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# miR-186, miR-216b, miR-337-3p, and miR-760 cooperatively induce cellular senescence by targeting α subunit of protein kinase CKII in human colorectal cancer cells

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ABSTRACT

We previously demonstrated that downregulation of protein kinase CKII induces cellular senescence in human colon cancer HCT116 cells. To investigate the role of microRNAs (miRNAs) in CKII downregulation during senescence, we employed computational algorithms. Four miRNAs (miR-186, miR-216b, miR-337-3p, and miR-760) were predicted to be miRNAs against *CKIIα* mRNA. Mimics of all four miRNAs jointly downregulated CKIIα expression in HCT116 cells. Reporter analysis and RT-PCR have suggested that these four miRNAs may stimulate degradation of *CKIIα* mRNA by targeting its 3′ untranslated regions (UTRs). The four miRNA mimics increased senescent-associated β-galactosidase (SA-β-gal) staining, p53 and $p21^{Cip1/WAF1}$ expression, and reactive oxygen species (ROS) production. In contrast, concomitant knockdown of the four miRNAs by antisense inhibitors increased the CKIIα protein level and suppressed CKII inhibition-mediated senescence. Finally, CKIIα overexpression antagonized senescence induced by the four miRNA mimics. Therefore, the present results show that miR-186, miR-216b, miR-337-3p, and miR-760 cooperatively promote cellular senescence through the p53–$p21^{Cip1/WAF1}$ pathway by CKII downregulation-mediated ROS production in HCT116 cells.



## 1. Introduction

Protein kinase (CKII) is a ubiquitous serine/threonine kinase that catalyzes the phosphorylation of a large number of cytoplasmic and nuclear proteins [1,2]. The holoenzyme of CKII is a heterotetramer composed of two catalytic (α and/or α′) subunits and two regulatory β subunits. The β subunit stimulates the catalytic activity of the α or α′ subunit, thereby mediating tetramer formation and substrate recognition. Overexpression of the CKIIα subunit leads to tumorigenesis in mice overexpressing Myc [3]. In addition, recent studies showing that CKII phosphorylates caspase substrate confirmed that CKII prevents apoptosis [4]. These findings suggest that CKII plays a critical role, not only in cell growth and proliferation, but also in anti-apoptotic activities.

In normal primary cells, the cell division cycle ceases after a finite number of divisions and the cells enter an irreversible state of proliferation-arrest designated replicative senescence or, more generally, cellular senescence [5]. Cellular senescence can also be induced by oxidative stress and oncogenic activation [6,7]. Senescence is characterized by several molecular and phenotypic characteristics, including large flat cell morphology and appearance of senescence-associated β-galactosidase activity (SA-β-gal) [8,9]. Previously, we have shown that CKII activity is downregulated in both senescent human lung fibroblast IMR-90 cells and aged rat tissues, and that CKII inhibition induces premature senescence of IMR-90 cells and HCT116 human colon cancer cells. The p53–$p21^{Cip1/WAF1}$–RB pathway is required for senescence induced by CKII inhibition [10,11]. Superoxide anion generation by NADPH oxidase activation as well as p53 acetylation by downregulation of SIRT1, a silent information regulator 2 (Sir2) homolog, both serve as upstream activators of p53 stabilization in cells made senescent by CKII inhibition [12,13]. DNA methylation is involved in silencing of *CKIIα* and *CKIIα′* genes during senescence [14]. However, the exact molecular mechanism behind *CKII* gene silencing during senescence remains obscure.

Gene expression can be regulated by a family of small regulatory RNAs termed microRNAs (miRNAs). These short non-coding RNAs ranging in size from 19 to 22 nucleotides mainly regulate

*Abbreviations:* CKII, protein kinase CKII (also known as casein kinase II); DMEM, Dulbecco's modified Eagle's medium; DHE, dihydroethidium; miRNA, microRNA; ETH, ethidium; HA, hemagglutinin; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; RT, reverse transcription; SA-β-gal, senescent-associated β-galactosidase; Sir2, silent information regulator 2; SDS, sodium dodecyl sulfate; siRNA, small-interfering RNA; UTR, untranslated region.

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gene expression at the posttranscriptional level through interaction with the 3′ UTR of mRNA. The binding of miRNA to the 3′ UTRs of their target mRNAs results in inhibition of translation or mRNA degradation, depending on the degree of complementarity between the two sequences. For effective repression, base-pairing between the miRNA-response elements and the first 2–7 nucleotides of the miRNA, or seed region, is important [15]. Bioinformatic analysis has predicted that miRNAs may modulate more than 30% of human protein-coding genes, and each miRNA is predicted to regulate over hundreds of target genes. Conversely, each target gene can be controlled by multiple miRNAs [16,17]. This correlation between miRNAs and their targets reflects the complexity of miRNA function. Recently reported data suggest that miRNAs are key regulators of many cellular processes such as development, differentiation, apoptosis, proliferation, and senescence [18].

