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Communication

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# The p53-p21$^{Cip1/WAF1}$ Pathway Is Necessary for Cellular Senescence Induced by the Inhibition of Protein Kinase CKII in Human Colon Cancer Cells

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**We have previously shown that the down-regulation of protein kinase CKII activity is tightly associated with cellular senescence of human fibroblast IMR-90 cells. Here, we examined the roles of p53 and p21$^{Cip1/WAF1}$ in senescence development induced by CKII inhibition using wild-type, isogenic p53-/- and isogenic p21-/- HCT116 human colon cancer cell lines. A senescent marker appeared after staining for senescence-associated β-galactosidase activity in wild-type HCT116 cells treated with CKII inhibitor or CKIIα siRNA, but this response was almost abolished in p53- or p21$^{Cip1/WAF1}$-null cells. Increased cellular levels of p53 and p21$^{Cip1/WAF1}$ protein occurred with the inhibition of CKII. CKII inhibition upregulated p53 and p21$^{Cip1/WAF1}$ expression at post-transcriptional level and transcription level, respectively. RB phosphorylation significantly decreased in cells treated with CKII inhibitor. Taken together, this study shows that the activation of the p53-p21$^{Cip1/WAF1}$ pathway acts as a major mediator of cellular senescence induced by CKII inhibition.**

## INTRODUCTION

Protein kinase CKII (formerly casein kinase II) is a ubiquitous and highly conserved serine/threonine protein kinase. CKII is a heterotetramer composed of two catalytic (α and/ or α') and two regulatory (β) subunits. The α and α' subunits contain the catalytic core and activity of the enzyme while the β subunit is the regulatory subunit that modulates the catalytic activity of α or α' subunits, and the β subunit also mediates tetramer formation and substrate recognition (Litchfield 2003; Pinna, 1990). Crystal structure of the α subunit has confirmed the constitutively active nature of CKII (Niefind et al., 1998). The catalytic activity of CKII is inhibited strongly by 5,6-dichloro-1-β-$_D$-ribofuranosylbenzimidazole (DRB) and apigenin (Sarno et al., 2002; Zandomeni, 1989). CKII is known to phosphorylate a broad spectrum of substrates which are involved in cell growth and proliferation including DNA-binding proteins, nuclear oncoproteins, and transcription factors (Litchfield 2003; Pinna, 1990). The expression level of CKII is greatly enhanced in a variety of tumor or leukemic cells (Daya-Makin et al., 1994; Faust et al., 1996; Münstermann et al., 1990). Overexpression of CKIIα in T cells of transgenic mice results in a high predisposition for lymphoma formation (Seldin and Leder, 1995). Overexpression of CKIIα or CKIIα' exhibits cooperativity with Ras in the transformation of rat embryo fibroblasts and Balb/c 3T3 cells (Orlandini et al., 1998). Analysis using temperature-sensitive yeast mutants for the CKII gene has shown that CKII is required for cell cycle progression in both G1 and G2/M phases of the cell cycle (Hanna et al., 1995). Microinjection of antibodies directed against either CKIIα or CKIIβ inhibits cell cycle progression in response to serum stimulation in human IMR-90 cells (Lorenz et al., 1993; Pepperkok et al., 1994). These observations suggest that CKII plays a significant role in the control of cell proliferation and transformation.

Recently, we have reported that CKII activity is down-regulated at the transcriptional level in both senescent human lung fibroblast IMR-90 cells and aged rat tissues. We have also shown that CKII inhibition in IMR-90 cells by CKII inhibitors or anti-CKIIα siRNA can induce premature senescence of the cells (Ryu et al., 2006). In addition, silencing of *CKIIα* and *CKIIα'* genes during cellular senescence is mediated by DNA methylation (Kim et al., 2009). In the present study, we show results from experiments designed to examine the mechanism by which CKII inhibition contributes to development of senescence. CKII inhibition induced senescence-associated β-galactosidase (SA-β-gal) activity in wild-type HCT116 colon carcinoma cells but not in HCT116 lines with a homozygous knockout of p53 or p21$^{Cip1/WAF1}$. This suggests that the p53-p21$^{Cip1/WAF1}$ pathway is involved in the development of senescence induced by CKII inhibition.

