Downregulation of Polo-Like Kinase 1 Induces Cellular Senescence in Human Primary Cells Through a p53-Dependent Pathway

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Polo-like kinase 1 (PLK1) plays a key role in various stages of mitosis from entry into M phase to exit from mitosis. However, its role in cellular senescence remains to be determined. Therefore, the effects of PLK1 on cellular senescence in human primary cells were investigated. We found that expression of PLK1 decreased in human dermal fibroblasts and human umbilical vein endothelial cells under replicative senescence and premature senescence induced by adriamycin. PLK1 knockdown with PLK1 small interfering RNAs in young cells induced premature senescence. In contrast, upregulation of PLK1 in old cells partially reversed senescence phenotypes. Cellular senescence by PLK1 inhibition was observed in p16 knockdown cells but not in p53 knockdown cells. Our data suggest that PLK1 repression might result in cellular senescence in human primary cells via a p53-dependent pathway.

Key Words: Polo-like kinase 1—Cellular senescence—p53—Human primary cells.

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NORMAL somatic cells show restricted cell proliferation, and irreversible growth arrest is one of the hallmarks of cellular senescence (1). In addition to irreversible growth arrest, senescent cells display characteristic phenotypes, including enlarged and flattened morphology; expression of senescence-associated β-galactosidase (SA-β-gal); robust secretion of numerous growth factors, cytokines, proteases, and other proteins (senescence-associated secretory phenotype); and nuclear foci containing DNA damage response proteins or senescence-associated heterochromatin foci (2). Various factors, such as telomere erosion, activation of oncogenes or tumor suppressor genes, oxidative stress, chemotherapeutic agents, and culture stress with or without a DNA damage response, have been reported to induce cellular senescence (3). Although diverse factors and phenotypic markers are associated with cellular senescence, the p53 and pRb/p16INK4A pathways are critically responsible for the regulation of cellular senescence (4). Cellular senescence contributes to tissue and/or organismal aging, tumor suppression, tumor progression, tissue repair, and age-related pathology (5).

Increasing evidence suggests that errors in the mitotic machinery of cells caused by altered expression of mitotic genes might be an underlying mechanism of aging. Alteration of the expression of several mitotic proteins that regulate centrosome and kinetochore integrity and mitotic checkpoint function was previously reported to induce premature senescence through a p53-dependent pathway (6). Several genes involved in the regulation of chromosomal processing and assembly, such as centromere protein A, centromere protein F, mitotic kinesin-like protein-1, and so on, have been reported to be altered in expression in fibroblasts isolated from the elderly people and from humans with progeria (7). The expression levels of centromere protein A are also reduced in senescent human fibroblasts, and downregulation of centromere protein A induces premature senescence through a p53-dependent pathway (8). Moreover, dysfunction of mitosis causes chromosomal abnormalities such as increased polyploidy in human endothelial cells (9), fibroblasts (10,11), and aortic vascular smooth muscle cells (12). The levels of chromosome-specific aneuploidy are increased with advancing donor age (13).

Polo-like kinases (PLK), a subfamily of serine or threonine kinases, play key roles in cell cycle progression (14). Mammals contain five PLK family members: PLK1, PLK2, PLK3, PLK4, and PLK5 (15). Among the five PLKs, PLK1 has crucial functions in various stages of mitosis from entry into M phase to exit from mitosis: activation of CDK1, centriole and centrosomes biogenesis, segregation of mitotic chromosomes, regulation of anaphase-promoting complex, and execution of cytokinesis (16). In addition, PLK1 is involved in microtubule dynamics, DNA replication, chromosome dynamics, p53 regulation,
and recovery from the G2 DNA damage checkpoint (17). In mammals, PLK1 is expressed mainly in dividing cells during late G2 and M phases, and its activity is regulated by transcription, phosphorylation, proteolysis, and protein interaction (16). Because PLK1 plays a principal role in the regulation of ordered execution of mitosis, alteration of PLK1 activity affects cell proliferation and chromosomal abnormalities, which in turn contributes to the development of cancer. PLK1 upregulation is observed frequently in diverse human cancers (18,19), supporting a role for elevated PLK1 activity as a tumor-promoting force. In contrast, inhibition of PLK1 activity has been revealed to induce mitotic spindle defects and aberrant cytokinesis, resulting in growth arrest and/or apoptosis in diverse cancer cells (20–22). Further, knockdown of PLK1 was reported to induce a significant increase in the tetraploid cell population (23), which is one of the features of cellular senescence. However, the role of PLK1 in cellular senescence has not been reported yet.

