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CDK2 differentially controls normal cell senescence and cancer cell proliferation upon exposure to reactive oxygen species

Chae Young Hwang, Seung-Min Lee, Sung Sup Park, Ki-Sun Kwon *

Laboratory of Cell Signaling, Aging Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahangno, Yusong, Daejeon 305-806, Republic of Korea

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ABSTRACT

Reactive oxygen species modulate cell fate in a context-dependent manner. Sublethal doses of H_2O_2 decreased the level of proliferating cell nuclear antigen (PCNA) in normal cells (including primary human dermal fibroblasts and IMR-90 cells) without affecting cyclin-dependent kinase 2 (CDK2) activity, leading to cell cycle arrest and subsequent senescence. In contrast, exposure of cancer cells (such as HeLa and MCF7 cells) to H_2O_2 increased CDK2 activity with no accompanying change in the PCNA level, leading to cell proliferation. A CDK2 inhibitor, CVT-313, prevented H_2O_2-induced cancer cell proliferation. These results support the notion that the cyclin/CDK2/$p21^{Cip1}$/PCNA complex plays an important role as a regulator of cell fate decisions.

1. Introduction

Reactive oxygen species (ROS), such as H_2O_2, act as potent second messengers to trigger signal transduction pathways involved in the regulation of cell growth, transformation, senescence, and apoptosis [1]. These distinct biological outcomes may reflect subtle differences in the level and duration of oxidative stimulation as well as cellular context. The relationship between intracellular oxidants and the senescence program is supported by the observation that treatment with exogenous H_2O_2 causes human fibroblasts to rapidly enter senescence [2,3]. Among the accompanying biochemical changes, increases in the levels of tumor suppressor protein, p53, and the cyclin-dependent kinase (CDK) inhibitor, $p21^{Cip1}$, a p53-dependent cell cycle regulator, are significant [4,5]. Inhibition of the kinase activity of the cyclin D–CDK complexes results in hypophosphorylation of retinoblastoma protein (Rb), which keeps E2F inactive, leading to G1 arrest [4,6].

Conversely, H_2O_2 can mimic growth factor-induced G1 → S cell cycle progression in cancer cells [7–9]. In particular, earlier studies have shown that low levels of ROS stimulate proliferation in various cell types [10], whereas antioxidant treatment inhibits proliferation of several cell lines [11–13]. Furthermore, the cellular levels of ROS are tightly regulated throughout the cell cycle [11,12,14]. Overexpression of NADPH oxidase producing ROS causes cellular transformation and tumor growth [8]. Moreover, fibroblasts transformed by constitutively active forms of Ras and Rac1 contain higher levels of ROS [15]. These findings collectively suggest that ROS play a role in cell proliferation, potentially influencing transformation and tumor progression. At the molecular level, ROS can inactivate many protein tyrosine phosphatases [16,17], activate specific kinases and transcription factors [18], and modulate ubiquitin ligase activity that controls the cyclin level [11], thereby affecting the cell cycle in cancer cells. Previously, we showed that low doses of H_2O_2 induce cytoplasmic translocation, ubiquitination and subsequent proteasome-dependent degradation of $p21^{Cip1}$ [19], a protein that functions in the cell cycle [20].

Cyclin-dependent kinases 2 (CDK2) is activated in response to mitotic stimulation of quiescent cells. Activation of CDK2 initiates DNA synthesis and progresses cell cycle from G1 through S phase. Multiple mechanisms exist to regulate CDK2 activation [21]. First, CDK2 activation requires expression of cyclin E or cyclin A. CDK2 activity is also regulated via phosphorylations by CDK-activating kinase (CAK) and Wee kinases. These phosphorylations are removed by CDK-associated phosphatase and CDC25A. Additionally, CDK2 activity is inhibited by inhibitory proteins such as $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. CDK2, complexed with proliferating cell nuclear antigen (PCNA) and cyclins, phosphorylates DNA ligase I and replication factor C as well as Rb protein, resulting in DNA replication and cell cycle progression [22].

