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The study of physiological role of p53R2

조선대학교 대학원

의학과

박 춘 매

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p53R2의 생리학적인 역할 연구

2008년 8월 25 일

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지도교수 유 호 진

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박 춘 매

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CHX	cycloheximide
DMSO	dimethyl sulfoxide
dNTP	Deoxyribonucleoside triphosphates
DTT	dithiothreitol
ERK	Extracellular signal-regulated kinase
FITC	Flurorescein isothiocynate
Jab1	Jun activation domain-binding protein 1
Hdm2	Human Doube Minute 2
IB	Immunoblotting, western blotting assay
IP	Immunoprecipitation
MAPK	Mitogen-activated protein kinase, Ras/Raf/mitogen-activated protein kinase
MEK2	ERK kinase2/MAP kinase kinase 2
MMP	Matrix metalloproteinase
p53R2	p53-dependent RR small subunit
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	pheylmethylsulfonyl fluoride
SCF	F-box-SKP1-culin1
siRNA	Small interfeing RNA
TBST	Tris buffer saline containing 0.05% Tween-20
TNT	Transcription/translation system
RR	Ribonucleotide reductase
UB	ubiquitin
UPP	ubiquitin -proteasome pathway

ABSTRACT

The study of physiological role of p53R2

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p53R2 is a newly identified small subunit of ribonucleotide reductase (RR) and plays a key role in supplying precursors for DNA repair and mitochondrial DNA replication. Many studies on physiological function of p53R2 were done recently, however not fully elucidated.

In this study, we found some very important proteins related to protein stability, and tumor metastasis, were interacted with p53R2 by Yeast-2-hybrid screening. We focused on these proteins interactions to understand molecular mechanism of physiological function for p53R2.

Firstly, we studied for two protein related with protein stability, Skp1A and Jab1. SKIP1A is one component of SCF(the Skip1-Cullin-F-box)complex(ubiquitin protein ligase). Jab1 is a nucleus exporter and inducer of cytoplasmic degradation for p53. We confirmed interactions of two proteins by immunoprecipitation in mammalian cells. p53R2 induced by some DNA damage agents, in p53-dependent manner, however little is known degradation of p53R2. We demonstrated that p53R2 is a short-lived protein, half-life is less than 30min in living HEK293T cells, and p53R2 degradation is inhibited by proteosome inhibitor MG132. *in vitro* and *in vivo* ubiquitination assay elucidate that p53R2 protein is degraded by ubiquitin pathway in HEK293T cell; and this degradation of

p53R2 were required two components of SCF complex, Skp1A and Culin1. p53R2, is regulated by tumor suppressor p53, and p53R2 contains a p53-binding sequence. In mammalian cells silencing p53R2, accelerated p53 degradation, and decreased the half-life of wild-type p53; and overexpression of p53R2 inhibited ubiquitination of exogenous p53 in a dose-dependent manner, whereas knocking down p53R2 by siRNA enhanced ubiquitination of endogenous p53. In addition to our study, p53R2 were binding with Jab1. We showed that silencing p53R2, increased transport of p53 from nucleus, and this exclusion mediated by Jab1. These results suggest that as the transcriptional target of p53, p53R2 regulated p53 stability via Jab1. ,

Secondly, we studied for MEK2 related with tumor metastasis. Although some studies are going on the metastasis-suppressing property of p53R2, recently, mechanism of metastasis-suppressing property of p53R2 is not clear yet. we confirmed that p53R2 interacted with MEK2 by immunoprecipitation, which is a component of Ras/Raf/mitogen-activated protein kinase (MAPK) which signal were associated with tumor invasion and metastasis. Then amino acids 160 to 306 of p53R2 are critical for interacting with MEK2. Binding of p53R2 with MEK2, modulate Erk-MAPKinse pathway, silencing p53R2 increased phosphorylation of erk1/2, while overexpression of p53R2 decreased phosphorylation of erk1/2; which susquencialy promote cell invasion by silencing of p53R2, while overexpression of p53R2 inhibit cell invasion, cell transformation. p53R2 Inhibited Erk-MAPKinase signal via inhibiting phosphorylation of MEK2 , which affect Erk-MAPKinase pahway resulted in p53R2 metastasis-suppression property.

All these results suggested that p53R2 is a multi-functional protein, which had many physiological functions based on interacting with some important proteins related with cell survival, DNA repair and so on. Understanding the molecular mechanism of p53R2 function will be significant, not only on DNA repair and replication, but also cell survival, tumor metastasis and so on.

I. INTRODUCTION

Mammalian cells need a balanced supply of deoxyribonucleoside triphosphates (dNTPs) for DNA replication and repair. The rate-limiting step in the formation of DNA precursors is the de novo reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates by the enzyme ribonucleotide reductase (RR) (Reichard, 1988). Classical human RR consists of two proteins, which together form a heterotetrameric active enzyme. a large subunit known as hRRM1 and a small subunit known as hRRM2, The large hRRM1 (90 kDa) carries the active site, whereas the small hRRM2(45 kDa) contains a diferric iron center generating a tyrosyl free radical necessary for catalysis (Thelander, 1994). An additional mammalian RR protein, p53R2, was identified in 2000 (Nakano et al., 2000; Tanaka et al., 2000). Like the homologous hRRM2 protein, p53R2 contains a tyrosyl free radical and forms an active RR complex with the R1 protein in vitro(Guittet et al., 2001). 351-amino-acid p53R2 is 80−90% identical to hRRM2 (Chabes et al., 2003). However, expression of p53R2, unlike that of hRRM2, is not cell-cyle dependent; rather, p53R2 is induced by DNA damage agents(Arner and Eriksson, 1995; Bradshaw and Deininger, 1984), such as γ-irradiation, UV light, and adriamycin, in a p53 dependent manner(Nakano et al., 2000).

p53R2 has been assumed to facilitate the repair of damaged DNA by providing an increased supply of dNTPs. Mammalian cells show a low constitutive expression of p53R2 protein (Mann et al., 1991; Sandrini and Piskur, 2005; Zhou and Elledge, 2000), and the original cDNA cloning was from a mouse skeletal muscle cDNA library supporting its expression in nondamaged cells(Mann et al., 1991). The exact physiological function of p53R2 is not known, but it is essential for cell survival because mice lacking functional p53R2 apparently grow normally up to 6 weeks but then die from kidney failure (Kimura et al., 2003; Powell et al., 2005).

A.p53R2 regulates p53 stability via ubiquitination pathway

The ubiquitin(UB) system is the most important proteolytic machinery in eukaryotic cells and is involved in the regulation of essential cellular processes such as cell cycle, signal transduction and antigen processing.

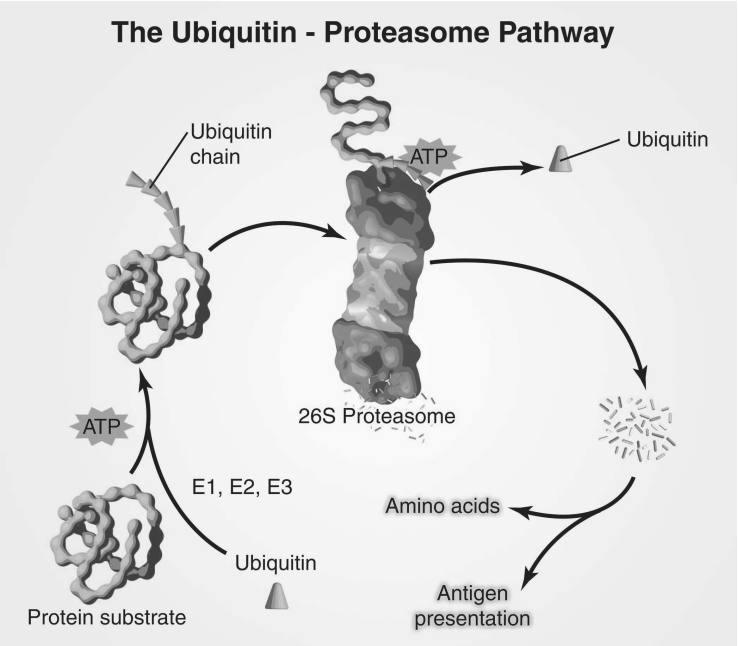

Figure 1. The ubiquitin (Ub)-proteasome pathway (UPP) of protein degradation.

Ub is conjugated to proteins that are destined for degradation by an ATP-dependent process that involves three enzymes. A chain of five Ub molecules attached to the protein substrate is sufficient for the complex to be recognized by the 26S proteasome. In addition to ATP-dependent reactions, Ub is removed and the protein is linearized and injected into the central core of the proteasome, where it is digested to peptides. The peptides are degraded to amino acids by peptidases in the cytoplasm or used in antigen presentation. Illustration by Josh Gramling—Gramling Medical Illustration.

The formation of ubiquitin-protein conjugates involves three components that participate in a cascade of ubiquitin transfer reactions, an ubiquitin-activation enzyme (E1),1 an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3) that acts at the last step of the cascade (Ciechanover, 1998). Ubiquitin is first sequentially transferred through a series of ubiquitin system enzymes, designated E1, E2, and E3. The E3 enzyme then transfers the ubiquitin molecule to one or more lysine residues in the substrate(Scheffner et al., 1995).

In the present study, we found that p53R2 bound with ubiquitin proteins(SKIP1A) by Y2H. Skp1A is one component of SCF(the Skip1-Cullin-F-box)complex. The SCF (Skp1, Cdc53/Cullin1, F-box protein) and SCF-like complexes are the largest family of ubiquitin ligases, one of ubiquitin protein ligase which has an important role in cell cycle regulations (DeSalle and Pagano, 2001). Interaction of two proteins confirmed and immunoprecipitation, in addition we also found that p53R2 is short life protein, degraded by ubiquitin pathyway, and this degradation required of two components of SCF, Skp1A, Cullin1. p53R2 degradation by ubiquitination pathway , mediated by Skp1A.

The tumor suppressor protein p53 is a transcription factor activated in response stress to induce expression of its target genes. The proteins encoded by these genes then mediate multiple cellular responses, such as cell cycle arrest, apoptosis, differentiation, cell senescence, or DNA repair. In normal, unstressed cell, the p53 protein is short-lived(t½~20min), reflecting a rapid turnover through ubiquitin-mediated proteolysis(Vousden, 2002). The p53 protein is stabilized, and its levels increase in response to various stresses, including DNA damage, hypoxia, and inappropriate oncogene signaling(Giaccia and Kastan, 1998). The induction of p53 involves several mechanisms including post-translational modifications such as ubiquitination, phosphorylation and acetylation (Giaccia and Kastan, 1998).

p53R2, which is regulated by tumor suppressor p53, and p53R2 contains a p53-binding sequence in intron 1 and encodes a 351-amino-acid peptide. p53 is known as “the genome gatekeeper”, the biological mechanisms for maintaining the basal level of p53 in

mormal cells require nuclear exclusion and cytoplasmic degradation. In normal condition it maintained at low steady-state level by ubquitination, in addition we confirmed p53R2 bound with Jab1 by immunoprecipitation. Jun activation domain-binding protein 1 (Jab1)/CSN5, the fifth member of COP9 signalosome (CSN) complexes, is a nucleus exporter and inducer of cytoplasmic degradation for p53, Jab1 facilitates p53 nuclear exclusion and its subsequent degradation in coordination with Hdm2(Oh et al., 2006). We hypothesized that p53R2 regulate p53 stablility by ubiquination pathway.

In mammalian cells silencing p53R2, accelerated p53 degradation, and decreased the half-life of wild-type p53; overexpression of p53R2 inhibited ubiquitination of exogenous p53 in a dose-dependent manner, whereas knocking down p53R2 by siRNA enhanced ubiquitination of endogenous p53. p53R2 regulated p53 stability may be affect export p53 from nucleus and cytoplasmic degradation. We demonstrated that silencing p53R2, increased exclusion of p53 from nucleus, mediated by Jab1. These results suggest that as the transcriptional target of p53, p53R2 regulated p53 stability in return.

This study showed that p53R2 is degraded by ubiquination pathway, and p53R2 involved in p53 cytoplasmic localization, which subsequently leads to the degradation of p53. These result suggested that maintenance of p53 homeostasis requires the presence of p53R2, which may be another useful target for p53-related cancer therapies.

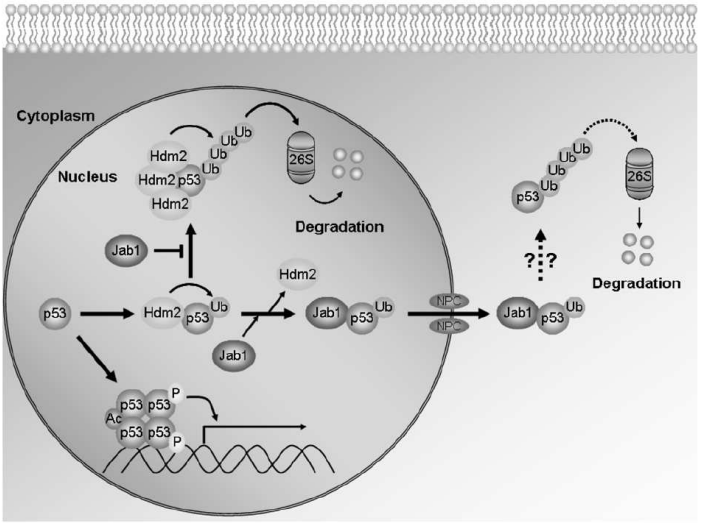

Figure 2. A schematic diagram of Jab1-dependent p53 nuclear transport and degradation in coordination with Hdm2. Jab1 might prevent polyubiquitination of p53 by competing with Hdm2. Jab1 might induce nuclear export of p53 from nucleus to cytoplasm in coordination with CRM1, RanGTP, etc. The Jab1-mediated translocation of p53 would result in the proteasome-dependent degradation of p53 by mediating polyubiquitination (Wei and Deng, 1999).

B. p53R2 suppress metastasis of cancer cell by inhibiting ERK/MAPK signal

MAPKs are involved in the regulation of a wide range of cellular responses including cell proliferation, differentiation and apoptosis(Gulli et al., 1996; Reddy et al., 1999; Su and Karin, 1996). Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway is a frequent event in tumorigenesis. MAPKs have been implicated in cell migration, proteinase induction, regulation of apoptosis, and angiogenesis, events that are essential for successful completion of metastasis(Reddy et al., 2003).

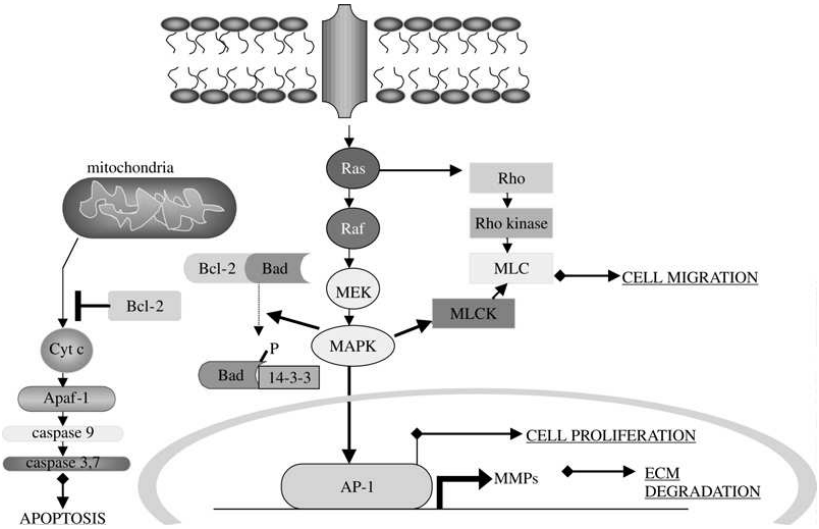

Figure 3. Schematic representation of intracellular signal transduction pathways emphasizing those pathways activated by ERK to regulate metastasis.

