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Role for p53 in Selenium-Induced Senescence

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Supporting Information

ABSTRACT: The tumor suppressor p53 and the ataxia-telangiectasia mutated (ATM) kinase play important roles in the senescence response to oncogene activation and DNA damage. It was previously shown that selenium-containing compounds can activate an ATM-dependent senescence response in MRC-5 normal fibroblasts. Here, the shRNA knockdown approach and other DNA damage assays are employed to test the hypothesis that p53 plays a role in selenium-induced senescence. In MRC-5 cells treated with methylseleninic acid (MSeA, 0−10 μM), depletion of p53 hampers senescence-associated expression of β-galactosidase, disrupts the otherwise S and G2/M cell cycle arrest, desensitizes such cells to MSeA treatment, and increases genome instability. Pretreatment with KU55933, an ATM kinase inhibitor, or NU7026, an inhibitor of DNA-dependent protein kinase, desensitizes MSeA cytotoxicity in scrambled but not p53 shRNA MRC-5 cells. These results suggest that p53 is critical for senescence induction in the response of MRC-5 noncancerous cells to selenium compounds.

KEYWORDS: selenium, p53, senescence, cancer

INTRODUCTION

Senescence limits the proliferation of mitotic cells and serves as an early barrier of tumorigenesis.[1,2] The p53 tumor suppressor and ataxia-telangiectasia mutated (ATM) kinase allow for induction of senescence by DNA replication stress and oncogene activation.[3−5] In response to DNA damage and oxidative stress, the ATM pathway is activated by autophosphorylation on Ser-1981 (pATM Ser-1981).[6,7] p53 is phosphorylated by ATM after DNA damage,[8] resulting in stabilization of p53 protein by interrupting ubiquitin-mediated proteolysis.[9] In addition, catalytic subunit of DNA-dependent protein kinase (DNA-PK$_{cs}$) is in the same family of kinase as ATM and can phosphorylate p53.[10,11]

The majority of adult human tumors carry p53 gene mutations.[12−16] In some cancer cells that express p53, such as the colorectal HCT 116 cells, the p53 pathway can be defective due to loss-of-function mutations of this protein.[17] Also, dysfunction of p53 promotes genome instability and tumor development.[18,19] As a guardian of the genome, p53 responds to DNA damage by arresting cell cycle progression and inducing an apoptotic response. At the transcriptional level, p53 up-regulates expression of an array of genes involved in DNA repair,[20,21] cell cycle arrest,[22,23] and induction of apoptosis.[22,24,25] Alternatively, p53 can induce apoptosis by nontranscriptional mechanisms, including inhibition of antiapoptotic factors in the mitochondria.[26]

Selenium is a promising chemoprevention agent. Selenium-containing compounds, including selenite and methylseleninic acid (MSeA), are known to induce the formation of reactive oxygen species (ROS) and p53-dependent apoptosis.[27,28] However, selenomethionine is not an effective chemoprevention agent and does not induce p53 phosphorylation.[29,30] We have recently shown that MSeA can activate a senescence response in a manner depending on ROS and ATM in MRC-5 normal fibroblasts but not in two lines of cancerous cells.[31] Because p53 is a downstream mediator of ATM and involved in senescence response, we hypothesized that p53 plays a role in the induction of senescence by selenium. To test the possibility, we generated stable p53 and scrambled knockdown cells and demonstrated that p53 could mediate MSeA-induced senescence in MRC-5 cells.

