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SF3B4 Regulates Cellular Senescence and Suppresses Therapy-induced Senescence of Cancer Cells

SEUNGYEON YANG[1,2*], MINBEOM KO[1,2*], SOOJUNG CLAIRE HUR[3], EUN KYUNG LEE[1,2] and SEUNG MIN JEONG[1,2]

[1]*Department of Biochemistry, Institute for Aging and Metabolic Diseases, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea;*
[2]*Department of Medical Sciences, Graduate School of The Catholic University of Korea, Seoul, Republic of Korea;*
[3]*Department of Mechanical Engineering, Johns Hopkins University, Baltimore, MD, U.S.A.*

Abstract. *Background/Aim: Cellular senescence is a state in which cells permanently exit the cell cycle, preventing tumor growth, but it can also contribute to aging and chronic inflammation. Senescence induced by cancer therapies, known as therapy-induced senescence (TIS), halts cancer cell proliferation and prevents metastasis. TIS has been investigated as an important therapeutic approach that could minimize cytotoxicity effects. This study aimed to elucidate the role of splicing factor 3B subunit 4 (SF3B4) in cellular senescence and TIS in cancer cells. Materials and Methods: β-galactosidase staining was used to examine senescence induction. SF3B4 and p21 expression were determined by RT-qPCR and western blot. Cell proliferation and cell death were evaluated. Results: SF3B4 expression decreases in replicative senescent human fibroblasts and its knockdown induces senescence via a p21-dependent pathway. In A549 non-small cell lung cancer (NSCLC) cells, SF3B4 knockdown also increased senescence markers. Notably, SF3B4 overexpression mitigated doxorubicin-induced senescence in A549 cells. Conclusion: SF3B4 regulates senescence, and this study highlights its potential as a therapeutic target for developing better cancer treatment strategies by leveraging TIS to suppress tumor growth and enhance treatment efficacy.*

*These Authors contributed equally to this work.

Correspondence to: Seung Min Jeong, Department of Biochemistry, College of Medicine, The Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul, 06591, Republic of Korea. Tel: +82 231478336, Fax: +82 25964435, e-mail: smjeong@catholic.ac.kr

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Cellular senescence refers to a state in which cells permanently stop dividing and enter a state of irreversible cell cycle arrest due to various cellular stresses, such as DNA damage, oxidative stress, and oncogene activation (1). Senescence is characterized by distinct morphological changes, altered gene expression, and the release of a wide range of pro-inflammatory cytokines, growth factors, and proteases, collectively termed the senescence-associated secretory phenotype (SASP) (2). This process serves as a crucial tumor suppressive mechanism by preventing the proliferation of damaged or mutated cells, and also plays significant roles in tissue repair and wound healing (1, 3). However, the accumulation of senescent cells over time leads to chronic inflammation and the development of age-related diseases by altering cellular and tissues functions, changing tissue microenvironments, and promoting degenerative processes (4, 5).

Senescence can be induced during various cancer therapies, such as chemotherapy, radiation therapy, and targeted therapy (6). When cancer cells receive non-lethal stress from these treatments, they enter a state known as therapy-induced senescence (TIS) (7). TIS offers several benefits in cancer treatment, acting as a tumor-suppressive mechanism by halting cancer cell proliferation and preventing metastasis (8). Moreover, the SASP factors produced by senescent cancer cells can recruit immune cells to the tumor regions, enhancing immune clearance (6). Additionally, TIS sensitizes cancer cells to further treatments and reduces tumor heterogeneity, making subsequent therapies more effective (6, 9). Thus, TIS limits cancer cells' ability to adapt and develop drug resistance. Consequently, TIS has been investigated as an effective method to achieve sustained suppression of cancer cell growth with minimal cytotoxicity and to enhance the effectiveness of combinational cancer treatments.

Splicing factor 3b subunit 4 (SF3B4) is a crucial component of the spliceosome, the complex responsible for the accurate removal of introns from pre-mRNA transcripts, ensuring proper gene expression (10). SF3B4 has been associated with numerous cellular processes beyond splicing, including cell cycle regulation and apoptosis (11). In cancer, the dysregulation and overexpression of SF3B4 have been linked to multiple malignancies, such as hepatocellular carcinoma, esophageal squamous cell carcinoma, cervical, and lung cancer with its high expression is correlated with poor prognosis (12-16). These alterations lead to aberrant splicing events that produce alternative isoforms of key regulatory proteins, promoting tumor growth and progression. SF3B4 influences the expression of oncogenes and tumor suppressor genes, affecting cancer cell survival, proliferation, and metastasis (17, 18). The important roles of SF3B4 in cancer highlight its potential as a therapeutic target.