In the current study, we investigated the differential regulation of cell fate by ROS in various cell types, taking into consideration a role of the CDK complex in this process. Our data show that

Abbreviations: ROS, reactive oxygen species; CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; FACS, fluorescence-activated cell sorter; BrdU, 5-bromo-2′-deoxyuridine; PI, propidium iodide.

* Corresponding author. Fax: +82 42 879 8596.
E-mail address: kwonks@kribb.re.kr (K.-S. Kwon).

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sublethal doses of H_2O_2 promote CDK2 activation in cancer cells but reduce PCNA levels in normal cells in a transient manner, probably through different states of the cyclin/CDK/$p21^{Cip1}$/PCNA complexes. We propose an additional mechanism linking ROS and cell fate between normal and cancer cells.

2. Materials and methods

2.1. Cell culture

HDFs, IMR-90, wild-type and $p21^{Cip1-/-}$ MEFs, MCF7, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 20 mM HEPES, and antibiotics (Life Technologies Corp., USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Immunoblotting

Immunoblotting was performed as described previously [20]. Cells were lysed in a buffer containing 20 mM HEPES, pH 7.2, 50 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 μg/ml aprotinin, 1 μg leupeptin, 1 mM Na_3VO_4, 1 mM NaF. Antibodies against $p21^{Cip1}$ (C19), β-actin, PCNA, Cyclin D1, Cyclin E, and CDK2 were obtained from Santa Cruz Biotechnology, and antibodies against anti-phospho-CDK2 from Cell Signaling Technology.

2.3. In vitro CDK2 kinase assays

Cells were lysed in lysis buffer (20 mM HEPES, pH7.9, 300 mM NaCl, 100 mM KCl, 10 mM EDTA, 0.1% NP-40, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg leupeptin, 1 mM Na_3VO_4, 1 mM NaF). Lysates were precleared with protein A-agarose (Sigma–Aldrich Inc., USA) and immunoprecipitated with an anti-CDK2 antibody. Immunoprecipitates were washed 3× in lysis buffer and once in CDK2 kinase reaction buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol). The reaction was carried out at 30 °C for 30 min in 40 μl of kinase reaction buffer containing 2.5 μg of histone H1, 10 μCi of [γ-^{32}P]ATP, and 0.01 mM ATP. Subsequently, the reaction was quenched by the addition of SDS–PAGE sample buffer containing β-mercaptoethanol, followed by boiling for 5 min. Kinase reaction mixtures were subjected SDS–PAGE, and exposed to X-ray film.

2.4. Cell cycle analysis

Cell cycle progression was assayed as previously described [20]. Cell cycle was performed with fluorescence-activated cell sorter (FACS; BD Biosciences, USA) using CellQuest software. Cell cycle phases (G1, S and G2/M) were detected and analyzed using ModFit LT.

2.5. BrdU incorporation

Cell proliferation was assessed via 5-bromo-2′-deoxyuridine (BrdU; BD Biosciences) incorporation. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Santa Cruz Biotechnology Inc.,) and incorporation of BrdU was measured using FACS.

2.6. Cell proliferation assay

Relative cell proliferation was assessed by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) assay using Cell Counting kit-8 (Dojindo, Japan). Briefly, WST-8 solution was added to cells for 2 h and then measured the absorbance at 450 nm using a VICTOR^{TM}X3 Multilabel Plate Reader (PerkinElmer Inc., USA).

2.7. Senescence-associated (SA) β-galactosidase activity

SA-β-galactosidase activity in cells was measured as described previously [23]. Blue cells were count under an inverted microscope (Axiovert25; Carl Zeiss, Germany).

