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SM22α-induced activation of p16^{INK4a}/retinoblastoma pathway promotes cellular senescence caused by a subclinical dose of γ-radiation and doxorubicin in HepG2 cells

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ABSTRACT

Smooth muscle protein 22-alpha (SM22α) is known as a transformation- and shape change-sensitive actin cross-linking protein found in smooth muscle tissue and fibroblasts; however, its functional role remains uncertain. We reported previously that SM22α overexpression confers resistance against anti-cancer drugs or radiation via induction of metallothionein (MT) isozymes in HepG2 cells. In this study, we demonstrate that SM22α overexpression leads cells to a growth arrest state and promotes cellular senescence caused by treatment with a subclinical dose of γ-radiation (0.05 and 0.1 Gy) or doxorubicin (0.01 and 0.05 μg/ml), compared to control cells. Senescence growth arrest is known to be controlled by p53 phosphorylation/p21$^{WAF1/Cip1}$ induction or p16^{INK4a}/retinoblastoma protein (pRB) activation. SM22α overexpression in HepG2 cells elevated p16^{INK4a} followed by pRB activation, but did not activate the p53/p21$^{WAF1/Cip1}$ pathway. Moreover, MT-1G, which is induced by SM22α overexpression, was involved in the activation of the p16^{INK4a}/pRB pathway, which led to a growth arrest state and promoted cellular senescence caused by damaging agents. Our findings provide the first demonstration that SM22α modulates cellular senescence caused by damaging agents via regulation of the p16^{INK4a}/pRB pathway in HepG2 cells and that these effects of SM22α are partially mediated by MT-1G.

1. Introduction

Cellular or replicative senescence is a process leading proliferating somatic cells to permanent growth arrest and is recognized as a response of cells against damage and stress from exogenous and endogenous sources [1]. Senescence is considered deleterious because it contributes to decrements in tissue renewal and function, and eventually to aging. However, induction of cellular senescence in tumor cells can be beneficial because it can function as a tumor-suppressive mechanism. Therefore, a number of studies on different cancer cell lines have attempted to induce replicative senescence by genetic, chemical, or biological treatments, and thus, provide preliminary evidence for considering senescence as an anti-cancer therapy [2–7].

Smooth muscle protein 22-alpha (SM22α) is a member of actin-binding proteins and is abundantly expressed in smooth muscle cells of normal adult vertebrates [8]. Although its functional role remains uncertain, abolition of SM22α gene expression by the oncogenic Ras has been shown to be an important early event in tumor progression and was suggested as a diagnostic marker for breast and colon cancer development [9]. However, SM22α is expressed in various tumor cell lines and is known to confer cellular resistance against anti-cancer drugs or gamma-radiation [10], which makes other cancer therapies necessary to suppress the proliferation of SM22-expressing tumor cells. Interestingly, SM22α was also reported to be increased in senescence-induced fibroblasts [11]. Given this, we tested the possibility of senescence-induced tumor suppression in SM22α-expressing tumor cells and showed that SM22α overexpression in HepG2 hepatocellular carcinoma cells promotes cellular senescence caused by cytotoxic agents via activation of the p16^{INK4a}/retinoblastoma protein (pRB) pathway.

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2. Materials and methods

2.1. Cell culture and chemicals

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone). Cells were treated with methylmethanesulfonate (MMS, Sigma–Aldrich, St. Louis, MO) and doxorubicin (Sigma–Aldrich) at the final concentration of 0.01–10 μM. For γ-irradiation, cells were cultured in a 35-mm dish (1×10^6 cells/dish) and after 24 h were irradiated with a single exposure to 0.05 Gy (^{60}Co γ-ray source; dose rate, 0.02 Gy/min) or 0.1 Gy (dose rate, 0.02 Gy/min). The cells were then cultured for the indicated time periods.