The majority of arrows represent possible mechanism by which ERK activates cell proliferation, proteinaseinduction, cell migration and prevention of apoptosis(Reddy et al., 2003).

In addition to their role in RR catalysis, hRRM1 and hRRM2 also appear to play a role in determining the malignant potential of tumor cells. Expression of hRRM1 suppresses tumor cell transformation, tumorgenesis, and metastasis(Cao et al., 2003; Fan et al., 1997), whereas expression of hRRM2 enhances the invasive potential of human and mouse cells(Fan et al., 1996; Zhou et al., 1998). Interestingly, recent studies of highly invasive cancer cells(oropharyngeal cancer KB, prostate cancer PC3, and pancreatic cancer MIA Paca-2 cells) have shown decreased levels of p53R2 and increased levels of hRRM2(Liu et al., 2006), in addition, overexpression of p53R2 reduces migration of KB and PC3 cells and suppresses invasion by colon cancer, KB and PC-3 cells(Liu et al., 2007). therefore, in contrast to hRRM2, p53R2 might function in suppression of cancer cell invasion and metastasis, although the mechanism of this suppression remains to be determined.

In the present study, we determined that MEK2(ERK kinase2/MAP Kinase kinase2)I a binding partner of p53R2, in our experiments, p53R2 knockdown markedly increased serum-stimulated phosphorylation of MEK1/2 and ERK1/2, and p53R2 inhibited ERK1/2 phosphorylation by suppressing MEK2 activity. Our results suggest that p53R2 regulates the invasive potential of human lung cancer H1299 cells by modulating MEK2 activity.

II. MATERIALS AND METHODS

1. Maintenance of Cell Lines

HEK 293 (Coriell Institute for Medical Research, Camden, NJ), Human pancreatic Cancer cell line Mia paca-2(p53 mutant type, ATCC) were maintained in Dulbecco's modified Eagle's medium. Human breast cancer epithelial cell lines MCF-7 and (Coriell Institute for Medical Research, Camden, NJ) were maintained in RPMI-1640 medium. Human lung carcinoma H460(p53 wild type), H1299 (p53-null) cells (ATCC number CRL-5803; Manassas, VA) were grown in RPMI 1640 medium. Human osteosarcoma cell line U2Os(p53 wild type, ATCC) was cultured in McCoys 5A medium. HCT116(p53+/+), HCT116(p53-/-) were maintained in IMDM medium. All cellline supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified incubator in an atmosphere containing 5 % CO_2 at 37 ^{o}C.

2. Plasmid constructions of p53R2

The human p53R2 cDNA was amplified by RT-PCR using the p53R2 oligo primer: sense 5'- GAA TTC ATG GGC GAC CCG GAA AGG C -3' and antisense 5'- AAA ATC TGC ATC CAA GGT GAA -3' from human fibroblast GM00637 cells. The amplified p53R2 cDNA construct was cloned into a pcDNA3.1/ V5-His TOPO mammalian expression vector(invitrogen), which was driven by the CMV promoter(Invitrogen) and confirmed the DNA sequence and orientation. The p53R2 construct was transfected into cells using Lipofectamine transfection reagent(Invitrogen) according to the manufacturer's instruction, after transfection, cells were incubated with complete medium containing 400μg/ml G418 for 5 weeks, the cell clones resistant to G418 were isolated and analyzed. Full-length human p53R2 cDNAs or truncated p53R2 (1-481, 1-618, 91-1,053, 619–1,053, and 946–1,053 bp cDNAs) cDNA fragments were amplified by PCR using PCR Master Mix (Promega, Madison, WI). All resulting PCR products were cloned into

pCR8/GW/TOPO vector (invitrogen), then for mammalian expression were inserted in pcDNA-DEST47 Gateway ® vector with LR reaction by Gateway® LR clonase TM II Enzyme Mix(invitrogen), resulting in mammalian expression constructs. All constructs confimed the sequence. The amplified p53R2 cDNA was cloned into a pcDNA-DEST47 Gateway vector(invitrogen);

Topo-p53R2EcoR1 F1: 5'- GAA TTC ATG GGC GAC CCG GAA AGG C -3'

Topo-p53R2 R end: 5'- AAA ATC TGC ATC CAA GGT GAA -3'

p53R2Entry F 5'- ACC ACC ATG GGC GAC CCG -3'

ENTRY P53R2: 5'- TCA TTC AAT TGC ATT AAA TAA AAA TTC C -3'

ENTRY P53R2 483R: 5'- TTC AAT TGC ATT AAA TAA AAA TCC C -3'

p53R2 F91 ATG : 5'-ACC ATG GAA GAG CCA CTC CTA AGA AAG AGT TC-3'

p53R2 F619 ATG: 5'-ACC ATG TGG CTA AAG AAG AGA GGT CTT ATG CC -3'

p53R2 F946 ATG: 5'-ACC ATG TCT TTG AGA AGG AAA AAC AAA TTT CTT TG-3'

p53R2 R618: 5'-GAA TAT AGC AGC AAA AGA TCC TG-3'

After confirming the DNA sequence and orientation, the p53R2 construct was transfected into cells using the Lipofectamine transfection reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. After transfection, cells were incubated with complete medium containing 200ug/ml G418 for 4 weeks. The cell clones resistant to G418 were isolated and analyzed.

3. Small interfeing RNA(siRNA) based experiments

Three target sites within human p53R2 genes were chosen from the human p53R2 mRNA sequence (Genbank accession no.AB ab036063), which was extracted from the National Center for Biotechnology Information Entrez nucleotide database. After selection, each target site was searched with National Center for Biotechnology Information BLAST

to confirm the specificity only to the human p53R2. The sequences of the 21-nucleotide sense and antisense RNA are as follows: hRRM2B si-RNA , 5'- UGA GUU UGU AGC UGA CAG AUU -3' (sense) and 5'- AAU CUG UCA GCU ACA AAC UCA -3'(antisense) These siRNAs were prepared using a transcription-based method with a Silencer siRNA construction kit (Ambion) according to the manufacturer's instructions. Cells were transiently transfected with siRNA duplexes using Oligofectamine (Invitrogen) and stably transfected with the constructed siRNA expression plasmid based on pSilence hygro vector (Ambion), which includes a human U6 promoter and a hygromycin resistance gene, using LipofectAMINE (Invitrogen). pSilence hygro vector was used as the negative control. If required, we selected several resistant colonies against 100 μg/mL hygromycin in the medium after transfection.

4. Western blot analysis

The cell were washed with phosphate-buffered saline (PBS) and lysed on ice for 10 minutes in the M-PER mammalian protein Extraction Reagent (PIERCE) added protease Inhibitor Cocktail tablet (Roche). After incubation, extracts were vortexed for 5min and centrifuged at 13000rpm for 15min. The supernatant was diluted with 5×SDS-sample buffer and boiled. After cellular protein concentrations were determined using the dye-binding microassay (Bio-Rad, Hercules, CA), and 20μg of protein per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred onto Hybon ECL membranes (Amersham Biosciences, Piscataway, NJ). After electroblotting, the membranes were blocked by 5% skim-milk in Tris buffer saline containing 0.05% Tween-20(TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) at room temperature for 2 hours. The membranes were rinsed with TBST and then incubated with appropriate primary antibodies in TBST at 4℃ overnight. All antibodies used in this study are anti-p53R2 polyclonal antibody (pAb) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-p44/42 pAb, anti-phospho-p44/42 pAb, anti-Akt pAb, anti-phospho-Akt pAb, anti-MEK1 pAb, anti-MEK2 pAb(Cell Signaling Technology,

Danvers, MA); anti-β-actin mAb (BD Phamingen, San Jose, CA); anti-a-tubulin mAb (BD Phamingen, San Jose, CA); anti-p53R2 mAb (Abnova, Taipei, Taiwan). We followed manufacturer's protocol for dilution of all primary antibodies. The membranes were then washed, incubated with the biotinylated secondary antibodies (1:4,000) in a blocking buffer for 2 hours at room temperature , and washed again. The blotted proteins were developed using an enhanced chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea).

5. Immunoprecipitation (IP)

Whole cell lysates were made in lysis buffer (50 mM HEPES at pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, 100 mM NaF, 1 mM PMSF, 1 mM DTT, 1× complete protease cocktail and 10% glycerol) and pre-cleared with protein A Sepharose beads for 1 h. The lysates were then immunoprecipitated with the indicated antibodies and isotype-matched control antibodies plus protein A Sepharose for at least 4 h or overnight. Beads were washed four times with lysis buffer, once with ice-cold PBS and boiled in 2× loading buffer. Protein samples were resolved by SDS−PAGE and transferred onto nitrocellulose membrane, which was blocked in 5% skim milk in PBST and probed with the indicated antibodies. The following antibodies were used for immunoprecipitation and western blotting: rabbit polyclonal MEK2 antibody, mouse GFP B-2 monoclonal antibody, goat p53R2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

6. Confocal Immunofluorescence Analysis.

Cells were grown on sterile glass coverslips at 37°C for 24 h and 16-24hrs serum starvation stimulation with FBS for 10-30min. At the designated times, cells were washed briefly with PBS and fixed with 4% parafomaldyhyde for 5 min. After fixed cells were washed with PBS three times, then 0.3% Triton X-100 10 min, Then blocked for 1 h in a blocking buffer (0.5% BSA in PBS), and then additional incubated for another hour in PBS with 0.5% BSA containing anti-p53, anti-Jab1, anti-MEK2 , anti-p53R2 4°C overnight. After washing three times in PBS, cells were incubated with Chicken anti-rabbit 488,

chicken anti-goat 563 secondary antibodies in PBS with 0.5% BSA for 1hr. Coverslips were washed five times with PBS and mounted. Images were acquired using a confocal microscope (Zeiss).

7. Nuclear-cytoplasmic fractionation

Cells were harvested and resuspended in 800 μl buffer A (25 mM Tris–HCl at pH 8.0, 10 mM KCl, 1 mM DTT and 0.5 mM PMSF) supplemented with complete protease inhibitor cocktail (Boehringer Mannheim). After incubation on ice for 15 min, 50 μl of 10% Nonidet P40 was added to the cells and vortexed for 10 sec. After centrifugation at 1,400*g* for 30 sec, the supernatants were collected and subsequently referred to as cytoplasmic extracts. The pellet was resuspended in 100 μl of ice-cold buffer C (50 mM Tris–HCl at pH 8.0, 400 mM NaCl, 1 mM DTT and 1 mM PMSF) supplemented with complete protease inhibitor cocktail (Roche). After centrifugation at 15,000*g* for 30 min, the supernatants were collectd and subsequently referred to as nuclear extracts

8. *In vitro* invasion assay.

In vitro invasion assay was performed using BD Biocoat Invasion Assay system(BD biosciences) according to manufacture. NCI-h1299 (5×10^4) were placed in the upper chamber of system with serum medium. the lower compartment was filled with serum-free media containing 5% bovine serum albumin. Cells were placed in the upper part of the transwell plate, incubated for 24h, fixed with methanol, and stained with Hematoxyline for 10min followed briefly by eosin. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at ×400, ten random filelds were accounted for each filter, and each sample was assayed in triplicate. Invasiveness was caculated ad the percentage of cells that had successfully invaded through the Matrix-coated membrane to the lower wells relative to the total number of the

cells seeded into the upper wells. The invasion assay were done in triplicate and repeated once.

9. Soft agar colony formation analysis

Cell transformation was evaluated with a soft-agar assay. Stable transfectant or control cells were plate 2×10^4 in duplicates in 60-mm tissue culture dishes containing 0.3% top low-melt agarose and 0.6% botton low-melt agarose(Bacto agar; Difco,Detroit, MI, USA) supplemented with DMEM 10% CS. Medium was replaced every 2 to 3 days, and the cells were left for 14 days after reaching confluence. Microscopically visible foci were then counted and photographed.

10. *In vitro* and *in Vivo* Ubiquitination Assay

In Vitro ubiquitination Assay, in a 30 μl reaction, 1μl of p53R2 protein obtained from TNT kit(TNT® T7 Quick Coupled Transcription/Translation System, Promega), was incubated with an ATP regenerating system (50 mM Tris [pH 7.4-7.6], 1 mM $MgCl_2$, 5 mM ATP, 200μM Hemin), 5 μg of methyl-ubiquitin, and 10 μg of S100 at 37°C for one hour. After terminating the reactions with SDS sample buffer, reaction products were fractionated by SDS-PAGE (10%) and analyzed by Western blotting with specific antibody.

In vivo ubiquitination assays were conducted as previously described (Kubbutat et al., 1997). Briefly, HEK 293 was transfected with combinations of the following plasmids as indicated in the figure legends: His6-ubiquitin (0.8μg), myc3-p53 (0.4μg), p53R2(0.4, 0.8, 1.2μg) using Metafectamine. For inhibition of proteasome-mediated protein degradation, the cells were treated with 20 mM MG132 for 4 h before harvest. Forty-eight hours after transfection cells from each plate were harvested and split into three aliquots, one for immunoblot and the other two for ubiquitination assays. For pulldown assay, Cell pellets were lysed in buffer I (6 M guanidinium-HCl, 0.1 mol/ liter Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl (pH 8.0), 10 mmol/liter β-mercaptoethanol) and incubated with Ni-NTA beads

at room temperature for 4 h. Beads were washed once each with buffer I, buffer II (8 mol/liter urea, 0.1 mol/liter Na_2HPO_4/NaH_2PO_4, 10 mmol/liter Tris-HCl (pH 8.0), 10 mmol/liter β-mercaptoethanol), and buffer III (8M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris- HCl (pH 6.3), 10 mM β-mercaptoethanol). Proteins were eluted from the beads in buffer IV (200 mM imidazole, 0.15 M Tris-HCl (pH 6.7), 30% (v/v) glycerol, 0.72 M β-mercaptoethanol, and 5% (w/v) SDS). Eluted proteins were analyzed by immunoblot with monoclonal anti-p53 (DO-1), anti-HA, antibodies. For Immunoprecipitation, cell lysates were IP with anti-p53(DO-1), and detected with anti-ubiquitin.

11. Statistical analysis

Results are expressed as mean ± standard deviation (SD). For statistical analysis, ANOVA with *p* values were performed for both the overall (*p*) and the pair-wise comparison as indicated by asterisks. Values of $p<0.05$ were considered to be significant.

