05% Tween20 in PBS). After the last antibody incubation, membranes were washed two additional times with PBS. Bound antibodies were visualized by enhanced chemo luminescence (PerkinElmer, Boston, U.S.A.)

Biotinylation of cell surface proteins

Cultured HUVECs were placed on ice, washed twice with ice-cold PBS, and subsequently labeled for 20 minutes with 100µg/ml sulfo-NHS-LC-LC-biotin (Pierce, Rockford, U.S.A.) in PBS. After labeling, cells were washed twice with 10mM lysine in PBS and subsequently incubated for 5 minutes in the same solution to block all remaining reactive NHS groups. Excess liquid was removed and cell lysates were prepared.

Immuno fluorescence

HeLa cells were grown on 15mm∅ cover slips till 70% confluency. Cells were washed twice with PBS at 37°C and subsequently fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. Cover slips were washed twice with PBS for 5 minutes and cells were subsequently permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. The wash steps were repeated and non-reacted aldehyde groups were quenched with 50mM glycine in PBS for 10 min. Cells were washed twice with 1% BSA in PBS (blocking buffer) and subsequently blocked in the same buffer for 30 minutes. Thereafter, cover slips were incubated with an anti-endoglin antibody, which was detected with GAM-ALEXA555. Each incubation with antibody was performed for one hour, subsequently followed by four wash steps with blocking buffer for 5 minutes. Finally, cells were washed twice with PBS, cover slips were mounted onto glass slides with Mowiol-PPD, air-dried and examined using a fluorescence and confocal microscope.

Results

Confirmation of replicative senescence

To confirm the induction of replicative senescence *in vitro*, a senescence-associated β-galactosidase assay was performed. Furthermore, changes in cell morphology were monitored (Figure 1). The senescent cells were enlarged, had a clear flattened cell morphology and increased granularity compared to the young cells. Furthermore, with increasing passages the number of positive cells in the senescence-associated β-galactosidase assay increased significantly (Figure 1 and 2).

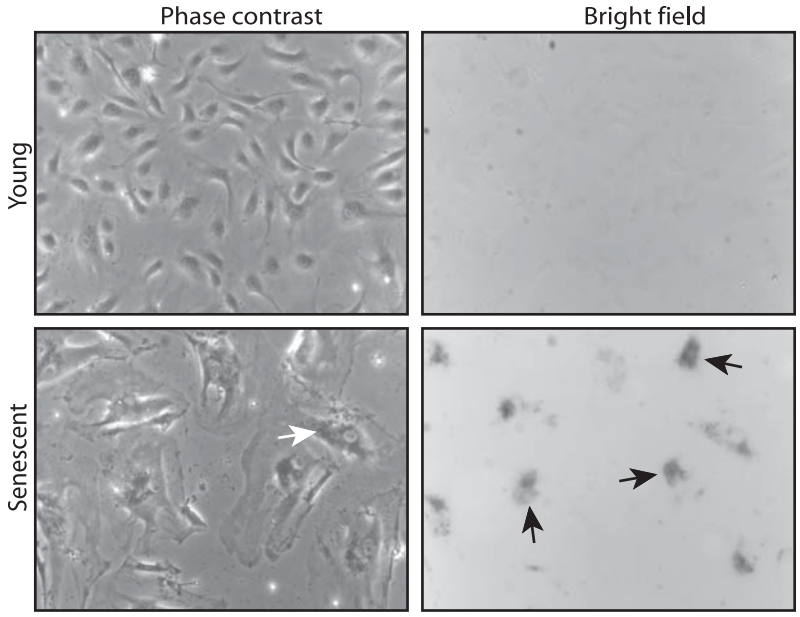

Figure 1: Apparent changes in cell morphology and an increase of β-galactosidase activity in senescent HUVEC cells. The morphology of late passage HUVEC cells is clearly enlarged and flattened with increased granularity (white arrow) compared to early passage cells (phase contrast). Furthermore, the late passage cell population reveals a clear increase in senescence-associated β-galactosidase assay positive cells (black arrows).

One feature of senescent cells described in literature is the expression of p21(Cip) with increasing passages (Antropova et al., 2002; Chen et al., 2006; Vaziri and Benchimol, 1996). This increased expression was also found in cell lysates of late passage cells in our senescent HUVEC model, which further confirmed induction of senescence (Figure 3A).