In this study, we found that the expression of PLK1 decreased in senescent human primary cells. PLK1 down-regulation in young cells induced premature senescence. In contrast, overexpression of PLK1 in old cells partially reversed cellular senescence. Senescence phenotypes induced by PLK1 knockdown were observed in p16 knockdown cells but not in p53 knockdown cells. These results suggest that PLK1 inhibition might induce cellular senescence through a p53-dependent pathway.

METHODS

Human dermal fibroblasts (HDFs), human umbilical vein endothelial cells (HUVECs), and endothelial cell basal medium-2 with growth factors and supplements (endothelial cell growth medium-2) were purchased from Lonza Inc, (Walkersville, MD). AD293 cells, pShuttle vector, pAdEasy-1 vector, and pAdEasy titer kit were purchased from Stratagene Corp, (La Jolla, CA). Oligonucleotides for PLK1, p53, p16, and glyceraldehyde-3-phosphate dehydrogenase and small interfering RNAs (siRNAs) against PLK1 (siPLK1 #1, aacgagcugcu-uaagacgaguu; siPLK1 #2, aagggcggcuuugccaagugcuu) were obtained from Bioneer Corp, (Daejeon, Republic of Korea). Stealth negative control RNAi and horseradish peroxidase–conjugated secondary rabbit or mouse antibody were from Invitrogen Life Technologies Inc, (Santa Cruz, CA). Antibodies against phospho-Rb (ser807/811) and p21 were from Cell Signaling Technology Inc, (Danvers, MA). The pRetroSuper-p53sh and pRetroSuper-p16sh vectors were kindly provided by Dr. R. Agami (Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, Netherlands).

Cell Culture

HDFs and AD293 cells were cultured in Dulbecco’s Modified Eagle Medium containing 10% fetal bovine serum and 1% antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) and HUVECs in endothelial cell basal medium-2 with endothelial cell growth medium-2. HDFs and HUVECs were seeded at 1 x 10^5 cells per 100-mm culture plate and incubated at 37°C in a 5% CO₂ humidified incubator. When cells reached 80%~90% confluence, they were trypsinized and subcultured. The number of population doublings (PDs) was calculated from the geometric equation: PD = log₂F/log₂I, where F is the final population number and I is the initial population number. For experiments, cells were used in either PD < 24 (young) or 39–45 PDs (mid-old) or PD ≥ 50 (old).

Adriamycin Treatment

HDFs and HUVECs (PD < 28) were seeded at 1 x 10^5 cells in 60-mm dishes and incubated overnight at 37°C in a 5% CO₂ humidified incubator. Cells were washed two times with Dulbecco’s Modified Eagle Medium containing 1% antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) and treated with 0.5 μM adriamycin for 4 hours. After washing, cells were further incubated in culture media for the indicated times.

Cell Counting

HDFs and HUVECs (2 x 10^4 cells) were plated in six-well plates and incubated overnight at 37°C in a 5% CO₂ incubator. After the indicated times, the cells were harvested, and the cell number was determined using a hemocytometer.

PLK1 Immunofluorescence Staining

Cells were treated with 150 ng/mL of nocodazole for 14 hours and fixed with 3.7% paraformaldehyde for 1 hour. Cells were treated with 0.05% Triton X-100 in phosphate-buffered saline (PBS) for 1 hour and blocked in 3% goat serum (Invitrogen Corp) for 45 minutes and then stained with a PLK1 (sc-17783) antibody (dilution 1:100) at 37°C for 3 hours. Cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (dilution 1:250; Molecular probes, Eugene, OR) for 1 hour and then 4',6-diamidino-2-phenylindole (DAPI) staining was performed. Cells were observed with a confocal microscope.