III.RESULTS

A. p53R2 degrades via ubiquitination pathway

1. Identification of p53R2 interacting with some ubiquitination protein.

To identify candidate molecular mediators of ubquintation pathway, we used full-length and partial (343-717) human p53R2 cloned in pGBT9 vector as bait in Yeast Two Hybrid screen. We identified two positive clones, one is highly homologous with human Skp1A, the other is with Jab1, re-Yeast 2 hybrid was done to confirm binding of p53R2 and Skp1A, Jab1 which cloned in pACT2AD vector was used as prey. Interaction of these two proteins results in the formation of colonies in −Leu/-Trp/-His plate. Interaction is confirmed by the formation of blue colonies in X-alpha gal −Leu/-Trp/-His plate after 3-5 days of incubation period at 30℃. Positive colonies were picked up and cultured in YPD broth. After overnight incubation, plasmid DNA was extracted, amplified by PCR and sequenced. Confirmed sequencing results prove that p53R2 binds to Skp1A, Jab1 in yeast cells (Fig. *4A*). In order to confirm these proteins binding in mammalian cells, immunoprecipitation-Western blot assay was done, input (10% of the lysate used for immunoprecipitation assay) indicated the presence of p53R2 in (Fig. *4B*, *4C*). The lysate of MCF7 cells was incubated with anti-p53R2, Skp1A antibody to selectively immunoprecipitate intracellular p53R2. The immunoprecipitates were then subjected to Westernblot analysis with anti-p53R2 antibody. As shown in Fig. *4B*, Skp1A interaction with p53R2 in MCF7 cells (Fig. *4B*, lane 2.3), while control experiments could not. These findings indicate that Skp1A may interact with p53R2 *in vivo.* The cell lysate of MCF7 cells was incubated with anti-Jab1 antibody to selectively immunoprecipitate intracellular p53R2. The immunoprecipitates were then subjected to Westernblot analysis with anti-p53R2 antibody. As shown in Fig. *4C*, Jab1 could bind p53R2 in MCF7 cells (Fig. *4C*, lane 3.4). Control experiments showed that immunoprecipitate mouse derived serum could

not. These findings indicate that Jab1 may interact with p53R2 *in vivo*. The result from Y2H, and immuoprecipitation, p53R2 interaction with some ubiquitin proteins, however there are no significant difference between MCF7 cells treated with or without 10J of UV, from the all above we can know these interactions independent of DNA damage in *vivo*.

A

pGBT9+ p53R2 (Full length)

Name of binding protein	Re-yeast-2-hybrid
SKP1A (NM_170679)	OK
p53R2/liver a486	OK
Jab1	OK

pGBT9+ p53R2 (343-717)

Name of binding protein	Re-yeast-2-hybrid
SKP1A	OK
Jab1	OK

B

C

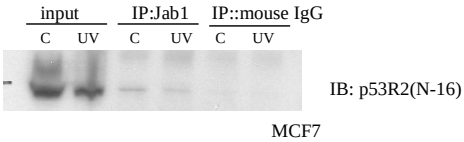

Figure 4. p53R2 interact with ubiquitination protein

(A) p53R2 binded with some ubiquination protein in Yeast-2-hybrid screening, p53R2 full length and partial (343-717) cloned in pGBT9 vector respectively used as bait and Skip1A, Jab1 cloned in pACT2AD vector was used as prey for Re-yeast Two Hybrid analysis.

(B) p53R2 binded with Skp1A *in vivo*, Hela cells were treated with or without UV damage 24h , and lysated. Proteins were immunoprecipitated from the lysates wityh anti-p53R2 and anti-Skp1A Ab, and the immunoprecipitates were subjected to Western blot analysis with an Ab specific for p53R2.

(C) MCF7 cells treated with or without 10J of UV then harvested after 24hrs and lysed. Proteins were immunoprecipitated from the lysates with anti-Jab1 Ab, and the immunoprecipitates were subjected to Western blot analysis with an Ab specific for p53R2. Lane1 and Lane 2 contain 10% input. Normal mouse IgG was used as an immunoprecipitation control.

2. p53R2 is degraded through ubiquitination pathway

p53R2 induced by DNA damage agent, in the p53 dependent manner, however little is known about how p53R2 degraded, we carried out pulse-chase experiment to assess the stability of the p53R2 protein *in vivo* after blocking protein synthesis with cycloheximide. The majority of protein substrates targeted by the ubiquitin system are short-lived. As shown in Fig. *5A*, the half-life of p53R2 protein in HEK293T cells is ~30 min. To examine whether tagging of p53R2 with ubiquitin leads also to degradation of the protein, *in vitro* and *in vivo* ubiquitination assay was done, we reconstituted a cell-free proteolytic system. p53R2 protein was obtained by TNT kit, then incubation, in reconstitiuted cell-free system with S100(Hela cell extract), Incubation of wt p53R2 in the presence of s100, ubiquitin, and ATP leads to complete degradation of the protein. p53R2 leads to accumulation of high molecular-mass ubiquitin conjugates of p53R2 from 2hrs(Fig *5B*). then *in vivo* ubiquitin assay was carried out, HEK293T cells were cotransfected with p53R2, His6-ubiquitin and SKIP1A vector, in the presence of p53R2, leads to accumulation of high molecular-mass ubiquitin conjugates of p53R2(Fig.*5C*). Altogether these data suggested that p53R2 degraded via ubiquitination pathway.

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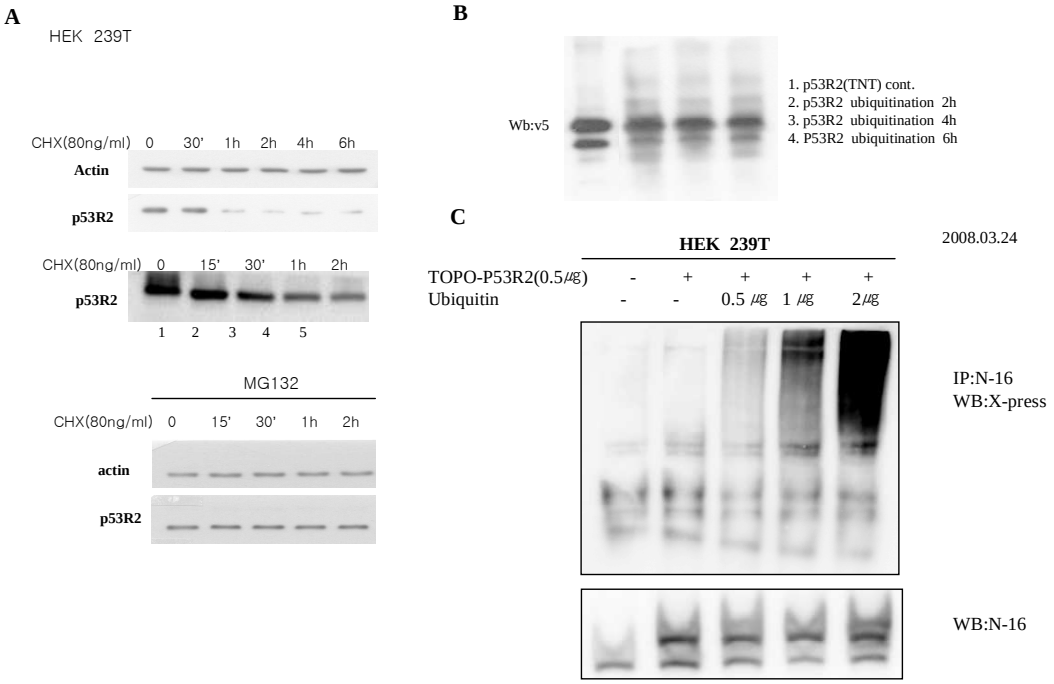


Figure 5. p53R2 p53R2 is degraded through ubiquitination pathway

(A) Stability of p53R2 in vivo. The half-life of p53R2 in HEK293T cells was measured in a pulse-chase experiment as described in Materials and Methods. The cells were treated with cycloheximide(CHX) to block protein synthesis, cell lysates were prepared at the indicated time and analyxed by Westernblotting with anti-p53R2, anti-actin, respectively.

(B) p53R2 ubiquitination assay *in vitro*, 1ul of p53R2 protein which obtained by TNT assay were incubated in Hela cell extract(s100) 2hrs, 4hrs, 6hrs in 37℃, reaction were stopped by the addition of 1/5 volume of 5×SDS-PAGE sample buffer, protein seperated by SDS-PAGE, Western blotted onto PVDF membranes, and probed with anti-p53, anti-p53R2.

(C) p53R2 ubiquitin assay *in vivo*, HEK293T cell were cotransfected either p53R2, ubiquitin vector, cells were disrupted and extracts were immunoprecipitated with anti-p53R2(N-16) antibody and resolved by SDS-PAGE. Proteins were detected by Western blot analysis anti-X press.

3. Mediation of p53R2 ubquination by the SCF complex(F-box-SKP1-culin1)

Ubiquitin-dependent proteolysis plays a critical role in the control of many cellular processes and is mediated by a cascade of enzymes involving ubiquitin activating (E1), conjugating (E2), and ligating (E3) activities(Ohta and Xiong, 2001). Cul1 is the best characterized member of the family and is the only one that interacts with Skp1, It is likely that the emerging role of Cul1 in SCFs is as a core component that supports the targeting of multiple substrates(DeSalle and Pagano, 2001). Posttranslational modifications of target proteins are implicated in the recognition by certain SCF-type E3 ubiquitin ligases and subsequent degradation by the 26 S proteasome(Ciechanover, 1998; Ivan et al., 2001; Yoshida et al., 2002).

To identify whether SCF complex(Fbox-Skp1-culin1) effect the degradation of p53R2, specific components of an E3 ubiquitin-protein ligase SCF, cul1 and Skp1A was tested by using p53R2 as a substrate *in vivo* ubiquitination assays. To test effect of cul1 on p53R2 ubiquitination, HEK293T cell were cotransfected either p53R2-V5-HIS, cul1 vector, cells lysed and extracts and probed specific antibody. as shown Fig. *6A*, p53R2 protein degraded by transfected by cul1 vector. Whether Skp1A effect p53R2 ubiquination too, HEK293T cells transfected with Skp1A, p53R2, and then p53R2 degraded in dose dependent of Skp1A(Fig. *6B*). For the further study, *in vivo* ubiquination assays were done. We cotransfected ubquitin, p53R2, Skp1A expression vector to HEK293T cells, accumulation of high molecular-mass ubiquitin conjugates of p53R2 might be increased in expressioned Skp1A when transfected with Skp1A expression vector, however ladder of ubiqination of p53R2 is not changed much, more delicate experiment will be designed to obtain good result(Fig. *6C*). It appears that p53R2 degraded by ubiquination and ubiquination of p53R2 require of SCF complex.

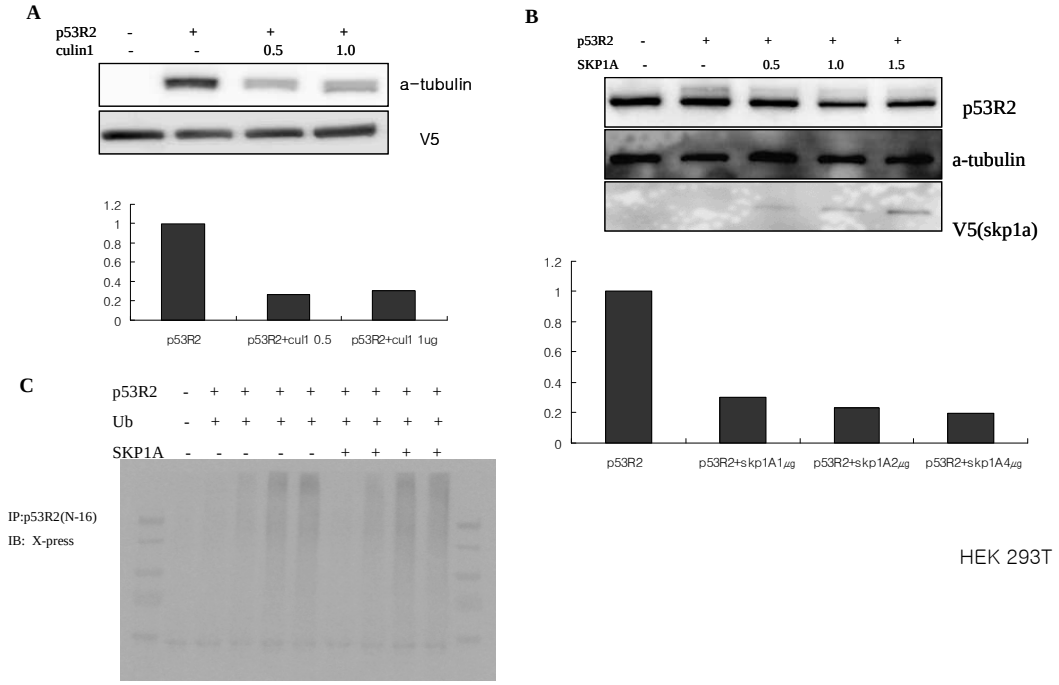


Figure 6. Degradation of p53R2 needs SCF complex(F-box-SKP1-culin1)

(A) Effect of cullin1 on p53R2 degradation, HEK293T cell were cotransfected either p53R2-V5-HIS, cul1 vector, cells were disrupted and extracts were immunoprecipitated with anti-V5 antibody and resolved by SDS-PAGE. Proteins were detected by Western blot analysis.

(B) Effect Skp1A on p53R2 degradation, HEK293T cell were cotransfected either p53R2-V5-HIS, SKIPIA vector, cells were disrupted and extracts were immunoprecipitated with anti-p53R2 antibody and resolved by SDS-PAGE. Proteins were detected by Western blot analysis.

(C) Effect of Skp1A on p53R2 ubiquitin assay *in vivo*, HEK293T cell were cotransfected either p53R2-V5-HIS, ubiquitin, SKIPIA vector, cells were disrupted and extracts were immunoprecipitated with anti-p53R2 antibody and subjected Westernblot by anti-X press for ubiquination.

4. p53R2 regulates p53 stability

p53 is known as “the genome gatekeeper”, the biological mechanisms for maintaining the basal level of p53 in mormal cells require muclear exclusion ans cytoplasmic degradation.

To determine whether the p53 level is regulated by p53R2 in wild-type p53 cell lines, including a pair of MCF7 and U2Os cells that are wild type (*p53+/+*), cells were treated with the p53R2 small interfering RNA (siRNA), treatment for 24 hours resulted in a significant decrease in p53 level (Fig. *7A*).

The effect of p53R2 on p53 steady-state levels is not due to changes in their transcription because p53R2 does not alter the abundance of p53 mRNA in human HCT116 cells (data not shown), but regulates the stability of p53 protein.

Effect of siRNA-mediated down-regulation of the stable GM00637 cellline transfected with p53R2 shRNA, lysates from indicated cells subjected to western blot with specific antibody. p53R2 regulate stability of p53 protein in both transient and stably transfected celllines(Fig.*7B*), p53R2 Stability *in vivo*. Cells were cultured in the presence of 80 μg/ ml CHX, and were subsequently analyzed by Western blot. The half-life of p53 in U2Os and MCF7 cells was measured in a pulse-chase experiment. Knocking down p53R2 by siRNA decreased the half-life of wild-type p53 in MCF7 or U2OS cells (Fig. *7C*).

All these data shown that p53R2, as the transcriptional target of p53, regulate p53 stability.

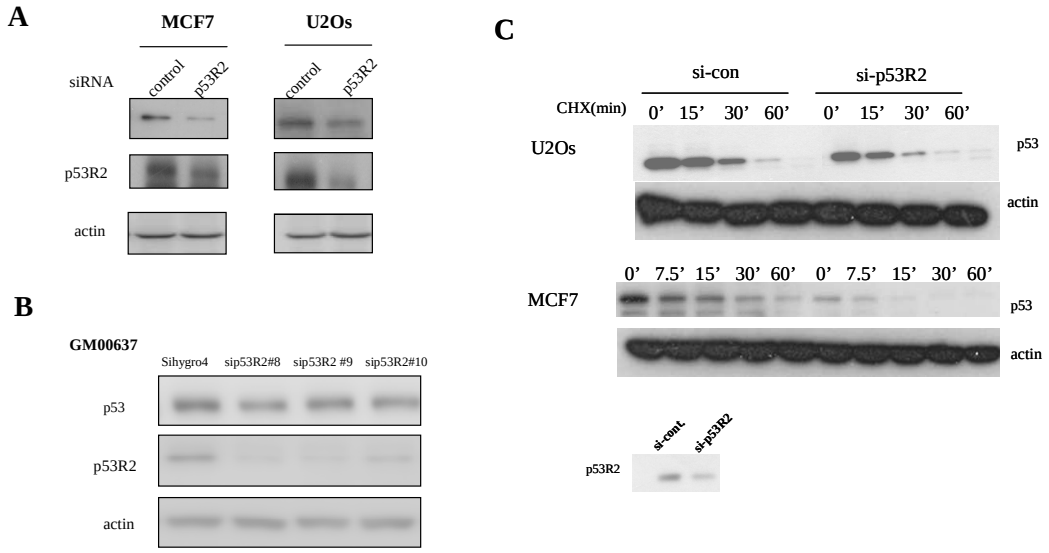


Figure 7. p53R2 regulates p53 stability

(A) Effect of siRNA-mediated downregulation of p53R2 on the steady-state levels of p53 in MCF7, U2Os cells. Lysates from indicated cells subjected to western blot with specific antibody.