MATERIALS AND METHODS

Cell Culture, p53 shRNA Knockdown, and Chemicals. The MRC-5 normal lung fibroblasts and HCT 116 cancerous colorectal epithelial cells were maintained as described previously.[31−33] We have previously demonstrated a selenium-induced senescence response in MRC-5, but not in HCT 116, cells.[31] HCT 116 cells are known to express p53.[17] The nontarget scrambled shRNA and p53 shRNA sequences (targeting p53 amino acids 231−237) were designed on the basis of Oligoengine 2.0 (Oligoengine Inc., Seattle, WA) and were cloned into the pLKO.1 puro vector (E3 clones, Supplemental Figure 1 of the Supporting Information). We employed a lentivirus-based, pLenti6/BLOCK-iT system and selected for puromycin-resistant clones to generate both the scrambled and p53 stable knockdown MRC-5 and HCT 116 cells following the manufacturer's instruction (Invitrogen, Carlsbad, CA). MSeA was obtained from Sigma-Aldrich (St. Louis, MO) and was dissolved in phosphate-buffered saline (PBS). KU55933 and NU7026 were purchased from Tocris (Ellisville, MO) and dissolved in DMSO.

RNA Isolation and Real-Time Quantitative RT-PCR. The p53 knockdown efficiency was confirmed by quantitative real-time RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized from 2 μg of total RNA using an

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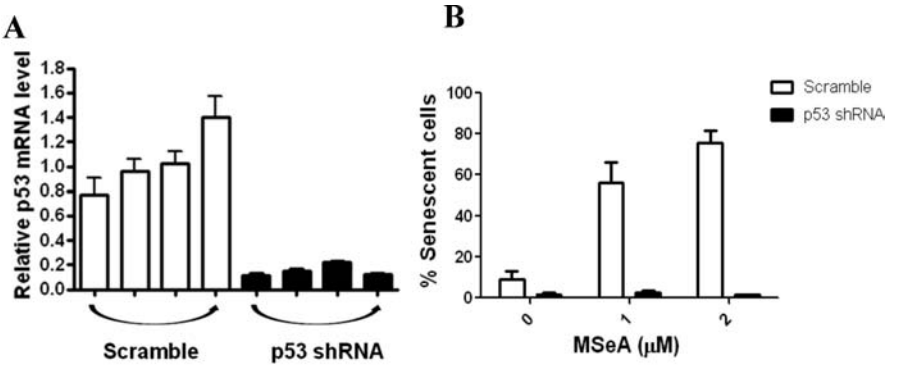

Figure 1. Selenium-induced senescence is suppressed in p53 shRNA MRC-5 cells. (A) p53 knockdown efficiency was determined using quantitative real-time RT-PCR in four clones of scrambled and p53 shRNA MRC-5 cells. (B) Scrambled and p53 shRNA MRC-5 cells were treated with 0−2 μM MSeA for 48 h, followed by a 7-day recovery. Percent SA-β-gal-positive cells are presented as means ± SD (n = 3).

AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer's protocol. Real-time quantitative RT-PCR was carried out using TaqMan Fast Universal PCR Master Mix (2×) (Applied Biosystems, Foster City, CA) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems) following the manufacturer's protocol. Human TaqMan probes and primers were purchased from Applied Biosystems using inventoried TaqMan gene expression assays: TP53 (assay ID: Hs99999147_m1), GAPDH (assay ID: Hs99999905_m1), and an endogenous control. Quantification of mRNA levels was performed using the delta Ct method as previously described.[34]

Senescence and Cell Survival Analyses. Senescence-associated β-galactosidase (SA-β-gal) was detected by using a Senescence Detection Kit (MBL Co. Ltd., Woburn, MA).[31] MRC-5 cells were seeded onto 6-well plates (10^5 cells per well) and incubated with MSeA (1−10 μM) for 48 h, followed by counting the cells using hemacytometers at day 7.

Immunofluorescence. Immunofluorescence analyses of pATM Ser-1981 and phospho-H2A.X on Ser-139 (γH2A.X) were performed as previously described.[31,35] All photos were taken using the same parameters and processed by deconvolution. The focus-positive cells are defined as those containing at least five foci.[36]

Flow Cytometric Analysis. Cells were treated with 2 μM MSeA for 24 h, followed by a 1−7 day recovery. Trypsinized cells were resuspended in 1 mL of propidium iodide solution (25−50 μg/mL) containing RNaseA (100 μg/mL) for 30 min at room temperature in the dark. The DNA was then analyzed by a FACScalibur cytometer with CELLQuest program (Becton Dickinson, San Jose, CA).