In the present study, we tested the hypothesis that *CKIIα* expression can be controlled posttranscriptionally by miRNAs. Here, we show for the first time that four miRNAs (miR-186, miR-216b, miR-337-3p, and miR-760) jointly downregulate CKIIα expression in colon cancer cells.

## 2. Materials and methods

### 2.1. Cell culture and transfection

HCT116 human colon cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum under a humidified atmosphere of 5% (v/v) $CO_2$ at 37 °C. Mimics for miR-186, miR-216b, miR-337-3p, miR-760 and control miRNA were purchased from Genolution Inc. (Seoul, Korea). Antisense inhibitors for four miRs were obtained from Panagene Inc. (Seoul, Korea). RNAs were transfected into HCT116 cells at a final concentration of 40 nM using Lipofectamine RNAi max (Invitrogen, Carlsbad, CA). Transfection of cells with CKIIα siRNA was performed as described previously [13]. At 48 h after the transfection, the cells were harvested.

### 2.2. Bioinformatics method

The miRNAs against human CKIIα mRNA predicted by computational algorithms were obtained from TargetScan Release 5.0 (http://www.targetscan.org/vert_50/), MicroRNA (http://www.microrna.org/microrna/home.do), Mami (http://mami.med.harvard.edu/), miRBase Targets (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi#Results) Segal lab (http://132.77.150.113/cgi-bin/software.pl?dir=mir07&page=mir07_prediction), RNA hybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html), and psRNATarget (http://plantgrn.noble.org/psRNATarget).

### 2.3. SA-β-gal activity and CKII activity assays

SA-β-gal activity was measured as described previously [10]. Standard assay for measuring the phosphotransferase activity of CKII was conducted in the presence of 1 mM synthetic peptide substrate (RRREEETEEE) as described previously [10].

### 2.4. Immunoblotting

Cells in 60 mm-dishes were washed with ice-cold PBS, collected by scraping with a rubber policeman, and lysed in 100 μl of ice-cold RIPA buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin]. Protein concentration in the supernatants was determined using Bradford protein dye reagent (Bio-Rad, Hercules, CA), and the volumes of the supernatants were adjusted for equal protein concentration. Immunoblotting was performed as described previously [13]. Antibodies specific to CKIIα, CKIIβ, p53, $p21^{Cip1/WAF1}$, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-HA antibody was obtained from Roche (Basel, Switzerland).

### 2.5. RT-PCR

RNAs were extracted from HCT116 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was reverse-transcribed using gene-specific reverse primers and reverse transcriptase (Takara, Japan), and the resulting cDNAs were PCR-amplified. PCR primer sequences for CKIIα were: CKIIαFwd (5′-GACAAGCTTATGTCGGG ACCC-3′) and CKIIαRev (5′-GACAAGCTTTTACTGCTGAGC-3′). Primers specific to β-actin RNA were used to standardize the amounts of RNA in each sample. PCR products were resolved on 1.5% agarose gel.

### 2.6. Construction of 3′ UTR reporter plasmid and luciferase assay

The *CKIIα* 3′ UTR reporter was generated by inserting the entire 3′ UTR of human *CKIIα* mRNA into XhoI/NotI sites of psiCHECK-2 vector (Promega) downstream of the *Renilla* luciferase gene. PCR primer sequences used for *CKIIα* 3′ UTR were as follows: Forward 5′-AATCTCGAGCGGCCCTATCTGTCTCCTGAT-3′ and Reverse 5′-TCGCGGCCGCCACCAAAGAATTCCAACACTGGATC-3′. For the luciferase assay, $1 \times 10^5$ cells were transfected along with the *CKIIα* 3′ UTR reporter and the four miRNA mimics (or antisense inhibitors) in a 24-well plate using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 24 h, firefly and *Renilla* luciferase activities were measured consecutively using Dual Luciferase Assay (Promega).

### 2.7. Measurement of intracellular ROS

Intercellular ROS was measured as described previously [12].

### 2.8. Statistical analysis

Statistical significance of the data was analyzed by one-way ANOVA with the SPSS package program. The results were considered significant if the value of $P$ was less than 0.05. Duncan's multiple-range test was also performed if the differences between the groups were identified as $\alpha = 0.05$.

