Identification of *NEEP21*, encoding neuron-enriched endosomal protein of 21 kDa, as a transcriptional target of tumor suppressor p53

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Abstract. NEEP21, also designated D4S234E or NSG1, is an endosomal protein expressed in neuronal cells under normal conditions. Here, we report that *NEEP21* is a direct transcriptional target gene of the tumor suppressor p53. *NEEP21* expression is inducible in non-neuronal human cancer cell lines by exposure to adriamycin, hydrogen peroxide, UV and γ-ray in a p53-dependent manner. Chromatin immunoprecipitation assay indicated that a potential p53-binding site (p53BS) is located in intron 1 of the *NEEP21* gene. A reporter assay confirmed that p53BS has p53-responsive activity. The heterologous luciferase gene containing p53BS is also transactivated by p73-ß and p63-γ. The introduction of the *NEEP21* gene into various cancer cell lines suppressed cell growth. Infection with an adenovirus vector containing *NEEP21* induced apoptotic cell death via caspase-3 activation in many cancer cell lines. The expression of *NEEP21* mRNA was remarkably induced by γ-ray irradiation in the spleen of $p53^{+/+}$ mice but not in that of $p53^{-/-}$ mice. These results suggest that NEEP21 may play a critical role in apoptosis as a mediator of p53.

Introduction

The tumor suppressor p53 is a transcription factor that regulates both cell cycle arrest and apoptosis. The activation of p53 after genotoxic stresses induces various downstream events that provide a complex network of signals, leading to tumor suppression (1,2). Analyses of many tumors have revealed that *p53* is mutated in more than half of all cancers, and most of the mutations occur in the region responsible for sequence-specific DNA binding (3). Therefore, it is important to investigate the downstream genes regulated by p53 in order to understand the mechanism for p53-dependent tumor suppression in detail. Dozens of p53 target genes have been reported, but many p53 target genes still remain to be identified (4,5).

NEEP21 was initially identified as the human *D4S234E* [DNA segment on chromosome 4 (unique) 234 expressed sequence] gene, which is located on chromosome 4p16.3, during the genomic analysis of the Huntington disease gene region (6). It encodes a 185 amino acid protein with a calculated molecular weight of 21 kDa. The similarity between the human and mouse sequences is 93.2% at the nucleotide level within the coding regions, and only three amino acids differ in the protein sequences (6). The encoded protein belongs to the NSG family of proteins and is named brain neuron cytoplasmic protein 1. The protein is also designated as NEEP21 (neuron-enriched endosomal protein of 21 kDa) because the expression is specific to neuronal cells (7).

The NEEP21 protein consists of three parts: a lumenal region (aa 1-82), a transmembrane region in the center (aa 83-103), and a cytosolic region (aa 104-185). The N-terminal lumenal region can form a complex with syntaxin 13 (7), and the cytosolic region can bind to GRIP1, a type II PDZ protein, at aa positions 129-164 (8). NEEP21 is considered a neuronal endosomal protein for correct receptor recycling in neurons, and is associated with learning and memory. NEEP21 has been reported to contribute to the regulation of synaptic transmission and plasticity in slice cultures by affecting the recycling and targeting of AMPA receptors to the synapse (8,9). NEEP21 suppression leads to impaired recycling of the internalized transferrin receptor (TfR) (7) and neurotensin receptor 2 (10).

In this study, we found that *NEEP21* expression is inducible in several non-neuronal cell lines under genotoxic conditions in a p53-dependent manner. Furthermore, we showed that the enforced expression of *NEEP21* suppressed colony formation of p53-deficient cancer cell lines by inducing apoptosis. These results suggested that NEEP21 plays a critical role in apoptosis as a novel transcriptional target of tumor suppressor p53.

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Materials and methods

Cell lines. Human cancer cell lines, HepG2 (liver, p53 wild-type), H1299 (lung, p53 mutant), U373MG (brain, p53 mutant), HT-29 (colon, p53 wild-type), LS174T (colon, p53 wild-type) MCF-7 (breast, p53 wild-type) and A549 (lung, p53 wild-type), SH-SY5Y (brain, p53 wild-type) were purchased from American Type Culture Collection (Manassas, VA, USA). Human cancer cell line T98G (brain, p53 mutant) was purchased from Human Science Research Resource Bank HSRRB (Osaka, Japan). Human cell line HEK293 (kidney fibroblast) was purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). All cell lines were cultured under the conditions recommended by their respective depositors.