(B) Effect of siRNA-mediated downregulation of the stable GM00637 cellline transfected with p53R2 shRNA, Lysates from indicated cells subjected to western blot with specific antibody.

(C) Stability of p53R2 in vivo. Cells were cultured in the presence of 80 μg/ ml CHX, and were subsequently analyzed by Western blot.The half-life of p53R2 in U2Os and MCF7 cells was measured in a pulse-chase experiment as described in Materials and Methods.

5. p53R2 regulates p53 stability via ubiquitination pathway

To determine if the effect of p53R2 on p53 steady-state levels is due to changes p53 ubiquitination, *in vitro* and *in vivo* ubiquitination assay were done, p53 ubiquitin assay *in vitro*, 1ul of p53 with or without p53R2 protein, both proteins which obtained by TNT assay were incubated in Hela cell extract(s100) 4hrs in 37℃, reaction were stopped by the addition of 1/5 volume of 5×SDS-PAGE sample buffer, protein separated by SDS-PAGE, Western blotted and probed with anti-p53 antibody. From the figure *8A*, there are ladders above the p53 band, while the control lane is none. Which means p53R2 blocked ubiquination of p53. *In vivo* ubiquitination assay, HEK 293 cells were transfected with expression DNAs encoding myc3-tagged wild-type p53, MDM2, His6-Ubiquitin or co-transfected with p53R2 DNA. Lysates from the transfected cells were immunoprecipitated with an anti-p53(DO-1) antibody, and the immunoprecipitates were examined with an antibody against ubiquitin, or immuno-precipitated with NTA-Ni bead, and the immunoprecipitates were examined with an antibody against p53(DO-1). As shown in Fig. *8B*, a high molecular weight ladder of p53 species that are ubiquitin-p53 conjugates was increased in p53R2 transfected cell types when myc3-tagged wild-type p53 was expressed with MDM2, indicating that overexpression of p53R2 increases ubiquitination of p53 in a dose-dependent manner.

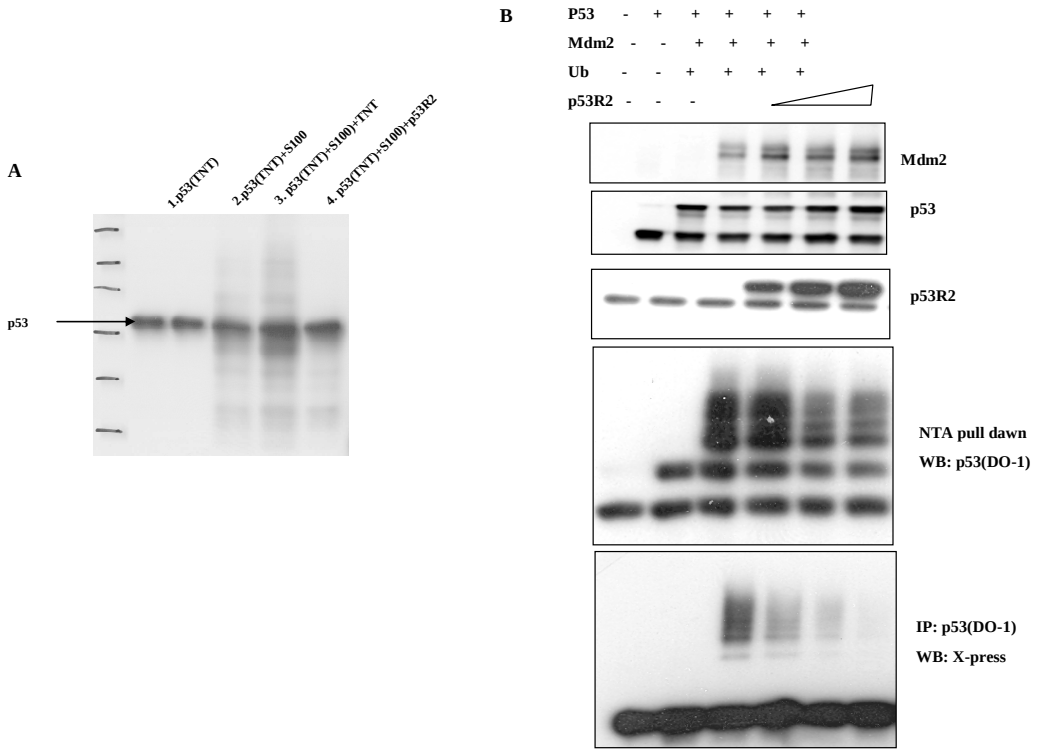


Figure 8. p53R2 regulates p53 stability via ubiquitination pathway

(A) p53 ubiquitin assay *in vitro*, 1μl of p53 with or without p53R2 protein, both proteins which obtained by TNT assay were incubated in Hela cell extract(s100) 4hrs in 37℃, reaction were stopped by the addition of 1/5 volume of 5×SDS-PAGE sample buffer, protein seperated by SDS-PAGE, Western blotted and probed with anti-p53 antibody.

(B) p53R2 ubiquitin assay *in vivo*, HEK293T cell were transfected either p53, ubiquitin vector, cells were lysed and immunoprecipitated with anti-p53 antibody and Western blotted with specific antibody.

6. p53R2 mediates nuclear export of p53

p53R2 regulate p53 stability via ubiquitin pathway, Hdm2 is considered a major factor that contributes the most to lowering the levels of p53 under normal conditions. However there is no change of Hdm2 level in both p53R2 silengcing and overexpression cells according to p53 level, so p53R2 regulate p53 stability by ubiquintin pathway is hdm2 independent. May be p53R2 regulate p53 stability through effecting at bridge ubiquitination and the nuclear export of p53.

To determine whether p53R2 related to nucleus exclusion of p53, MCF7 cells were transfected with p53R2-targeted siRNA and control siRNA, treated with or without 10J of UV, 24hrs later stained with labeled anti-p53. p53R2 silencing, nucleus p53 increased(Fig *9A*), MCF7 stably trasfected with p53R2 shRNA control-shRNA as control, cell lysed, separated cytosol and nucleus fraction and subjected to Western blot with specific antibody. Histograms indicate the percentage of p53 in nucleus increased in p53R2 knocking down cells and cytosol portion were increased in normal condition (Fig *9B*). As UV condition, nucleus increased in p53R2 knocking down cells and cytosol portion were increased (Fig *9A*). All these result confirmed the p53R2 regulate p53 stability, by mediates p53 exclusion from nucleus to cytoplasm.

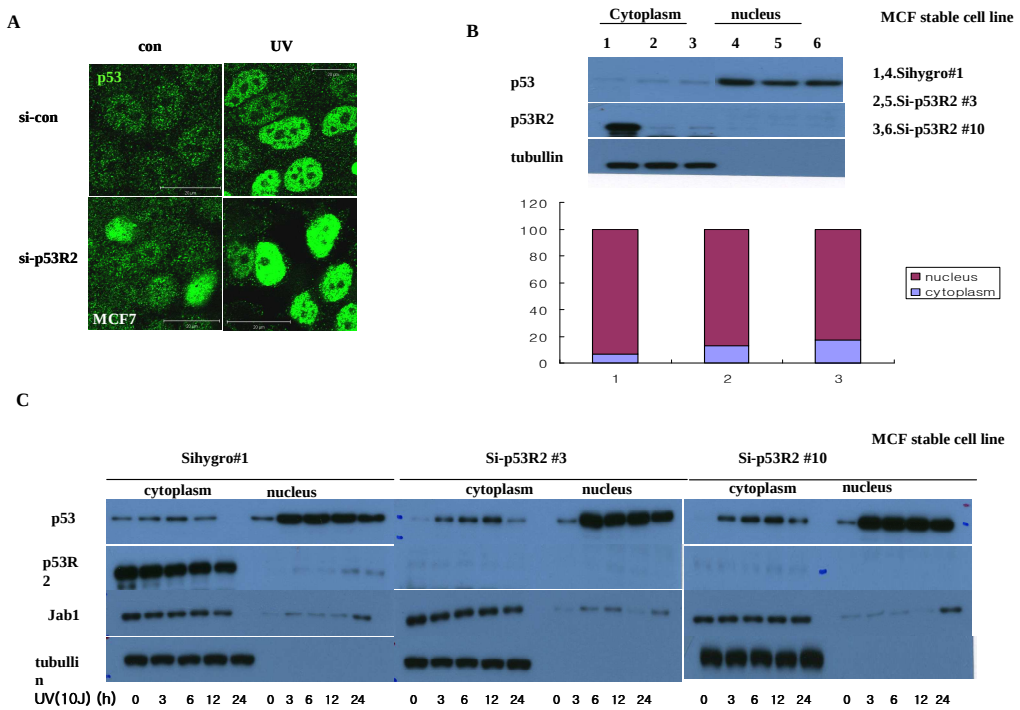


Figure 9. p53R2 mediates nuclear export of p53

(A) MCF7 cells were transfected with p53R2-targeted siRNA and control siRNA, treated with or without 10J of UV, 24hrs later stained with labled anti-p53(green). Scale bars, 10μm..

(B) MCF7 stably trasfected with p53R2 shRNA control-shRNA as control, cell lysed, separated cytosol and nucleus fraction and subjected to Western blot with specific antibody. Histograms indicate the percentage of p53 in nucleus and cytosol.

(C) MCF7 stably trasfected with p53R2 shRNA control-shRNA as control, cells treated with or without UV 10J, 0hr, 3hr, 6hr, 12hr, 24hr incubated with 10% FBS, cell lysed . Separated cytosol and nucleus fraction and subjected to Western blot with specific antibody.

7. p53R2 mediated p53 nuclear exclusion via Jab1

Recent studies determined that Jab1 functions as a nuclear exporter and inducer of cytoplasmic degradation for p53(Lee et al., 2006a). To estimate whether Jab1 and p53R2 interaction effect p53 translocation, we transfected HEK293T cells with p53, jab1and p53R2, then cell lysed, separated cytoplasm and nucleus fraction. and subjected to Western blotting with anti-p53 Ab. Remarkedly p53R2 inhibit p53 nucleus exclusion and degraded in cytosol(Fig. *10A*), this result is similar with the result of confocal microscope assay, MCF7 cells transfeted with p53R2-siRNA, and control siRNA, treated with UV damage, p53 and Jab1 interaction were increase p53R2 silencing cells compared with control siRNA(Fig. *10B*).

Interaction of p53R2 and Jab1 effect the p53 nuclear exclusion, furthermore decreasing p53 degradation in cytoplasm.

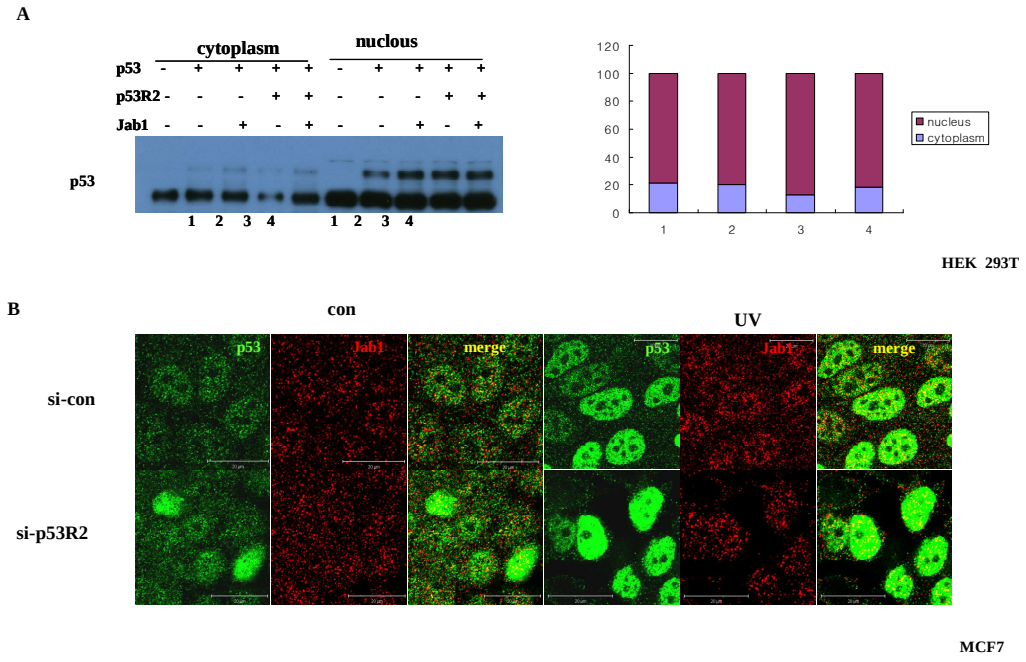


Figure 10. p53R2 mediates p53 nuclear exclusion via Jab1

(A) HEK 293T cells transfeted with p53, p53R2, Jab1 vector, 24hrs later, cells were lysated and extracts were separated cytoplasm and nucleus fraction, then subjected to Western blot analysis with an Ab specific for p53.

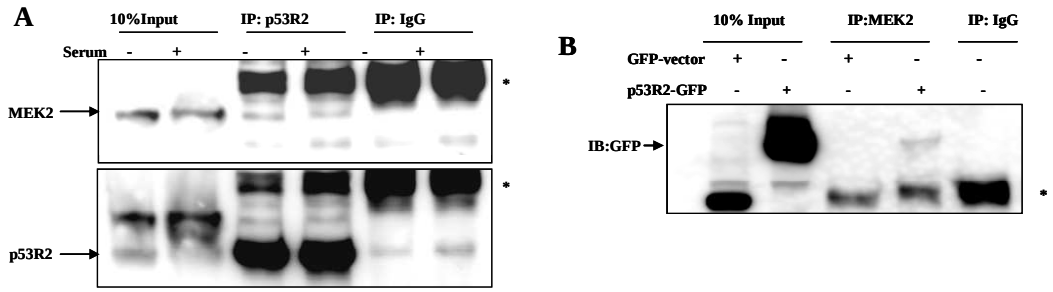
(B) MCF7 cells were transfected with p53R2-targeted siRNA and control siRNA, treated with or without 10J of UV, 24hrs later stained with labled anti-p53(green) and anti-JAB1(red)Abs. Colocalization of Jab1 and p53 in cells is shown as yellow in the merged images. Scale bars, 10μm.

