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Article

Innovative Treatment for Reducing Senescence and Revitalizing Aging Cells through Gene Silencing

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SUMMARY

Many studies explore whether harnessing a cellular state known as senescence holds the solution to revitalizing aging tissues. Senescent cells are unique in that they stop cell division but are resistant to apoptosis, a programmed cell death. Senescent cells continue to release chemicals that can trigger inflammation. mTOR inhibitors are currently the only known pharmacological interventions that increase lifespan and revitalize aging tissues in animal models. However, the side effect profiles of mTOR inhibitors are a major cause of concern because mTOR inhibitors inhibit the genes that regulate cell proliferation and immune cell differentiation. To explore a possible solution to the side effects of an mTOR inhibitor, we specifically targeted two mTOR downstream genes, RPS6KB1 (G1 cell cycle inducer) and PPARGC1A (mitochondria energy metabolism regulator) using a small interfering RNA (siRNA) to inhibit gene expression. We hypothesized that inhibiting expression of RPS6KB1 and PPARGC1A would decrease senescence caused by hydrogen peroxide (H_2O_2). We used human skin cells (Detroit 551) to test our hypothesis and induced senescence by H_2O_2 treatment. Our result indicates that when both genes were knocked down by siRNA on Detroit 551 cells pretreated with H_2O_2, the number of senescent cells significantly decreased compared to scrambled siRNA transfected control cells. Our results suggest that inhibiting gene expression of both RPS6KB1 and PPARGC1A may inhibit senescence. Our results may support the development of novel treatments for revitalizing aging tissues and inhibiting inflammation triggered by senescent cells.

INTRODUCTION

Cellular senescence is a state in which a cell undergoes a permanent cell cycle arrest but continues to live beyond its expected life span (1). In other words, it refers to a cell that can no longer divide but has continued metabolic activity (2). Senescence is involved in normal development, tissue homeostasis, and tumor progression control (2). However, senescent cells that remain in the body are hazardous as they constantly release chemicals that incite inflammation (3). In addition, senescent cells can induce inflammation in neighboring cells, thereby spreading the condition and damaging neighboring cells (4). In living organisms, senescence is characterized by the permanent, continuous deterioration of their biological functions and mechanisms (5). Senescence is caused by various mechanisms such as telomere shortening, environmental and internal stress factors, DNA damage, and mitochondrial malfunction that induce irreversible cell cycle arrest (6). Therefore, senescence is a major cause of age-related disease (7).

Recent experimental evidence shows that senescence is closely associated with aging (8). For example, researchers investigated the effects of senescent cells on the physical aspects of aging in mice (8). The researchers implanted a small number of senescent fat cells into healthy, young, and middle-aged mice to observe their effect on strength, endurance, and other indicators of physical health (8). The results showed that, in comparison to control mice, mice given senescent cells had slower walking speeds, worse physical endurance, and lower grip strength (8). This study, along with many others, indicated that the genetic or pharmaceutical removal of senescent cells can extend the life span and improve the health span (6). As a treatment strategy targeting senescence, some previous researchers used senolytic agents to induce apoptosis in senescent cells that show a senescence-associated secretory phenotype, or cells that excrete signals that turn senescent fibroblasts into pro-inflammatory cells (7). As a person ages, the efficiency of the immune system declines and causes the number of senescent cells to grow, resulting in stress responses caused by insults associated with aging (7). Because senescent cells in the brain can damage cognitive abilities, this can affect a person's capacity to overcome stress or disease, recover from injuries, and learn new things (9). Cellular senescence is closely connected to various age-related diseases like cancer, diabetes, osteoarthritis, Alzheimer's disease, stroke, osteoporosis, dementia, and cardiovascular disease. It is also associated with mobility, eyesight, and cognitive ability (10).