SA-β-Gal Activity

HDFs and HUVECs were plated at 2 x 10^4 cells in six-well plates and incubated overnight. Following washing twice with PBS, cells were fixed with 3.7% (v/v) formaldehyde in
RNA Extraction

RNAs were extracted from cells using TRIzol reagent (Bio Science Technology, Daegu, Republic of Korea) according to the manufacturer’s instructions. The concentration of RNA was determined by measuring the absorbance at 260 nm using a NanoDrop (Thermo Fisher Scientific Inc, Waltham, MA).

Reverse Transcription-Polymerase Chain Reaction

Complementary DNAs (cDNAs) were prepared from RNAs (1 µg) using 2.5 µM oligo-dT primers, 1 mM dNTPs, and moloney murine leukemia virus reverse transcriptase (Promega Corp, Madison, WI). PLK1, p53, and p16 were amplified from cDNAs with Super-Therm DNA polymerase (SR Product, Kent, United Kingdom) and gene-specific primers (Table 1). Glyceraldehyde-3-phosphate dehydrogenase primers were used to standardize the amount of RNA in each sample. PCR products were separated on 1%–2% agarose gels, and the DNAs were visualized by SYBR green staining with a LAS-3000 image system (Fujifilm Life Science, Stamford, CT).

Real-Time Quantitative PCR Analysis

Real-time quantitative PCR analysis was conducted using SYBR Green PCR master mix (Applied Biosystems Inc, Carlsbad, CA) and the LightCycler 2.0 Real-Time PCR system (Roche Diagnostic Corp, Indianapolis, IN). The PCR protocol was as follows: 50°C for 2 minutes, 94°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, 60°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute. Expression levels were determined with the LightCycler software, version 3.5.3 (Roche Diagnostic Corp).

Table 1. Primer Sequences for Reverse Transcription-Polymerase Chain Reaction Analyses

<table>
<thead>
<tr>
<th>Genes</th>
<th>Nucleotide Sequences</th>
<th>Size (bp)</th>
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<tr>
<td>PLK1</td>
<td>Forward: GCCCCTCTACACAGTCTCCAATA</td>
<td>261</td>
</tr>
<tr>
<td></td>
<td>Reverse: TACCCAAAGGCGCTACTCTGTC</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>Forward: CCCTGAGGTGGCTGCTGTTGA</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTGTGAGCCTCCCTCCTTTT</td>
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<tr>
<td>p16</td>
<td>Forward: CTTCCTGGACACGCTGTTGA</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACCTTCCCAGGCACATCTAT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: CAGGACACTTTGGCAAGCTCA</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGGCGTCTCAGTGGCAACACTG</td>
<td></td>
</tr>
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Notes. PLK1 = polo-like kinase 1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Protein Extraction

Cells were washed with ice-cold PBS containing 1.0 mM CaCl₂ and 0.5 mM MgSO₄. Cells were disrupted with ice-cold radioimmunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM Na₃VO₄, 5 mM NaF, and 1 mM phenylmethylsulfonyl fluoride) and harvested by scraping with a cell scraper. After vortexing three times for 30 seconds on ice, cell lysates were centrifuged at 13,200 rpm for 15 minutes. Protein concentrations of the supernatants were determined by the bicinchoninic acid method (Thermo Fisher Scientific, Rockford, IL) using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Proteins (30–50 µg) were resolved on sodium dodecyl sulfate-polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were incubated with TWEEN 20–tris-buffered saline containing 5% nonfat dry milk for 1 hour at room temperature. The membranes were incubated overnight at 4°C with one of the specific antibodies in 5% nonfat dry milk. After washing three times in TWEEN 20–tris-buffered saline, horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit antibodies were applied for 1.5 hours at 4°C. The proteins were visualized using Western blotting luminol reagent (Santa Cruz Biotechnology Inc) with a LAS-3000 image system (Fujifilm Life Science). Some membranes were stripped with antibody stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 100 mM β-mercaptoethanol) at 37°C for 30 minutes with shaking. Glyceraldehyde-3-phosphate dehydrogenase was used as a control for protein loading.