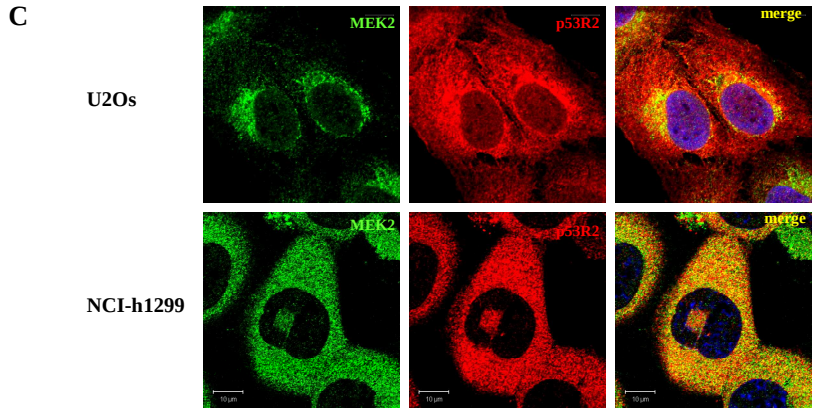
B. p53R2 suppresses MEK/ERK activity and tumor cell invasion by binding to ERK Kinase2

1. MEK2 is a binding partner of p53R2 *in vivo.*

To identify proteins binding to p53R2, particularly those that might be involved in cancer progression, a yeast two-hybrid screen of an adult human liver cDNA library was performed using a pGBT9-p53R2 as the bait. Of several resulting candidates, one was identified as a partial MEK2 cDNA sequence. To determine whether MEK2 would interact with p53R2 in a cell endogenously expressing both proteins, we used immunoprecipitation assays. After U20S cells were serum-starved for 16 h and incubated with or without 20% FBS for 30 min, the cells were lysed, and the cellular proteins were immunoprecipitated with a p53R2-specific Ab and subjected to Western blotting with anti-MEK2 Ab. Immunoprecipitation with anti-p53R2 Ab revealed that endogenous p53R2 bound MEK2, and stimulation with 20% FBS did not significantly change the amount of p53R2 that bound to MEK2 (Fig. *11A).* Binding was specific, as shown by the fact that no MEK2 was detected in samples immunoprecipitated with mouse IgG (lanes 5 and 6). Endogenous MEK2 and p53R2 form a complex under the conditions of both serum starvation and serum stimulation. A common theme in growth factor signaling cascades is the induction

of specific intracellular protein-protein interactions as a consequence of of phospho-ERK1/2 in these cells after incubation in 20% FBS medium for 24 h were determined by probing blots with an ERK1/2 Ab. As shown in Fig. *14B* and *C*. In a reciprocal co-immunoprecipitation assay, HEK293T cells were transiently transfected with an expression vector for full-length p53R2-GFP. The cell lysates were immunoprecipitated with anti-MEK2 Ab. As shown in Fig. *11B,* endogenous MEK2 was readily immunoprecipitated with the GFP-specific Ab (lane 4), but not with a control Ab (lane 5). Next, to determine the site of endogenous p53R2 and MEK2 localization in cells, we used immunofluorescence staining. As shown in Figure *11C*, MEK2 and p53R2 were co-localized in the cytoplasms of U2Os and Hl299 cells.





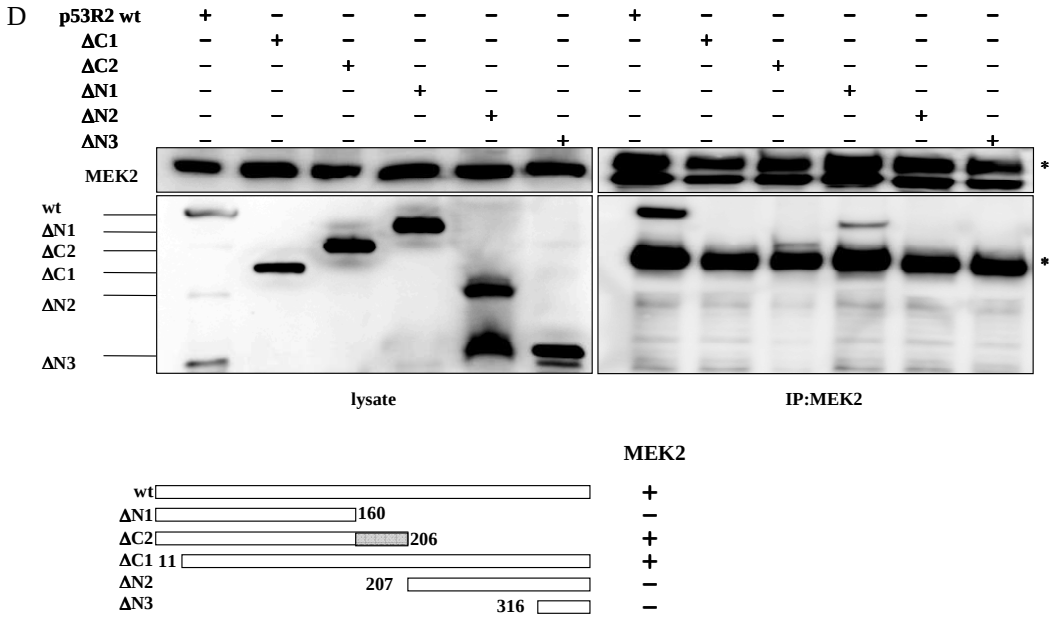


Figure 11 . Interaction of p53R2 with MEK2 in intact cells

(A) U2Os cells were serum starved for 16h, stimulated with or without 20% FBS for 30min, and lysed. Proteins were immunoprecipitated from the lysates wityh anti-p53R2 Ab, and the immunoprecipitates were subjected to Western blot analysis with an Ab specific for MEK2 and p53R2. Lane1 and Lane 2 contains 10% input. Normal mouse IgG was used as an immunoprecipitation control.*, Ig heavy chain.

(B) HEK293T cells were transfected with a control or p53R2-GFP expression vector as indicated. Cell lysates were subjected to immunoprecipitation with anti-MEK2 Ab, and the resulting precipitates were immunobloted with anti-GFP Ab. . Lane1 and Lane 2 contains 10% input. . Normal rabbit IgG was used as an immunoprecipitation control.*, Ig heavy chain.

(C) U2Os and H1299 cells were stained with labled anti-MEK2(green) and anti-p53R2(red)Abs. Colocalization of mek2 and p53R2 in cells is shown as yellow in the merged images. Scale bars, 10μm.

(D) Lysates of HEK293T cells expressing GFP-p53R2(p53R2 wt) or p53R2 deletion mutants (ΔC1- ΔN3) were subjected to immunoprecipitation with anti-MEK2

Ab, and the resulting precipitates were immunobloted with anti-GFP Ab. .*, Ig heavy chain. And arrow show the p53R2 and its deletion mutants. The ability of p53R2 and its deletions to interact with MEK2 were shown as “+”or “—”.

We next mapped the p53R2 region required for interaction with MEK2 by immuno-precipitation. HEK293T cells transfected with full-length GFP-p53R2(p53R2 wt) or p53R2 deletion mutants (ΔC1- ΔN3) expression vector, and cell lysates were immunoprecipitated with anti-MEK2 Ab. As shown in Fig. *11D,* endogenous MEK2 was readily immunoprecipitated with the GFP-specific Ab(p53R2-wt,ΔC2 and ΔN1), but not with ΔC1 and ΔN2-3. the region spanning p53R2 amino acid 160-206 is required for MEK2 binding.

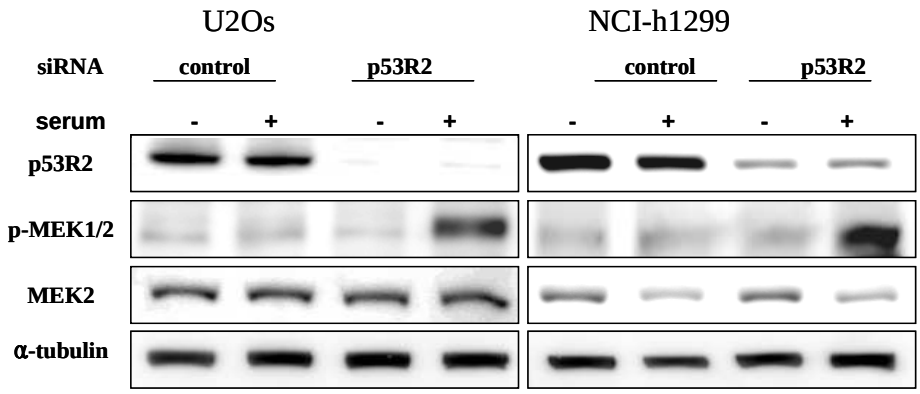
2. p53R2 Modulates Serum-Stimulated MEKl/2 Phosphorylation.

To determine whether the binding of p53R2 to MEK2 affects MEK phosphorylation and activation, we examined serum-stimulated phosphorylation of MEK2 in cells expressing various levels of p53R2. When p53R2 siRNA was transfected into U2Os and Hl299 cells, the endogenous p53R2 protein level was knocked down by about 90%, as shown by Western blotting (Fig. *12A).* We used these cells to analyze the effects of p53R2 knockdown on serum-stimulated MEK activation, which normally occurs through phosphorylation of MEK residues Ser-217 and Ser-221. The cells were serum-starved for 16 h and then stimulated with 20% FBS for 10 to 30 min. Immunoblot analysis revealed that serum-induced phosphorylation of MEKl/2 was significantly increased in cells transfected with p53R2-targeted siRNA, but not in those transfected with control siRNA (Fig. *12A* and *B).*

We also analyzed serum-induced MEK1/2 activation in U2Os and Hl299 cells expressing p53R2. Cells transfected with a control or p53R2 expression vector were incubated in 20% FBS medium for 24 h to activate MEK, and the level of MEK1/2

phosphorylation was measured. As shown in Fig. *12C* and *12D,* the induction of phosphoMEK1/2 by serum was significantly suppressed in U2Os and Hl299 cells expressing p53R2, compared with vector-transfected cells.

A



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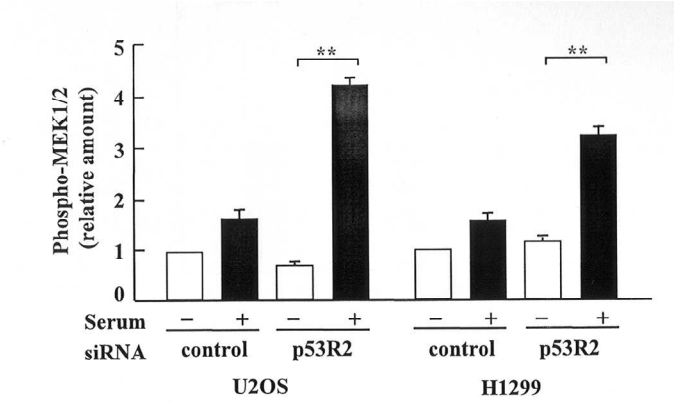

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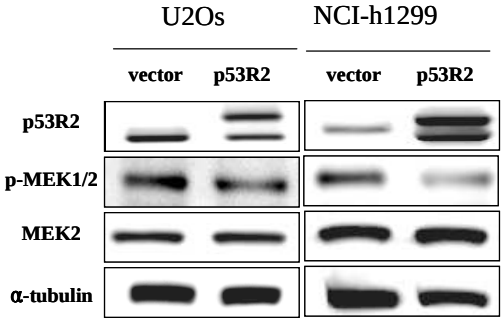

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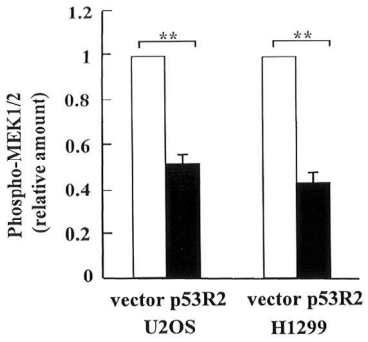


Figure 12 . Effect of p53R2 on serum-stimulated MEK1/2 phosphorylation.

(A) MEK1/2 phosphorylation was examined in U2Os and H1299 cells transiently transfected with control or p53R2-targeted siRNA. U2Os and H1299 cells were starved of serum for 16h and then incubated with or without 20% FBS for 10min and 30min,respectively, the whole cell lysates were probed with anti-phospho-MEK1/2, anti-MEK2, Anti-p53R2, α-tubulin Ab.

(B) The amount of phopho-MEK1/2 was quantifiedy by densitometry and corrected for the amount of MEK2 in the corresponding lysate. Levels of phosphor-MEK1/2 are expressed relative to its level in non-serum-treated clls transfected with control siRNA. The data shown are the means±S.D. from three separate experiments. **, $p<0.01$.

(C) U2Os and H1299 cells were transfected with a control or p53R2-V5-His expression vector as indicated. Cells were cultured with 20%FBS for 24h, and the cell lysates wre immunoblotted with anti-p53R2 and anti-phospho-MEK1/2 Abs. Expression of total MEK2 was also determined.

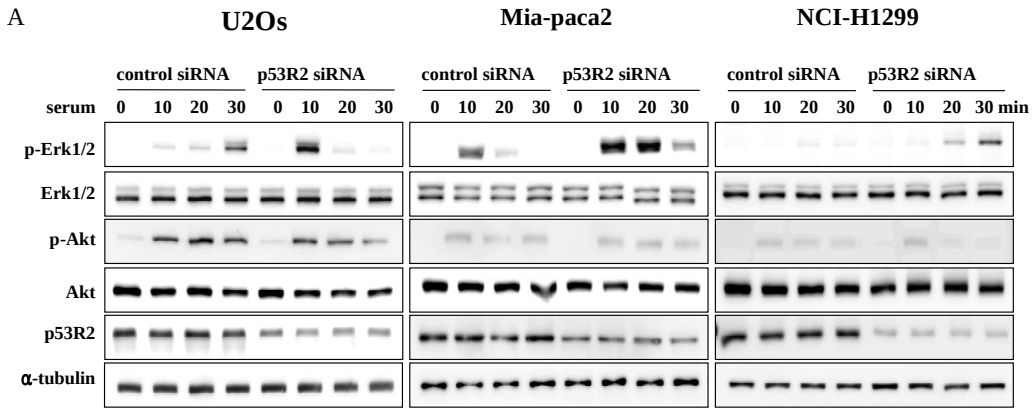
(D) The amount of phopho-MEK1/2 was quantifiedy by densitometry and corrected for the amount of MEK2 in the corresponding lysate. Levels of phosphor-MEK1/2 are expressed relative to its level in non-serum-treated clls transfected with control vector. The data shown are the means±S.D. from three separate experiments. **, $p<0.01$.