## MATERIALS AND METHODS

### Cells and culture media

The HCT116 human colon cancer cell lines (wild-type, p53-/-, and p21-/-) were generously provided by Dr. YC Chang (Catholic University of Daegu). Human diploid fibroblast IMR-90 cells were obtained from ATCC (Manassas, USA) at a population doubling level (PDL) of 24. Cells were cultured in Dulbecco's

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modified Eagle's medium containing 10% (v/v) fetal bovine serum under a humidified atmosphere of 5% (v/v) $CO_2$ at 37°C. The number of population doublings (PD) for IMR-90 cells was calculated using the formula PD = log($N_f$/$N_i$)/log2 where $N_f$ is the final cell number and $N_i$ is the initial number of seeded cells.

**SA-β-gal activity assay**

SA-β-gal activity was measured as described previously (Dimri et al., 1995) with minor modifications. Cells in sub-confluent cultures were washed with ice-cold phosphate-buffered saline (PBS), fixed in 3% (v/v) formaldehyde in PBS for 10 min at room temperature, and then incubated with a stain solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside, 40 mM citric acid-sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$ for 24 h at 37°C. The number of blue-stained cells was counted in at least 10 fields at 400× magnification, and expressed as percentage of positive cells.

**Preparation of cell extracts**

Cells in 60mm 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 aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The particulate debris was removed by centrifugation at 12,000 × *g* for 10 min. The protein concentration for the supernatants was determined using the Bradford assay (Bio-Rad, USA) and the protein concentration was made the same for all samples by adjusting the supernatant volumes.

**Immuno-blotting**

Proteins were separated by polyacrylamide gel electrophoresis in the presence of SDS, and then transferred by electrophoresis to nitrocellulose membranes. The membrane was blocked with 5% (w/v) non-fat, dry skim milk in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 2 h and then incubated with specific antibodies in 1% (w/v) non-fat, dry skim milk for 1 h. The membrane was washed 3 times in TBST, and then analyzed with the ECL system (Amersham Pharmacia Biotech, Korea). Some membranes were stripped in stripping buffer (2% SDS, 100 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.0) at 50°C for 1 h with gentle shaking and reprobed with anti-β-actin antibody as a control for protein loading. Anti-p53, -$p21^{Cip1/WAF1}$, and -β-actin antibodies were obtained from Santa Cruz Biotechnology Inc., CA. Anti-CKIIα and anti-RB antibodies were obtained from CalBiochem Co., (USA) and BD Biosciences (USA), respectively.

**Assay for CKII activity**

The standard assay for the phosphotransferase activity of CKII was conducted in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, and 100 μM [γ-$^{32}$P]ATP in the presence of 1 mM synthetic peptide substrate (RRREEETEEE) in a total volume of 30 μl at 30°C. The reactions were started by the addition of cell lysates and incubated for 15 min. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10% and centrifuged, and 10 μl of supernatant was then applied to P-81 paper. The paper was washed in 100 mM phosphoric acid and the radioactivity was measured by scintillation counting.

**Reverse transcription-PCR**

Total RNA was extracted from HCT116 cells. RNA was reverse transcribed using gene-specific reverse primers and reverse transcriptase (Takara, Japan), and the resulting cDNAs were PCR-amplified. The PCR primer sequences used for p53 were: p53Fwd (5′-CCTCACCATCATCACACTGG-3′) and p53Rev (5′-CCTCATTCAGCTCTCGGAAC-3′). The PCR primer sequences used for $p21^{Cip1/WAF1}$ were: p21Fwd (5′-GTGAGCGATGGAACTTCGACT-3′) and p21Rev (5′-CGAGGCACAAGGGTACAAGAC-3′). Primers for β-actin RNA were used to standardize amounts of RNA in each sample. PCR products were resolved on a 1.5% agarose gel. Quantification of the RT-PCR bands was done using densitometry.