## 3. Results

### 3.1. Prediction of miRNAs against CKIIα mRNA and downregulation of CKIIα expression by miR-186, miR-216b, miR-337-3p, and miR-760

We hypothesized that the elevated miRNAs against *CKIIα* mRNA might lower CKIIα expression in human colon cancer cells. To investigate this, seven different, commonly used computational algorithms (MicroRNA, TargetScan, miRBase, Mami, Segal lab, psRNATarget, and RNA hybrid) were employed to predict miRNAs against *CKIIα* mRNA. Several miRNAs were obtained as possible regulators of CKIIα. As a result, we found that miR-186, miR-216b, miR-337-3p, and miR-760 could target the 3′ UTR of *CKIIα* mRNA (Fig. 1A). It was found that the 5′ UTR and coding region of *CKIIα* mRNA did not contain putative binding sites for these four miRNAs.

To test the possible role of miR-186, miR-216b, miR-337-3p, and miR-760 in the regulation of CKIIα, HCT116 cells were transfected with mimics of these miRNAs (sequences shown in Table 1). Western blot analysis revealed no significant differences in CKIIα protein level between control and the different mimics. Further,

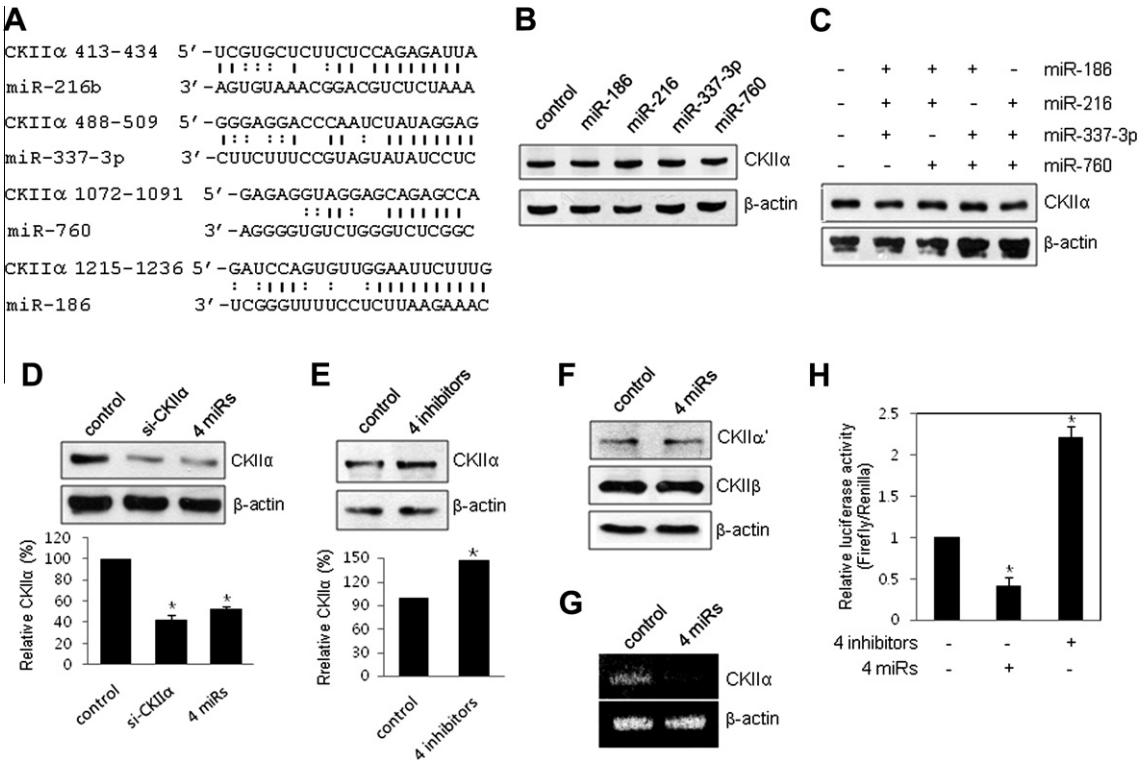


**Fig. 1.** Prediction of miRNAs for *CKII*α mRNA and downregulation of CKIIα expression by miR-186, miR-216b, miR-337-3p, and miR-760. (A) The sequence of four miRNAs and their potential matching sites in *CKII*α 3′ UTR. Watson–Crick and wobble–base (G–U) pairings are indicated by solid and dashed vertical lines, respectively. (B-D) HCT116 cells were transfected with control miRNA (control) or different combinations of mimics for miR-216b, miR-337-3p, miR-760, and miR-186. After 2 days, effects of each mimic (B), different combinations of three mimics (C), or a combination of the four mimics (4 miRs) (D) on the protein level of CKIIα were determined by immunoblot analysis. CKIIα siRNA (si-CKIIα) was used as a positive control. Quantification of each band was carried out by a densitometer analysis. Data are shown as the means ± SEM. $^{*}P < 0.05$. (E-G) HCT116 cells were transfected with the four antisense inhibitors (E) or the four mimics (F and G) for miR-216b, miR-337-3p, miR-760, and miR-186. After 2 days, the protein levels of the CKII subunits were determined by immunoblot analysis (E and F). Quantification of each band was carried out by a densitometer analysis (E). Data are shown as the means ± SEM. $^{*}P < 0.05$. Total RNA was extracted from cells and reverse-transcribed using CKIIα-specific primers and reverse transcriptase (G). Primers for β-actin RNA were used as a control. PCR products were resolved on a 1.5% (w/v) agarose gel, and (H) HCT116 cells were co-transfected with *CKII*α 3′ UTR reporter and the four miRNA mimics or four antisense inhibitors, after which firefly and *Renilla* luciferase activities were measured after 24 h. Data are shown as the means ± SEM. $^{*}P < 0.05$.