2.2. Construction of SM22α and metallothionein (MT)-1G expression vectors

To construct the SM22α expression vector, a 636-bp insert of human SM22α was amplified from poly(A) mRNA of HepG2 cells by reverse transcription polymerase chain reaction (RT-PCR) using the following primers: HindIII (forward), 5′-AGCTTAAGCTTGACATGGCCAACAAG-3′; and BamHI (reverse): 5′-GCGGATCCTCTCCGCTCTAACTG-3′. To construct the MT-1G expression vector, a 294 bp insert of human MT-1G was also amplified from poly(A) mRNA of HepG2 cells by RT-PCR with the following primers: HindIII (forward), 5′-ACTCCAAGCTTCACGTGCACCCA-3′; and EcoRI (reverse), 5′-CTGAATTCACTTG GGAGCAGGGC-3′. The SM22α and MT-1G cDNA inserts were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen). To prepare SM22α-overexpressing HepG2 cell clones, SM22α overexpression vector (SM22α/pcDNA3.1) was transfected into the HepG2 cell using Lipofectamine 2000 (Invitrogen), followed by selection with 500 μg/mL G418 (Calbiochem, San Diego, CA).

2.3. Small interfering RNA transfection

The small interfering RNAs (siRNA) against SM22α were obtained from the siGENOME SMARTpool TAGLN (D-003714-02, 03, 05, 18; Dharmacon, Lafayette, Co., Table 1), and the negative control Medium GC was obtained from Invitrogen. Cells were seeded at 2×10^5 cells per well in six-well plates and allowed to reach ~50% confluence on the day of transfection. Cells were then transfected with 100 nM siRNA in Opti-MEM medium (Invitrogen) using Lipofectamine™ RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. The cells were incubated for 72 h post-transfection, and the efficiency of siRNA was measured by Western blot analysis.

2.4. cDNA synthesis and PCR amplification

Total RNA was isolated from the cells using easy-BLUE™ Reagent (Intron Biotechnology, Sungnam, Korea) according to the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA was used in a 20-μl reaction mixture constituted using Maxime RT PreMix kit (Intron Biotechnology), and 1 μl of the cDNA was used for synthesis of the PCR product in a 20-μl reaction mixture constituted using Maxime PCR PreMix Kit (i-Taq; Intron Biotechnology) and specific primers (SM22α, forward: 5′-TCCCAAGCTTATGGCCAACAAGGG TCCTTCC-3′; reverse: 5′-GCGGATCCCTAACTGATGATCTGCCGAGG TC-3′; MT-1G, forward: 5′-ACGAATTCTTCCACGTGCA CCCA-3′; reverse: 5′-CTATTAAGCTTTGGGAGCAGGGC-3′; $p16^{INK4a}$, forward: 5′-ATGGAGCCGGCGGCGGGGAGC-3′; reverse: 5′-TCAATCGGGGATG TCTGAGGGACCTTCCG-3′; β-actin, forward: 5′-CATCCTCACCCTGAA GTACCC-3′; reverse: 5′-AGCCTGGATAGCAACGTACATG-3′). PCR was carried out as follows: 94 °C for 4 min; 30 cycles at 94 °C for 1 min, 65 °C ($p16^{INK4a}$) or 64 °C (MT-1G) for 1 min, and 72 °C for 1 min; and 72 °C for 5 min. The PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining.

2.5. Western blot analysis

Anti-phospho-pRB (serine 608, 780, and 807/811), anti-$p21^{WAF1/Cip1}$, anti-$p16^{INK4a}$, and anti-phospho-p53 (serine 14) antibodies for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the anti-SM22α antibody from Abcam (Cambridge, UK). All reagents used in this study were reagent-grade or better. Protein concentration was determined with a Lowry kit (Bio-Rad, Hercules, CA). Equal amounts of protein were separated on a 10% or 15% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond, Amersham Bioscience, UK). The blots were blocked for 1 h at room temperature with blocking buffer (10% nonfat milk in phosphate-buffered saline PBS containing 0.1% Tween 20). The membrane was incubated overnight at 4 °C with the specific primary antibody solution. After washing with TBS, the membrane was probed with a horseradish peroxidase-labeled secondary antibody and visualized with the Westzol enhanced chemiluminescence detection kit (Intron Biotechnology).

2.6. Senescence-associated β-galactosidase (SA-β-Gal) activity staining

SA-β-Gal activity staining was performed using bromo-4-chloro-3-indolyl-β-D-galactosidase according to the manufacturer's instructions (Intron Biotechnology). Quantification of SA-Gal-positive cells was obtained by counting five random fields per dish and assessing the percentage.