3. Results

3.1. H_2O_2 induces cellular senescence in normal cells, but triggers proliferation in cancer cells

To determine whether cell fate is differentially regulated by H_2O_2 in normal and cancer cells, HDFs (human dermal fibroblasts), IMR-90 (human fetal lung fibroblast), HeLa (human cervical carcinoma) and MCF7 (human breast adenocarcinoma) cells were treated with 0.2 mM H_2O_2 (representing the sublethal concentration) [19], and cell proliferation and cell cycle progression examined via BrdU incorporation and FACS analyses after PI staining, respectively. Consistent with previous reports [4,24], normal cells (HDFs and IMR-90) G1 arrest (Fig. 1A) with no change in BrdU-positive cell number (Fig. 1B) after H_2O_2 treatment. At 5 days after H_2O_2 exposure, HDFs showed senescent-like morphology, including flattened, irregular shape and enlarged size [6], as well as senescence associated (SA)-β-galactosidase activity (Fig. 1D). However, the cancer cell lines, HeLa and MCF7, exhibited increased BrdU-positive cell number (Fig. 1B) and S-phase entry (Fig. 1C), with no significant changes in cell morphology or senescence-associated (SA)-β-galactosidase activity (Fig. 1D) after H_2O_2 exposure. Accordingly, we suggest that H_2O_2 treatment at the sublethal dose induces senescence in normal cells, and conversely, proliferation in cancer cells.

3.2. $p21^{Cip1}$ is not necessary for H_2O_2-induced senescence in normal cells

$p21^{Cip1}$ is the first identified gene displaying a progressive increase in expression during cellular senescence [25]. $p21^{Cip1}$ has been shown to induce permanent growth arrest and senescence, both in a p53-dependent and independent manner [26,27]. A previous study reported that the level of $p21^{Cip1}$ protein increases about 18 h after H_2O_2 exposure and remains elevated for at least 21 days, resulting in senescence of HDFs [4]. In contrast, we found that mild H_2O_2 exposure at a similar concentration leads to a transient decrease in $p21^{Cip1}$ protein levels (for ~2 h) in MEFs, HDFs, and IMR-90 cells as well as in HeLa and MCF7 (Fig. 2B). To further assess whether $p21^{Cip1}$ plays a role in H_2O_2-induced senescence in normal cells, wild-type and $p21^{Cip1-/-}$ MEFs were treated with 0.2 mM H_2O_2 for 12 h, and the cell cycles analyzed. Both wild-type and $p21^{Cip1-/-}$ MEFs displayed growth arrest after H_2O_2 treatment (Fig. 2A), consistent with previous findings [28], while a human epithelial carcinoma cell line, HeLa, showed a dramatic increase in S phase entry after H_2O_2 treatment (Fig. 2A). This result indicates that $p21^{Cip1}$ is not necessary for H_2O_2-induced cellular senescence in normal cells.

3.3. H_2O_2 differentially regulates CDK2 activities in normal and cancer cells

To determine whether CDK2 plays a critical role in H_2O_2-induced cell fate decision (senescence or proliferation) in normal and cancer cells, we examined the effects of H_2O_2 on the CDK2 kinase activities in both cell types. Surprisingly, CDK2 kinase activity remained at the basal level in IMR-90 cells after H_2O_2 exposure