Although the pivotal roles of SF3B4 in modulating splicing events under various cellular conditions have been extensively investigated, its function in cellular senescence has not been well defined. In this study, we aimed to elucidate the role of SF3B4 in cellular senescence and investigate its impact on TIS in cancer cells following chemotherapy.

Materials and Methods

Cell culture. Human diploid fibroblasts, IMR90 and A549, were grown as previously described (19). Replicative senescence (RS) was triggered by repeatedly passaging human fibroblasts and IMR-90 cells until they reached replicative exhaustion. For DIS, A549 cells were incubated with 300 nM Dox for 4 days, followed by 4 days without the drug. Human SF3B4 was cloned into a retroviral pBabe vector (20) and utilized to generate stable A549 cell lines.

Antibody and reagents. The antibodies used in this study were the following: SF3B4 (ab157117, Abcam, Cambridge, UK), p21 (ab109199, Abcam) and GAPDH (CSB-PA00025A0Rb, Cusabio biotech, Wuhan, PR China). Doxorubicin (44583) was obtained from Sigma (St. Louis, MO, USA).

Cell death assay. Following siRNA transfection, cells were trypsinized, centrifuged and washed with PBS, and resuspended with PBS. Cell death was evaluated by flow cytometry using propidium iodide (PI: 0.5 μg/ml) staining.

Senescence associated β-gal (SA-β-gal) assay. SA-β-gal assay was performed as previously reported (19). Briefly, cells were fixed in 4% paraformaldehyde for 5 min and then incubated with a β-gal staining solution [150 mM NaCl, 2 mM $MgCl_2$, 40 mM citric acid-sodium phosphate (pH 6.0), 5 mM potassium hexacyanoferrate trihydrate, 5 mM potassium hexacyanoferrate, 1 mg/ ml X-gal] at 37˚C for 18 h in the dark. After fixing cells, β-gal positive cells were examined with a microscope (Olympus, Center Valley, PA, USA).

Quantitative RT-PCR. Total RNA was extracted from cells using Trizol (Takara Bio Inc, Kyoto, Japan, 9100), following the manufacturer's instructions. cDNA was synthesized from 0.5 μg of total RNA using the RT master mix (Takara Bio Inc, RR036a). Real-time quantitative PCR was conducted using the SYBR Green Mastermix (Roche, Basel, Switzerland, 04887352001) on a Light Cycler 480 (Roche). Primer sequences were the following: Forward: GGATGAGAAGGTTAGTGAACCGC and Reverse: GGCATAGT CAGCATCTTCCTCAC for human SF3B4, Forward: GGCAGACC AGCATGACAGATTT and Reverse: GGCGGATTAGGGC TTCCTCT for human p21, Forward: CTACGTCGCCCTGGACT TCGAGC and Reverse: GATGGAGCCGCCGATCCACACGG for β-actin. Gene expression levels were normalized to β-actin.

Western blotting. Western blotting was conducted as previously described (21). Cell lysates were prepared by using RIPA lysis buffer (ATTO, Tokyo, Japan, WSE-7420) and protease/phosphatase inhibitors on ice for 15 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK), and blocked for 1 h with either 3% BSA or 5% non-fat milk. Membranes were incubated with primary antibodies, washed with TBST, and incubated with secondary antibodies. Protein bands were visualized using LAS 4000 (Fujifilm, Tokyo, Japan). Bands were normalized to the GAPDH loading control.

siRNA transfection. Transfections were carried out using lipofectamine RNAiMAX (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocols. Cells were transfected with 20 nM siRNAs immediately after being seeded at 50% confluency. Cells were harvested 72 h post-transfection. The siRNA sequences were: 5'-GCAGUACCUCUGUAACCGU-3' for SF3B4, 5'-GGAACAAGGAGUCAGACAUTT-3' for p21 and 5'-UUC UCCGAACGUGUCACGUTT-3' for control.

Statistical analysis. Sample numbers are represented by dots. The graphs show the mean values from three independent experiments. Statistical significance was determined using a two-tailed Student's *t*-test, one-way ANOVA with Dunnett's multiple comparisons test or two-way ANOVA with Sidak's multiple comparison test, as appropriate. All experiments were conducted at least twice independently, with each experiment including more than three samples. *p*-Values are detailed in each figure legend, with a *p*-value of <0.05 considered statistically significant.