2.7. Colony-forming assay

For the colony-forming assay, cells were plated in 35-mm culture dishes at a density of 5×10^3 cells per plate. Cells were incubated for 10–20 days and stained with 0.5% crystal violet. Colonies, defined as groups of 50 or more cells, were counted. Clonogenic survival of tested cells was expressed as a percentage relative to the untreated controls.

3. Results

3.1. SM22α overexpression promoted cellular senescence caused by γ-radiation and doxorubicin

For the study of cellular senescence induced by DNA damage, a human hepatocellular carcinoma cell line HepG2 was selected because it expresses wild-type $p53/p21^{WAF1/Cip1}$ and $p16^{INK4a}$/pRB systems, which are known to be involved in cell growth arrest and cellular senescence. SM22α expression is also relatively very low in HepG2 cells compared to other tumor cell lines. When HepG2 cells were treated with MMS, an alkylating agent that methylates DNA, cellular senescence with cell growth inhibition

Table 1
siRNA sequences against SM22α (TAGLN).

Primer (siSM22α)	Size	Sequence (5′ → 3′)
D-003714-02	19 mer	AGAAAGCGCAGGAGGAUAA
D-003714-03	19 mer	CCAGACUGUUGACCUCUUU
D-003714-05	19 mer	CCAAAAUCGAGAAGAAGUA
D-003714-18	19 mer	UGUCCUCCUUGGCGGCAAA

was induced, as indicated by the increase in SA-β-Gal activity staining (Fig. 1A). In these cells, the expression level of SM22α was also highly increased, an occurrence reported to be associated with senescent cells [12–14] (Fig. 1B). To investigate whether SM22α would be involved in the signaling pathway regulating cell growth or cellular senescence in HepG2 cells, SM22α/pcDNA3.1 expression vector was transfected into HepG2 cells, and two stable SM22α-overexpressing cell lines were selected (SM22α(+) S4 and S6; Fig. 2A). Cell growth of these SM22α-overexpressing cells was measured by the colony-forming assay. As shown in Fig. 2B, SM22α overexpression significantly induced the inhibition of cell growth of HepG2 cells. Conversely, when SM22α-overexpressing cells were transfected with siRNA, which suppresses the expression of SM22α (Fig. 2C), the cell growth was partially restored to that of control cells (Fig. 2D). These results suggest that SM22α is closely involved in regulation of cell growth and senescence.

Overexpression of SM22α in S6 or S4 clone cells, however, did not immediately or directly cause cellular senescence (Fig 3). Cellular senescence can be induced by treatment of DNA-damaging agents such as γ-radiation or doxorubicin, an inducer of DNA adduct [15,16]. When vector-control HepG2 cells were treated with doxorubicin (0.01 and 0.05 μg/ml) or γ-radiation (0.05 and 0.1 Gy), cellular senescence was induced in 2–5% of total cells. In contrast, significant portions of S6 and S4 cells showed a dose-dependent induction of a senescent state (Fig. 3), suggesting that SM22α functions as a promoter of cellular senescence.

3.2. SM22α promoted cellular senescence via activation of the $p16^{INK4a}$/pRB pathway and MT-1G acted as a mediator of SM22α signaling

To identify downstream targets of SM22α that regulate cell growth arrest and thus promote cellular senescence, we analyzed cellular states of $p53/p21^{WAF1/Cip1}$ and $p16^{INK4a}$/pRB, which are known to be primarily responsible for cell growth inhibition and cellular senescence induction [4]. The phosphorylation of p53 is one of the common steps in growth arrest or the induction of senescence arrest. However, in SM22α-overexpressing HepG2 cells, phosphorylation of p53 (serine 14) was not detected (Fig 4A). In addition, p53-dependent activation of $p21^{WAF1/Cip1}$, which is particularly important for cell cycle arrest after DNA damage due to γ-radiation and chemotherapy, was not significantly changed (Fig 4A). Therefore, other mechanisms of senescence that are apparently driven by DNA damage or other signals were investigated. Of particular