**RNA interference**

The siRNA for CKIIα was 5′-UCAAGAUGACUACCAGCUGdTdT. The siRNA for the negative control was 5′-GCUCAGAUCAAUACGGAGAdTdT. Both siRNAs were used for transfection using Lipofectamine (Invitrogen, USA) as described by the manufacturer. The 5 h after the transfection, the medium was changed and the cells were grown for another 3 days before harvesting or SA-β-gal staining.

**Growth curves**

HCT116 cells were seeded in six-well dishes at a starting density of 5,000 cells/well in the absence or presence of 20 μM apigenin with duplicate wells for each sample. Every 24 h, cells were trypsinized and counted in triplicate using a hemocytometer. Trypan blue was used to distinguish viable cells from non-viable cells.

**Statistical analysis**

Results were presented as means with their standard errors. Statistical analysis was performed using SPSS version 11.0 (SPSS Inc., USA). Data were analyzed by one-way ANOVA, and Duncan's multiple-range test was performed if differences were identified among groups at $p < 0.05$. Analysis between two groups was determined using the unpaired Student's *t*-test. Differences with $p < 0.001$, $p < 0.01$ or $p < 0.05$ were considered significant.

## RESULTS

**CKII inhibition induces senescence through the p53-dependent pathway in human colon cancer cell**

Activation of p53 is required for cells to establish and maintain cellular senescence (Dimri et al., 1995). To examine the role of p53 in senescence induced by CKII inhibition, we used sublines of the human colon cancer cell line HCT116 colon carcinoma cells where the *p53* gene was inactivated by homozygous knockout (Bunz et al., 1998). To detect senescence, p53-/- and wild-type HCT116 cells were treated with CKII inhibitors, apigenin and DRB, and then stained for SA-β-gal activity. In the p53 positive HCT116 cells, there was a significant dose-dependent increase in SA-β-gal activity in response to apigenin and DRB. However, p53 negative HCT116 cells showed only slight signs of senescence demonstrating that p53 is required for the induction of senescence through CKII inhibition (Fig. 1A). We examined if CKII activity decreased in CKII inhibitor-induced senescent HCT116 cells. When CKII activity in these cells was assessed using [γ-$^{32}$P]ATP and CKII peptide substrate RRREEETEEE, the acceleration of senescence by CKII inhibitor treatment resulted in decrease of CKII activity (data not shown). To confirm the role of p53 in cellular senescence induced by CKII down-regulation, we knocked down CKIIα in HCT116 cells by gene silencing using siRNA duplexes. As shown in Fig. 1B, transfection of CKIIα siRNA markedly reduced the protein level of CKIIα in both p53 positive and nega-