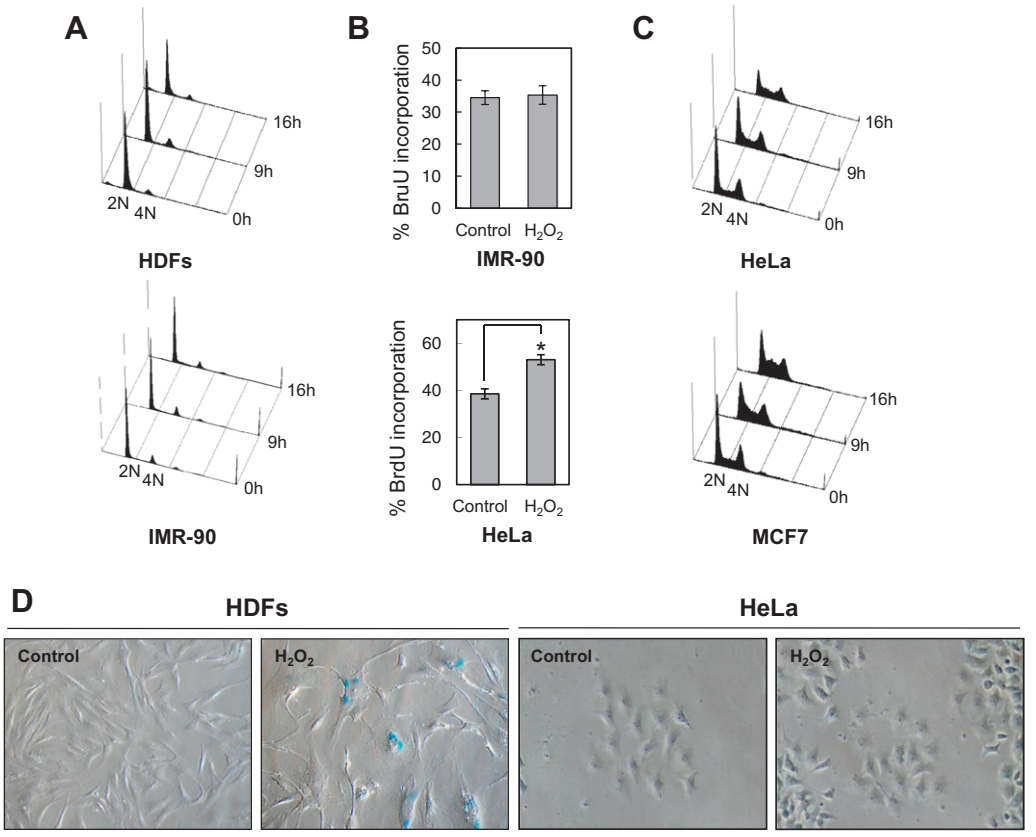

Fig. 1. Sublethal dose of H_2O_2 induces G1 arrest in normal cells but S-phase progression in cancer cells. (A and C) Cell cycle analysis. HDFs, IMR-90, HeLa, and MCF7 cells were starved and treated with 0.2 mM H_2O_2, as described. At 9 and 16 h after H_2O_2 treatment, cells were harvested, permeabilized, and stained with PI, prior to FACS analysis. The percentages of cells within each cell cycle phase (G1, S, and G2/M) were determined based on the DNA content. (B) BrdU incorporation assay. HeLa and IMR-90 cells were starved in serum-free medium for 16 h and treated with or without 0.2 mM H_2O_2 in normal medium (10% FBS in DMEM) for an additional 6 h. Next, cells were labeled with 10 μM BrdU for 1 h prior to harvest. Cells were trypsinized, collected, fixed, and stained with a FITC-conjugated anti-BrdU antibody. The BrdU incorporation percentage of cells was determined via FACS analysis. Values are means ± SD of two independent experiments ($^*P < 0.05$). (D) SA-β-galactosidase activity. HDFs and HeLa cells were treated with or without 0.2 mM H_2O_2 and cultured for an additional 5 days. Cells were subjected to SA-β-galactosidase activity analysis.