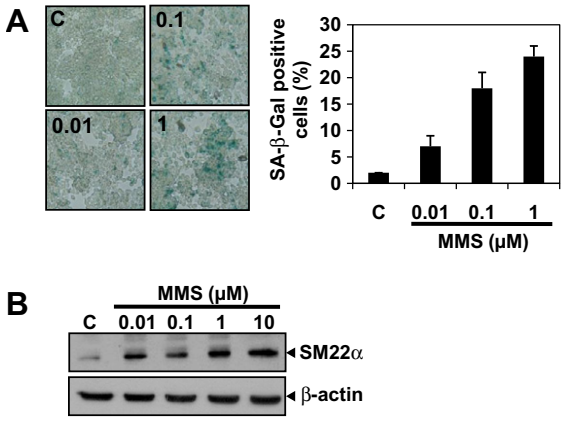

Fig. 1. Methylmethanesulfonate (MMS) induced cellular senescence and the elevation of SM22 in HepG2 cells. (A) SA-β-Gal activity staining analysis after MMS treatment on HepG2 cells. Cells (1.5×10^5 cells per well of 6-well plate) were treated with MMS for 48 h at indicated concentrations. (B) Western blot analysis of SM22α in HepG2 cells after MMS treatment for 48 h at indicated concentrations. Lysates were also analyzed by immunoblotting with anti-β-actin as a loading control.

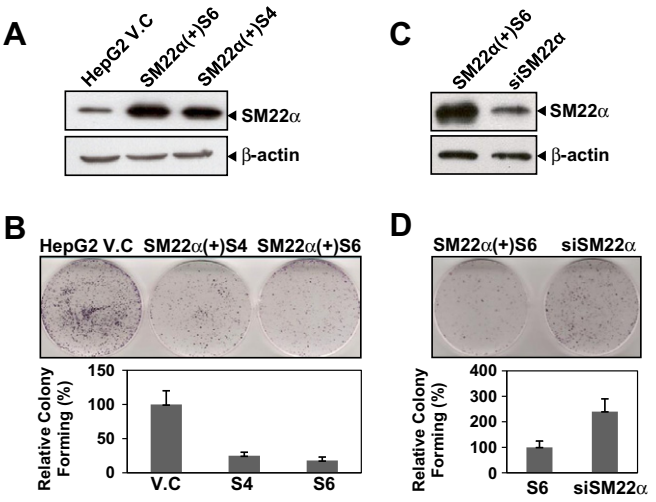

Fig. 2. Comparison of colony forming capacity of SM22α-overexpressing or suppressed cells. (A) Two selected SM22α-overexpressing clones (S4 and S6) were confirmed by Western blot analysis. Empty vector-transfectant cells (HepG2V.C) were used as control. (B) Colony forming capacity of SM22α-overexpressing cells. For the colony-forming assay, cells were plated in 35-mm culture dishes at a density of 5×10^3 cells per plate. Cells were incubated for 10 d and stained with 0.5% crystal violet. (C) Overexpressed-SM22α in SM22α(+)S6 cells was re-suppressed by using siSM22α, which was confirmed by Western blot analysis. (D) Change of colony forming capacity by re-suppression of SM22α with siRNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

importance is the INK4 gene encoding $p16^{INK4a}$. As a cyclin-dependent kinase inhibitor, $p16^{INK4a}$ connects some senescence-initiating signals to the pRB pathway, which is independent of p53 activation. SM22α overexpression resulted in a significant elevation of $p16^{INK4a}$ when analyzed by RT-PCR and Western blot (Fig. 4A). In wild-type HepG2 cells, serine 780 and 807/811 residues of pRB were highly phosphorylated. However, SM22α overexpression in these cells significantly inhibited the phosphorylation of serine 780 and 807/811 residues in pRB, which may prevent the detachment of E2F from pRB and thus suppress cell proliferation. Phosphorylation of serine 608 residue in pRB was also significantly inhibited (Fig. 4A). On the other hand, when SM22α-overexpressing cells were transfected again with siRNA to suppress SM22α expression, serine 608, 780, and 807/811 residues in pRB were re-phosphorylated (Fig 4B), thereby restoring cell growth to a certain level (Fig. 2D). These results suggest that the activation $p16^{INK4a}$/pRB by SM22α is involved in cellular senescence promotion.