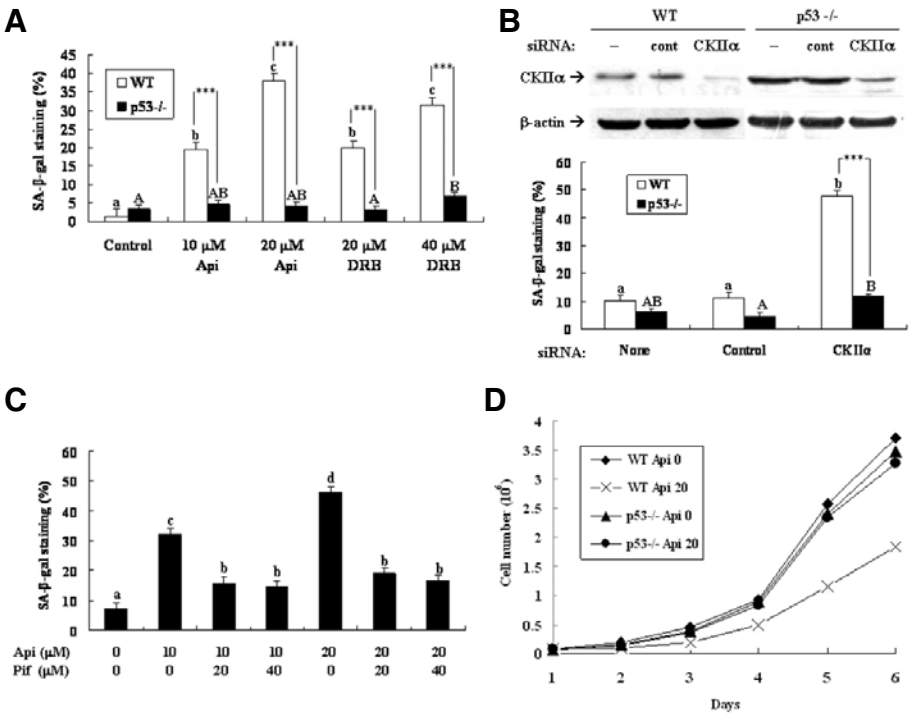


**Fig. 1.** Effect of p53 on cellular senescence induced by CKII down-regulation in HCT116 cells. (A) Wild-type (WT) and p53-/- HCT116 cells were treated with CKII inhibitors apigenin (Api) or DRB for 6 d. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside as described in "Materials and Methods". The percentage of positively stained cells was measured. Values are mean ± S.E. [abc]Means not sharing a common letter are significantly different among groups within WT at $p < 0.05$. [AB]Means not sharing a common letter are significantly different among groups within p53-/- at $p < 0.05$. ***Means are significantly different between WT and p53-/- at $p < 0.001$. (B) HCT116 cells were transfected with either CKIIα or control siRNAs using Lipofectamin as described by the manufacturer. At 5 h after the transfection, the medium was changed and the cells were grown for another 3 days. Cells were lysed, electrophoresed on a 12% (w/v) SDS-polyacrylamide gel, and visualized by immuno-blot with anti-CKIIα and -β-actin antibodies (upper panel). Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (bottom panel). Values are mean ± S.E. [ab]Means not sharing a common letter are significantly different among groups within WT at $p < 0.05$. [AB]Means not sharing a common letter are significantly different among groups within p53-/- at $p < 0.05$. ***Means are significantly different between WT and p53-/- at $p < 0.001$. (C) Wild-type HCT116 cells were treated with or without p53 inhibitor pifithrin (Pif) in the presence of apigenin for 6 days. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside. Values are mean ± S.E. [abcd]Means not sharing a common letter are significantly different among groups within WT at $p < 0.05$. (D) HCT116 cells ($5 \times 10^3$) were seeded in six-well dishes in the absence or presence of 20 μM apigenin (Api). Every 24 h, cells were trypsinized and counted using a hemocytometer. These experiments were performed twice with similar results.

tive HCT116 cells. When these transfectants were stained for SA-β-gal activity, p53 negative HCT116 cells exhibited an apparently lower rate of SA-β-gal activity compared to p53 positive HCT116 cells. To investigate whether the DNA binding activity of p53 is necessary for senescence induced by CKII inhibition, wild-type HCT116 cells were exposed to pifithrin-α, an inhibitor for DNA binding of p53. Treatment of wild-type HCT116 cells with pifithrin-α resulted in a significant decrease in SA-β-gal activity induced by CKII inhibitors (Fig. 1C). These results suggest that the DNA binding activity of p53 is involved in the development of senescence induced by CKII inhibition. To test whether the senescent effect of CKII inhibition were caused by reduction in cell proliferation, growth curves were performed on wild-type and p53-/- HCT116 cells. Apigenin treatment induced an apparent decrease in proliferation of wild-type, but not p53-/-, HCT116 cells (Fig. 1D).