Statistical Analysis. The data were analyzed by using SAS 9.0 software (SAS Institute Inc., Cary, NC). A two-tailed Student's t test was applied to determine statistical significance between the treatments. The linear regression was computed to confirm the selenium dose-dependent senescence response in Figure 3A ($p < 0.0001$).

RESULTS

Selenium-Induced Senescence Was Suppressed in p53 shRNA MRC-5 Cells. We generated p53 shRNA MRC-5 and HCT 116 cells to test a role for p53 in selenium-induced senescence. Results from real-time quantitative RT-PCR demonstrated 80−95% knockdown efficiency of p53 in four different E3 clones (Figure 1A and Supplemental Figure 2 in the Supporting Information), from which we selected one clone with the greatest p53 knockdown efficiency for subsequent assays. Whereas scrambled MRC-5 cells showed a senescence response 7 days after recovery from MSeA exposure (1 and 2 μM), p53 shRNA MRC-5 cells were resistant to MSeA-induced senescence (Figure 1B and Supplemental Figure 3 in the Supporting Information). In contrast, MSeA treatment did not induce SA-β-gal expression in HCT 116 cells.[31] Thus, p53 is needed for selenium-induced senescence in MRC-5 normal fibroblasts.

Knockdown of p53 Potentiates MSeA-Induced Expression of pATM Ser-1981 and γH2A.X in MRC-5 Cells. The majority of cancer incidence involves loss-of-function mutations of p53 and shows increased genome instability. Next, we tested a role for p53 in selenium-induced DNA damage response. MSeA treatment (2 μM) resulted in a significant increase of pATM Ser-1981 focus formation, the extent of which was greater in p53 shRNA than in scrambled shRNA MRC-5 cells (Figure 2A and Supplemental Figure 4 in the Supporting Information). Treatment of the scrambled shRNA MRC-5 cells with MSeA (2 μM) resulted in a significant increase in the population of cells expressing γH2A.X foci (Figure 2B and Supplemental Figure 5 in the Supporting Information). Although essentially all of the p53 shRNA MRC-5 cells expressed γH2A.X in the presence or absence of MSeA treatment, these foci were larger after MSeA treatment. Therefore, loss of p53 exacerbates MSeA-induced ATM pathway activation and DNA break formation in MRC-5 cells.

Loss of p53 Rendered MRC-5 Cells Resistant to MSeA-Induced Cytotoxicity. Because p53 drives cells to apoptosis or cell cycle arrest in the stress response, we tested whether or not p53 deficiency affects the sensitivity of MRC-5 cells to MSeA treatment. Compared to scrambled shRNA cells, p53 shRNA cells were more resistant to MSeA treatment in a dose-dependent manner (1−5 μM, Figure 3A). Pretreatment of KU55933 (5 μM, an ATM kinase inhibitor) or NU7026 (20 μM, a DNA-PK$_{cs}$ kinase inhibitor) for 24 h desensitized scrambled shRNA cells to MSeA cytotoxicity (1 μM, Figure 3B). In contrast, the treatment sensitized p53 shRNA cells to MSeA treatment. Consequently, KU55933 and NU7026 pretreatment rendered p53 shRNA cells increased sensitivity to MSeA treatment. This suggests that kinase activities of ATM and DNA-PK$_{cs}$ contribute to the p53-dependent cytotoxicity in MSeA-treated MRC-5 cells.