Results

SF3B4 expression is decreased in replicative senescent cells. SF3B4 functions as an important regulator of cell growth by controlling the expression of its target proteins. It has been shown that the depletion of SF3B4 suppresses proliferation and triggers cell cycle arrest in various types of cancer, including hepatocellular carcinoma, cervical cancer, and non-small cell lung cancer (NSCLC) (12-14). However, cell death was not induced by SF3B4 depletion (13), indicating that SF3B4 may regulate cellular senescence. To explore the role of SF3B4 in replicative senescence (RS), we first examined whether its expression changes during cellular senescence. Replicative senescent cells were produced by repeated passaging of human diploid fibroblasts or IMR-90 cells. These cells showed heightened senescence-associated β-galactosidase (SA-β-Gal)

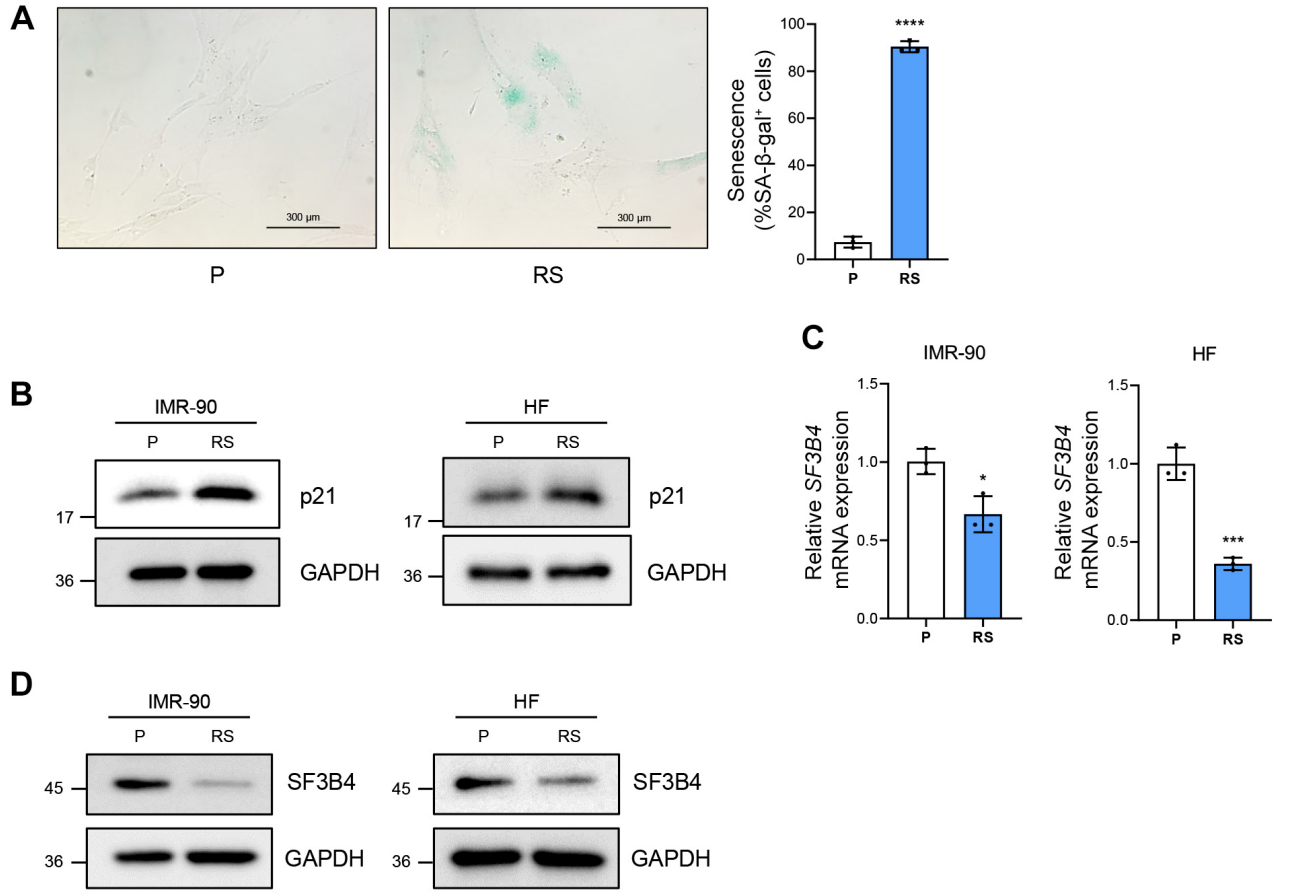

Figure 1. *SF3B4 is decreased in replicative senescent human fibroblasts. (A) Representative images of SA-β-gal staining (left) and quantification (right) in proliferating (P) and replicative senescent (RS) IMR-90 cells. Scale bar represents 300 μm. (B) Protein levels of p21 in proliferating and RS cells (IMR-90 cells or human fibroblasts). (C and D) SF3B4 mRNA (C) and protein (D) levels in proliferating and RS cells (IMR-90 cells or human fibroblasts). GAPDH served as a loading control. Statistical analysis was based on two-tailed Student's t-test. Error bars show standard deviation (SD); *p<0.1, ***p<0.001 and ****p<0.0001.*