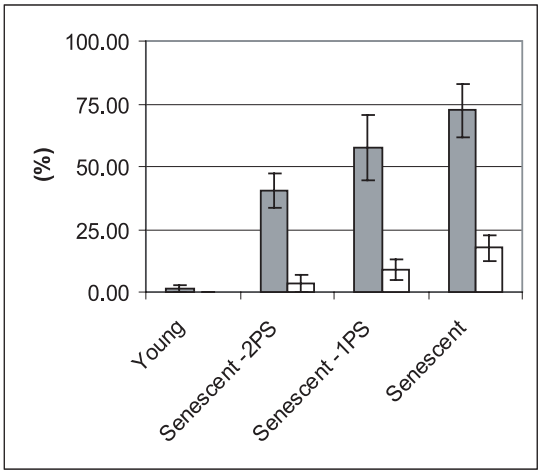

Figure 2: There is an increased prevalence in the late passage HUVECs (two passages prior to senescence (-PS and -2PS) and the senescent population) of β-galactosidase positive cells (grey bars), followed by an increased prevalence of cells that stain negative for endoglin (white bars). HUVECs were labeled with the senescence-associated β-galactosidase assay, followed by IF with a monoclonal anti-endoglin antibody. The percentage of β-galactosidase positive and endoglin negative cells were determined in five randomly chosen microscopic fields.

Changes in expression pattern of ER-resident proteins in senescent HUVECs

To evaluate whether ER functionality is affected upon replicative senescence, the expression of several ER-resident proteins was evaluated with increasing passages. Cell lysates from early passage and the last three passages of cultured HUVECs were evaluated on Western blot with an anti-KDEL antibody (Klooster et al., (Submitted)-b) that recognized the proteins endoplasmin, glucose regulated protein 78kDa (GRP78), calreticulin, PDI A1, PDI A6, ERp46, collagen binding protein 2 and thioredoxin domain-containing protein 4 (Figure 3B). A clear difference in expression pattern was observed between the early passage and senescent cell population (Figure 3B). The expression of the protein bands corresponding to endoplasmin and collagen binding protein2/thioredoxin domain-containing protein 4 appeared to decrease upon induction of replicative senescence. In contrast, the expression of calreticulin and especially PDI A1 appeared to increase, while the expression of the remaining detected ER-resident proteins remained relatively constant. These expression differences were also observed with two additional HUVEC isolations (data not shown), although some exceptions were observed. PDI A1 was not up-regulated in one of the HUVEC isolations, which was linked to a decreased expression of the protein band corresponding to the proteins PDI A6 and ERp46.