Effect of p53 Knockdown on Cell Cycle Profiles in MSeA-Treated MRC-5 Cells. DNA damage checkpoint halts cell cycle from entering the next phase before the damage is fixed. A role for p53 in MSeA-induced cell cycle arrest was determined by examining cell cycle profiles 0−7 days after recovery (2 μM). Prior to MSeA treatment, knockdown of p53 resulted in significantly

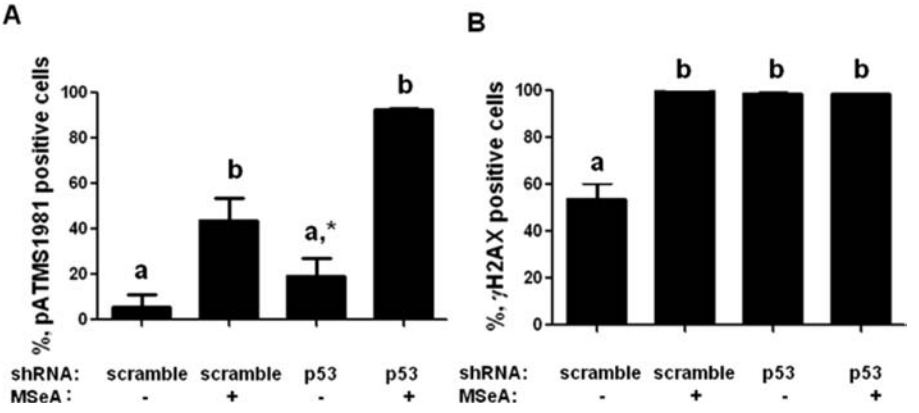

Figure 2. Role for p53 in MSeA-induced phosphorylation of ATM on Ser-1981 (pATMS1981) and H2A.X on Ser-139 (γH2AX) in MRC-5 cells. Scrambled and p53 shRNA MRC-5 cells were treated with MSeA (2 μM, 48 h), followed by a 7-day recovery. Five pictures were randomly taken in each of the slides, and cells expressing pATMS1981 (A) or γH2A.X (B) are presented as means ± SD (n = 3). Bars without the same letter are different (p < 0.05). ∗, p = 0.10, compared with scrambled shRNA cells without MSeA treatment.

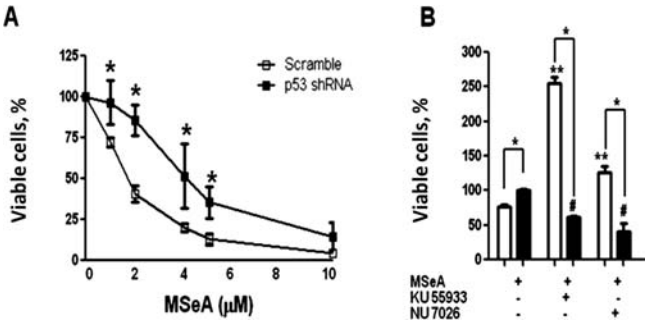

Figure 3. Role for p53 in the sensitivity of MRC-5 to MSeA exposure. Cell viability was determined by counting the number of cells. (A) Cells were treated with MSeA (1−10 μM, 48 h), followed by a 7-day recovery (n = 3). The number of cells in the untreated conditions was set as 100%. (B) Cells were treated with MSeA (1 μM, 48 h) with or without pretreatment with KU55933 (5 μM) or NU7026 (20 μM) for 1 day, followed by a 7-day recovery (n = 3). The number of cells in those without KU55955 or NU7027 treatment was set as 100%. ∗, p < 0.05, and ∗∗, p < 0.05, compared with scrambled shRNA cells treated with MSeA only; #, p < 0.05, compared with p53 shRNA cells treated with MSeA only.

(p < 0.05) increased distribution of MRC-5 cells in S and G2/M phases. By 24 h after MSeA treatment (2 μM), significantly greater p53 shRNA cells appeared at G0/G1 phase (Figure 4A), whereas greater scrambled cells were arrested in S and G2/M phases (Figure 4B,C). One day after recovery, scrambled and p53 shRNA cells showed decreased G0/G1 phase and increased S phase cells. Seven days after recovery, p53 shRNA cells resumed cell cycle distribution reminiscent of that in untreated condition, whereas scrambled shRNA cells continued to arrest in G2/M phase. Therefore, p53 plays an essential role in S and G2/M arrest after MSeA treatment and in G2/M checkpoint during the recovery stage in MRC-5 cells.