Small interfering RNAs (siRNA) are highly specialized RNA molecules that inhibit the translation of specific messenger RNAs (mRNAs). Therefore, siRNA can control the translation of selective proteins (11). siRNAs are double-stranded RNAs that have been transcribed and then cut into smaller fragments in the nucleus before being released into the cytoplasm (12). siRNA gene silencing is a promising novel therapeutic strategy that can theoretically be utilized to silence any disease-related gene. This can be done by

chemically synthesizing a corresponding siRNA for a specific gene sequence. siRNA gene therapy allows the development of new remedies for previously incurable diseases with chemical antibodies or drugs (13). Also, the short process and cost-effectiveness of editing siRNA sequences have advantages over antibody-based treatment. Furthermore, constructing a new sequence of siRNA to deal with variants and mutations found in target regions can be easily verified as an effective treatment compared to modifying antibodies (14).

siRNA therapy has previously been tested to target proximal tubular cells (PTCs), a cell type in the kidney that exhibits senescence as a result of aging and pathology, to preserve kidney functions (15). It was well documented that ischemia and acute kidney injuries caused by cisplatin, an anti-cancer chemotherapy drug, induce senescence in the kidney (16). In mouse models, researchers showed that intravenous administration of siRNA targeting the p53 gene in PTC cells successfully reduced cellular p53, thereby reducing p53-mediated apoptosis (17). A phase 3 clinical trial testing p53 siRNA for renal function maintenance after major cardiothoracic surgery is now being conducted to determine the validity of this technique (18). This study did not directly target senescent cells but showed the applicability of siRNA therapy. However, there are no published studies on using siRNA to target senescent cells to reduce senescence. To our knowledge, our study seems to be one of the first to use siRNA to target and inhibits senescent cells.

The mammalian target of rapamycin (mTOR) regulates cell proliferation, autophagy, and apoptosis (19). The mTOR signaling system regulates cell proliferation and immune cell differentiation by regulating gene transcription and protein synthesis (20). mTOR regulates protein synthesis through a variety of downstream targets (20). Among many downstream target genes of mTOR signaling, S6K1 (p70-S6 Kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E (eIF4E) binding protein 1) are the two best-characterized mTOR downstream targets (20).

mTOR also controls cellular senescence by regulating mitochondrial metabolism, autophagy, and protein translation, making it an essential regulator of aging (21). The mTOR pathway may hold the key to treatment for aging (21). The only known pharmacological treatment that increases lifespan is the inhibition of mTOR complex 1 with rapamycin (22). Rapamycin targets an evolutionarily conserved mTOR, a serine-threonine kinase that integrates various intracellular signals that respond to growth factors and nutrients (23). However, since this drug alters the expression level of hundreds of different genes, it also causes severe side effects, such as decreasing the number of red blood cells. Because of the limitations of rapamycin, we focused on specific genes in the mTOR pathway, namely two mTOR downstream molecules, Ribosomal Protein S6 Kinase B1 (RPS6KB1) and PPARG Coactivator 1 Alpha (PPARGC1A). RPS6KB1 encodes p70S6K, a serine-threonine kinase that promotes protein synthesis, cell growth, and proliferation (24). P70S6K is a G1 cell cycle inducer that controls cell growth (25). PPARGC1A encodes the PGC-1α protein, a transcriptional