### Up-regulation of p53 in senescent HCT116 cells mediated by CKII inhibition

Next, we wanted to determined whether p53 would be over-expressed in wild-type HCT116 cells treated with CKII inhibitor or CKIIα siRNA. When the protein level of p53 was determined by immuno-blot, the expression of p53 was up-regulated in both apigenin- and CKIIα siRNA-treated HCT116 cells. However, immuno-blotting analysis using a serine-392 phosphorylation-specific antibody revealed that CKII-mediated p53 phosphorylation decreased regardless of p53 up-regulation in these cells (Fig. 2A). The protein level for β-actin was measured as a control. We wanted to know if there was a correlation between the increase in the total amount of p53 protein and an increase at the RNA level. We extracted total RNA from HCT116 cells. We then reverse-transcribed the RNA and performed PCR by using specific primers for the human p53. The level of p53 mRNA in both apigenin- and CKIIα siRNA-treated cells was unchanged compared to the untreated control cells (Fig. 2B). These results suggest that CKII inhibition up-regulates p53 expression at the post-transcriptional level in cells and that the over-expression of p53 in CKII-inhibited cells enable them to enter senescence.

### CKII inhibition induces senescence through the $p21^{Cip1/WAF1}$-dependent pathway in HCT116 cells

To examine the role of $p21^{Cip1/WAF1}$ in senescence induced by CKII inhibition, we used sublines of HCT116 cells with the *p21*$^{Cip1/WAF1}$ gene inactivated by homozygous knockout (Brown et al., 1997; Waldman et al., 1995). In $p21^{Cip1/WAF1}$ positive HCT116 cells, there was a significant increase in SA-β-gal activity in response to apigenin and DRB. However, $p21^{Cip1/WAF1}$ negative HCT116 cells showed only slight signs of senescence demonstrating that $p21^{Cip1/WAF1}$ is required for the induction of senescence via CKII inhibition (Fig. 3A). We examined whether $p21^{Cip1/WAF1}$ is over-expressed in wild-type HCT116 cells treated with CKII inhibitor. Cells treated with apigenin showed an increase both in the $p21^{Cip1/WAF1}$ protein and mRNA levels, suggesting that CKII inhibition up-regulates $p21^{Cip1/WAF1}$ expression at the transcriptional level (Figs. 3B and 3C).

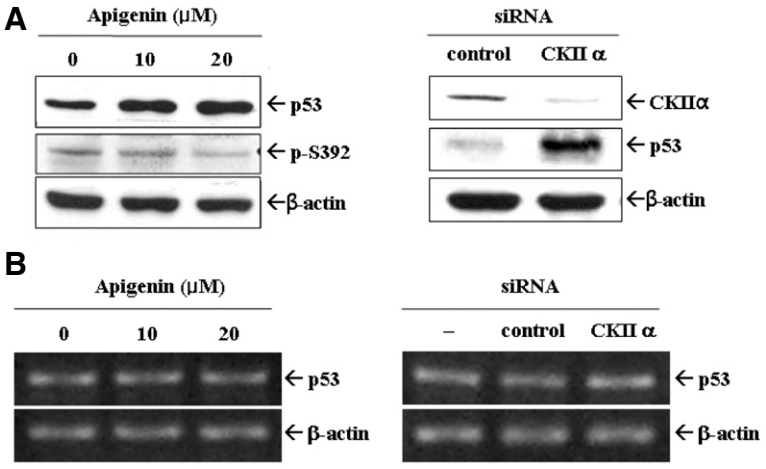


**Fig. 2.** Effect of CKII down-regulation on p53 expression in HCT116 cells. (A) Wild-type HCT116 cells were treated with apigenin (left) or transfected with CKIIα siRNA (right). Cells were lysed, and subjected to electrophoresis on a 12% (w/v) SDS-polyacrylamide gel, and visualized by immuno-blotting with anti-p53, -phosphoserine 392, -CKIIα, and -β-actin antibodies. (B) Total RNA was extracted from cells and reverse transcribed using p53-specific primers. PCR products were resolved on a 1.5% (w/v) agarose gel.