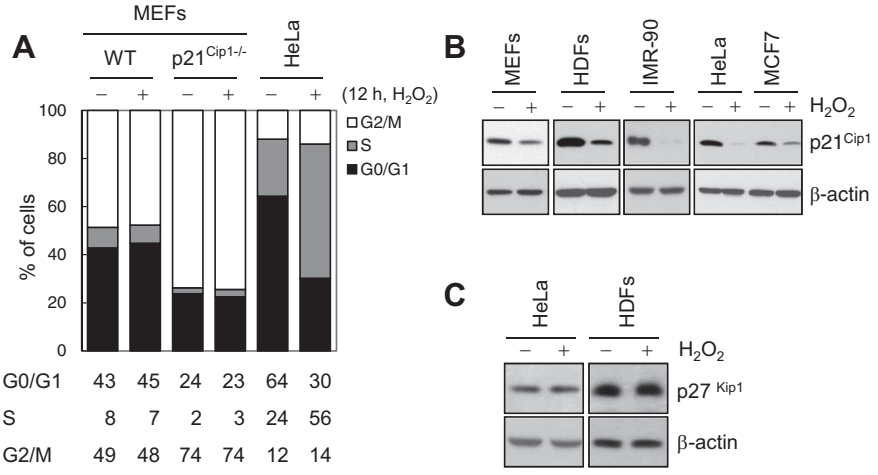

Fig. 2. H_2O_2-induced senescence is not $p21^{Cip1}$-dependent in normal cells. (A) Wild-type MEFs, $p21^{Cip1-/-}$ MEFs, and HeLa cells were starved in serum-free medium for 16 h and treated with 0.2 mM H_2O_2 for 12 h before collecting. Cells were permeabilized and stained with PI, prior to FACS analysis. The percentages of cells within each cell cycle phase (G1, S, and G2/M) were determined based on the DNA content. (B) Western blotting for $p21^{Cip1}$ and -actin. MEFs, HDFs, IMR-90, HeLa, and MCF7 cells were treated with 0.2 mM H_2O_2 for 30 min. (C) Western blotting for $p27^{Kip1}$ and -actin. HDFs and HeLa cells were treated with 0.2 mM H_2O_2 for 30 min.

(Fig. 3A), but was stimulated transiently in HeLa cells reaching peak levels at 30 min after exposure and returning to the basal level at 2 h (Fig. 3A and B). Then we tested whether CDK2 inhibition prevented H_2O_2-induced cell proliferation. HeLa cells pretreated with a CDK2 inhibitor, CVT-313 [29], did not proliferate as good as untreated control cells after H_2O_2 exposure (Fig. 3C). These results suggest that CDK2 plays a pivotal role in H_2O_2-dependent cell fate decision in normal and cancer cells. Next, we examined

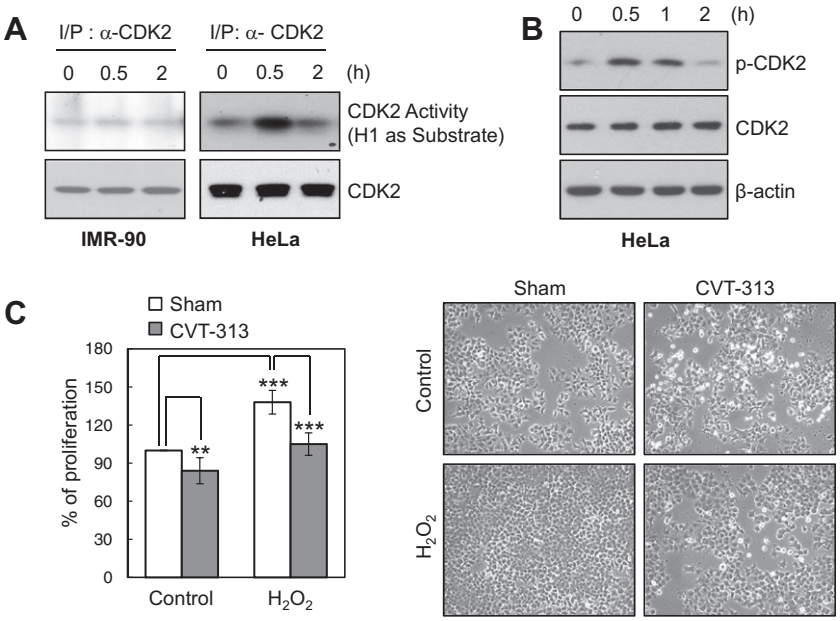

Fig. 3. H_2O_2 differentially regulates CDK2 activities in normal and cancer cells and CDK2 inhibitor hinders H_2O_2-induced cell proliferation in cancer cells. (A) CDK2 kinase assays. (B) Western blotting for phospho-CDK2, CDK2, and β-actin. HeLa cells were treated with 0.2 mM H_2O_2 for the indicated times. (C) Effect of CDK2 inhibitor. HeLa cells were grown with or without H_2O_2 and CVT-313. Values are means ± SD of three independent experiments ($^{**}P < 0.001$, $^{***}P < 0.0001$).