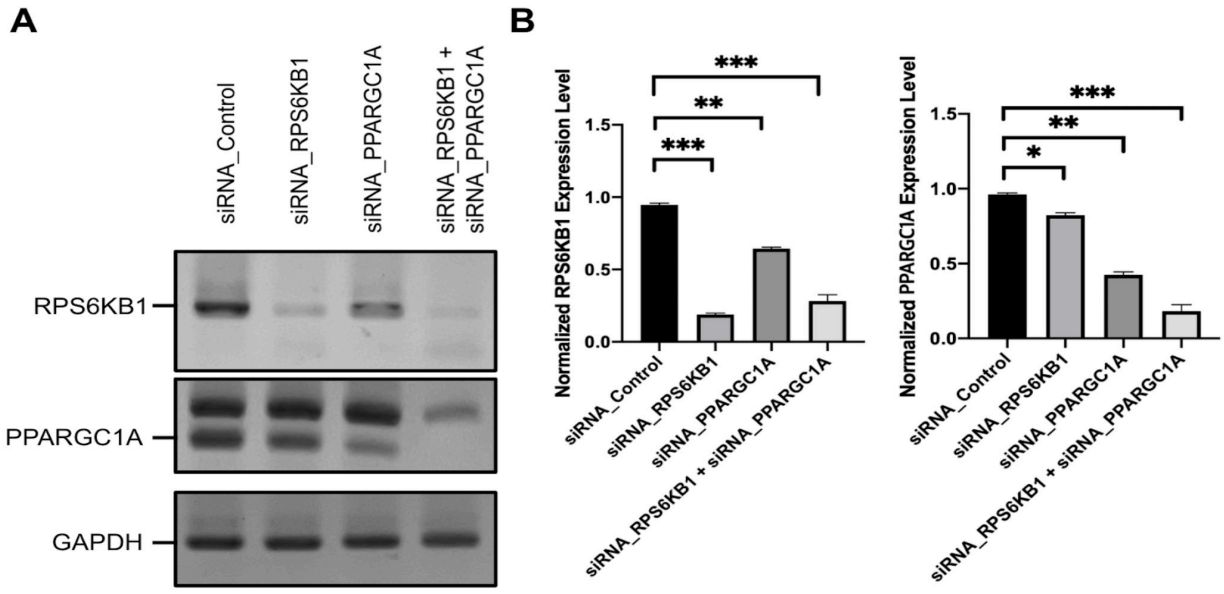

Figure 1. siRNA targeting either RPS6KB1 or PPARGC1A efficiently inhibited the mRNA expression level of target genes. (A) Amplified RPS6KB1, PPARGC1A, and GAPDH genes from cDNA templates using agarose gel electrophoresis. Detroit 551 cells were transfected with either negative control (siRNA_control), siRNA targeting RPS6KB1 (siRNA_RPS6KB1), siRNA targeting PPARGC1A (siRNA_PPARGC1A), or a combination of both siRNAs (siRNA_RPS6KB1 + siRNA_PPARGC1A) for 48 h. (B) Bar graph showing mean ± SD of either normalized RPS6KB1 or PPARGC1A expression level (n=2) quantified by band intensity of agarose gel. RPS6KB1 and PPARGC1A expressions were normalized with the GAPDH expression level. One-way ANOVA with Tukey's post hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