PLK1 cDNA Cloning

Full-length PLK1 cDNA was amplified by PCR using total RNA isolated from HDFs with Takara HS DNA polymerase (Shiga, Japan) and primers (ATGAGTGGCTGAGTGTAGTGGAG and TTAGGAGCCCTTGGAGACCGGTGTC). The PCR products were purified using a gel extraction kit (SolGent Corp Ltd, Daejeon, Republic of Korea) and ligated into pCR2.1-TOPO vector (Invitrogen Life Technologies Inc). Cloned cDNA sequence was confirmed by dideoxy DNA sequencing (SolGent Corp).

Preparation of Recombinant PLK1 Adenovirus

PLK1 cDNA subcloned into pCR2.1-TOPO vector was digested with XhoI and SpeI (Takara Bio Inc), and the resulting PLK1 cDNAs were ligated into the same restriction enzyme sites of the pShuttle vector. The recombinant PLK1 adenoviral vectors were prepared by a double recombination event in BJ5183 bacteria between the
pAdEasy-1 vector and pShuttle/PLK1 vector. Briefly, the pShuttle/PLK1 vectors were treated with Pmel and alkaline phosphatase (New England Biolabs Inc, Ipswich, MA) and then cotransformed into BJ5183 cells by electroporation with pAdEasy-1 vector. Bacterial colonies containing pAd/PLK1 vectors were selected, and the presence of pAd/PLK1 vectors were confirmed by PacI (New England Biolabs Inc) digestion. The recombinant pAd/PLK1 vectors were linearized with PacI digestion and transfected into AD293 cells using Fugene HD transfection reagent (Roche Diagnostic Corp) according to the manufacturer’s protocol. Recombinant adenovirus was amplified in AD293 cells and purified by an adenovirus purification kit (CellBiolabs, San Diego, CA). Virus titers were determined using the pAdEasy titer kit in AD293 cells.

**Transduction of Recombinant PLK1 Adenovirus**

Old HDFs and HUVECs (PD > 50; 1.5×10^5 cells) were seeded in 60-mm dishes and incubated overnight. Cells were treated with 5, 10, and 15 multiplicity of infection (MOI) of recombinant PLK1 virus for 24 hours. After discarding the media, cells (2×10^4) were further incubated for 5 days. Cell number and SA-β-gal staining were analyzed. The protein levels were measured by Western blot analysis.

**Measurement of BrdU Incorporation**

Intracellular BrdU incorporation in old cells transduced with recombinant PLK1 adenovirus was measured using the BrdU flow kit. Cells transduced with recombinant PLK1 adenovirus were incubated for 5 days and treated with BrdU for 1 day. The BrdU-positive cells in 10,000 cells were estimated using a Becton-Dickinson FACS Canto II flow cytometer (BD Bioscience).

**Measurement of PDs by Live-Cell Time-Lapse Microscopy**

HUVECs and HDFs (1×10^4 cells) were seeded in 96-well plates and transduced with 15 MOI of recombinant PLK1 adenovirus for 24 hours. After discarding the media, cells were further incubated and live cell images were obtained every 2 hours for 96 hours with a Leica AS-MDW microscope (Leica Microsystems GmbH, Wetzlar, Germany). The number of PDs was calculated by counting cells in each image.

**Transfection of PLK1 siRNAs**

Young HDFs and HUVECs (2×10^4 cells) were seeded in 60-mm dishes and incubated overnight. Two different siRNAs against PLK1 (10 pmol) or Stealth negative control RNAi were transfected into young HDFs and HUVECs (PD < 24) using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies Inc) according to the manufacturer’s instructions. After 24 hours of incubation, media were changed. siRNA-transfected cells (2×10^4 cells) were further incubated for 5 days, after which cell counting and SA-β-gal-positive cells were analyzed. The protein levels were measured by Western blot analysis.