DNA microarray. Total cellular RNA was extracted at various time points (0, 6, 12, 24 and 48 h) from HepG2 cells after infection with adenovirus vectors at a multiplicity of infection (MOI) of 30 designed to express either wild-type p53 (Ad-p53-WT), p53-46F (Ad-p53-46F) or EGFP (Ad-EGFP) (11). For gene expression profiling, GeneChip Human Genome U133A and U133B microarrays (Affymetrix, Santa Clara, CA, USA) were used that contain 22,215 and 22,577 probe sets, respectively, to examine a total of about 39,000 transcripts. Target cRNA for microarray hybridization was prepared from 5 μg of total RNA according to the manufacturer's instructions using a BioArray RNA transcript labeling kit (Enzo Diagnostics, Farmingcale, NY, USA). Hybridization to the microarrays, washing and staining with the antibody amplification procedure, and scanning were also carried out according to the manufacturer's instructions. The expression value of each gene was calculated and normalized using Affymetrix Microarray Suite software version 5.0.

Semi-quantitative RT-PCR. HepG2 and H1299 cells were infected with recombinant adenoviruses expressing p53 (Ad-p53) or EGFP (Ad-EGFP) as negative controls. HepG2 (control/p53si) and A549 (control/p53-si) cells were subjected to adriamycin (1 μg/ml, 2 h), hydrogen peroxide (100 μM, 1 h), UV-irradiation (30 J/m^2), γ-irradiation (50 Gy) using a ^{60}Co source. Total RNA was isolated from the cells 0, 6, 12, 24 and 48 h after treatment using TRIzol (Invitrogen). cDNAs were synthesized from 5 μg of total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). The RT-PCR was run in the exponential region (14-30 cycles) to allow semi-quantitative comparisons among cDNAs developed from identical reactions. Each PCR regime involved a 94˚C, 5-min initial denaturation step followed by cycles indicated in figures at 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Oligonucleotide primer sequences were as follows: *NEEP21*-forward; 5'-GTTGGG GAACAATTTCGCAG-3', *NEEP21*-reverse; 5'-GGTGACA CCCTCCGTGATG-3'. mouse-*NEEP21* -forward; 5'-TCACC GAGGGTGTCACCG-3' mouse-*NEEP21* -reverse; 5'-ACAT CCATGGCGACACGGA-3'.

RNA interference. We established the p53-knockdown (p53-si) and the control (control) cell lines as described previously (12-14), which were derived from HepG2 and A549 cell lines. Briefly, HepG2 cells and A549 cells were infected with SI-MSCV-puro-H1R-p53Ri retrovirus for down-regulation of p53 expression and with SI-MSCV-puro-H1R-control retrovirus for negative control. Then the infected cells were selected with 1 μg/ml puromycin for 1 week, and the single clones were isolated. The cells were maintained with 0.1 μg/ml puromycin.

Plasmids. The entire coding sequence of *NEEP21* cDNA was amplified by PCR using KOD-plus DNA polymerase (Toyobo) and inserted into pCR-Blunt II-TOPO vector (Invitrogen). The construct plasmid was confirmed by sequencing. Then the EcoRI-digested DNA fragment from the pCR-Blunt II-TOPO plasmid, which includes the entire coding region of *NEEP21*, was digested by EcoRI and inserted into the EcoRI site of pcDNA 3.1(+) (Invitrogen) to prepare the plasmids of pcDNA3.1(+) sense or antisense *NEEP21*.