different combinations of three mimics had no significant effects on the protein level of CKIIα (Fig. 1B and C). However, quantification by densitometry revealed that transfection with all four of the miRNA mimics decreased the CKIIα protein amount by 50% compared with samples transfected with control miRNA. CKIIα siRNA was used as a positive control (Fig. 1D). To confirm the inhibitory effects of miR-186, miR-216b, miR-337-3p, and miR-760 on CKIIα expression, HCT116 cells were transfected with antisense inhibitors of these miRNAs (sequences shown in Table 1). As shown in Fig. 1E, concomitant knockdown of the four miRNAs by antisense inhibitors increased the CKIIα protein amount in cells. To explore whether or not the four miRNAs regulate the catalytic subunit CKIIα′, HCT116 cells were transfected with the four miRNA mimics. Co-transfection of the four miRNA mimics did not affect the level of CKIIα′ protein. Likewise, the protein level of CKIIβ was not affected by co-treatment with the four mimics (Fig. 1F). We next tested whether or not the miRNA mimics regulate stability of *CKII*α mRNA. RT-PCR analysis demonstrated that co-transfection of cells with the four mimics decreased the level of *CKII*α mRNA (Fig. 1G). To investigate whether or not the 3′ UTR of *CKII*α carries binding sites for the four miRNAs, we used a *CKII*α 3′ UTR luciferase reporter vector. Co-transfection of the four mimics with *CKII*α 3′ UTR reporter into cells led to nearly 55% inhibition of luciferase activity in comparison with samples transfected with control miRNA. In contrast, co-treatment of the four antisense inhibitors with *CKII*α 3′ UTR reporter increased luciferase activity by 220% compared with control (Fig. 1H). Again, different combinations of three mimics had no significant effects on luciferase activity (data not shown). Taken together, these results indicate that miR-186, miR-216b, miR-337-3p, and miR-760 jointly function to repress

**Table 1**
Sequences of oligonucleotides used in this study.

| | | |
|---|---|---|
| mimics | miR-186 | Forward: 5′-CAAAGAAUUCUCCUUUUGGGCU-3′ Reverse: 5′-AGCCCAAAAGGAGAAUUCUUG-3′ |
| | miR-216b | Forward: 5′-AAAUCUCUGCAGGCAAAUGUGA-3′ Reverse: 5′-UCACAUUUGCCUGCAGAGAUUU |
| | miR-337-3p | Forward: 5′-CUCCUAUAUGAUGCCUUUCUUC-3′ Reverse: 5′-GAAGAAAGGCAUCAUCUAGGAG-3′ |
| | miR-760 | Forward: 5′-CGGCUCUGGGUCUGUGGGGA-3′ Reverse: 5′-UCCCACAGACCCAGAGCCG-3′ |
| | Negative control | Forward: 5′-ACGUGACACGUUCGGAGAAUU-3′ Reverse: 5′-UUCUCCGAACGUGUCACGUUU-3′ |
| Antisense inhibitors | miR-186 | RRRQRRKKRR-OO-CCAAAAGGAGAATTCTTT |
| | miR-216b | RRRQRRKKRR-OO-TTGCCTGCAGAGATT |
| | miR-337-3p | RRRQRRKKR-OO-GAAAGGCATCATATAGGA |
| | miR-760 | RRRQRRKKRR-OO-TCCCCACAGACCCAGAGCCG |
| | Negative control | RRRQRRKKRR-OO-CTCCCTTCAATC |

RRRQRRKKRR, cell penetrating peptide; OO, AEEA linker–.