We also investigated whether MT-1G regulates cellular levels of $p16^{INK4a}$/p-pRB. In a previous study, we showed that SM22α overexpression elevated the expression of MT isozymes, especially MT-1G, which confer cell resistance and thus inhibit apoptotic cell death [10]. In two selected SM22α-overexpressing clones, the levels of MT-1G were also elevated (Fig. 4C), and forced overexpression of MT-1G in HepG2 cells also increased the $p16^{INK4a}$ level, inhibiting phosphorylation of pRB (Fig. 4D). We also confirmed that MT-1G overexpression in HepG2 cells induced cell growth arrest by a colony-forming assay (Fig. 4E). In these cells, senescence was promoted by a low dose of doxorubicin (Fig. 4F). Taken together, these data suggest that overexpression of MT-1G mediates cell growth arrest and promotion of cellular senescence induced by γ-radiation or doxorubicin in SM22α-overexpressing cells and is associated with pRB activation.

4. Discussion

Cellular senescence was first investigated by Hayflick et al. [15] when they observed that the *in vitro* lifetime of normal human

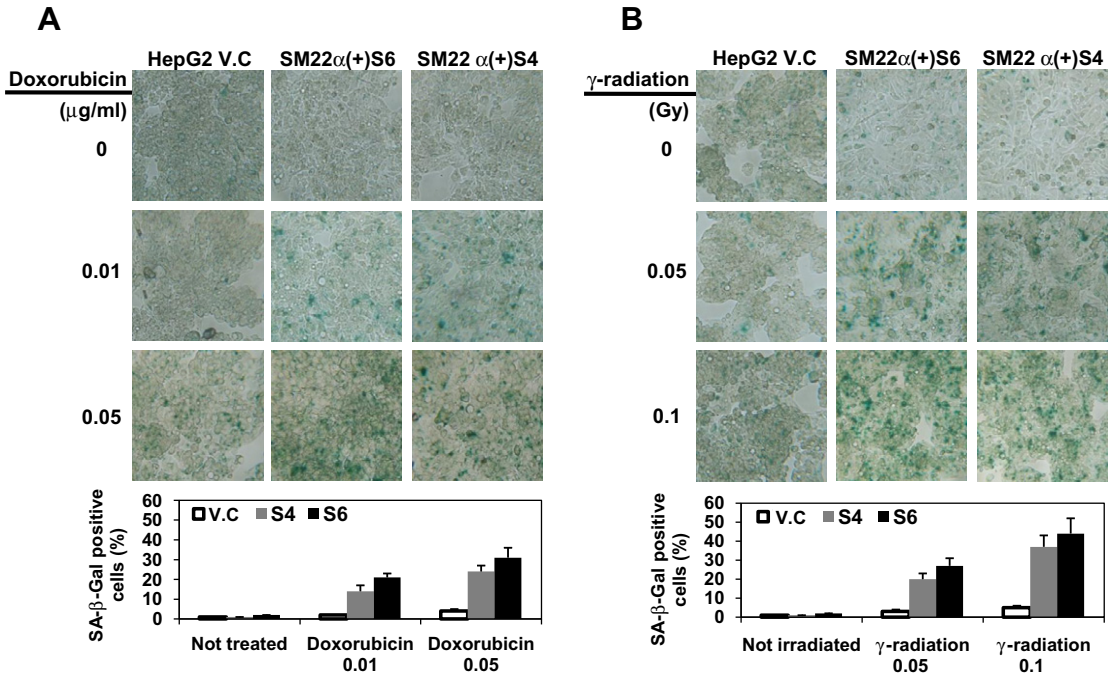

Fig. 3. Comparison of cellular senescence induced by cytotoxic agents using β-gal staining assay in SM22α-overexpressing HepG2 cells. (A) Cellular senescence promotion by doxorubicin (0.01 and 0.05 μg/ml) in stable SM22α-transfectant HepG2 cells. SA-β-Gal activity staining analysis was performed after cells were treated with doxorubicin for 48 h at indicated concentrations. (B) Cellular senescence promotion by γ-radiation (0.05 and 0.1 Gy) in stable SM22α-transfected HepG2 cells. Cells were plated in 35-mm culture dishes at a density of 1×10^6 cells per plate and, after 24 h, were irradiated with a single exposure to a dose of 0.05 and 0.1 Gy. After 72 h, SA-β-Gal activity staining was performed.