activity and adopted a large, flattened shape (Figure 1A). Consistent with these results, the cyclin-dependent kinase inhibitor p21 (CDKN1A), a major marker for cellular senescence (22, 23), was increased in these cells (Figure 1B). Importantly, we observed a significant reduction in the mRNA levels of SF3B4 in both RS human fibroblasts and IMR-90 cells (Figure 1C). The reduction of SF3B4 was also observed at the protein level in these cells (Figure 1D). These results indicate that SF3B4 may play a significant, previously unrecognized role in cellular senescence.

Depletion of SF3B4 induces cellular senescence. Given our observation that SF3B4 is decreased in senescent cells, we next investigated whether SF3B4 knockdown promotes cellular senescence. The expression of SF3B4 was efficiently reduced using a specific siRNA (Figure 2A). As previously reported, we found that SF3B4 knockdown significantly suppressed cell growth (Figure 2B) without causing cell death (Figure 2C), suggesting that the decrease in cell proliferation may result from the induction of senescence. Indeed, SF3B4 knockdown markedly increased the levels of SA-β-Gal positive human fibroblasts compared to control cells (Figure 2D). We also found that the expression of p21 was significantly increased both at the mRNA and protein levels in SF3B4 knockdown cells compared to control cells (Figure 2E and F). Taken together, these results suggest that SF3B4 regulates cellular senescence and that its depletion alone can induce senescence in human fibroblasts.

Depletion of SF3B4 elicits p21-mediated senescence. As SF3B4 is reduced in senescent human fibroblasts and its knockdown induces senescence, we aimed to investigate the mechanisms by which SF3B4 modulates senescence. Previous studies have shown that SF3B4 regulates the expression of p21 (13). Many studies have demonstrated that p21 plays a crucial role in the induction and maintenance of cellular senescence (22, 23).