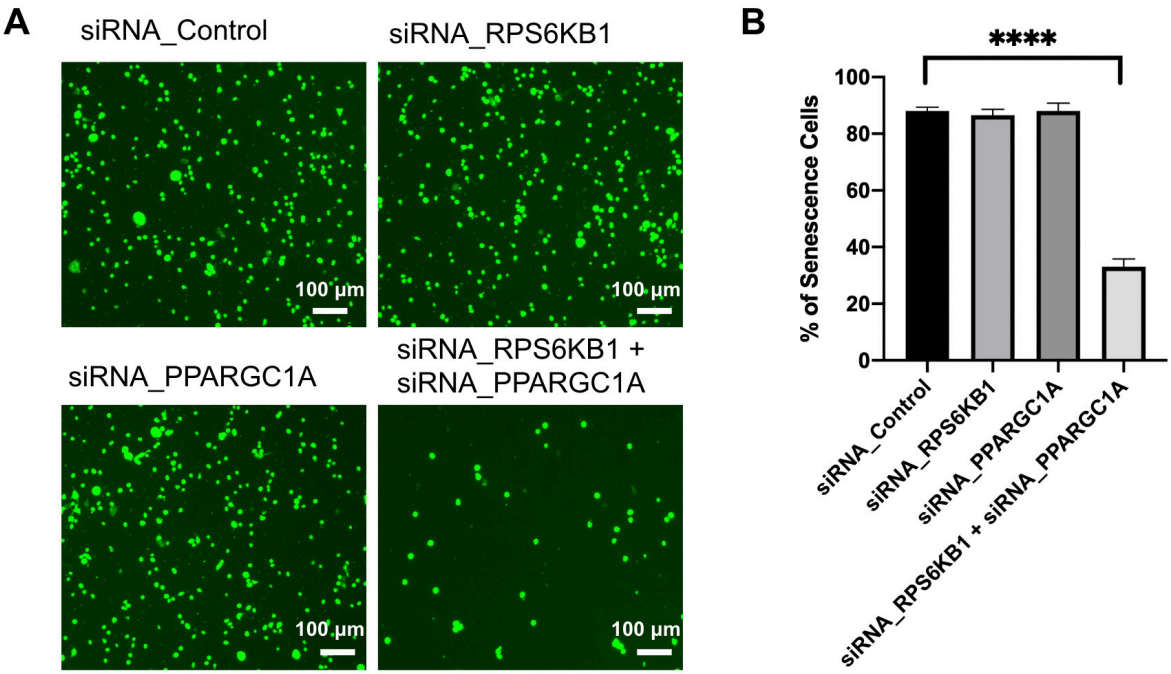

Figure 2. Combination treatment of siRNAs targeting RPS6KB1 and PPARGC1A inhibits cellular senescence in Detroit 551 skin cells. (A) CellEvent Senescence Green probe staining images in Detroit 551 cells. Senescent cells were stained as green fluorescent cells. Detroit 551 cells were transfected with either negative control (siRNA_control), siRNA targeting RPS6KB1 (siRNA_RPS6KB1), siRNA targeting PPARGC1A (siRNA_PPARGC1A), or a combination of both siRNAs (siRNA_RPS6KB1 + siRNA_PPARGC1A) for 48 h. Then, the cells were incubated with 150 µM H_2O_2 for 2 h. (B) Bar graph showing mean ± SD of percentage of senescent cells (n=2) quantified by the fluorescence image. One-way ANOVA with Tukey's post hoc test, **** $p < 0.0001$.

coactivator that regulates the genes involved in mitochondrial energy metabolism (26). This protein regulates mitochondrial biogenesis by controlling the crucial mitochondrial genes (27).

In this study, we hypothesized that inhibiting two mTOR downstream molecules, RPS6KB1 and PPARGC1A, would reduce senescence caused by H_2O_2. These targets were chosen for their impacts on reducing the number of senescent cells. We transfected siRNA targeting RPS6KB1 and PPARGC1A genes and measured the number of senescent cells induced by H_2O_2, one of the primary causes of senescence. Our result indicated that targeting both genes efficiently decreased the number of senescent cells induced by H_2O_2.

RESULTS

We hypothesized that silencing two mTOR downstream molecules, RPS6KB1 and PPARGC1A, would reduce senescence caused by H_2O_2. This experiment aimed to test the effect of siRNAs that inhibit gene expression levels of RPS6KB1 and PPARGC1A in Detroit551 cells (human skin cells) (**Figure 1**). We transfected human skin cells with a control siRNA, one of the gene-targeted siRNAs, or a combination of siRNAs targeting two genes.

We tested four conditions in this experiment (**Figure 1A**). For negative control, we used a scrambled sequence that does not lead to the specific degradation of any human mRNAs (siRNA_control). We also used siRNA targeting either RPS6KB1 (siRNA_RPS6KB1) or PPARGC1A (siRNA_PPARGC1A) for target gene silencing. siRNA_RPS6KB1 inhibits the translation of RPS6KB1 mRNA, the messenger RNA that encodes p70S6K protein. siRNA_PPARGC1A inhibits the translation of PPARGC1A mRNA, the messenger RNA that encodes PGC1-α protein. Lastly, we tested the effect of combining both siRNAs (siRNA_RPS6KB1 + siRNA_PPARGC1A). siRNA_RPS6KB1 significantly decreased the expression level of RPS6KB1 compared to siRNA control ($p = 0.001$, one-way ANOVA, **Figure 1B**). Unexpectedly, siRNA_PPARGC1A significantly decreased the expression level of RPS6KB1 compared to siRNA control ($p = 0.002$, one-way ANOVA, **Figure 1B**). siRNA_RPS6KB1 + siRNA_PPARGC1A significantly decreased the expression level of RPS6KB1 compared to siRNA control ($p = 0.0002$, one-way ANOVA, **Figure 1B**), but combining both showed a lower efficiency of silencing RPS6KB1 than using siRNA_RPS6KB1 alone.

For PPARGC1A, siRNA_RPS6KB1 significantly decreased the expression level of PPARGC1A compared to siRNA_control ($p = 0.047$, one-way ANOVA, **Figure 1B**), an unexpected result. Furthermore, for PPARGC1A, siRNA_PPARGC1A significantly decreased the expression

level of PPARGC1A compared to siRNA_control (p = 0.0011, one-way ANOVA, **Figure 1B**). siRNA_RPS6KB1 + siRNA_PPARGC1A significantly decreased the expression level of PPARGC1A compared to siRNA_control (p = 0.0003, one-way ANOVA, **Figure 1B**), which showed that combining both was most efficient in silencing PPARGC1A. This result indicates that transfection of siRNA_RPS6KB1, siRNA_PPARGC1A, or siRNA_RPS6KB1 + siRNA_PPARGC1A effectively decreased the expression level of RPS6KB1 and PPARGC1A in Detroit 551 skin cells.