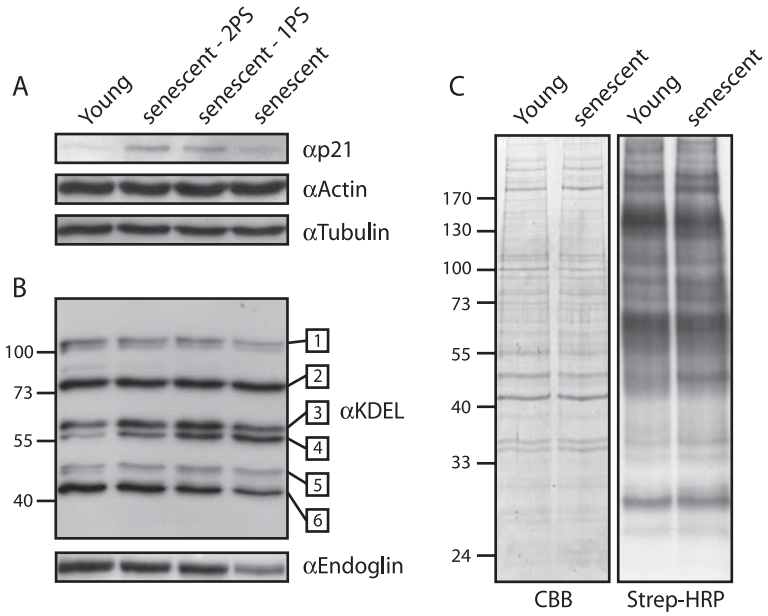

Figure 3: Upon induction of replicative senescence, HUVEC cells revealed differential expression of several intracellular and membrane bound proteins. Cell lysates were obtained from early passage and the last three passages of isolated HUVECs and subsequently probed with protein specific antibodies on Western blot. (A) The up-regulation of p21(Cip) with increasing passages, which is described in literature, confirmed induction of replicative senescence. Detection of tubulin and actin confirmed that equal amounts of protein were loaded. (B) The expression of several ER-resident proteins detected with an anti-KDEL antibody changed with increasing passages. The protein bands corresponding to endoplasmin (1) and collagen binding protein2/thioredoxin domain-containing protein4 (6) showed an apparent decrease in expression, while the protein bands corresponding to the proteins GRP78 (2) and PDI A6/ ERp46 (5) appeared to remain relatively constant. In contrast, the expression of calreticulin (3) and especially PDI A1 (4) appeared to increase. Detection of endoglin expression showed a clear decrease in the senescent cell population, while in the passages prior to senescence there is no apparent decrease in expression compared to the early passage cells. (C) Labeling of membrane bound proteins with biotin and subsequent analysis on Western blot with HRP conjugated streptavidin revealed that the cell morphological changes of senescent cells induced no apparent ratio difference between total (CBB) and surface protein (Strep-HRP) levels.

Decreased endoglin expression in senescent HUVECs

To evaluate whether the loss of proliferative ability of senescent cells correlated to the expression of endoglin, a membrane protein needed for cell proliferation, cell lysates of HUVECs cells were evaluated on Western blot with an endoglin specific antibody (Figure 3B). This revealed that endoglin expression was clearly decreased in the senescent HUVEC culture compared to

the early passage cells, while almost no difference was found in the two passages before senescence. As cell morphology changes during replicative senescence, the ratio between membrane and intracellular protein levels might change, resulting in the observed expression differences on Western blot. Therefore, surface proteins of young and senescent HUVEC cells were biotinylated and subsequently detected on Western blot to evaluate whether the ratio between surface and total protein levels changed or were constant (Figure 3C). No apparent differences in signal intensities were observed, indicating that the decreased expression of endoglin in the senescent population was not caused by cell morphological changes. These same results were obtained with two additional HUVEC isolations (data not shown).

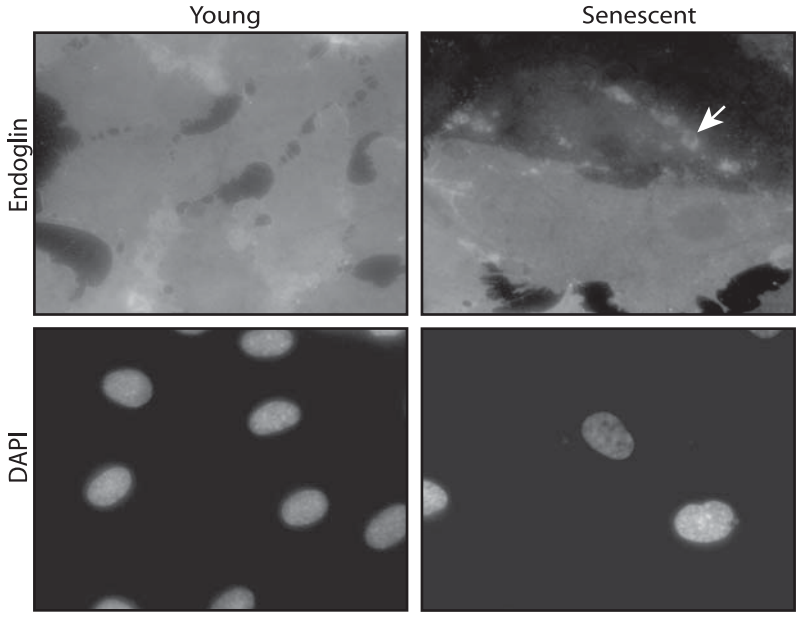

Figure 4: Decreased endoglin labeling in part of the senescent HUVEC population. Cultured cells were labeled with DAPI to visualize the nuclei and a monoclonal anti-endoglin antibody. While all young cells revealed an even and equal membrane staining, there was a clear decreased expression in some of the late passage cells. In some of the endoglin negative cells clear aggregate-like structures were observed that revealed autofluorescence (arrow).