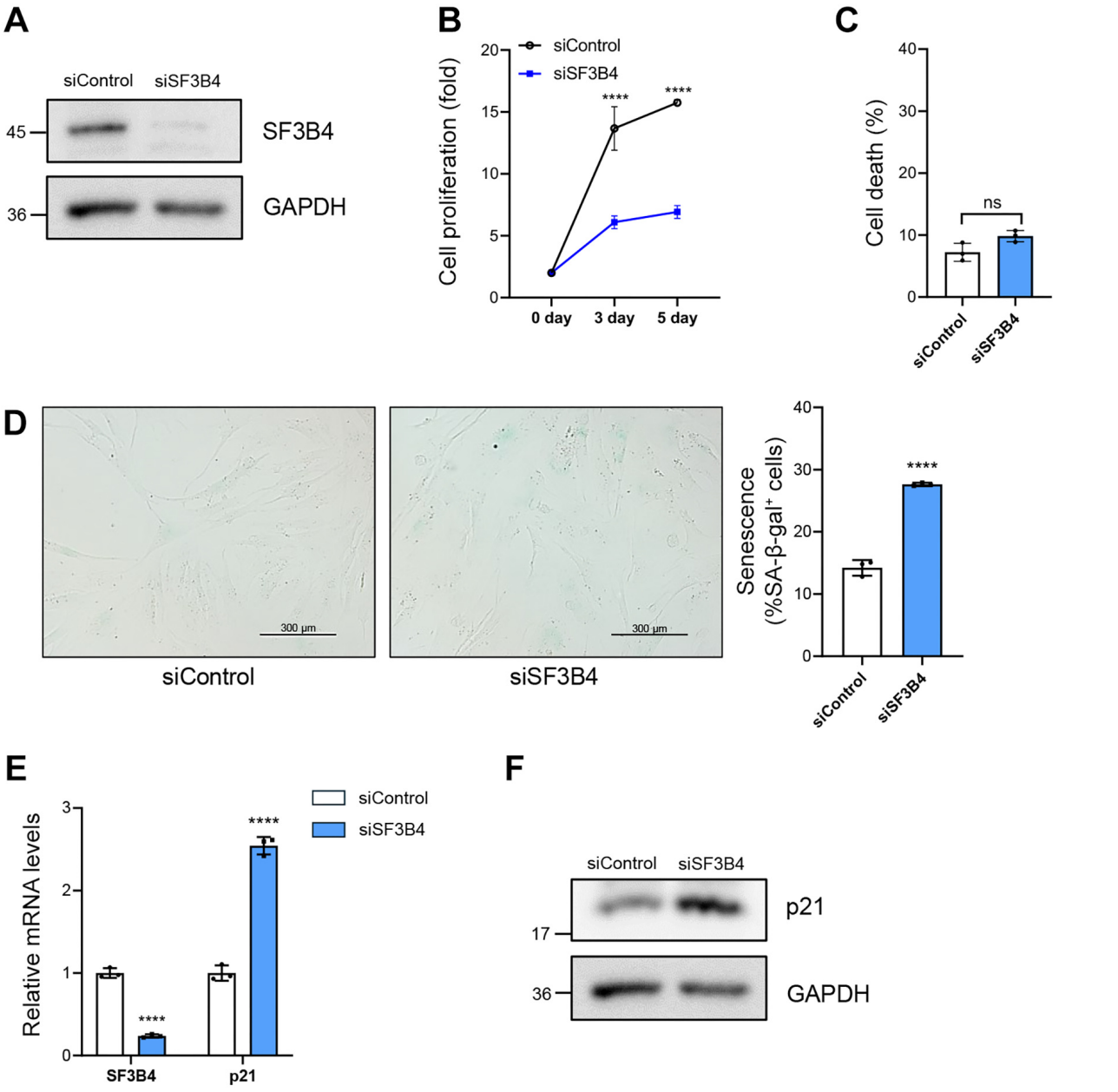

Figure 2. *Knockdown of SF3B4 induces senescence in human fibroblasts. (A) Protein levels of SF3B4 in human fibroblasts transfected with non-targeting siRNA (siControl) or siRNA against SF3B4. GAPDH served as a loading control. (B) Growth curves of SF3B4 knockdown cells. Statistical significance was calculated using a two-way ANOVA with Sidak's multiple comparison test. (C) Percentage of cell death in SF3B4 knockdown cells, measured using PI staining. Statistical analysis was based on two-tailed Student's t-test. (D) Representative images of SA-β-gal staining for control and SF3B4 knockdown cells (left) and the percentage of SA-β-gal positive cells (right). Scale bar represents 300 µm. Statistical analysis was based on two-tailed Student's t-test. (E) Relative mRNA levels of indicated genes in control and SF3B4 knockdown cells. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test. (F) Protein levels of p21 in control and SF3B4 knockdown cells. GAPDH serves as a loading control. Error bars show standard deviation (SD); ns, not significant, ****p<0.0001.*

During the development of senescence, p21 levels increase, and the removal or enhancement of p21 can respectively prevent or trigger the onset of senescence (5, 24). Thus, we hypothesized that the depletion of SF3B4 might promote senescence by increasing the expression of p21. Consistent with the induction of senescence by SF3B4 knockdown (Figure 2), we observed that the protein levels of p21 were significantly increased in SF3B4-depleted human fibroblasts compared to control cells (Figure 3A). Importantly, the induction of the SA-β-Gal positive population in human fibroblasts following SF3B4