Next, we aimed to analyze the effect of siRNA silencing on cellular senescence. We used CellEvent senescence green probe, which stains the senescent cells to generate green fluorescence by targeting β-galactosidase. This well-known enzyme is produced in senescent cells, which we used as a criterion to determine senescent cells (28). First, Detroit 551 cells were transfected with the four conditions (siRNA_control, siRNA_RPS6KB1, siRNA_PPARGC1A, siRNA_RPS6KB1 + siRNA_PPARGC1A) for 48 h. Then, the cells were treated with 150 μM H_2O_2 for 2 h. Finally, cells were stained with a senescence green probe, and the cell image was captured (Figure 2A). A similar percentage of green positive fluorescent cells was observed in three conditions (siRNA_Control, siRNA_RPS6KB1, and siRNA_PPARGC1A), indicating that knockdown of RPS6KB1 or PPARGC1A individually did not reduce senescence (**Figure 2A**). Surprisingly, the concentration of green positive fluorescent cells treated with siRNA_RPS6KB1 + siRNA_PPARGC1A was significantly less than the concentration observed in the control condition ($p < 0.0001$, one-way ANOVA, **Figure 2B**). This result indicates that silencing both RPS6KB1 and PPARGC1A together efficiently inhibits the H_2O_2-dependent transformation of Detroit 551 cells to senescent cells.