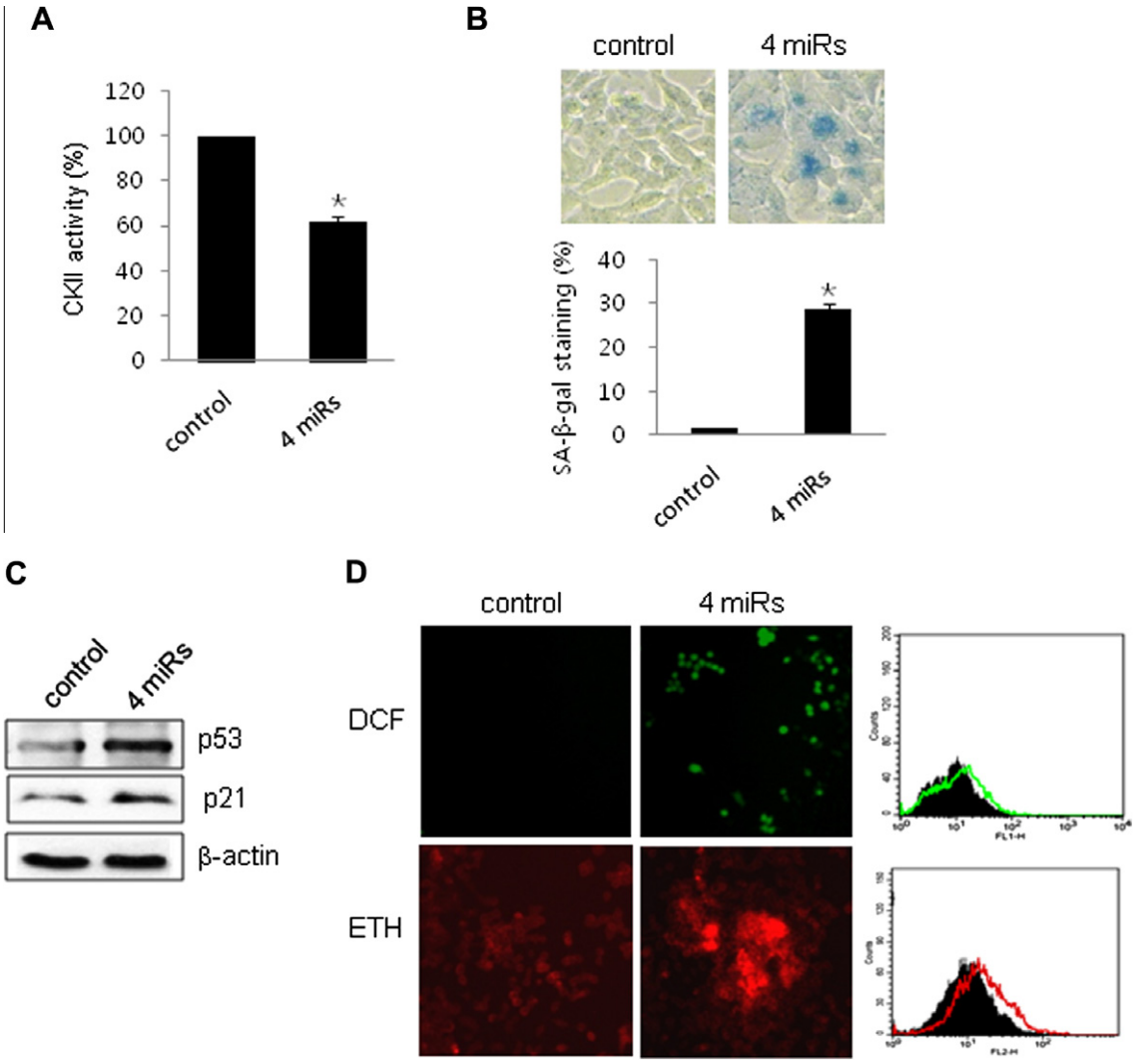


**Fig. 2.** miR-186, miR-216b, miR-337-3p, and miR-760 stimulate cellular senescence. (A–D) HCT116 cells were transfected with control miRNA or the four mimics (4 miRs) for 2 days. (A) Lysates from cells were utilized in kinase assays using specific CKII substrate peptide. $^{32}$P incorporation into the substrate peptide was measured by scintillation counting. Data are shown as the means ± SEM. $^{*}P < 0.05$. (B) Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside and the percentage of positively stained cells was measured. Data are shown as the means ± SEM. $^{*}P < 0.05$. (C) Cells were lysed, electrophoresed on a 12% (w/v) SDS–polyacrylamide gel, and visualized by immunoblotting with anti-p53 and anti-p21$^{Cip1/WAF1}$ antibodies, and (D) Cells were incubated with CM-$H_2$DCFDA or DHE as described in Section 2. Representative images illustrate the green fluorescence of DCF produced by ROS (left upper panel) and the red fluorescence of ETH produced by superoxide anion (left bottom panel). Fluorescence intensity was determined by flow cytometry analysis (right panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CKIIα expression by reducing the stability of *CKIIα* mRNA through specific targeting of the 3′ UTR of *CKIIα*mRNA in HCT116 cells.