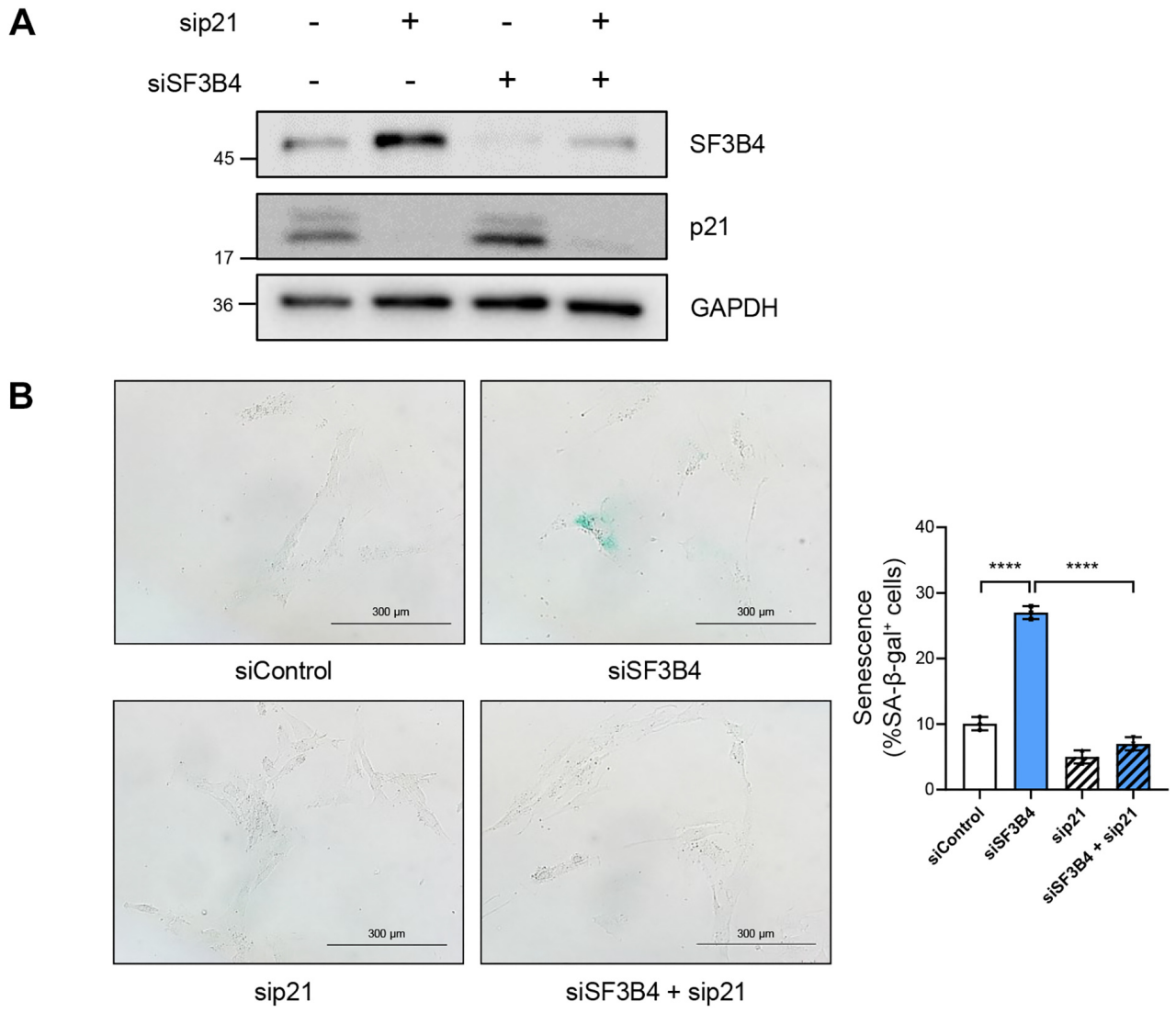

Figure 3. *Knockdown of SF3B4 promotes p21-dependent cellular senescence. (A) Protein levels of p21 and SF3B4 in human fibroblasts transfected with non-targeting siRNA (siControl) or siRNAs against p21 or SF3B4. (B) Representative images of SA-β-gal staining for cells transfected with control, SF3B4 and/or p21 siRNAs as indicated (left) and the percentage of SA-β-gal positive cells (right). Scale bar represents 300 µm. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test. Error bars show standard deviation (SD). ****p<0.0001.*

knockdown was almost completely reversed by p21 knockdown (Figure 3B). Collectively, these findings demonstrate that the depletion of SF3B4 triggers cellular senescence via a p21-dependent pathway.

SF3B4 regulates the induction of senescence in NSCLC cells following chemotherapy. There is accumulating evidence that the induction of senescence by therapy is associated with enhanced clinical results and has therapeutic potential without cytotoxicity (3, 25). Given the important role of SF3B4 in the induction of senescence, we examined whether SF3B4 is involved in TIS. First, we found that SF3B4 knockdown increases the percentage of SA-β-Gal positive cells and elevates p21 expression at the mRNA level in A549 NSCLC cells (Figure 4A and B), indicating that SF3B4 also regulates senescence induction in cancer cells.

To determine the relevance of SF3B4 in TIS, we examined its expression in senescent A549 cells following chemotherapy. To induce senescence, A549 cells were treated with doxorubicin (DOX), a topoisomerase II inhibitor and widely used chemotherapeutic agent known for inducing senescence across various cancer cell lines (24, 26). A549 cells were exposed to 300 nM DOX for 4 days, followed by 4 days without drug. This regimen induced DOX-induced senescence (DIS) in the majority of cells, with cell death rarely observed (Figure 4C). In line with previous results, the mRNA and protein levels of SF3B4