**Transfection of p53 or p16 shRNA Retroviral Vectors and PLK1 siRNAs**

Cells were plated at 50%–80% confluency in 60-mm dishes and transfected with 3 μg of pRetroSuper vectors, pRetroSuper-p16sh vectors, or pRetroSuper-p53sh vectors using FugeneHD transfection reagent (Roche Diagnostic Corp). After 36 hours of incubation, cells were transfected with PLK1 siRNAs and incubated for 4 days. Expression levels of p16, p53, p21, and PLK1 proteins were determined by Western blotting. Cell proliferation and SA-β-gal activity were observed.

**Statistical Analysis**

The results are represented as means ± SD. p values for determining statistical significance were calculated using an unpaired two-tailed Student’s t test.

**RESULTS**

**Downregulation of PLK1 Levels During Cellular Senescence in Human Primary Cells**

Initially, we tried to identify novel senescence-associated genes in HDFs or HUVECs under replicative senescence using cDNA microarray technology and found that PLK1 expression decreased in both cell types (data not shown). To verify downregulation of PLK1 during replicative senescence in HDFs and HUVECs, the expression levels of PLK1 mRNA and protein were measured by semiquantitative reverse transcription-polymerase chain reaction (data not shown), real-time PCR, and Western blotting. Replicative senescence in cells was confirmed by increases in SA-β-gal staining and the levels of p53, p21, and p16 proteins as well as enlarged, flattened cell morphology (Figure 1A). The expression levels of PLK1 mRNA and protein decreased in old cells compared with young cells (Figure 1B and C). PLK1 immunoreactivity was stronger in young cells than in old cells and PLK1 was localized in the nucleus or chromosomes of young cells under mitosis (Figure 1D). Furthermore, the level of PLK1 protein also decreased during adriamycin-induced premature cellular senescence (Figure 1E).

**Induction of Cellular Senescence by PLK1 Knockdown**

Because the level of PLK1 decreased in senescent cells, we measured the effect of PLK1 knockdown on cellular senescence in HDFs and HUVECs. Knockdown of PLK1 down with two different PLK1 siRNAs
Figure 1. Reduction of polo-like kinase 1 (PLK1) levels in cellular senescence of human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVECs). (A) Senescence-associated β-galactosidase (SA-β-gal) staining in HDFs and HUVECs (×100). Replicative senescence was induced by serial cultivation of cells. Cells were fixed and incubated with SA-β-gal staining solution. The proportion of cells positive for SA-β-gal was determined by counting 100 cells in four different fields. (B) Real-time PCR for PLK1 mRNA in cells under replicative senescence. (C) Western blot analysis for PLK1, p53, p21, and p16 proteins in cells under replicative senescence. The numbers indicate the relative amounts of each protein normalized with the GAPDH level and compared with young cells using the ImageJ densitometry software. (D) Immunofluorescence staining of PLK1 in young and old cells. Young and old cells treated with 150 ng/mL nocodazole for 14 hours were stained with a PLK1 antibody and observed with a fluorescence microscope. (E) Expression levels of PLK1 protein in cells under adriamycin-induced cellular senescence. Cells were treated with 0.5 μM adriamycin for 4 hours and then washed. The cells were further incubated for the indicated times, and expression levels of PLK1 protein were detected by Western blotting. Representative data are shown from three independent experiments. Values are means ± SD of three independent experiments. Y = young cells; O = old cells; NT = not treated cells; p53 = tumor protein p53; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.
(#1 or #2: Figure 2A) resulted in inhibition of cell proliferation (Figure 2B) and increased SA-β-gal staining (Figure 2C). In addition, downregulation of PLK1 decreased the phosphorylation of Rb at serine 807 and serine 811 as well as the level of cyclin A and increased the level of p53 protein (Figure 2D). However, the p16 protein was not detected in PLK1-siRNA cells by Western blotting (data not shown). Since PLK1 siRNA #2 had a greater effect on PLK1 downregulation than PLK1 siRNA #1, senescence phenotypes induced by PLK1 siRNA #2 were more obvious than those induced by PLK1 siRNA #1. We observed that the levels of PARP1/2 and caspase 3 did not change (Figure 2D), and their cleaved fragments were not observed in PLK1 siRNA-treated cells (Supplementary Figure 1), indicating that inhibition of cell proliferation by PLK1 knockdown was not due to apoptotic cell death. Our results suggest that down-expression of PLK1 induced premature cellular senescence in young human cells.