Heterogenous endoglin expression in the senescent HUVEC population

Immuno fluorescence (IF) experiments were performed, to evaluate whether all or part of the HUVECs in the senescent population showed a decrease in endoglin expression. This analysis clearly showed an even and equal membrane staining of endoglin in all early passage cells, while in the late

passage cells the membrane staining was very heterogenous (Figure 4). A large portion of the late passage cells showed an expression that was comparable to the young cells, whereas others showed an apparent decrease, or no detectable endoglin expression. The appearance of these cells increased significantly in the last three passages (Figure 2).

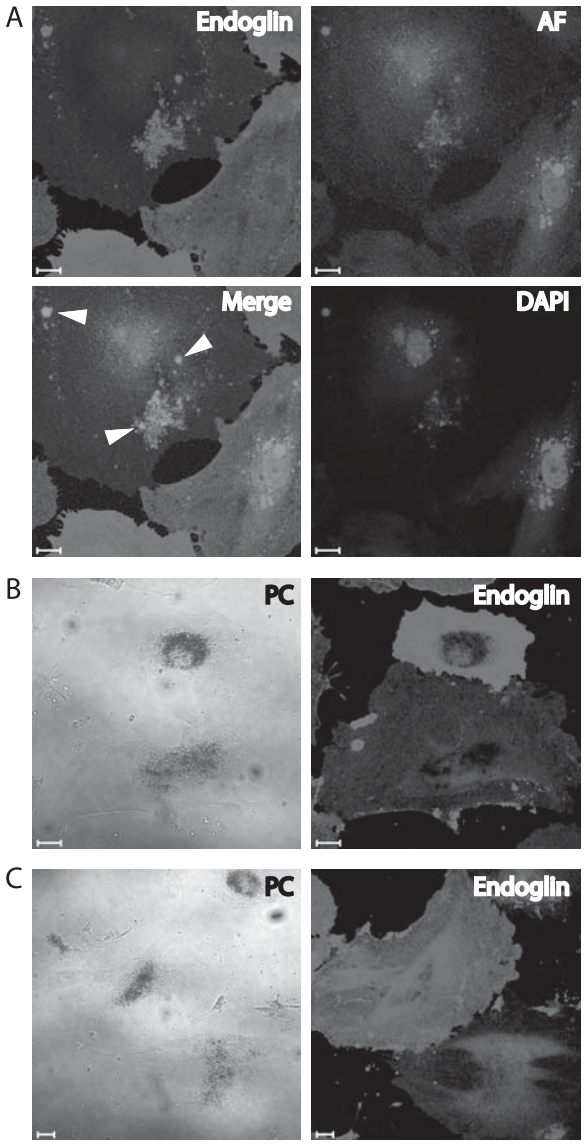

Figure 5: Heterogenous endoglin labeling in senescent HUVECs. HUVECs cells were stained with the β-galactosidase activity assay kit and subsequently labeled with a monoclonal anti-endoglin antibody. Several endoglin negative cells revealed clear aggregate-like structures that showed auto-fluorescence (AF). This suggested that these structures were protein aggregates containing lipofuscin (Merge, white arrowheads). The extent of aggregate formation differed per cell (A). Phase contrast pictures (PC) revealed that several β-galactosidase positive cells were also positive when labeled with the monoclonal anti-endoglin antibody. However, all endoglin negative cells stained positive in the senescence-associated β-galactosidase assay (Phase contrast). Even cells with reduced levels of endoglin were β-galactosidase positive (B, C). Bar represents 20μm.

Intriguingly, in part of the late passage cells staining was observed in aggregate like structures (Figure 4). These structures revealed auto-fluorescence suggesting that these were protein aggregates containing

lipofuscin (Figure 5A), which are known to accumulate in post-mitotic and aging cells (Seehafer and Pearce, 2006; Terman and Brunk, 2004).

Detection combining the senescence-associated β-galactosidase assay and the monoclonal anti-endoglin antibody revealed that several β-galactosidase positive cells did not show a decreased expression of endoglin (Figure 5B). However, all HUVEC cells with a decreased expression or a negative staining pattern for endoglin were positive in the senescence-associated β-galactosidase assay (Figure 5B and 5C).

Discussion

Here, we show that induction of replicative senescence in HUVECs is associated with changes in expression of several ER-resident proteins and with a decreased expression of the transmembrane protein endoglin.