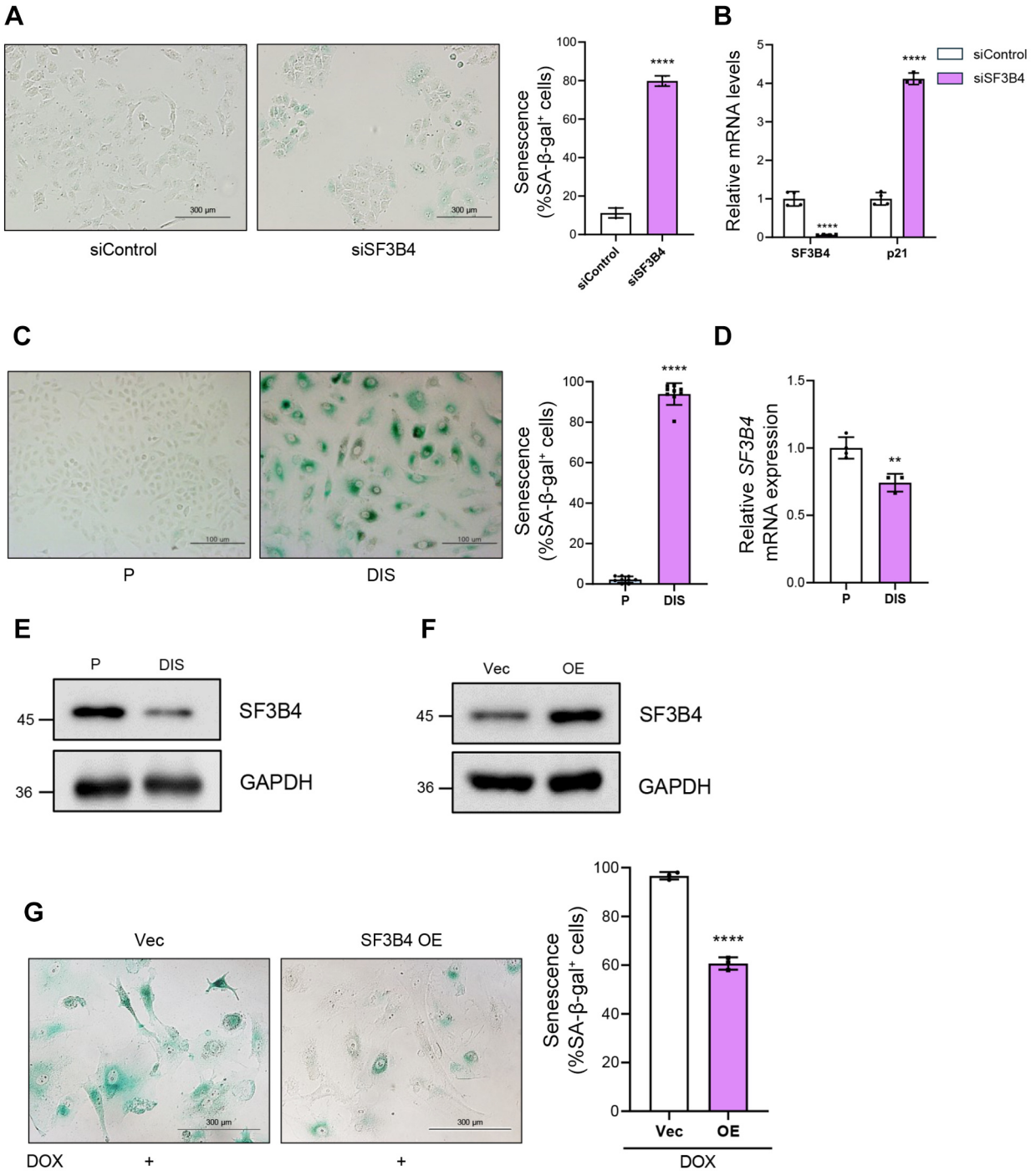

Figure 4. *SF3B4 inhibits senescence induction in A549 cancer cells following chemotherapy. (A) Representative images of SA-β-gal staining for A549 cells transfected with non-targeting siRNA (siControl) or siRNA against SF3B4 (left) and the percentage of SA-β-gal positive cells (right). Scale bar represents 300 µm. Statistical analysis was based on two-tailed Student's t-test. (B) Relative mRNA levels of indicated genes in control and SF3B4 knockdown cells. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test. (C) Representative images of SA-β-gal staining for proliferating and DOX-induced senescence (DIS) A549 cells (left) and the percentage of SA-β-gal positive cells (right). Scale bar represents 100 µm. Statistical analysis was based on two-tailed Student's t-test. (D and E) SF3B4 mRNA (D) and protein (E) levels in proliferating and DIS A549 cells. Statistical analysis was based on two-tailed Student's t-test. (F) Protein levels of SF3B4 in A549 cells stably expressing empty vector or SF3B4. GAPDH served as a loading control. (G) Representative images of SA-β-gal staining for control and SF3B4-overexpressing A549 cells following DOX treatment (left) and the percentage of SA-β-gal positive cells (right). Scale bar represents 300 µm. Statistical analysis was based on two-tailed Student's t-test. Error bars show standard deviation (SD); **p<0.01 and ****p<0.0001.*

decreased in senescent A549 cells (Figure 4D and E), suggesting that the reduction of SF3B4 is required for TIS. To directly test this idea, we examined the levels of senescence induction upon chemotherapy in A549 cells overexpressing SF3B4 (Figure 4F). Notably, SF3B4 overexpression clearly and reproducibly reduced the extent of DIS (Figure 4G). Taken together, our results suggest that SF3B4 functions as an important regulator of senescence and suppresses TIS in A549 NSCLC cells.