3. p53R2 modulate serum-stimulated phosphorylation of Erk1/2

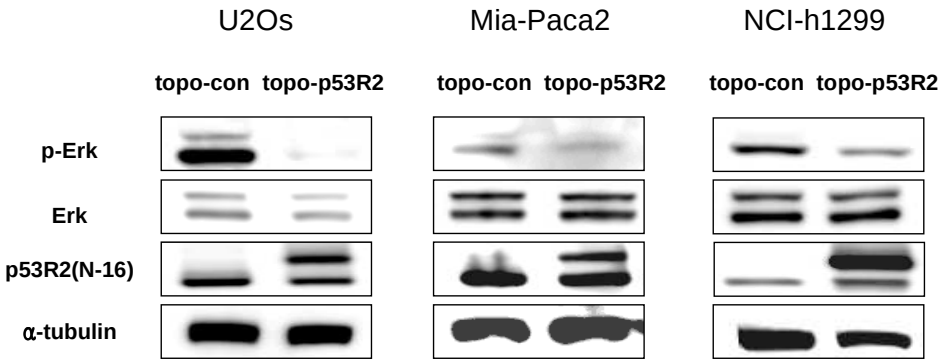
Since MEK can phosphorylate serine, threonine, and tyrosine residues of its ERKl/2 substrate (Dhillon et al., 2007), we next analyzed the effect of p53R2 on ERK activity in U20S and Hl299 cells. The activity of ERK1/2 was monitored in cells by measuring serum-stimulated changes in ERKl/2 phosphorylation. U2Os, H1299, and MIA PaCa-2 cells transfected with control or p53R2 siRNA were starved for 16 h and then stimulated with 20% FBS. Lysates were immunoblotted with an Ab specific for Thr-202/Tyr-204-

phosphorylated ERK1/2. Time course analysis showed that serum-induced phosphorylation of ERKl/2 in U2Os and MIA PaCa-2 cells reached a maximum at 30 min and 10 min, respectively, (Fig. *13A),*whereas no significantly increasing phosphorylation ofERK1/2 in Hl299 cells occurred for 30 min. However, down-regulation of p53R2 significantly increased the stimulatory effect of serum on ERKl/2 phosphorylation, and phosphorylation of ERKl/2 was increased at 10 min in U2Os and MIA PaCa-2 cells, and at 30 min in Hl299 cells. In contrast to our results regarding the ERK pathway, down-regulation of p53R2 did not affect induction of Akt phosphorylation by serum, suggesting that p53R2 specifically modulates the MEK/ERK signaling pathway. The effect of p53R2 on serum-stimulated ERK activity was confirmed in p53R2overexpressing or control U2Os, MIA PaCa-2, and Hl299 cells. The relative amounts of phospho-ERK1/2 in these cells after incubation in 20% FBS medium for 24 h were determined by probing blots with an ERK1/2 Ab. As shown in Fig. *13B* and *C*, ERK1/2 phosphorylation in the cells expressing p53R2 was 40 to 60% lower than in the vector transfected cells.

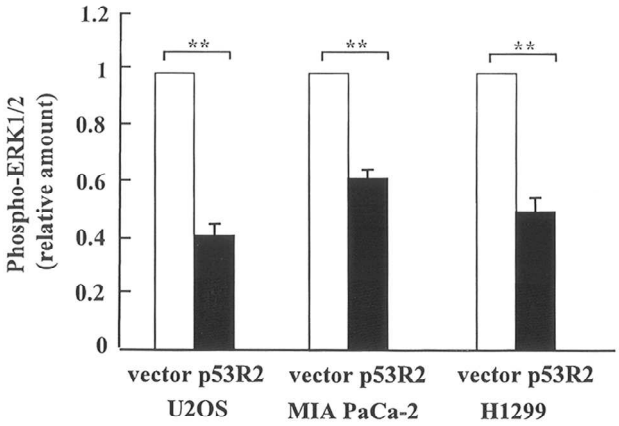
To confirm that p53R2-modulated activation of MEK2 contributes to the activation of ERK by serum stimulation, MEK2- and p53R2-targeted siRNAs were cotransfected into U20S, MIA PaCa-2, and Hl299 cells, and the level of serum-induced phosphorylation of ERK1/2 was measured. Remarkably, serum was unable to activate ERK1/2 in those p53R2-knockdown cells with reduced levels of endogenous MEK2 (Fig. *13D*), indicating that p53R2 suppresses the ability of MEK to activate ERK1/2.



B


U2Os
Mia-Paca2
NCI-h1299
topo-con topo-p53R2
topo-con topo-p53R2
topo-con topo-p53R2
p-Erk
Erk
p53R2(N-16)
α-tubulin

C


Phospho-ERK1/2
(relative amount)
1.2
1
0.8
0.6
0.4
0.2
0
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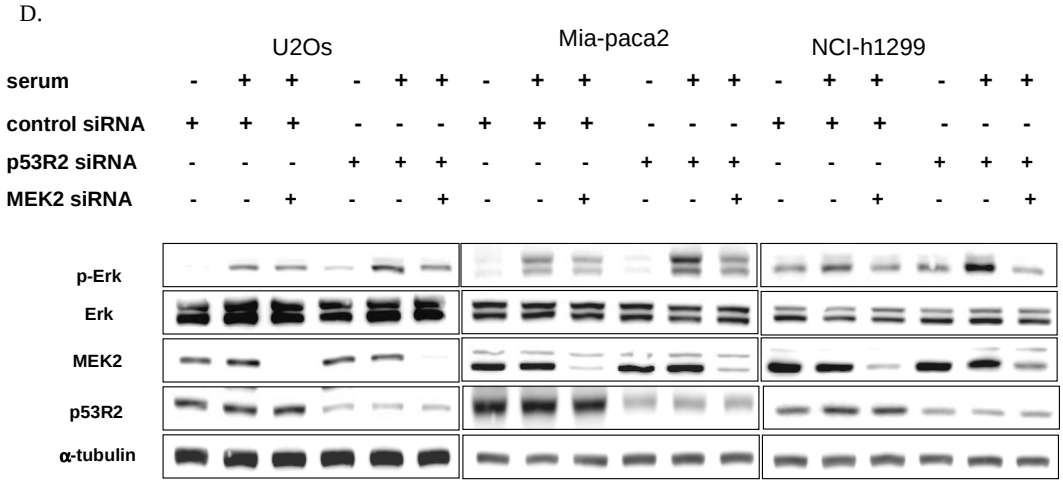
**
**
vector p53R2
U2OS
vector p53R2
MIA PaCa-2
vector p53R2
H1299

D.


U2Os
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serum - + + - + + - + + - + + - + + - + +
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p53R2 siRNA - - - + + + - - - + + + - - - + + +
MEK2 siRNA - - + - - + - - + - - + - - + - - +
p-Erk
Erk
MEK2
p53R2
α-tubulin

Figure 13. p53R2 modulates serum-stimulated phosphorylation of ErK1/2

(A) U2Os, MIA PaCa-2, and H1299 cells were transiently transfected with control or p53R2-targeted siRNA.24hours later, cells were serum-starved for 16h and then stimulated with 20% FBS for the indicated length of time. Whole cell lysates were probed with an Ab specific for phosphor-Erk1/2, Erk1/2, phosphor-Akt, Akt, p53R2, or α-tubulin.

(B) U2Os, MIA PaCa-2, and H1299 cells were transiently transfected with control vector or p53R2-V5-His expression vector, Cells were cultured with 20%FBS for 24h, and the cell lysates wre immunoblotted with Ab specific for phosphor-Erk1/2, Erk1/2, phosphor-Akt, Akt, p53R2, or α-tubulin.

(C) The amount of phopho-Erk1/2 was quantified by densitometry and corrected for the amount of Erk1/2 in the corresponding lysate. Levels of phosphor- Erk1/2 are expressed relative to its level in non-serum-treated cells transfected with control vector. The data shown are the means±S.D. from three separate experiments. **, $p<0.01$.

(D) U2Os, MIA PaCa-2, and H1299 cells were transiently transfected with control or p53R2-targeted or MEK2-targeted siRNA. 24hours later, cells were serum-starved for 16h and then stimulated with 20% FBS for 10-30min. Whole cell lysates were probed with an Ab specific for phosphor-Erk1/2, Erk1/2, MEK2, phosphor-Akt, Akt, p53R2, or α-tubulin.

4. Effect of p53R2 on the colony formation in H1299 cells

Cell transformation potential is often resulted from constitutive activation of components of signal pathway, Oncogenes such as ras, src, raf, and mos have been proposed to transform cells by prolonging the activated state of MAPKK and of components downstream in the signaling pathway(Mansour et al., 1994). p53R2 modulated MEK/ERK signaling pathway, so might be important for cancer cell transformation. Soft agar assay is a classical in vitro expreriment to determine its

transformation potential.

To determine whether p53R2 effect cell transformation, we used H1299 cells stably transfected with a p53R2 expression plasmid, p53R2-targeted shRNA, or a control plasmid, control shRNA. We confirmed that the level of p53R2 transcripts was markedly reduced in p53R2 shRNA-transfected cells(Fig.*14A*) and was markedly increased in p53R2-overexpression cells(Fig.*14B*), compared with the level in mock and control vector-transfected cells, respectively(Fig.*14A* and *14B*).

To investigate the potential inhibitory effects of p53R2 on H1299 cell transformation, we performed an soft agar assay using 60-mm tissue culture dishes containing 0.3% top low-melt agarose and 0.6% bottom low melt agarose. After 2 Weeks of incubation, colonies of >1mm size were counted. p53R2-silenced cells were much more colony formated than control siRNA-transfected cells(Fig.*14B*). In addition, overexpression of p53R2 significantly decreased colony formation (Fig.14A). Thus based on the result from colony formation we concluded that p53R2 suppressed colony formation.

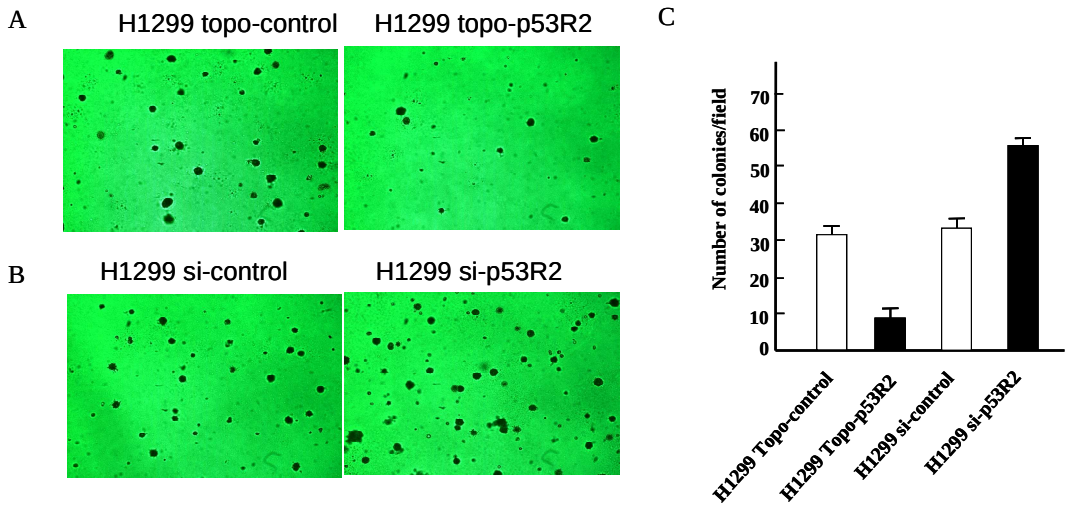

Figure 14 . Effect of p53R2 on the colony formation in H1299 cells

(A) The H1299 cells were stably transfected with control empty vector or p53R2-V5-His expression vector, cells were plated 2×10^4 in duplicates in 60-mm tissue culture dishes containing 0.3% top low-melt agarose and 0.6% bottom low melt agarose. After 2 Weeks of incubation, clonies of >1mm size were counted. Cellular migration was observed with light microscope(×40).

(B) Stable transfectant of H1299 cells expressing control shRNA(Mock) or p53R2-shRNA were were plated 2×104 in duplicates in 60-mm tissue culture dishes containing 0.3% top low-melt agarose and 0.6% bottom low melt agarose. After 2 Weeks of incubation, clonies of >1mm size were counted. Cellular migration was observed with light microscope(×40).

(C) Histograms indicate the average number of colony in H1299 cells were stably transfected with a control or p53R2 expression vector or with control or p53R2-shRNA, Each value shown is the mean±S.D. from three separate experiments. **, $p<0.01$.

5. p53R2 decreases th invasive potential of H1299 cells by blocking MEK2 activity.

The MEK/ERK pathway is critical for cell migration and invasion in various types o cancer cells(Reddy et al., 2003). Thus p53R2 mediated suppression of the MEK/ERK signaling pathway might be important for p53R2-mediated inhibition of tumor invasion.

To determine whether p53R2 supprssses cancer cell invasion by inbibiting MEK activation, we used H1299 cells stably transfected with a p53R2 expression plasmid. With p53R2 shRNA, or a control plasmid , control shRNA. We confirmed that the level of p53R2 transcripts was markedly reduced in p53R2 shRNA-transfected cells(Fig.*15A*) and was markedly increased in p53R2-overexpression cells(Fig.*15B*), compared with the level in mock and control vector-transfected cells, respectively(Fig.*15A* and *15B*).

To investigate the potential inhibitory effects of p53R2 on H1299 cell invasion, we performed an in vitro invasive assay using 24-well units with polycarbonate filter coated

on the upper side with Martrigel. Quantitation of invasive cells were found on the lower surfaces of the inserts after 48h showed that p53R2-silenced cells were much more invasive than control siRNA-transfected cells. In addition, overexpression of p53R2 significantly decreased cell invasiveness(Fig.*15C*).

If p53R2 binding of MEK2 is required for inhibition of invasion, the knockdown of MEK2 should decrease the invasive potential of p53R2-depleted cells. Thus, p53R2 knockdown H1299 cells transfected with either control or MEK-targeted siRNA and assessed for their invasive ability. Down-regulation of MEK2 significantly decreased the invasive potential of p53R2-knockdown H1299 cells, as compared with control cells(Fig.*15D*). The number of cells that crossed the Matrigel barrier to the lower surface of the insert was approximately 2.6-fold lower in the MEK siRNA-transfected cells than in the control siRNA-transfected cells. Thus, based on the effect of MEK2 siRNA. We conclude the p53R2 blocks invasion by suppressing the MEK signaling pathway.

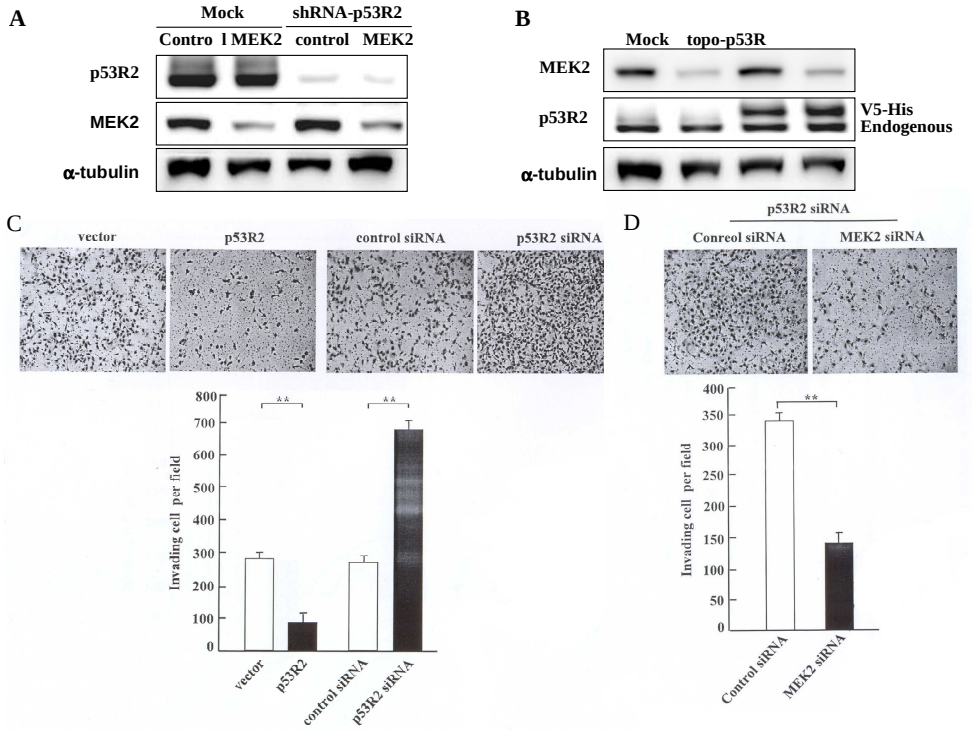

Figure 15. p53R2 suppression of invasion is dependent on MEK2 signaling

(A) Stable transfectant of H1299 cells expressing control shRNA(Mock) or p53R2-shRNA were transiently transfected with control or MEK2-targeted siRNA. 24hours later, whole cell lysates were probed with anti-p53R2, anti-MEK2, or anti-α-tubulin Ab.