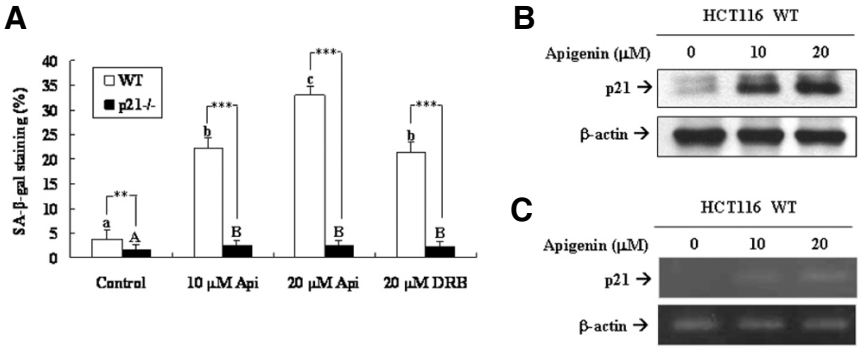


**Fig. 3.** Effect of p21 on cellular senescence induced by CKII down-regulation in HCT116 cells. (A) Wild-type (WT) and p21-/- HCT116 cells were treated with apigenin (Api) or DRB for 6 d. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-D-galactoside. The percentage of positively stained cells was measured. Values are mean ± S.E. [abc]Means not sharing a common letter are significantly different among groups within WT at $p < 0.05$. [AB]Means not sharing a common letter are significantly different among groups within p21-/- at $p < 0.05$. **Means are significantly different between WT and p21-/- at $p < 0.01$. ***Means are significantly different between WT and p21-/- at $p < 0.001$. (B) Wild-type HCT116 cells were treated with apigenin, and then the lysates were subjected to electrophoresis on a 12% (w/v) SDS-polyacrylamide gel and visualized by immuno-blotting with anti-p21 and -β-actin antibodies. (C) Total RNA was extracted from cells and reverse transcribed using p21-specific primers. PCR products were resolved on a 1.5% (w/v) agarose gel.

**Hypophosphorylation of the RB protein during senescence mediated by CKII inhibition**

It has been reported that $p21^{Cip1/WAF1}$ mediates cell cycle arrest primarily by inhibiting cyclin-dependent kinases (CDKs) that phosphorylate RB (Harper et al., 1993). The down-regulation of CDK activity in senescent cells induced by CKII inhibition was investigated by the phosphorylation status of the RB protein. As shown in Fig. 4, the level of hyperphosphorylated RB protein decreased while hypophosphorylated form increased in wild-type HCT116 cells treated with CKII inhibitor. Hypophosphorylation of the RB protein was also shown both in replicatively senescent (PDL51) and apigenin-treated proliferating (PDL32) IMR-90 cells. However, the hypophosphorylated form was hardly detected in apigenin-treated p53-/- or p21-/- HCT116 cells.

## DISCUSSION

We have previously shown that CKII inhibition in IMR-90 cells can induce premature senescence in the cells (Kim et al., 2009; Ryu et al., 2006). In the present study, we have examined the role of p53 and $p21^{Cip1/WAF1}$ in premature senescence in HCT116 cells induced by CKII inhibitors and siRNA-mediated gene knockdown. We have found that senescence mediated by CKII inhibition is strongly attenuated in p53-/- or p21-/- HCT116 colon carcinoma cells. Expression of both p53 and $p21^{Cip1/WAF1}$ was up-regulated in cells treated with CKII inhibitor. Levels of hypophosphorylated Rb protein increased in wild-type HCT116 and IMR-90 cells treated with CKII inhibitor. These results indicate that activation of p53-$p21^{Cip1/WAF1}$ pathway acts as a major mediator of senescence when mediated by CKII inhibition.