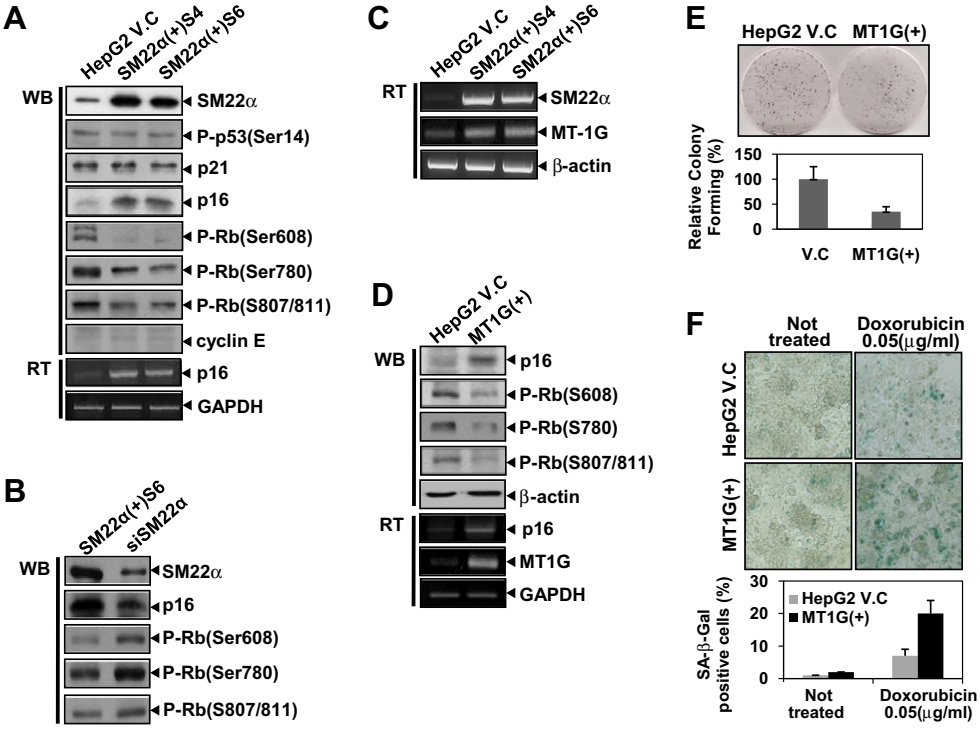

Fig. 4. Activation of the $p16^{INK4a}$/pRB senescence signaling pathway by SM22α overexpression and MT-1G-mediated SM22α signaling. (A) Western blot and RT-PCR analysis of the expression of proteins related to cellular senescence in SM22α-overexpressing HepG2 cells. (B) Western blot analysis of the $p16^{INK4a}$/pRB pathway in SM22α-suppressed S6 cells using siRNA. (C) RT-PCR analysis of MT-1G in SM22α-overexpressing cells. (D) Western blot and RT-PCR analysis of the $p16^{INK4a}$/pRB pathway in MT-1G-overexpressing HepG2 cells. Cells (1×10^5) were analyzed 72 h after transfection of MT-1G/pcDNA3.1 expression vector (1 μg). (E) Colony forming capacity of MT-1G-overexpressing HepG2 cells. (F) Cellular senescence promotion by doxorubicin in MT-1G-overexpressing HepG2 cells. Cells (1.5×10^5) were transfected with MT-1G expression vector, grown for 72 h, and then treated with doxorubicin for 48 h, followed by SA-β-Gal activity staining.