Partial Reversal of Cellular Senescence by PLK1 Overexpression in Old Cells

Because PLK1 knockdown induced premature cellular senescence, we tried to determine whether upregulation of PLK1 in old cells might reverse senescence phenotypes. Following transduction of old cells with recombinant PLK1 adenovirus, upregulation of PLK1 protein levels was confirmed by Western blot analysis (Figure 3A). PLK1 overexpression in old cells increased cell proliferation (Figure 3B), BrdU incorporation (Figure 3C), and PDs (Figure 3D). PLK1 upregulation decreased SA-β-gal staining (Figure 3E) in a dose-dependent manner. These results suggest that upregulation of PLK1 lengthened the replicative lifespan of senescent human cells and partially reversed cellular senescence in old human cells.

Involvement of a p53-Dependent Pathway in Cellular Senescence Induced by PLK1 Knockdown

It is well known that cellular senescence induced by diverse factors such as oncogene activation, telomere shortening, oxidative stress, and chemotherapeutic agents converge into two tumor suppressor pathways mediated by p53 and pRb/p16INK4A (4). Therefore, we investigated which pathway might play a crucial role in the regulation of cellular senescence by PLK1 knockdown. The levels of p53 or p16 proteins were downregulated by transfection with p53 or p16 shRNA retroviral vectors, followed by PLK1 knockdown with PLK1 siRNAs. Downregulation of p53, p21, and PLK1 was confirmed by Western blotting (Figure 4A). However, we could hardly detect the p16 protein in our experimental condition because young cells were known to express very low level of p16 protein in normal condition (25). Instead, we confirmed the knockdown of p16 by transfecting old cells with the p16 shRNA vector since old cells show an increase in the p16 level (Figure 4B). Although p16 shRNA–treated cells showed senescence phenotypes induced by PLK1 knockdown, such as decreased cell proliferation and increased SA-β-gal staining, p53 shRNA–treated cells did not (Figure 4C and D). These data indicate that cellular senescence by PLK1 knockdown was mediated through a p53-dependent pathway.

Discussion

This study demonstrated that PLK1 repression resulted in cellular senescence in human primary cells based on the following findings: (i) the expression levels of PLK1 were decreased in senescent primary cells (Figure 1B–E) and (ii) downregulation of PLK1 in young cells induced premature senescence (Figure 2). To our knowledge, this is the first report to reveal the involvement of PLK1 in cellular senescence in human primary cells. However, some indirect evidence has suggested that PLK1 might have functions in the regulation of cellular senescence. Elevated expression of PLK1 has been observed in human cancers such as those derived from the colon (18), thyroid (26), prostate (27), pancreas, breast (28), and stomach (19,29). Overexpression of PLK1 in tumors often correlates with tumor stages and poor prognosis in many cancers (27,28,30), suggesting that PLK1 could have tumor-promoting activity. In contrast, inhibition of PLK1 activity has been reported to induce growth arrest and/or apoptosis in diverse cancer cells (20–22), showing impairment of mitosis, failure of cytokinesis, and defects in centrosome integrity and maintenance (31). Systemic application of shRNA against PLK1 previously resulted in inhibition of tumor growth in nude mice carrying xenograft tumors (32). A reversible, ATP-competitive inhibitor of PLK1, GSK461364A, was shown to inhibit cell proliferation of most proliferating cancer cell lines, inducing prometaphase arrest with characteristic collapsed polar spindle (33). However, PLK1 knockdown in nontransformed cells has no effect on proliferation compared with tumor cells (34,35). Repression of PLK1 activity by a chemical inhibitor, BI 2536, was reported to temporarily arrest primary cardiac fibroblasts in mitosis and generate aneuploidy in vitro (36), as well as activate the DNA damage checkpoint and induce apoptosis in MCF10A and hTERT-RPE1 cells (37). These reports imply that the effect of PLK1 inhibition in normal cells is still controversial compared with that in cancer cells.