Endoglin, a TGF-β type III receptor, fulfills a regulating role in TGF-β signal transduction. Endoglin is a negative regulator of TGF-β/ALK5 signaling. This pathway is a negative regulator of EC migration and angiogenesis via induction of plasminogen activator inhibitor type 1 (PAI-1) expression (Stefansson and Lawrence, 1996; Stefansson et al., 2001). In addition, endoglin is a positive regulator of TGF-β/ALK1 signaling, which promotes EC proliferation and migration. Thus, endoglin is thought to be a modulator of the balance between the ALK1 and ALK5 signaling pathways and consequently endoglin protein levels determine the growth capacity of ECs (Lebrin et al., 2005).

The observation that the majority of the cells in the senescent population were still endoglin positive (Figure 4) suggests that the decreased expression of endoglin is a secondary effect during replicative senescence induction. This is further supported by the increased expression of p21(Cip), which is already observed in the two passages prior to senescence (Figure 1A). However, the differential endoglin expression could have an enormous impact on endothelial functioning. As it regulates ALK1 and ALK5 signaling pathways, expression of the fibrinolysis inhibitor PAI-1 (Stefansson and Lawrence, 1996; Stefansson et al., 2001) and the tight junction component claudin 5 (Watabe et al., 2003) might be affected by this decreased endoglin expression. An increased expression of PAI-1 *in vivo* could induce a local more pro-coagulant environment, which could lead to an increased risk of developing a blockage of the vasculature. Furthermore, the disturbed regulation of claudin 5 could affect the barrier function of the endothelial cell, causing enhanced exchange of blood components, such as LDL, with the rest of the vascular wall, which could increase the chance of developing atherosclerosis.

In addition, the expression of several ER-resident proteins was changed with increasing passages. These expression differences did not resemble the expression differences observed during acute ER stress, when the UPR is activated, e.g. up-regulation of GRP78 and endoplasmin (Klooster et al., (Submitted)-b). However, the changed expression pattern does indicate that the functionality of the ER changes upon induction of senescence. Especially

the increased expression of PDI A1 is intriguing, as increased expression was already observed two passages prior to senescence, while the changes in expression of the other detected ER-resident proteins was, for the most extent, visible only in the senescent population. As PDI A1 is involved in proper disulphide bond formation, this increased expression indicates that with increasing passages the capacity for proper disulphide bond formation is increased, which might indicate that this process is disturbed in senescent cells.
As with the decreased expression of endoglin, the precise mechanisms that cause these changes in ER-resident protein expression are unclear, as protein expression is controlled on several levels and could therefore be caused by changes in gene expression, RNA processing, translational control and protein degradation. Consequently, to understand the processes that contribute to induction of senescence, identification of both gene and protein expression differences are essential.

Although the differential expression of several ER-resident proteins was observed in three different HUVEC isolations, exceptions were observed. These differences could be caused by differences in growth conditions, but also by the different genetic makeup of the donors. The genetic makeup of an organism ultimately determines the efficiency to adapt to different environmental conditions, e.g. some individuals are better equipped than others to withstand oxidative stress. This heterogeneity is also illustrated by the huge variations in maximal passage number between different HUVEC isolations, which can vary from 10 to almost 25 passages. As senescence can be induced by different mechanisms, variation in differential protein expression between different isolations might depend on which mechanisms are primarily involved in senescence induction. Furthermore, heterogeneity is also observed in the senescent HUVEC culture itself, illustrated by the differences in cell morphology, β-galactosidase staining, amount of aggregate formation and endoglin labeling between cells in a single senescent HUVEC culture. These differences are most likely caused by the random and multi-factorial nature of the mechanisms that cause replicative senescence. The heterogeneity illustrates one of the challenges that are faced upon analysis of EC senescence or aging phenomena per definition. Recognition of this

challenge underscores the need for mapping the different RNA and protein expression differences in biological samples of different individuals.
However, this heterogeneity does pose us with an experimental sensitivity issue, as differential expression of specific proteins caused by senescence in one cell is diluted in a pool of cells that might reveal different expression patterns. This could explain the small protein expression differences found in a proteomics study that compared protein expression levels in young and senescent HUVECs (Eman et al., 2006). Therefore, if we really want to understand the changes and consequences of cellular senescence, analysis of single cell expression levels with regard to time and localization will be crucial. In addition, potential senescence membrane markers, such as endoglin, could be exploited to fractionate senescent cells in populations that have similar expression profiles.

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