### 3.2. miR-186, miR-216b, miR-337-3p, and miR-760 stimulate senescence in HCT116 cells

To examine the role of miR-186, miR-216b, miR-337-3p, and miR-760 in cellular senescence, we knocked down CKIIα in HCT116 cells by co-treatment with the same miRNA mimics. When CKII activity was assessed using [γ-$^{32}$P]ATP and CKII peptide substrate, extract from the cells transfected with the four mimics contained lower than 40% CKII activity compared to control extract (Fig. 2A). To assess the effect of miRNA knockdown on senescence, after transfection for 2 days the transfectants were then stained for SA-β-gal activity. HCT116 cells transfected with the four mimics exhibited a higher rate of SA-β-gal staining compared with control cells (Fig. 2B). Immunoblot data showed that the expression levels of p53 and p21$^{Cip1/WAF1}$ were upregulated in HCT116 cells treated with the four miRNA mimics in comparison with control cells (Fig. 2C). Recently, we showed that ROS generation is an upstream activator of p53 stabilization in cells made senescent by CKII inhibition [12]. Therefore, to examine whether or not miR-186, miR-216b, miR-337-3p, and miR-760 induce ROS production in cells, HCT116 cells were incubated with CM-$H_2$DCFDA or DHE. Fig. 2D shows representative images of DCF and ETH fluorescence in control cells as well as in HCT116 cells co-treated with the four miRNA mimics. Transfection with the four mimics significantly increased the levels of hydrogen peroxide and superoxide anion in cells. Taken together, these results suggest that miR-186, miR-216b, miR-337-3p, and miR-760 may induce senescence through CKII inhibition-mediated ROS generation.

### 3.3. Antisense inhibitors against miR-186, miR-216b, miR-337-3p, and miR-760 antagonize CKII inhibition-mediated cellular senescence

Next, we assessed the effects of antisense inhibitors of miR-186, miR-216b, miR-337-3p, and miR-760 on CKII inhibition-induced senescence in HCT116 cells. As shown in Fig. 3A, SA-β-gal activity