Phosphorylation of Thr-210 (T210) in the T-loop is crucial for activating PLK1 in mitosis (38). Initial phosphorylation of PLK1 at T210 by Aurora kinase A in collaboration with Bora is critical for the activation of PLK1 during the G2-M transition (39,40). Alteration of Aurora A activity was reported to be involved in cellular senescence. Aurora A overexpression induces cellular senescence in mammary gland hyperplastic tumors developed in p53-deficient mice (41). MLN8054, an inhibitor of Aurora A kinase, causes senescence in human
tumor cells both in vitro and in vivo (42). Downregulation of Aurora B kinase induces cellular senescence in human fibroblasts and endothelial cells through a p53-dependent pathway (43). BubR1 kinase, as a component of the spindle assembly checkpoint, is phosphorylated by PLK1 to facilitate kinetochore–microtubule interaction and chromosome alignment (44). Mice deficient in BubR1 revealed early aging-associated phenotypes along with

Figure 2. Effect of polo-like kinase 1 (PLK1) downregulation on cellular senescence in human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVECs). (A) Western blot analysis for PLK1 levels. Cells were transfected with PLK1 or negative control small interfering RNAs (siRNAs; 10 pmol) and incubated for 24 hours at 37°C. The numbers indicate the relative amounts of each protein normalized with the GAPDH level and compared with young cells using the ImageJ densitometry software. (B) Cell counting: siRNA-transfected cells (2 × 10^4) were incubated for 5 days, after which cells were harvested and counted. (C) Senescence-associated β-galactosidase (SA-β-gal) activity staining: siRNA-transfected cells were incubated for 5 days and then SA-β-gal-positive cells were analyzed. (D) Western blot analysis for cyclin A, pRb, p53, PARP1/2, and caspase 3 in HDFs and HUVECs. Representative data are shown from three independent experiments. Values are means ± SD of three independent experiments. Statistical significance was determined by the Student’s t test (*p < .05 and **p < .01 vs. siNC). Y = young cells; O = old cells; siNC = negative control siRNA; siPLK1 = PLK1 siRNA; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.
Figure 3. Effect of polo-like kinase 1 (PLK1) overexpression on cellular senescence in old human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVECs). (A) Western blot analysis: Old cells were transduced with recombinant PLK1 adenovirus, and PLK1 overexpression was confirmed by Western blotting in old HDFs and HUVECs. (B) Cell counting: Cell proliferation was measured by cell counting. (C) Bromodeoxyuridine (BrdU) incorporation: Cells were stained with BrdU and BrdU-positive cells were analyzed by flow cytometry. (D) The number of population doublings (PDs): Old cells transduced with 15 multiplicity of infection (MOI) of recombinant PLK1 adenovirus were incubated and cell proliferation was observed by live-cell time-lapse microscopy. The number of PDs was calculated (*p < .05 vs. old cells). (E) Senescence-associated β-galactosidase (SA-β-gal) staining: Cells were fixed with 3.7% paraformaldehyde for 5 minutes and incubated in SA-β-gal staining solution. The proportion of cells positive for SA-β-gal activity was determined. Representative data from three independent experiments are shown. Values are means ± SD of three independent experiments. Statistical significance was determined by the Student’s t test (*p < .05 and **p < .01 vs. 0 MOI). Y = young cells; Ad/PLK1 = recombinant PLK1 adenovirus.
Figure 4. Effects of p53 or p16 knockdown on cellular senescence induced by polo-like kinase 1 (PLK1) downregulation. (A) Western blot analysis for PLK1, p53, and p21 proteins. Young cells were transfected with p53 or p16 shRNA retroviral vectors and incubated for 24 hours at 37°C. Cells were then transfected with PLK1 or negative control small interfering RNAs (siRNAs). (B) Western blot analysis of p16 protein in old cells. Old cells were transfected with p16 shRNA or control retroviral vectors. Following 4 days, the p16 protein levels were measured with Western blotting. The numbers indicate the relative amounts of each protein normalized with the GAPDH level and compared with young cells using the ImageJ densitometry software. (C) Cell counting: shRNA- and siRNA-transfected cells were incubated for 3 and 6 days. Cell proliferation was measured by cell counting. (D) Senescence-associated β-galactosidase (SA-β-gal) staining in human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVECs). shRNA- and siRNA-transfected cells were incubated for 6 days, after which SA-β-gal-positive cells were analyzed. Representative data from three independent experiments are shown. Values are means ± SD of three independent experiments. Statistical significance was determined by the Student’s t test (***p < 0.001 vs. siNC). Y = young cells; O = old cells; siNC = negative control siRNA; siPLK1 = PLK1 siRNA; shVec = shRNA retroviral vector; shp53 = p53 shRNA retroviral vector; shp16 = p16 shRNA retroviral vector; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.
progressive aneuploidy and reduction of BubR1 levels in mouse embryonic fibroblasts resulted in aneuploidy and premature senescence (45). Consistent with our results, these reports suggest that alteration of PLK1 activity might be involved in the regulation of cellular senescence. In addition to their involvement in the regulation of cellular senescence, PLK1 reduction in senescent cells of human primary cells might be a secondary effect due to decreased proliferation in old cells. Furthermore, PLK1 inhibition results in lack of chromosome segregation and tetraploidy, which might contribute to induction of cellular senescence. Therefore, further study would be necessary to elucidate the PLK1 inhibition in cellular senescence.