ChIP assay. ChIP assay was performed using the chip assay kit (Upstate Biotechnology) as recommended by the manufacture. HepG2 cells (3x10^5 cells) were seeded on a 10-cm dish, and infected with Ad-p53-WT and Ad-EGFP at an MOI of 30. After 24 h, genomic DNA and protein were cross-linked by adding formaldehyde (1% final concentration) directly into the culture medium and incubated for 15 min at 37˚C. Cells were lysed in 200 μl SDS lysis buffer with a protease inhibitor cocktail and sonicated to generate DNA fragments 200-1,000 bp long. After centrifugation, the cleared supernatant was diluted 10-fold with ChIP assay buffer and incubated at 4˚C overnight with the specific antibody with rotation. Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating to 65˚C for 5 h. DNA was phenol-extracted, ethanol-precipitated and resuspended in 30 μl of DW. We used 1 μl of each sample as a template for PCR amplification. PCR amplifications of *NEEP21* intron 1, containing a predictive p53 binding-site (BS1) and *p21/waf1* binding-site as a positive control were performed on immunoprecipitated chromatin using specific primers: 5'-TGGAGC CCAATCGTTCGCT-3' (BS1, forward) and 5'-GCGACC CGCTGTTCACAG-3' (BS1, reverse), or 5'-ACCTTTCACC ATTCCCCTAC-3' (p21/waf1 BS, forward) and 5'-GCCCAA GGACAAAATAGCCA-3' (p21/waf1 BS, reverse).

Gene reporter assay. The plasmid including the putative binding-site of *NEEP21* for p53 was termed pGL-BS1. A 243-bp DNA fragment BS1 was amplified from genomic DNA by PCR and cloned into pGL3-promoter vector (Promega, Madison, WI). The same primers were used for PCR as for the ChIP assay. H1299 cells were plated in 35 mm tissue culture dishes (1x10^5 cells/dish) 24 h before transfection. pGL-BS1, pGL-WAF (p21/waf1 BS), or pGL-empty vector was co-transfected with either of the wild-type p53, mutant-p53, p73-α, p73-ß, p63-α, or p63-γ expression constructs. Twenty-four hours after transfection, cells were lysed in a passive lysis buffer and subjected to the Dual Luciferase assay system (Promega). Quantification of both luciferase activities and calculations of relative ratios were carried out manually with a luminometer.

Colony formation assay. H1299 cells (10^6 cells), T98G cells (10^6 cells) or HT-29 cells (1.5x10^6 cells) on the 10-cm dishes were transfected with 5 μg of plasmids with sense-*NEEP21*