DISCUSSION

Previous studies have established selenium-induced apoptosis responses in cancer cells.[37−39] By targeting early barriers of tumorigenesis, our laboratory is the first to demonstrate a role for

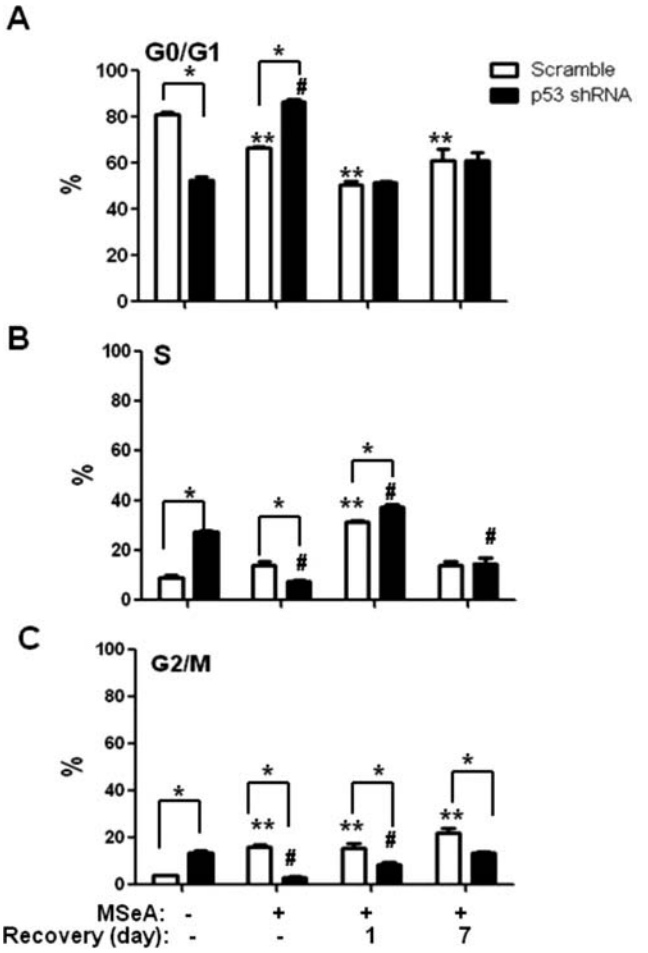

Figure 4. Role for p53 in cell cycle profiles in MRC-5 cells treated with MSeA (2 μM). Scrambled and p53 shRNA MRC-5 cells were treated with MSeA (2 μM, 1 day), followed by 1 or 7 days of recovery. Cell cycle profiles were analyzed by flow cytometry (n = 3). Cells in G0/G1 (A), S (B), and G2/M (C) phases were quantified and presented. ∗, p < 0.05, ∗∗, p < 0.05, compared with scrambled shRNA cells treated with MSeA only; #, p < 0.05, compared with p53 shRNA cells treated with MSeA only.

selenium in the activation senescence and DNA damage response in a manner dependent on ATM and ROS.[31] In light of the fact that p53 is a critical substrate of ATM phosphorylation and a regulator of senescence response, we hypothesized that p53 is involved in selenium-induced senescence and genome instability. To test this possibility, we generated p53 stable knockdown MRC-5 normal cells and identified an indispensible role for p53 in selenium-induced senescence.