(B) The H1299 cells were stably transfected with control empty vector or p53R2-V5-His expression vector, whole cell lysate were probed with anti-p53R2 or anti-α-tubulin Ab .

(C) H1299 cells were stably transfected with a control or p53R2 expression vector or with control or p53R2-shRNA, placed in the upper part of a trans-well unit, and incubated for 48h. Cells that penetrated to the bottom surface of the membrane were fixed,stained, and counted under a microscope. Figures are representive for cell invasion in each group, and histograms indicate the average number of invading cells. Each value shown represents the mean±S.D. from three separate experiments. **, $p<0.01$.

(D) Stable transfectants of H1299 cells expressing p53R2 targeted-siRNA were transiently transfected with control or MEK2-targeted siRNA. And cells were allowed to pass through Matrigel-coated filters. Histograms indicate the average number of invading cells. Each value shown is the mean±S.D. from three separate experiments. **, $p<0.01$.

IV. DISCUSSION

The p53R2 has been shown to participate in the regulation of multiple cellular functions, including DNA repair(Kimura et al., 2003; Tanaka et al., 2000)reactive oxygen species(ROS) regulation(Xue et al., 2006), cell cycle checkpoint(Xue et al., 2007; Yamaguchi et al., 2001), cell survival(Lin et al., 2004; Tsai et al., 2006), and cancer cell migration and invasion.

In this study, using Y2H screening system to identify the proteins interacted with p53R2, study multiple cellular function of p53R2. From all proteins binding with p53R2, we select three proteins, related with protein stability and tumor metastasis Skp1A, Jab1, MEK2. We have provided direct biological evidence that p53R2 binding with Skp1A, Jab1 and thereby not only induces its ubiquitin-dependent proteolysis but also regulate p53 degradation by ubiquitination. Furthermore we identified p53R2 binding with MEK2, a component of ERK-MAPKinase signal, suppress ERK/MEK activity to inhibit tumor metastasis.

A. p53R2 regulates p53 stability via ubiquitination pathway

p53R2 is induced by DNA damage agents (Arner and Eriksson, 1995; Bradshaw and Deininger, 1984), such as γ-irradiation, UV light, and adriamycin, in a p53 dependent manner(Nakano et al., 2000). It is really interested how p53R2 degraded, when its function were finished.

We investigated the degradation of p53R2 and p53. We have provided direct biological evidence that p53R2 interacts with Skp1A, Jab1, Skp1A is components of SCF complex, E3 ubquitin-proein ligase. While as Jab1 facilitate p53 nuclear exclusion and its subsequent degradation in coordinatation with Hdm2(Oh et al., 2006). We also demonstrated that p53R2 is short-life protein which degraded ~30min after blocking protein synthesis with CHX. Degradation of p53R2 blocked by MG132, proteosome inhibitor, which hypothesize that p53R2 degraded by UPP. And *in vitro* and *in vivo*

ubiquitination assay confirmed that p53R2 degraded by ubquination pathway, and ubiquination of p53R2, is necessary of Skp1A and cul1, two components of SCF complex.

p53 is often referred to as the ‘cellular gatekeeper’ or the ‘guardian of the genome’ and its importance is emphasized by the discovery of mutations of p53 in over 50% of all human tumors. Details of the events that occur after this reaction, including the recognition of a growing polyubiquitin chain and subsequent degradation by the 26S proteasome, have remained somewhat elusive (Bulavin et al., 1999). Recent studies have indicated that the level of ubiquination by Hdm2 are closely related to the nuclear or cytoplasmic localization of p53(Li et al., 2003). Jab1 facilitates p53 nuclear exclusion and its subsequent degradation in coordination with Hdm2(Oh et al., 2006). We found that p53R2 controls the stability of p53; knocking down p53R2 decreases p53 protein level , whereas overexpression of p53R2 increases p53 protein level in many mammalian cells in transient and stale trasfectant celllines. In addition knocking down p53R2 decreases the half-life of wild-type p53 in several of the human cell lines. From all above we can identified that p53R2 regulate p53 stability. Degradation of p53 mediated by p53R2 via ubiquitin pathway by *in vitro* and *in vivo* ubquination assay. Interaction p53R2 with Jab1 eluted that the mechanism of p53R2 regulate p53 by ubquitination pathway. p53R2 regulate p53 stability is interaction p53R2 with Jab1, subsequently regulate p53 nucleus export to cytoplasmic degradation.

Under normal conditions, however, p53 is generally be maintained at low steady-state levels, mainly through ubiquitination followed by proteasome-dependent proteolysis, which result in rapid turnover of p53 (O'Brate and Giannakakou, 2003; Yang et al., 2004). If levels of p53 are not carefully controlled, cells under normal conditions would go through cell cycle arrest and apoptosis (Vousden and Lu, 2002). One of the major interests in studying p53 is to determine how its endogenous levels are controlled under normal conditions. Although p53R2 is rapidly induced by a wide range of genotoxic stresses(Nakano et al., 2000), no significant difference between two proteins interaction with p53R2 with or without UV damage, hence p53R2 degradation and p53R2 regulation

p53 stability may be no concern with genotoxic stress.

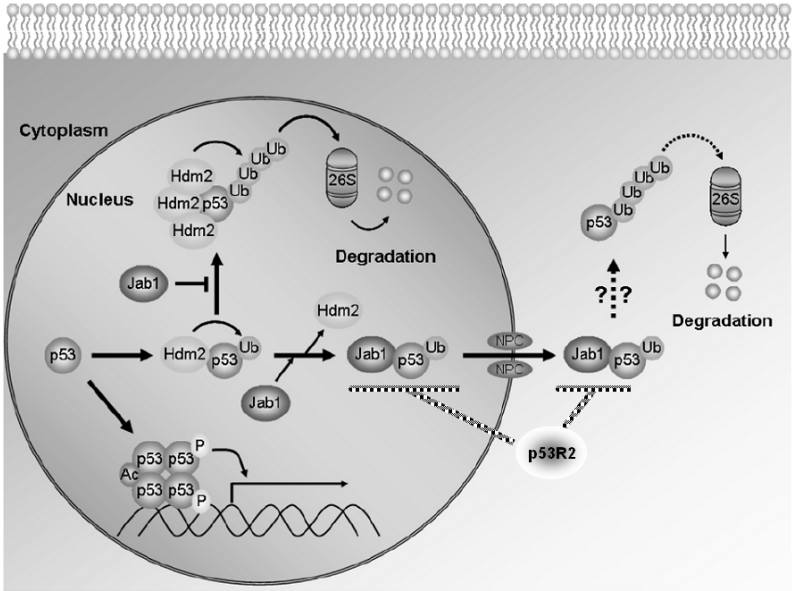

Figure 16. A schematic diagram of Jab1-dependent p53 nuclear transport and degradation in coordination with p53R2.

In conclusion, we have identified a p53 inducible protein; p53R2 is regulator of p53 stability. p53R2 interact with p53 to block ubiquination , mediated by Jab1. Our experiment argues for a model in which, in normal condition p53R2 expression is increasing its interaction with Jab1 and protecting p53 degradation through exclusion from nucleus to cytoplasm, and subsequent degradation. We propose that this novel mechanism provide the cell with means of maintaining p53 level in normal condition for cell homeostasis.

B. p53R2 suppresses MEK/ERK activity and tumor cell invasion by binding to ERK Kinase2

The MAPKs comprise a family of protein that mediate series of distinct signaling cascades that are targeted by a multitidue of extracellular stimuli(Dhillon et al., 2007). Activated MAPKs translocate to the nucleus where they phosphorylate their target molecules, including various transcription factors. An activated MAPK pathway has been detected in many human tumors, suggesting that MAPK is involved in many human tumors, suggesting that MAPK is involved in tumor progression and metastasis(Dhillon et al., 2007). Among the major groups of MAPKs(JNK, p38, and ERK1/2), ERK signaling plays the major role in regulating the invasive and metastatic potential of various cancer cells(McCubrey et al., 2007). ERK1/2 fosters increased transcription of gene encoding matrix metalloproteinase(MMPs) and urokinase plasminogen activator and its receptor. It can also promote invasiveness by reducing the transcription of tight junction proteins and of metastasis suppressor genes(Reddy et al., 2003). In addition, the extranuclease activaties of ERK1/2, including activation of cell mobility machinaery and disruption of focal adhesions, may contribute to increased invasiveness.

By Y2H, we found that p53R2 is associated with MEK2, and serves as a negative regulator of the MEK/ERK signaling pathway. MEK2, a component of Ras-RAF-Erk pathway, which known as involved in tumor invasion and metastasis(Reddy et al., 2003). The interaction of two protein confirmed by immunoprecipitation and colocolazation assays, p53R2 and MEK2 interaction was endogeneous in U2Os cells and in p53R2 overexpressioned HEK239T cells. And both endogenous protein located in cytoplasm, and colocalized. These studies suggested the interaction of two protein should be physiologyical and important.

Since mutational activation of Ras protein promotes tumor proliferation and metastasis , the inhibition of Ras signaling including Raf and MEK is likely to be an

effective strategy for anti-tumorigenesis and antitumor metastasis(Campbell and Der, 2004; Dudley et al., 1995; Shields et al., 2000).

The Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt pathways interact with each other to regulate growth and in some cases tumorigenesis. For example, in some cells, PTEN mutation may contribute to suppression of the Raf/MEK/ERK cascade due to the ability of activated Akt to phosphorylate and inactivate different Rafs. Although both of these pathways are commonly thought to have anti-apoptotic and drug resistance effects on cells, they display different cell lineage specific effects(McCubrey et al., 2007). In our result, there was no significant change of Akt activity in p53R2 silencing cells. Our data suggested that p53R2 specially inhibit the Raf/MEK/ERK signaling pathways, but not Ras/PI3K/PTEN/Akt pathways.

Reactive oxygen species (ROS) are well known to induce the activation of the Raf/MEK/ERK signaling pathways. Oxidative stress-induced ERK1/2 activation is reported in a variety of cell types(Conde de la Rosa et al., 2006; Schoemaker et al., 2004; Schoemaker et al., 2003). Recent study revealed that recombinant hRRM2 protein had a prooxidant potential to oxidize carboxy-H2DCF whereas p53R2 had a peroxide removal capacity. In a gene transfection study, an increase in p53R2 enhanced the hydroxyl free radical removal capacity and protected cells from H2O2 attacks (Xue et al., 2006). Although these studies showed that metastasis suppressing property of p53R2 may be concerned with ROS removal capacity of p53R2. Some studies also showed that MEK1 and 2 inhibitors U0126 and PD98059 both block oxidative stress-induced ERK1/2 activation, indicating that activating actions of oxidative stress do not occur directly on ERK1/2 but instead are localized at upstream targets(Lee et al., 2006b; Lee et al., 2005).

Expression of the p53R2 was induced by γ- and UV-irradiation, and also by adriamycin treatment in a wild-type p53-dependent manner, while a previously known R2, that plays an important role in DNA synthesis during cell division, was not(Nakamura, 2004). And there were several genes that can functionally link the p53 to MAPKinase pathway. Using a p53-inducible system, found that inducible expression of p53 in human

bladder cancer cells leads to activation of MEK and its downstream kinase Erk, but not p38(Lee et al., 2000). Activation of Erk by p53 requires transcriptionally active p53 because mutants that lack transcriptional activity fail to do so this suggests that p53 can transcriptionally activate Erk/MAPK signaling(Wu, 2004). And hRRM2 expression levels and p53 dysfunction might affect the metastasis-suppressing ability of p53R2 in colon cancer(Liu et al., 2006). All these studies suggested that p53 may be concerned with malignancy-suppressing ability of p53R2. however, the result showed that there were no significant difference between p53 wild type and p53 mutant, p53-null tumor cells.

Altogether, we conclude that interference with MEK/ERK signaling accounts for the effect of p53R2 on tumor cell invasion, and that this effect is probably mediated by blockage of the MEK/ERK activity and the resulting transcriptional activity. Because of the privotal role of MEK/ERK1/2 signal and invasion in many cancers, our findings suggest that the forced expression of p53R2 may offer a means to change tumor cell behavior. Furthermore, p53R2 is a new therapeutic target.

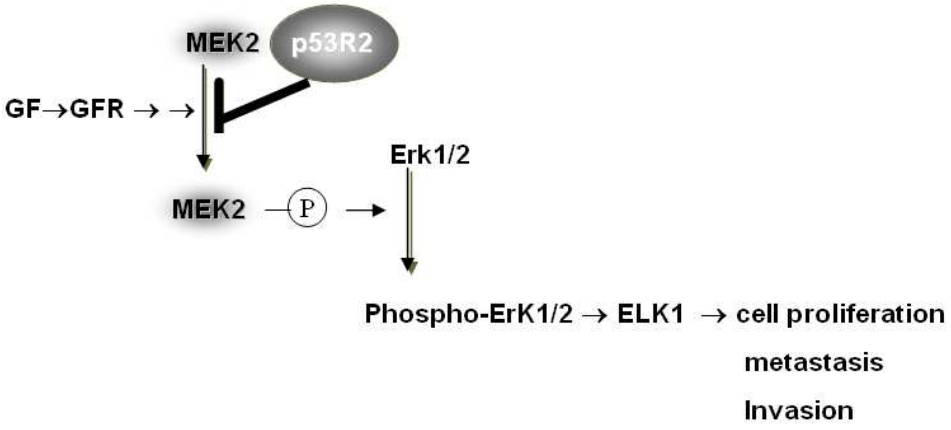

Figure 17. A schematic diagram of p53R2 suppresses MEK/ERK activity and tumor cell invasion by binding to ERK Kinase2

REFERENCES

Arner, E.S. and Eriksson, S. (1995) Mammalian deoxyribonucleoside kinases. *Pharmacol Ther*, **67**, 155-186.

Bradshaw, H.D., Jr. and Deininger, P.L. (1984) Human thymidine kinase gene: molecular cloning and nucleotide sequence of a cDNA expressible in mammalian cells. *Mol Cell Biol*, **4**, 2316-2320.

Bulavin, D.V., Saito, S., Hollander, M.C., Sakaguchi, K., Anderson, C.W., Appella, E. and Fornace, A.J., Jr. (1999) Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J*, **18**, 6845-6854.

Campbell, P.M. and Der, C.J. (2004) Oncogenic Ras and its role in tumor cell invasion and metastasis. *Semin Cancer Biol*, **14**, 105-114.

Cao, M.Y., Lee, Y., Feng, N.P., Xiong, K., Jin, H., Wang, M., Vassilakos, A., Viau, S., Wright, J.A. and Young, A.H. (2003) Adenovirus-mediated ribonucleotide reductase R1 gene therapy of human colon adenocarcinoma. *Clin Cancer Res*, **9**, 4553-4561.

Chabes, A.L., Pfleger, C.M., Kirschner, M.W. and Thelander, L. (2003) Mouse ribonucleotide reductase R2 protein: a new target for anaphase-promoting complex-Cdh1-mediated proteolysis. *Proc Natl Acad Sci U S A*, **100**, 3925-3929.

Ciechanover, A. (1998) The ubiquitin-proteasome pathway: on protein death and cell life. *Embo J*, **17**, 7151-7160.

Conde de la Rosa, L., Schoemaker, M.H., Vrenken, T.E., Buist-Homan, M., Havinga, R., Jansen, P.L. and Moshage, H. (2006) Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases. *J Hepatol*, **44**, 918-929.

DeSalle, L.M. and Pagano, M. (2001) Regulation of the G1 to S transition by the ubiquitin

pathway. *FEBS Lett*, **490**, 179-189.