embryonic fibroblast and human diploid cell strains was limited. After serial passage in culture, normal fibroblasts enter a permanent growth arrest state that was termed "replicative senescence." Considerable effort has been put into elucidating the mechanism that triggers senescence [2,3], and persistent DNA damage is considered the most common cause. Because hepatocyte senescence occurs *in vivo* in patients with chronic hepatitis and cirrhosis, senescence, a fail-safe mechanism, has been proposed to play an important role in protecting against tumorigenesis [16,17]. The cellular response to DNA damage and stress is largely controlled by the tumor suppressor p53 and one of its target genes, $p21^{WAF1/Cip1}$, which was first discovered as a gene that is overexpressed in cellular senescence [18]. Since $p21^{WAF1/Cip1}$ is a universal cyclin-dependent kinase inhibitor, the $p21^{WAF1/Cip1}$ protein functions as a mediator of p53 in controlling cell growth arrest and $p53/p21^{WAF1/Cip1}$ is the typical signaling pathway for the induction of cellular senescence. However, in p53 mutant cell lines, $p21^{WAF1/Cip1}$ can also be induced by stresses, including chemicals. Muristeron-induced p53-independent $p21^{WAF1/Cip1}$ induction in H1299 (p53 mutant) cell lines promotes cellular senescence by γ-radiation [19]. But in SM22α-overexpressing cells, $p21^{WAF1/Cip1}$ as well as p53 activation was not significantly induced. These results indicate that cell growth suppression caused by SM22α overexpression may not be accompanied by accumulation of activated p53 or $p21^{WAF1/Cip1}$. In HepG2 cells, $p16^{INK4a}$ as well as p53 and $p21^{WAF1/Cip1}$ are normal, and $p16^{INK4a}$ can lead to pRB activation, which is associated with cellular senescence irrespective of p53. Therefore, we investigated another possible senescence mechanism, the $p16^{INK4a}$/pRB pathway, and confirmed that this pathway was operative. Our findings suggest that SM22α-induced $p16^{INK4a}$/pRB activation is a novel pathway for cell growth inhibition and senescence.

MTs are low-molecular-weight and cysteine-rich proteins with metal ion contents. Increased expression of MT has been associated with malignancy in some tumors. They play multiple functions, including the protection against various injuries such as DNA damage resulting from anti-cancer drugs or γ-radiation and promotion of tumor survival [20–22]. However, MT is also down-regulated in other tumors such as hepatocellular carcinoma. MT overexpression is not universal to all tumor cells but may depend on the differentiation status and proliferative index. Because the MT expression pattern in human tumors is influenced by various factors such as the type of tumor, cell origin, histological type, degree of differentiation, stage of growth, and the presence of subpopulations, it is difficult to study the role and mechanism of MT functions. In hepatocellular carcinoma HepG2 cells, enforced MT-1F overexpression showed a growth suppression effect [23]. We also showed that SM22α-induced MT-1G, which was not significantly expressed in HepG2 cells, also induced cell growth inhibition. Recent studies have suggested that MT-1G acts as a tumor suppressor gene, and MT-1G silencing is associated with hypermethylation of the promoter region in hepatocellular carcinoma [24]. MT protein has a zinc finger domain that regulates the transcription of many downstream target genes by binding to the GC-rich element (GCE) in the promoter region, resulting in modulation of gene transcription [25]. Therefore, a mechanism of tumor suppression by MT could be that MT competes with transcription activator SP1 by binding to an overlapping consensus binding motif in the promoter region of GCE, which commonly exists in oncogene and tumor suppressor genes, and abolishes the function of SP1 thereby inhibiting tumor cell growth [26,27]. SM22α-induced MT-1G may partially function as a mediator of cell growth arrest via a similar pathway, and further studies are required to investigate this.

Senescence induction is now recognized as a novel mechanism contributing to the cessation of growth of premalignant or benign neoplasm to prevent malignant cancer development. Abolition of SM22α gene expression by the oncogenic Ras may be an important early event in tumor progression and a diagnostic marker for breast and colon cancer development [9]. Therefore, recent studies have continuously suggested that SM22α functions as a tumor suppressor or is involved in protection against anti-cancer drugs [10,28–31]. In this study, we first showed that SM22α overexpression, induced by DNA damages or forced overexpression, promotes cellular senescence caused by cytotoxic agents via regulation of the $p16^{INK4a}$/pRB pathway. Both senescence and cell death are fail-safe mechanisms for cancer therapy. However, because resistance or tolerance to chemotherapy and radiotherapy is a major obstacle in treating malignant cancer, induction of premature senescence using low doses of ionizing radiation or anti-cancer drugs could be an alternative proposal for cancer therapy. Despite stress-induced SM22α being an obstacle to chemo- or radiation cancer therapy, $p16^{INK4a}$/pRB activation and growth arrest by increments of SM22α would sensitize cellular senescence, which can be induced by even a low dose of cytotoxic agents or radiation.

In summary, these results are the first to provide the possible function of SM22α in cellular senescence via the SM22α/MT-1G/$p16^{INK4a}$/pRB signaling pathway and thus provide greater insight into the merits of SM22α in cancer therapy.

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