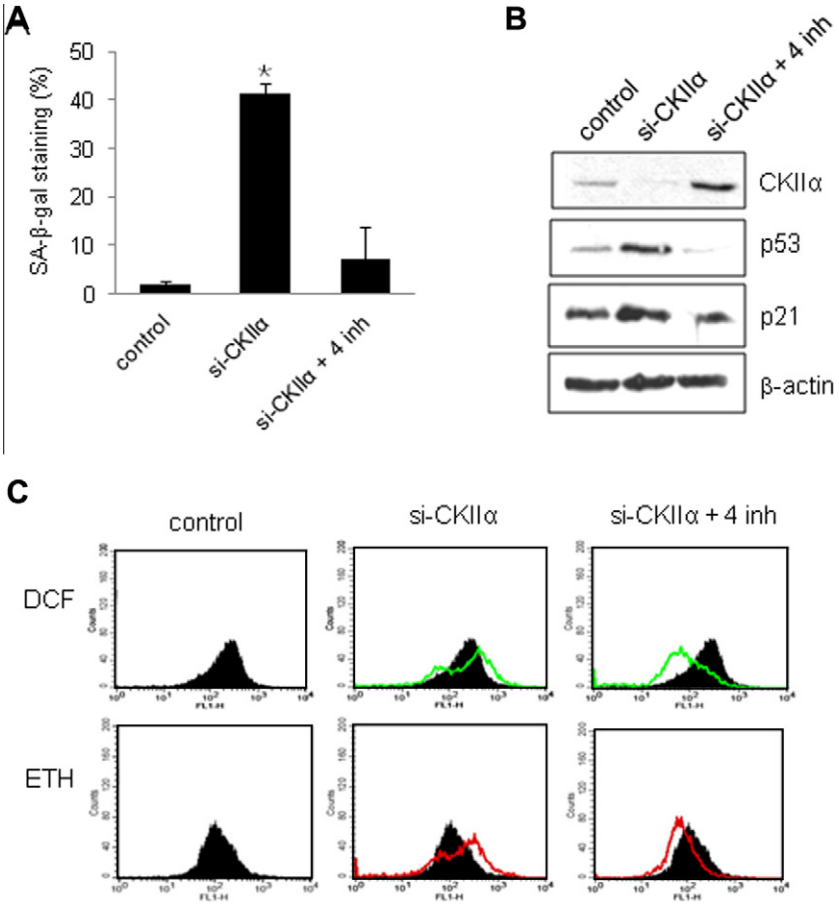


**Fig. 3.** Antisense inhibitors against miR-186, miR-216b, miR-337-3p, and miR-760 antagonize CKII inhibition-mediated cellular senescence. HCT116 cells were co-transfected with CKIIα siRNA (si-CKIIα) with or without the four antisense inhibitors (inh) for 2 days. (A) Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside and the percentage of positively stained cells was measured. Data are shown as the means ± SEM. $^{*}P < 0.05$. (B) Cells were lysed, electrophoresed on a 12% (w/v) SDS–polyacrylamide gel, and visualized by immunoblotting, and (C) Cells were incubated with CM-$H_2$DCFDA or DHE as described in Section 2 . The green fluorescence of DCF (upper panel) and the red fluorescence of ETH (bottom panel) were determined by flow cytometry analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly increased in cells transfected with CKIIα siRNA in comparison with scrambled siRNA-treated control cells. However, co-transfection with the four antisense inhibitors resulted in an apparent decrease in SA-β-gal activity, along with recovery of the CKIIα protein level in cells treated with CKIIα siRNA. Moreover, expression levels of p53 and $p21^{Cip1/WAF1}$ were up-regulated in cells treated with CKIIα siRNA, whereas co-treatment with the four antisense inhibitors resulted in lower expression of both p53 and $p21^{Cip1/WAF1}$ proteins (Fig. 3B). While CKIIα siRNA treatment significantly increased ROS production, co-treatment of the cells with the four antisense inhibitors apparently prevented the green and red fluorescent signals (Fig. 3C). Taken together, these results indicate that CKII inhibition-mediated senescence can be blocked by antisense inhibitors of miR-186, miR-216b, miR-337-3p, and miR-760 in HCT116 cells.

### *3.4. CKIIα overexpression suppresses miR-186, miR-216b, miR-337-3p, and miR-760-mediated senescence*

We next examined whether or not CKII activation antagonizes the cellular senescence induced by miR-186, miR-216b, miR-337-3p, and miR-760. For this, HCT116 cells were transfected with or without pcDNA-HA-CKIIα in the presence of the four miRNA mimics. Again, there was a significant increase in SA-β-gal activity in response to the four mimics. However, co-transfection of these cells with CKIIα resulted in a decrease in SA-β-gal activity (Fig. 4A). Similarly, co-treatment of cells with CKIIα resulted in lower expression of p53 and $p21^{Cip1/WAF1}$ proteins (Fig. 4B). We next examined whether or not CKIIα overexpression suppresses the ROS production induced by the four mimics. Co-transfection with CKIIα resulted in a decrease in the rate of DHE staining (Fig. 4C). Taken together, these results strongly indicate that miR-186, miR-216b, miR-337-3p, and miR-760 jointly produce ROS and induce senescence through CKIIα downregulation.

## 4. Discussion

We have demonstrated that CKII activity decreases in senescent human lung fibroblast cells and aged rat tissues, and that CKII inhibition induces premature senescence of human fibroblast and colon cancer cells [10,11]. This notion is also supported by findings from other groups. Downregulation of CKII leads to senescence in human mesenchymal stem cells [19]. Further, CKII activity is downregulated in rat chondrocytes with advancing age [20]. However, the mechanism of CKII downregulation in cells remains to be defined. In this study, we predicted four miRNAs (miR-186, miR-216b, miR-337-3p, and miR-760) as novel regulators of CKIIα using different computational algorithms. We showed here that these four miRNA mimics simultaneously decreased the levels of CKIIα protein and CKII activity in human colon cancer HCT116 cells, but not those of CKIIα′ and CKIIβ. Inhibition of these four miRNAs