whether the protein levels of cyclins are different in normal and cancer cells upon H_2O_2 treatment. There was no evident change in the protein levels of cyclin D1 and E in both HDFs and MCF7 cells after H_2O_2 treatment (Fig. 4A). This result suggests that cyclins may not determine the cell fates at least in response to H_2O_2 stimuli.

PCNA originally characterized as a DNA polymerase processivity factor coordinates complicated processes in DNA replication, DNA damage repair, and cell cycle control [30]. PCNA and $p21^{Cip1}$ serve as universal components of cyclin-CDK complexes, forming multiple independent quaternary complexes in nontransformed cells [31]. Cellular transformation is associated with selective subunit rearrangement of the cyclin-CDK complexes [32]. We further examined whether the PCNA protein levels were changed after treatment of HDFs and IMR-90 cells with sublethal concentrations of H_2O_2. PCNA levels were decreased at 30 min, reached lowest levels at 1 h, and returned to normal levels at 2 h after H_2O_2 exposure, consistent with both cell types (Fig. 4B). This result suggests that transient PCNA downregulation cause cell cycle arrest (Fig. 1A) and inhibition of DNA synthesis (Fig. 1B) in normal cells. However, PCNA levels remained constant in HeLa and MCF7 cells after H_2O_2 exposure (Fig. 4C). Taken together, we propose that the cyclin/CDK/$p21^{Cip1}$/PCNA complex is critical in the H_2O_2-dependent cell fate decision in normal and cancer cells.

In summary, H_2O_2 induces senescence in normal cells, and conversely, proliferation in cancer cells. While $p21^{Cip1}$ levels are decreased and $p27^{Kip1}$ levels appear constant in both cell types after H_2O_2 treatment, CDK2 activities are increased specifically in cancer cells and PCNA levels decreased in normal cells. In view of these findings, we propose that CDK2 and PCNA, rather than $p21^{Cip1}$ and/or $p27^{kip1}$ protein levels, play critical roles in H_2O_2-induced cell fate decision.

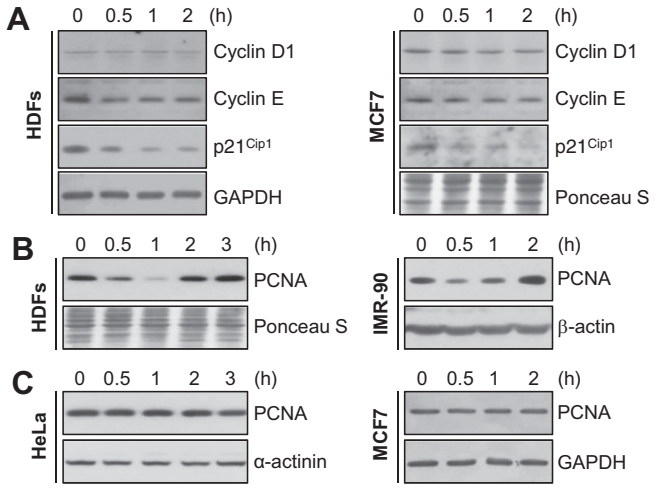

Fig. 4. H_2O_2 differentially regulates PCNA protein levels in normal and cancer cells. (A) Western blotting for cyclin D1, E, $p21^{Cip1}$, and GAPDH. HDFs and MCF7 cells were treated with 0.2 mM H_2O_2 for the indicated times. (B and C) Western blotting for PCNA, -actin, GAPDH, and α-actinin. HDFs, IMR-90, HeLa, and MCF7 cells were treated with 0.2 mM H_2O_2 for the indicated times.