The tumor suppressor protein p53 is a potent transcription factor playing a key role in cell cycle regulation. p53 is activated in response to a variety of cellular stress signals including DNA damage and triggers cell cycle arrest or apoptosis to prevent cells from undergoing transformation (Horn and Vousden, 2007). How can the inhibition of CKII mediate p53 activation in cells? First, CKII phosphorylates and activates the oncoprotein MDM2 (Hjerrild et al., 2001). Since MDM2 promotes the ubiquitination and proteasomal degradation of p53 (Kubbutat et al., 1997), we propose that the underlying mechanism of p53 activation may be related to hypophosphorylation of MDM2 mediated by CKII inhibition. Another possible explanation is that the down-regulation of CKII may be involved in the direct activation of p53. Although its function remains to be elucidated, it has been shown that p53 is phosphorylated by CKII at serine 392 (Meek et al., 1990). In fact, the present study also showed that phosphorylated-p53 at serine 392 decreased in cells treated with CKII inhibitor. Finally, an increase in hydrogen perox-

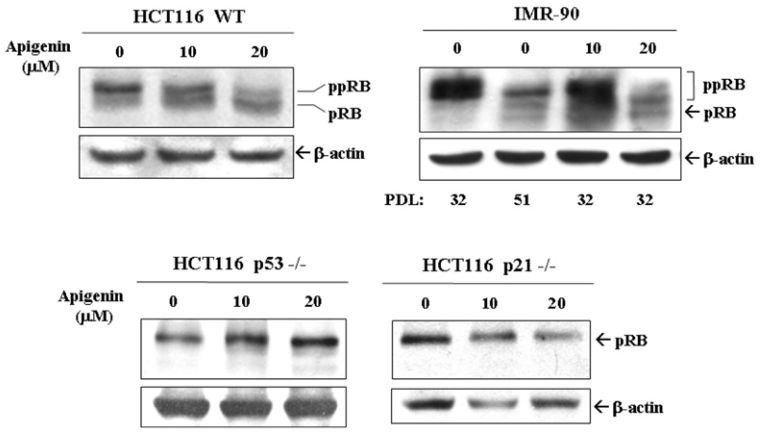


**Fig. 4.** Effect of CKII inhibitor on RB phosphorylation in HCT116 and IMR-90 cells. HCT116 and IMR-90 (PDL32 and 51) cells were treated with or without apigenin for 6 days. Cells were lysed, subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel, and visualized by immuno-blotting with anti-RB antibody. The slow-migrating differentially hyper-phosphorylated forms of RB (ppRB) and the fast-migrating hypophosphorylated form of RB (pRB) are indicated.

ide production has recently been reported in cells treated with CKII inhibitor (Ahmad et al., 2006). Since reactive oxygen species (ROS) stabilizes p53 (Horn and Vousden, 2007), down-regulation of CKII might induce cellular senescence via p53 stabilization by ROS production.

The gene encoding $p21^{Cip1/WAF1}$, *CDKN1A*, is a downstream target of p53 (Stein et al., 1999). CKII inhibition-mediated p53 upregulation also increased the mRNA level of $p21^{Cip1/WAF1}$ in this study. Although the expression of $p21^{Cip1/WAF1}$ is mostly regulated at transcriptional level, recent study suggests that post-transcriptional control of $p21^{Cip1/WAF1}$ is also important. E3 ubiquitin ligase complexes including SCFSKP2 (SKP1-CUL1-SKP2), CRL4CDT2 (CUL4-DDB1-CDT2) and APC/CCDC20 promote the ubiquitination and degradation of $p21^{Cip1/WAF1}$ (Kitagawa et al., 2009). Thus, we can not exclude the possibility that CKII inhibition may repress component(s) of the E3 ubiquitin ligase complexes, leading to stabilization of $p21^{Cip1/WAF1}$.

The present study also showed that CKII down-regulation induced cellular senescence even in p53-/- and p21-/- cells suggesting that this phenomenon is also mediated by some p53 and $p21^{Cip1/WAF1}$-independent pathways. The nature of senescence mediated CKII inhibition via p53- and $p21^{Cip1/WAF1}$-independent pathways remains to be elucidated.

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