It is well known that cellular senescence depends critically on two tumor suppressor pathways mediated by p53 and pRb/p16INK4A (5). Therefore, we investigated which
tumor suppressor pathway is involved in cellular senescence induced by PLK1 knockdown. Our study showed that the p53-dependent pathway might be involved in the regulation of cellular senescence induced by PLK1 downregulation: (i) PLK1 knockdown decreased cell proliferation in p16 shRNA-treated cells, but not in p53 shRNA-treated cells (Figure 4C) and (ii) PLK1 knockdown increased SA-β-gal activity in p16 shRNA-treated cells but not in p53 shRNA-treated cells (Figure 4D). Recent data described a role for PLK1 as a negative regulator of p53 function. PLK1 inhibits p53 transcription and proapoptotic function by physically binding to the DNA-binding domain of p53 in cultured cells (46). PLK1 also leads to ubiquitinylination and later degradation of p53 through phosphorylation of the topoisomerase I-binding protein torpers (47). GSTE1 (G2 and S phase expressed 1) phosphorylated by PLK1 binds with p53, which is then shuttled out of the nucleus, resulting in p53 inactivation (48). A persistent DNA damage response caused by telomere erosion, genomic damage at nontelomeric sites, histone deacetylase inhibitors, and strong mitogenic signals is known to be critical for the initiation and maintenance of senescence growth arrest (5). PLK1 was reported to play a central role in recovery from the G2 DNA damage checkpoint and is indispensable for the onset of mitosis after DNA damage is repressed (49). PLK1 was revealed recently to inhibit two DNA damage response signaling pathways: the ATR-claspin-CHK1 and ATM-53BP1-CHK2 pathways (17). In addition, PLK1 activity is negatively regulated by several checkpoint pathways, such as those involving ATM, ATR, BRCA1, and CHK1 (50). In response to DNA damage in G2 phase of the cell cycle, PLK1 is degraded by CDH1 (CDC20 homologue 1)-dependent ubiquitinylination, thus preventing cells from entering into mitosis and instead initiating DNA repair (51). p53 is also a negative regulator of PLK1 transcription (52). Collectively, these reports indicate that a complex autoregulatory mechanism between PLK1 and p53 might be involved in the regulation of cellular senescence induced by PLK1 knockdown in human primary cells.

In conclusion, our results imply that during cellular senescence, downregulation of PLK1 might cause growth arrest and cellular senescence due to defective mitosis in human primary cells, thus contributing to tissue homeostasis, organismal aging, and age-related diseases. Furthermore, PLK inhibition during cellular senescence seems to play a role in preventing proliferation of cells with aberrant chromosomes and protecting cellular transformation from chromosomal abnormalities (8).

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Supplementary Material
Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

References