4. Discussion

Data from the present study have provided insights into the molecular mechanisms underlying the distinct effects of H_2O_2 on cell fate decision between senescence and proliferation in normal and cancer cells. Several reports have shown that treatment with sublethal concentrations of H_2O_2 cause primary cells to enter senescence due to the transient increase in p53 protein (at 1.5 h after treatment) and sustained elevation of $p21^{Cip1}$ (at 18 h after treatment, remaining elevated for more than 21 days) [4,5]. Early responses are possibly crucial for fate decision, since cells subcultured immediately after treatment with H_2O_2 show irreversible phenotypes, including sustained elevation of $p21^{Cip1}$ and subsequent senescence [4]. Here, we propose that early responses of CDK activity and PCNA level are possibly critical for proliferation and senescence that occur days after. Our experiments here disclose that ROS differentially regulate DNA synthesis and G1 → S cell cycle progression in normal (HDFs and IMR-90) and cancer (HeLa and MCF7) cells (Fig. 1). Cancer cells progress through the cell cycle dramatically following H_2O_2 treatment, possibly via a

decrease in $p21^{Cip1}$ levels, in turn, increasing CDK2 activity (Fig. 3). Consistently, low levels of ROS stimulate proliferation in various cell types, while antioxidant treatment inhibits proliferation [11–13].

Normal cells do not proliferate but enter senescence following H_2O_2 treatment, probably through modulation of putative senescence factors that regulates CDK2 activity and/or DNA replication. Several possible candidates are putative senescence factors in normal cells. Firstly, we considered $p27^{Kip1}$ as a putative senescence factor, in view of its function as a critical negative regulator of CDK2, similar to $p21^{Cip1}$, and the finding that its overexpression results in G1 arrest in a variety of cell types [33,34]. However, since the $p27^{Kip1}$ protein levels were not affected in HDFs following treatment with H_2O_2 (Fig. 2C), we conclude that $p27^{Kip1}$ is not likely to regulate CDK2 activity in normal cells subjected to H_2O_2 treatment. Secondly, we considered CAK that phosphorylates Thr160 of CDK2 [35]. If H_2O_2 inhibits CAK, inactive CDK2 may induce senescence in normal cells during oxidative stress. However, this theory is yet to be examined. Thirdly, based on the general assumption that p53 activation signals senescence, the p53 protein levels were monitored in HDFs after H_2O_2 treatment. However, we observed no changes in p53 levels (data not shown). Thus, it appears unlikely that p53 activation is involved in H_2O_2-induced cellular senescence, at least in our experimental condition. Finally, we examined whether PCNA, an accessory protein of DNA polymerase δ and regulator of G1/S transition, is a senescence factor activated by H_2O_2. PCNA interacts directly with CDK2 and regulates its activity [22], and its knockdown induces G1 arrest [36]. Interestingly, in our experiments, mild H_2O_2 treatment decreased PCNA protein levels in HDFs and IMR-90, but not HeLa and MCF7 cells (Fig. 4B and C). It is possible that H_2O_2 exposure results in oxidative modification of PCNA, which is subsequently targeted for degradation, particularly in normal cells. Oxidized PCNA proteins are preferentially degraded by the proteasome [37]. Accordingly, we suggest that PCNA downregulation following H_2O_2 treatment results in senescence of normal cells.

Although the mechanism underlying variable regulation of cell fate by ROS through CDK2 modulation in normal and cancer cells remains to be established, our data provide new insights into the pathways of senescence and tumor progression, and support the theory that CDK2 is a promising target for developing therapeutic agents that inhibit tumor progression or increase the sensitivity of tumor cells to chemotherapy.

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