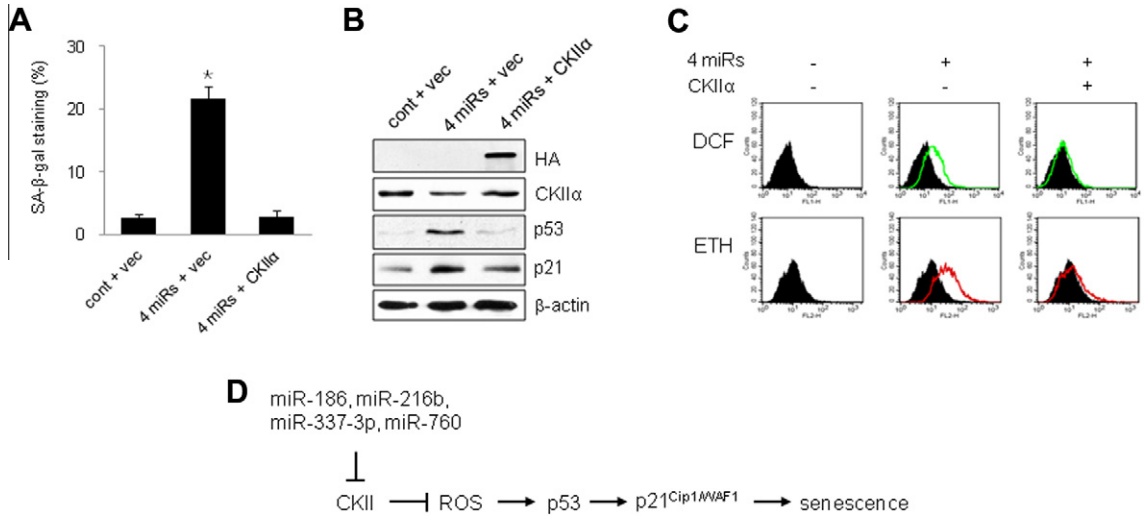


**Fig. 4.** CKIIα overexpression suppresses miR-186, miR-216b, miR-337-3p, and miR-760-mediated senescence. HCT116 cells were co-transfected with control miRNA (cont) or the four mimics (4 miRs) in the presence of empty vector (Vec) or pcDNA-HA-CKIIα for 2 days. (A) Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside and the percentage of positively stained cells was measured. Data are shown as the means ± SEM. $^{*}P < 0.05$. (B) Cells were lysed, electrophoresed on a 2% (w/v) SDS–polyacrylamide gel, and visualized by immunoblotting. (C) Cells were incubated with CM-$H_2$DCFDA or DHE as described in Section 2. Green fluorescence of DCF (upper panel) and red fluorescence of ETH (bottom panel) were determined by flow cytometry analysis, and (D) possible model illustrating cellular senescence mediated by miR-186, miR-216b, miR-337-3p, and miR-760. miR-186, miR-216b, miR-337-3p, and miR-760 may activate p53 by generating ROS through CKII knock-down. This process results in activation of the $p21^{Cip1/WAF1}$-Rb-dependent cellular senescence pathway [11,13]. ROS, reactive oxygen species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by synthetic antagonists resulted in upregulated CKIIα expression and CKII activity. Moreover, the present study suggested that the four miRNAs may promote destabilization of *CKIIα* mRNA by specifically targeting the 3′ UTR of *CKIIα*mRNA.

In many cases, one target gene is regulated by multiple miRNAs. For example, Marasa et al. reported that four miRNAs (miR-15b, miR-24, miR-25, and miR-141), which decrease in level during senescence, jointly lower expression of the kinase MKK4 [21]. The present study also demonstrated that the combined action of multiple miRNAs might be essential to achieve efficient downregulation of CKIIα. Previously, treatment of senescent human fibroblast IMR-90 cells with the demethylating agent 5-aza-2′-deoxycytidine was found to induce CKIIα expression, suggesting that DNA hypermethylation might be involved in the silencing of *CKIIα* gene in senescent cells [14]. Thus, we propose here that both miRNAs and DNA methylation may contribute to the regulation of CKIIα expression in cells (Fig. 4D). The combined effect of miRNAs and DNA methylation may afford cells with the means of regulating the level of CKII activity with a high degree of accuracy.

Each miRNA is thought to control hundreds of target genes, reflecting the complexity of miRNA function. To date, it has been reported that miR-216b suppresses tumor growth and invasion by targeting *KRAS* mRNA [22]. miR-186, in cooperation with miR-150, downregulates expression of the pro-apoptotic purinergic P2X7 receptor [23], and miR-760 expression is regulated in breast cancer MCF-7 cells by 17 β-estradiol [24]. Further, miR-337-3p is downregulated in the brains of mice infected with mouse-adapted scrapie [25]. However, the cellular function of these miRNAs remains to be clarified. Here, we suggest the possibility that miR-186, miR-216b, miR-337-3p, and miR-760 induce cellular senescence. In our results, these four miRNAs stimulated ROS production, upregulated p53 and $p21^{Cip1/WAF1}$ expression, and increased SA-β-gal activity. Importantly, antisense inhibitors of these four miRNAs suppressed cellular senescence and ROS production induced by CKIIα knockdown. Furthermore, overexpression of CKIIα rescued cellular senescence and ROS production induced by the four miRNA mimics. Therefore, on the basis of these results, we can conclude that miR-186, miR-216b, miR-337-3p, and miR-760 promote cellular senescence through the p53–$p21^{Cip1/WAF1}$ pathway by CKII downregulation-mediated ROS production. The present study is consistent with previous reports, in which CKII inhibition induced by CKIIα siRNA and CKII inhibitors was shown to stimulate senescence through the p53–$p21^{Cip1/WAF1}$ pathway in a NADPH oxidase- and ROS-dependent manner [12]. The p53 and $p21^{Cip1/WAF1}$ proteins are usually upregulated during senescence and induce cell cycle arrest. In this regard, our data will be helpful to identify universal and specific markers of aged tissues and senescent cells. miR-216b and miR-337-3p have been shown to increase during senescence by recent miRNA profiling [26,27]. miR-186 and miR-760 were also upregulated in senescent IMR-90 cells compared with young IMR-90 cells (S.Y. Kim et al., unpublished data).

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