The dependence of MSeA-induced senescence on p53 in MRC-5 cells may be attributed to changes in the profiles of cell cycle and gene expression. Senescence is known as an early barrier of tumorigenesis.[2,4] Nonmalignant adenomas removed from ovarian cancer patients undergoing chemotherapy express wild-type p53 and p53-inducible proteins that accelerate G1-to-S transition.[40] Likewise, MSeA treatment (2 μM, 2 days) results in S and G2/M arrest in senescing MRC-5 cells (Figure 4C). In the senescence-devoid p53 shRNA MRC-5 cells after MSeA treatment, the cell cycle may not appropriately arrest, thus progressing with unrepaired DNA damage. Interestingly, treatment of MRC-5 cells with H_2O_2 (500 μM, 45 min) results in induction of p53 protein and G1 cell cycle arrest in the senescent stage 7−10 days after recovery.[41] The difference in cell cycle profiles between these two studies may be explained by duration and doses of the treatment. Furthermore, the superoxide scavenger Tempo, but not the H_2O_2-eliminating NAC, greatly sensitizes scrambled shRNA MRC-5 cells to MSeA treatment (data not shown). Thus, ROS in forms other than H_2O_2 may play a major role in selenium-induced DNA damage response. Selenium in excess can generate ROS and activate the ATM pathway, which in turn causes G1, S, and G2 checkpoint responses.[42,43] It is likely that selenium-induced genotoxic stress could induce DNA oxidation and the subsequent formation of DNA breaks or DNA replication fork collapse, the extent of which is increased in p53-deficient noncancerous cells that do not senesce after selenium treatment.

Loss-of-function mutations of p53, especially for those occurring at the DNA-binding domain, may hamper p53-mediated transactivation and cell cycle arrest in MSeA-treated HCT 116 cells.[44] Moreover, ATM pathway activation by MSeA occurs only in the HCT 116 cancerous cells that express hMLH1 coupled with DNA mismatch repair complementation.[32] This suggests that a defective ATM pathway may preclude a potential role for p53 in selenium-induced senescence in HCT 116 cells. Interestingly, HCT 116 cancerous cells are known to express wild-type p53, but show G1, instead of S or G2/M, arrest after treatment with methylselenol, an active MSeA metabolite.[45] Therefore, both p53-dependent and p53-independent pathways are likely to be defective in HCT 116 cancerous cells, resulting in resistance to MSeA treatment. Consistent with this notion, cell cycle profiles do not significantly differ between scrambled and p53 shRNA HCT 116 cells after MSeA treatment (data not shown). It is of future interest to test this hypothesis by generating a model system that re-expresses wild-type and mutant p53 in p53 shRNA HCT 116 cells.

DNA damage response and senescence serve as early barriers of tumorigenesis that block precancerous and cancerous cells from inappropriate proliferation.[2,4,46] Because ATM is involved in both cell proliferation regulation and DNA repair, the ATM kinase is of great interest in cancer research.[47,48] In selenium-induced senescence, ATM activation and increased γH2A.X formation were observed. In p53 shRNA MRC-5 cells, γH2A.X level was already high prior to MSeA treatment (Figure 2B). Thus, p53 shRNA MRC-5 cells exhibit significant intrinsic genomic instability, which is similar to the observation of high γH2A.X background in PC-3 cancerous cells.[31] In addition to DNA double-strand breaks, ATM kinase can also be activated by various forms of chromosome alterations.[6] Importantly, our previous results indicate that inhibition of ATM kinase activity attenuates, but does not prevent, selenium-induced γH2A.X.[31] Apparently, kinases other than ATM phosphorylate H2A.X and contribute to increased γH2A.X expression in scrambled and p53 shRNA MRC-5 cells. Whether increased genomic instability in p53-deficient cells can be rescued or attenuated by antioxidants or DNA damage kinase inhibitors awaits further investigation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

ATM, ataxia-telangiectasia mutated; DNA-PK_{cs}, catalytic subunit of DNA-dependent protein kinase; pATM Ser-1981, phosphorylation of ATM on Ser-1981; ROS, reactive oxygen species; MSeA, methylseleninic acid; γH2A.X, phospho-H2A.X on Ser-139; SA-β-gal, senescence-associated β-galactosidase.

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