DISCUSSION

We found that siRNA knockdown targeting both RPS6KB1

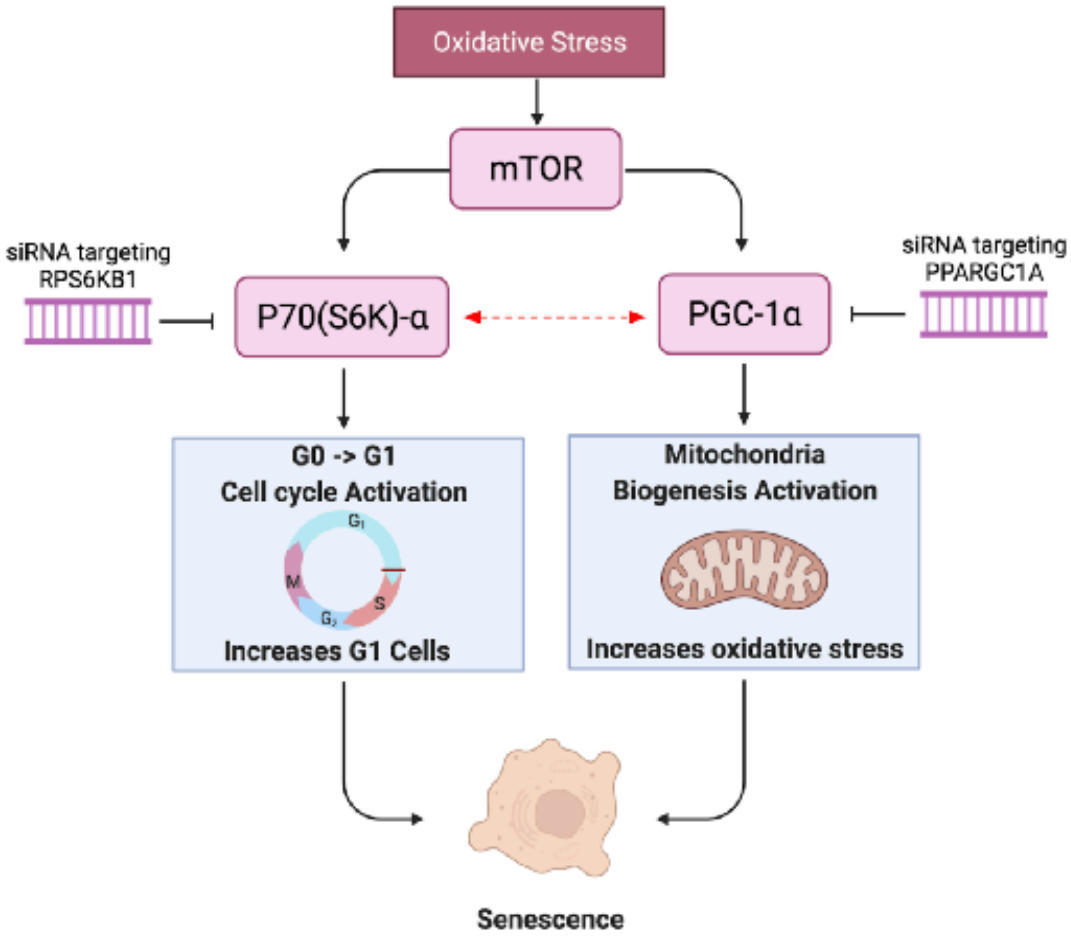

Figure 3. Schematic model of siRNAs targeting RPS6KB1 and PPARGC1A, which effectively inhibit cellular senescence. Silencing P70(S6K)-α reduces the proportion of G1 cells, which can transform into senescent cells. Silencing PGC-1α blocks oxidative stress, a cellular condition that damages the cells and induces senescent cells. The red dotted line indicates the possible interaction of two genes in the same pathway.

and PPARGC1A genes effectively inhibits the number of senescent cells induced by H_2O_2 in Detroit 551 cells. Also, we observed unexpected results of siRNA targeting RPS6KB1 decreased PPARGC1A expression level, and siRNA targeting PPARGC1A decreased RPS6KB1 expression level (**Figure 1**). Therefore, we speculated that RPS6KB1 and PPARGC1A may share a similar pathway and that knockdown of either gene affects the other gene's expression level.

From our experiment, we were able to derive several interesting conclusions. mTOR genes, triggered by oxidative stress, activate RPS6KB1 and PPARGC1A genes, which may cause increased activation of P70(S6K)-α and PGC-1α proteins (**Figure 3**). P70(S6K)-α proteins induce cell cycle activation from G0 phase to G1 phase which increases the number of G1 cells (29). Growth arrest in senescent cells mainly occurs in the G1 phase of the cell cycle (30). Therefore, P70(S6K)-α proteins increase the chance of cellular senescence in cells by activating their cell cycle (**Figure 3**).

PGC-1α proteins activate mitochondrial biogenesis, which is the division of pre-existing mitochondria to increase mitochondrial numbers. The mitochondria not only synthesize ATP to generate energy for the cell but also cause the accumulation of reactive oxygen species (ROS), thereby potentially increasing oxidative stress (31). Reactive oxygen species (ROS) are unstable molecules consisting of radical and non-radical oxygen species formed by partial oxygen reduction (32). ROS are either generated via mitochondrial oxidative metabolism or as a cellular response to xenobiotics, cytokines, and bacterial invasion (33). Excessive amounts of ROS can cause oxidative stress, which is when the cellular antioxidant defense system is overwhelmed by the plethora of ROS, and unable to produce an effective antioxidant response (34). During oxidative stress, ROS can react with macromolecules and cause damage to lipids, proteins, and DNA (34). Macromolecular damage generated by oxidative stress is linked to aging and several disease states, including atherosclerosis, diabetes, cancer, neurodegeneration, and aging. In oxidative stress, ROS activates the mTOR signaling pathway and its downstream genes, some of which play a crucial role in inducing senescence and amplifying oxidative stress within the cell (34). One typical example of a reactive oxygen species would be H_2O_2, which was used to induce senescence in Detroit 551 cells (**Figure 3**). Oxidative stress is a circumstance in the cell where there is an imbalance between the number of reactive oxygen species and antioxidants that stabilize these species (35). Oxidative stress is a key cause of cellular senescence as reactive oxygen species act as reducing agents for proteins and DNA, which can deform their chemical structure (36). Mitochondria biogenesis activation can directly give rise to senescence through oxidative stress or formulate a positive feedback loop by inducing the activation of the mTOR pathway (**Figure 3**).