Dhillon, A.S., Hagan, S., Rath, O. and Kolch, W. (2007) MAP kinase signalling pathways in cancer. *Oncogene*, **26**, 3279-3290.

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J. and Saltiel, A.R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci U S A*, **92**, 7686-7689.

Fan, H., Huang, A., Villegas, C. and Wright, J.A. (1997) The R1 component of mammalian ribonucleotide reductase has malignancy-suppressing activity as demonstrated by gene transfer experiments. *Proc Natl Acad Sci U S A*, **94**, 13181-13186.

Fan, H., Villegas, C. and Wright, J.A. (1996) Ribonucleotide reductase R2 component is a novel malignancy determinant that cooperates with activated oncogenes to determine transformation and malignant potential. *Proc Natl Acad Sci U S A*, **93**, 14036-14040.

Giaccia, A.J. and Kastan, M.B. (1998) The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev*, **12**, 2973-2983.

Guittet, O., Hakansson, P., Voevodskaya, N., Fridd, S., Graslund, A., Arakawa, H., Nakamura, Y. and Thelander, L. (2001) Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. *J Biol Chem*, **276**, 40647-40651.

Gulli, L.F., Palmer, K.C., Chen, Y.Q. and Reddy, K.B. (1996) Epidermal growth factor-induced apoptosis in A431 cells can be reversed by reducing the tyrosine kinase activity. *Cell Growth Differ*, **7**, 173-178.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin, W.G., Jr. (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*, **292**, 464-468.

Kimura, T., Takeda, S., Sagiya, Y., Gotoh, M., Nakamura, Y. and Arakawa, H. (2003) Impaired function of p53R2 in Rrm2b-null mice causes severe renal failure through attenuation of dNTP pools. *Nat Genet*, **34**, 440-445.

Kubbutat, M.H., Jones, S.N. and Vousden, K.H. (1997) Regulation of p53 stability by Mdm2.

Nature, **387**, 299-303.

Lee, E.W., Oh, W. and Song, J. (2006a) Jab1 as a mediator of nuclear export and cytoplasmic degradation of p53. *Mol Cells*, **22**, 133-140.

Lee, J.S., Kim, S.Y., Kwon, C.H. and Kim, Y.K. (2006b) EGFR-dependent ERK activation triggers hydrogen peroxide-induced apoptosis in OK renal epithelial cells. *Arch Toxicol*, **80**, 337-346.

Lee, S.W., Fang, L., Igarashi, M., Ouchi, T., Lu, K.P. and Aaronson, S.A. (2000) Sustained activation of Ras/Raf/mitogen-activated protein kinase cascade by the tumor suppressor p53. *Proc Natl Acad Sci U S A*, **97**, 8302-8305.

Lee, W.C., Choi, C.H., Cha, S.H., Oh, H.L. and Kim, Y.K. (2005) Role of ERK in hydrogen peroxide-induced cell death of human glioma cells. *Neurochem Res*, **30**, 263-270.

Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323-331.

Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R. and Gu, W. (2003) Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science*, **302**, 1972-1975.

Lin, Z.P., Belcourt, M.F., Cory, J.G. and Sartorelli, A.C. (2004) Stable suppression of the R2 subunit of ribonucleotide reductase by R2-targeted short interference RNA sensitizes p53(-/-) HCT-116 colon cancer cells to DNA-damaging agents and ribonucleotide reductase inhibitors. *J Biol Chem*, **279**, 27030-27038.

Liu, X., Zhou, B., Xue, L., Shih, J., Tye, K., Lin, W., Qi, C., Chu, P., Un, F., Wen, W. and Yen, Y. (2006) Metastasis-suppressing potential of ribonucleotide reductase small subunit p53R2 in human cancer cells. *Clin Cancer Res*, **12**, 6337-6344.

Liu, X., Zhou, B., Xue, L., Yen, F., Chu, P., Un, F. and Yen, Y. (2007) Ribonucleotide reductase subunits M2 and p53R2 are potential biomarkers for metastasis of colon cancer. *Clin Colorectal Cancer*, **6**, 374-381.

Mann, G.J., Graslund, A., Ochiai, E., Ingemarson, R. and Thelander, L. (1991) Purification and characterization of recombinant mouse and herpes simplex virus ribonucleotide reductase R2 subunit. *Biochemistry*, **30**, 1939-1947.

Mansour, S.J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande

Woude, G.F. and Ahn, N.G. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science*, **265**, 966-970.

McCubrey, J.A., Steelman, L.S., Chappell, W.H., Abrams, S.L., Wong, E.W., Chang, F., Lehmann, B., Terrian, D.M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A.M. and Franklin, R.A. (2007) Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta*, **1773**, 1263-1284.

Nakamura, Y. (2004) Isolation of p53-target genes and their functional analysis. *Cancer Sci*, **95**, 7-11.

Nakano, K., Balint, E., Ashcroft, M. and Vousden, K.H. (2000) A ribonucleotide reductase gene is a transcriptional target of p53 and p73. *Oncogene*, **19**, 4283-4289.

O'Brate, A. and Giannakakou, P. (2003) The importance of p53 location: nuclear or cytoplasmic zip code? *Drug Resist Updat*, **6**, 313-322.

Oh, W., Lee, E.W., Sung, Y.H., Yang, M.R., Ghim, J., Lee, H.W. and Song, J. (2006) Jab1 induces the cytoplasmic localization and degradation of p53 in coordination with Hdm2. *J Biol Chem*, **281**, 17457-17465.

Ohta, T. and Xiong, Y. (2001) Phosphorylation- and Skp1-independent in vitro ubiquitination of E2F1 by multiple ROC-cullin ligases. *Cancer Res*, **61**, 1347-1353.

Powell, D.R., Desai, U., Sparks, M.J., Hansen, G., Gay, J., Schrick, J., Shi, Z.Z., Hicks, J. and Vogel, P. (2005) Rapid development of glomerular injury and renal failure in mice lacking p53R2. *Pediatr Nephrol*, **20**, 432-440.

Reddy, K.B., Keshamouni, V.G. and Chen, Y.Q. (1999) The level of tyrosine kinase activity regulates the expression of p21/WAF1 in cancer cells. *Int J Oncol*, **15**, 301-306.

Reddy, K.B., Nabha, S.M. and Atanaskova, N. (2003) Role of MAP kinase in tumor progression and invasion. *Cancer Metastasis Rev*, **22**, 395-403.

Reichard, P. (1988) Interactions between deoxyribonucleotide and DNA synthesis. *Annu Rev Biochem*, **57**, 349-374.

Sandrini, M.P. and Piskur, J. (2005) Deoxyribonucleoside kinases: two enzyme families

catalyze the same reaction. *Trends Biochem Sci*, **30**, 225-228.

Scheffner, M., Nuber, U. and Huibregtse, J.M. (1995) Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature*, **373**, 81-83.

Schoemaker, M.H., Conde de la Rosa, L., Buist-Homan, M., Vrenken, T.E., Havinga, R., Poelstra, K., Haisma, H.J., Jansen, P.L. and Moshage, H. (2004) Tauroursodeoxycholic acid protects rat hepatocytes from bile acid-induced apoptosis via activation of survival pathways. *Hepatology*, **39**, 1563-1573.

Schoemaker, M.H., Gommans, W.M., Conde de la Rosa, L., Homan, M., Klok, P., Trautwein, C., van Goor, H., Poelstra, K., Haisma, H.J., Jansen, P.L. and Moshage, H. (2003) Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to nuclear factor-kappa B activation. *J Hepatol*, **39**, 153-161.

Shields, J.M., Pruitt, K., McFall, A., Shaub, A. and Der, C.J. (2000) Understanding Ras: 'it ain't over 'til it's over'. *Trends Cell Biol*, **10**, 147-154.

Su, B. and Karin, M. (1996) Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr Opin Immunol*, **8**, 402-411.

Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y. and Nakamura, Y. (2000) A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature*, **404**, 42-49.

Thelander, L., and Gra¨slund, A. . (1994) *Metal Ions in Biological Systems (Sigel, H., and Sigel, A., eds)*

Tsai, M.H., Chen, X., Chandramouli, G.V., Chen, Y., Yan, H., Zhao, S., Keng, P., Liber, H.L., Coleman, C.N., Mitchell, J.B. and Chuang, E.Y. (2006) Transcriptional responses to ionizing radiation reveal that p53R2 protects against radiation-induced mutagenesis in human lymphoblastoid cells. *Oncogene*, **25**, 622-632.

Vousden, K.H. (2002) Activation of the p53 tumor suppressor protein. *Biochim Biophys Acta*, **1602**, 47-59.

Vousden, K.H. and Lu, X. (2002) Live or let die: the cell's response to p53. *Nat Rev Cancer*, **2**, 594-604.

Wei, N. and Deng, X.W. (1999) Making sense of the COP9 signalosome. A regulatory protein complex conserved from Arabidopsis to human. *Trends Genet*, **15**, 98-103.

Wu, G.S. (2004) The functional interactions between the p53 and MAPK signaling pathways. *Cancer Biol Ther*, **3**, 156-161.

Xue, L., Zhou, B., Liu, X., Heung, Y., Chau, J., Chu, E., Li, S., Jiang, C., Un, F. and Yen, Y. (2007) Ribonucleotide reductase small subunit p53R2 facilitates p21 induction of G1 arrest under UV irradiation. *Cancer Res*, **67**, 16-21.

Xue, L., Zhou, B., Liu, X., Wang, T., Shih, J., Qi, C., Heung, Y. and Yen, Y. (2006) Structurally dependent redox property of ribonucleotide reductase subunit p53R2. *Cancer Res*, **66**, 1900-1905.

Yamaguchi, T., Matsuda, K., Sagiya, Y., Iwadate, M., Fujino, M.A., Nakamura, Y. and Arakawa, H. (2001) p53R2-dependent pathway for DNA synthesis in a p53-regulated cell cycle checkpoint. *Cancer Res*, **61**, 8256-8262.

Yang, Y., Li, C.C. and Weissman, A.M. (2004) Regulating the p53 system through ubiquitination. *Oncogene*, **23**, 2096-2106.

Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K. and Tai, T. (2002) E3 ubiquitin ligase that recognizes sugar chains. *Nature*, **418**, 438-442.

Zhou, B.B. and Elledge, S.J. (2000) The DNA damage response: putting checkpoints in perspective. *Nature*, **408**, 433-439.

Zhou, B.S., Tsai, P., Ker, R., Tsai, J., Ho, R., Yu, J., Shih, J. and Yen, Y. (1998) Overexpression of transfected human ribonucleotide reductase M2 subunit in human cancer cells enhances their invasive potential. *Clin Exp Metastasis*, **16**, 43-49.

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배움의 길은 끝이 있을 가요? 공부를 하면서 항상 이런 고민을 해왔습니다. 막연히 졸업 즈음에는 아마 답을 알게 되지 않을까 하면서……졸업하게 된 지금 제가 느끼기에는 그 종착역은 아마 우리의 삶이 끝나는 날 까지 아닌가 싶습니다. 배움의 길에서 졸업은 그 어떤 끝이 아니라 삶이 다음 단계를 위한 시작이라는 것을 알게 되였습니다.

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박춘매

<국문초록>

p53R2의 생리학적인 역할 연구

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p53R2는 새로 발견된 ribonucleotide reductase의 작은 subunit로서 p53에 의존하는 방식으로 DNA 의 손상 수복 및 사립체 DNA복제에 전구물질을 제공하는데 중요한 역할을 한다. p53R2의 생리적 역할에 대한 연구가 최근에 활발이 진행되지만 아직까지는 완벽히 해석이 되지 않고 있다.

본 연구에서 우리는 단백질의 안정성 및 종양의 전이 등 역할과 연관된 단백질들이 p53R2와 상호 결합한다는 것을 Y2H선별을 통해서 발견하였고, 이런 단백질이 상호결합을 초점으로 p53R2의 생리학적 역할의 분자생물학적 기전을 밝히려고 했다.

우선 우리는 p53R2가 단백질이 안정성에 연관된 단백질인 Skp1A, Jab1과 결합을 연구하였다. Skp1A는 ubquitin 단백질분해효소인 SCF복합체의 구성성분중의 하나이다. Jab1은 세포핵에서 p53을 운반해 내오고 세포질에서 p53을 분해시키는 작용을 한다. 이 두 가지 단백질과 p53R2와의 결합을 면역침전 방법으로 확인하였다. p53R2은 DNA 손상시키는 물질에 의해서 p53에 의존하는 방식으로 생성되지만 어떻게 분해되는지는 아직 알려진 바가 별로 없다. 우리는 HEK293T세포에서 p53R2가 생체에서 존재하는 시간이 짧은 단백질이고 반감기가 30분 미만이며 세포에서 proteasome 억제제 MG132를 사용시 이런 p53R2의 분해가 억제된다는 것을 증명하였다. 체내와 체외 ubquitin 검측을 통해서 우리는 p53R2는 분해는 ubiquitin 경로를 통해서

진행된다는 것을 증명 하였다. 이러한 p53R2의 ubquination은 SCF 복합체 조성성분인 Skp1A와 Cullin1이 필요하다. p53R2는 p53에 의해 조절되며 p53과의 결합서열을 갖고 있다. 추가로 우리는 p53R2가 Jab1과 결합하는 것을 이미 면역침전방법으로 확인했다. 포유동물의 세포에서 p53R2의 발현을 억제시켰을 경우 여러 가지 사람세포에서 p53의 반감기가 줄어들었고 과발현 시켰을 경우 p53의 ubiquitination은 p53R2양에 따라 증가하는 것을 볼 수가 있었다. 우리는 siRNA을 이용하여 p53R2발현을 억제시켰을 경우 핵에서 세포질로의 p53의 운반은 증가된다는 것을 보여주었고 이러한 운반은 Jab1에 의해 연관 되였다는 것을 증명하였다. 이로부터 우리는 p53R2는 p53의 전사타깃인 동시에 또 p53의 안정성을 조절한다는 것을 알게 되였다.

그 다음으로 우리는 Y2H에 의해서 선별된 MEK2라는 종양의 전이와 연관있는 단백질이 p53R2와 상호결합을 연구하였다, 최근까지 p53R2가 종양의 전이와 연관이 있을 거라는 연구가 진행 되여 왔지만 그 메커니즘은 아직 밝혀진 바가 없다. 우리는 p53R2가 종양의 전이나 침범에 연관된 MAPK의 한 개 조성성분인 MEK2와 결합하는 면역침전방법으로 확인하였다. 두 단백질의 결합은 p53R2의 아미노산 160-306사이에 결합하고, Erk-MAPKinase 활성을 조절한다, p53R2발현을 억제시키면 Erk1/2의 인산화가 증가되고 p53R2를 과발현 했을 경우 인산화가 억제된다, 순차적으로 p53R2발현을 억제시키면 세포의 침범을 증가하고 p53R2를 과발현하면 종양세포의 침범을 억제한다. p53R2의 Erk-MAPKinase 신호경로에 대한 억제작용은 MEK2의 인산화를 억제시켜서 한다. 이는 p53R2의 종양의 전이와 침범을 억제하는 메커니즘이다.

위의 결과로부터 p53R2는 여러 가지 역할을 가진 단백질로서 어떤 단백질과 결합하는가에 따라 세포의 생존, DNA 손상 수복, 종양세포의 성장과 침범 등에 중요한 역할을 한다. p53R2의 분자생물학적 역할을 연구하는 것은 이러한 생리학적 기능의 연구에 도움이 될 것 이다.

저작물 이용 허락서

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- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
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동의여부 : 동의(O) 반대( )

2008년 7 월 10일

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