Discussion

Here, we investigated the role of SF3B4 in replicative senescence and therapy-induced senescence (TIS). Our findings reveal that SF3B4 expression is significantly reduced in senescent human fibroblasts both at the mRNA and protein levels. Knockdown of SF3B4 induced senescence in human fibroblasts, evidenced by increased SA-β-Gal activity, elevated p21 levels, and upregulation of SASP-related genes. Mechanistically, SF3B4 depletion led to p21-mediated senescence, as reducing p21 levels reversed the senescent phenotype. In A549 NSCLC cells, SF3B4 knockdown similarly increased senescence markers and p21 expression. Decreased SF3B4 expression was also observed in DOX-induced senescent A549 cells. Additionally, overexpression of SF3B4 reduced DOX-induced senescence, indicating that SF3B4 suppresses TIS. Collectively, these results highlight SF3B4 as an important factor in regulating senescence and suggest its use as a therapeutic target to improve cancer therapy efficacy by modulating TIS.

The oncogenic properties of SF3B4 have been extensively investigated. Dysregulation and overexpression of SF3B4 are commonly observed in various cancer types, including hepatocellular carcinoma, cervical cancer, clear cell renal cell carcinoma, and lung cancer (12-14, 17). Its activity contributes to the malignant phenotypes by modulating the expression of oncogenes and tumor suppressor genes (27). Consequently, SF3B4 promotes tumor growth and progression, and its expression is negatively correlated with clinical outcomes (13, 17). However, the potential involvement of SF3B4 in cellular senescence had not yet been determined. Our results demonstrate that SF3B4 suppresses senescence induction by regulating p21 expression. Since senescence serves an important tumor-suppressive role, these findings align with the oncogenic functions of SF3B4. The activation of an oncogene or the inhibition of a tumor suppressor gene typically promotes senescence, and an additional mutation is often required for tumorigenesis, a concept known as the two-hit hypothesis (28, 29). Given its prominent role in senescence, SF3B4 may support tumorigenesis by suppressing senescence induction during oncogene activation or tumor suppressor inhibition.

We found that SF3B4 expression is decreased in both replicative senescence and TIS, and that the reduction of SF3B4 is required for both types of senescence. Thus, identifying the factors that regulate SF3B4 expression is essential for understanding of how SF3B4 contributes to tumorigenesis and for developing therapeutic approaches for cancer. Given that SF3B4 is overexpressed in various types of cancer and that abnormal alternative splicing is accompanied by tumorigenesis (30), oncogenic signals might upregulate SF3B4 to sustain its expression and bypass senescence induction. Recently, it was demonstrated that knockdown of SRSF3, a member of a conserved family of splicing factors, induces SF3B4 expression at both the mRNA and protein levels (31). However, since SRSF3 depletion promotes p21-dependent cellular senescence (32), these findings may not align with the observed decrease of SF3B4 in senescent cells. Interestingly, we observed that knockdown of p21 induces SF3B4 expression (Figure 3A), suggesting a feedback loop in which p21 and SF3B4 mutually inhibit each other. Considering that senescence profoundly impacts not only tumorigenesis but also aging and age-related diseases, future research should investigate the regulation of SF3B4 expression during senescence induction in mechanistic detail.

Conclusion

In conclusion, our findings elucidate a novel role for SF3B4 in cellular senescence. We have demonstrated that SF3B4 expression is diminished in senescent cells and that its knockdown promotes senescence. This role of SF3B4 is particularly pertinent in cancer cells following chemotherapy, where SF3B4-overexpressing cells exhibit resistance to TIS. These findings underscore the potential of targeting SF3B4 for cancer diagnosis and therapy, highlighting its significance as a therapeutic target to modulate senescence and improve the therapeutic efficacy of cancer treatments.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Seungyeon Yang and Minbeom Ko conducted the experiments and drafted the manuscript under the supervision of Seung Min Jeong. Seung Min Jeong conceived the idea for this study, wrote and edited the manuscript. Soojung Clair Hur and Eun Kyung Lee provided conceptual advice. All Authors read and approved the final manuscript.

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