The results of this study must be seen in the context of some limitations. First, our treatment was imposed on only one cell type: Detroit 551 cells. Given that there are approximately 200 different types of cells in our body, further investigation is needed as our study results only investigated the senescence of Detroit 551 cells (37). Another limitation is that we solely used H_2O_2 to induce senescence in our cells. Though H_2O_2 is the most common inducer of senescence, other reactive oxygen species in the body also cause senescence. Hence, we could not completely recreate the conditions of the cell environment in the human body. Since we used only one type of siRNA, we did not completely control for possible off-target effects. Additionally, the small sample size (n=2) is a limitation of this study. Therefore, increasing the sample size should be further investigated in the future.

The most interesting result from our experiment is the effectiveness of the treatment when testing siRNA_RPS6KB1 + siRNA_PPARGC1A. The inhibition of both of our target genes was necessary to decrease the percentage of senescent cells significantly. In conclusion, this study can be applied to targeted gene therapy that blocks the senescent cells produced by H_2O_2.

MATERIALS AND METHODS

Cell culture and maintenance

Detroit 551 (normal human skin cells) were purchased from the Korea Cell Line Bank. The cells were maintained in the cell culture medium RPMI 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% penicillin and streptomycin antibiotic reagents (Gibco). The cells were maintained in the 5% CO2 incubator at 37°C. To passage and plate cells, 0.25% Trypsin-EDTA solution was used to dissociate the cells from the old culture plate. Then, the detached cells were moved into a new culture plate with fresh RPMI1640 cell culture media.

siRNA transfection

Since siRNA cannot be directly delivered inside the cell cytosol through the cell membrane, we used liposomes to carry the siRNA into the cells (11). siRNA has a negative charge, and liposomes have a positive charge. Therefore, when mixed together, they form a complex where the liposome encases the siRNA. To perform siRNA transfection, safely delivering the transaction reagent into the cell using non-viral methods, we amalgamated siRNA and liposome to form a siRNA-liposome complex (12). In a six-well culture plate, 0.6 x 106 cells were added to each well. The control liposome-siRNA complex consisted of 7 µL of liposome RNAimax transfection reagent (Invitrogen) and 3 µL of siRNA control. We used the AccuTarget™ Negative Control siRNA (Bioneer) for the control siRNA. This negative control siRNA is a non-targeting siRNA consisting of sequences with low homology with humans, mice, and rats, thereby preventing the binding of the negative control siRNA with the mRNA of human cells. For the siRNA solutions targeting either RPS6KB1 or PPARGC1A, we combined 7 µL of liposome and 3 µL of the relevant siRNA. To test the effect of dual

RPS6KB1 or PPARGC1A knockdown on the number of senescent cells, we combined 14 µL of liposome, 3 µL of RPS6KB1 siRNA, and 3 µL of PPARGC1A siRNA. After the siRNA and liposome complex was added to each well, the cells were incubated for 48 hours. The predesigned siRNA targeting RPS6KB1 (Bioneer, catalog # 6198-1) was used. For PPARGC1A siRNA the following sequence was used: Sense: AAGUGCAGAAUCAACUUGAAG=tt; Anti-Sense: CUUCAAGUUGAUUCUGCACUU=tt.

Total RNA extraction from cells

AccuPrep Universal RNA Extraction Kit (Bioneer) was used to isolate total RNA from cultured cells. RNA was extracted according to the manufacturer's manual of AccuPrep Universal RNA Extraction Kit (Bioneer).

cDNA synthesis

We used a reverse transcriptase kit (Enzynomics) to synthesize cDNA from extracted RNA. For each of our four samples of extracted RNA (7 µL each), we added 1 µL of 10x TOPscript™ RT Buffer, 0.5µL of TOPscript™ Reverse Transcriptase (200 units/µL), 1 µL of dNTP Mixture, and 0.5µL of oligo dT primer. We then put our four solutions into a thermal cycler (BioRad) to synthesize cDNA. The following temperature and time were used in the thermal cycler: 1) 45°C for 60 min, 2) 95°C for 5 min, 3) 4°C for infinite (sample storage).

Polymerase Chain Reaction (PCR)

The synthesized cDNA was amplified using a thermal cycler. For each sample, RPS6KB1, PPARGC1A, GAPDH genes were synthesized. We prepared 20 µL PCR reactions for each gene. We used a premixed PCR solution (Bioneer) and added 1 µL cDNA, 1 µL forward primer (1 µM), 1 µL reverse primer (1 µM), and 17 µL water. The following temperature and time were used in the thermal cycler: 1) 95°C for 3 min, 2) 95°C for 30 sec, 3) 62°C for 30 sec, 4) 72°C for 20 sec, 5) repeat step 2 to 4 for 34 cycles, 6) 72°C for 5 min, 7) 12°C infinite (sample storage).

Agarose electrophoresis

We used agarose electrophoresis to compare the amount of amplified cDNA. To prepare the agarose gel, we mixed 0.6509g of agarose powder with 50mL of Tris-Borate-EDTA (TBE) buffer to create a 1.3% agarose solution. In order to melt the agarose inside the solution, we microwaved the solution for 3 min so that it mixed thoroughly with the buffer. After the agarose powder was completely melted into the solution, the solution was poured into the gel caster. After the gel was solidified for 30 min, the gel was placed into the gel tank (Mupid). Then, 1 x TBE buffer was poured over the gel to cover it fully. Each 7 µL of amplified DNA solution was loaded into each well. After the sample loading, the gel was run at 100 V for 25 min. Then, the gel was placed on the blue light illuminator to visualize the DNA amplified band.

Senescence assay

CellEvent Senescence Green Detection Kit (Invitrogen, C10850) was used to quantify the percentage of senescent cells. The cells were plated into a flat-bottom 96 well plate and incubated overnight. After the cells were transfected with the indicated siRNA, they were incubated for 48 h. Then, 150 µM H_2O_2 was added to each sample for 2 h because H_2O_2 is widely used to achieve oxidative stress-induced premature senescence. The cells were washed with phosphate-buffered saline (PBS). Then, the cells were fixed with the methanol for 10 min at room temperature. After the fixation solution was removed, the cells were washed with 1% bovine serum albumin (BSA) in PBS. The prewarmed Working Solution was added to each well of the 96-well plate. Then, the plate was covered with plastic film to prevent moisture loss and incubated for 2 h at 37°C. After the incubation, the working solution was removed and the samples were washed three times with PBS. The cells were detached from the 96-well plate and placed on the cell counting slide. A LUNA-FL cell counter (Logos Biosystems) was used to photograph the image of green fluorescence positive cells. Then, LUNA-FL cell counter quantifies the percentage of the number of green fluorescence cells among the total number of cells within the sample.

ImageJ Data Analysis

We used ImageJ's Gels analysis tools to measure the intensity of pixels in selected regions on the gel, which were displayed as a continuous graph classifying pixel intensity by horizontal location. Then, using the "Straight Tool," we divided the continuous graph into four separate areas representing siRNA_Control, siRNA_RPS6KB1, siRNA_PPARGC1A, and siRNA_RPS6KB1 + siRNA_PPARGC1A. By clicking on each region with a "wand tracing tool," the area of the region was calculated and displayed on the "Results" interface. This procedure was followed twice each for RPS6KB1, GAPDH, and PPARGC1A to collect the appropriate data stored in an Excel file. We used GAPDH as a housekeeping gene, which represents the total amount of RNA in a given sample. Experimental errors can cause the total amount of RNA to change across samples, so GAPDH plays a crucial role in allowing us to normalize the data obtained from RPS6KB1 and PPARGC1A. For each intensity data obtained from siRNA_Control, siRNA_RPS6KB1, siRNA_PPARGC1A, siRNA_RPS6KB1 + siRNA_PPARGC1A, we divided the quantified intensity data of RPS6KB1, PPARGC1A by the corresponding GAPDH intensity to normalize our data. To further analyze the normalized data, we used Prism 8 to graph our data and verify its statistical significance. The mean and standard error of deviation were calculated. For quantifying the PPARGC1A gene, we quantified the upper band because our primers were designed to amplify 175 bp and the upper band size was relatively similar to the lower bands.

Statistical analysis

We used the graphing tool Prism 8 to analyze the statistical significance. We used a one-way ANOVA with a Tukey post hoc test. A P-value less than 0.05 was considered statistically significant. "n" indicates the biological replicates.

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