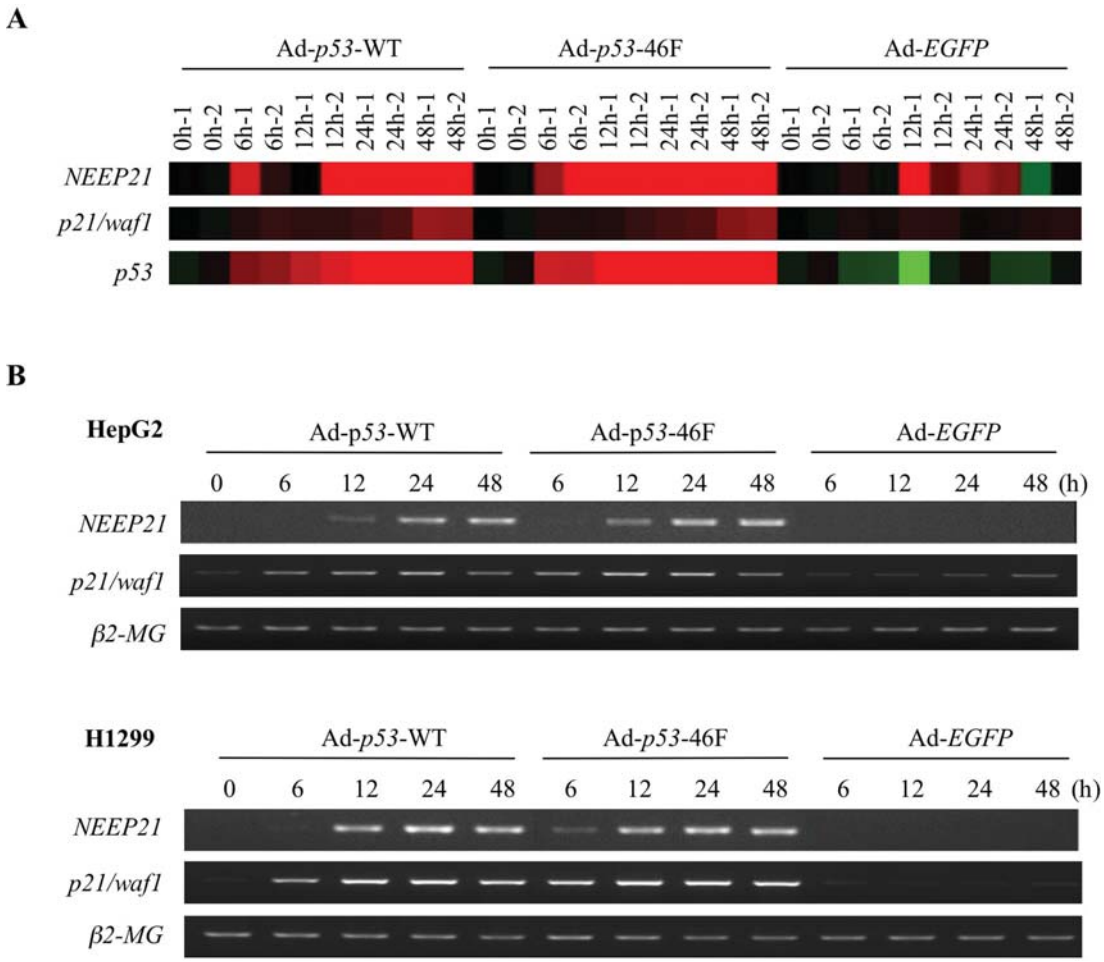

Figure 1. Identification of *NEEP21* as a p53-inducible gene. (A) Microarray analysis. The expressions of *NEEP21*, *p21/waf1*, and *p53* mRNA in HepG2 cells at the indicated times after infection with Ad-*p53*-WT, Ad-*p53*-46F, and Ad-*EGFP* are shown by the green and red intensities of the microarray analysis. The red and green intensities indicate up- and down-regulated expression of the genes, respectively. (B) RT-PCR analysis. HepG2 or H1299 cells were infected with Ad-*p53*, Ad-*p53*-46F or Ad-*EGFP* at an MOI of 30. *p21/waf1* and *β2-MG* were used as the positive and quantity controls, respectively.

(S), antisense- *NEEP21* (AS) and empty vector (E). After 48 h, the cells were split at 1:10, 1:100, 1:1000 ratios and the G418-resistant colonies were selected with G418 (0.8 mg/ml for H1299, 1 mg/ml for T98G and HT-29) for 2 weeks. The cells were fixed in 10% formaldehyde and stained with 0.125% crystal violet. The average numbers of colonies were determined from the experiments performed in triplicate.

Recombinant adenoviruses. The adenovirus vector expressing *NEEP21* was prepared using Takara adenovirus expression vector kit by the full length DNA insertion method, according to the manufacturer's instructions. Briefly, blunt-ended fragments of *NEEP21* cDNA were cloned into the SwaI site of the cosmid pAxCAwtit (Takara). Recombinant adenoviruses were generated in HEK293, by transfection with the cosmid DNA after digestion by BspT104I. Viruses were propagated in HEK293 cells and purified by two rounds of CsCl density centrifugation; viral titers were measured in a limiting-dilution bioassay using HEK293 cell.

Apoptosis assay. HepG2, LS174T, U373MG, A549, SH-SY5Y and H1299 cells were trypsinized, washed, collected and fixed with 70% ethanol at the indicated times after infection with Ad-*NEEP21*. Fixed samples were centrifuged, treated with RNase (0.2 mg/ml), and resuspended in propidium iodide (50 μg/ml). The stained cells were analyzed on a Becton-Dickinson FACScan flow cytometer. The subG1 fraction of the cells was counted as the apoptotic cells, and the proportion of the apoptotic cells to the total cells was indicated as percentage.

Caspase-3 activity assay. The labeled synthetic substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Cell lysate from HepG2 cells 36 h after infected with Ad-NEEP21 or Ad-EGFP at an MOI of 100 was reacted with Ac-DEVD-pNA in the assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 5 mM DTT) at 30˚C. The free pNA cleaved from the substrate was detected as the caspase-3 activity by monitoring the optical density at wavelength 405 nm. In this system, caspase activity is presented as a ratio of the sample activity to the negative control activity from uninfected cells, indicated as (-).

p53 knock-out mice. p53-deficient mice were a gift of Dr S. Aizawa, Center for Developmental Biology, RIKEN (15). $p53^{+/+}$ and $p53^{-/-}$ mice were irradiated by 10 Gy of γ-rays, and

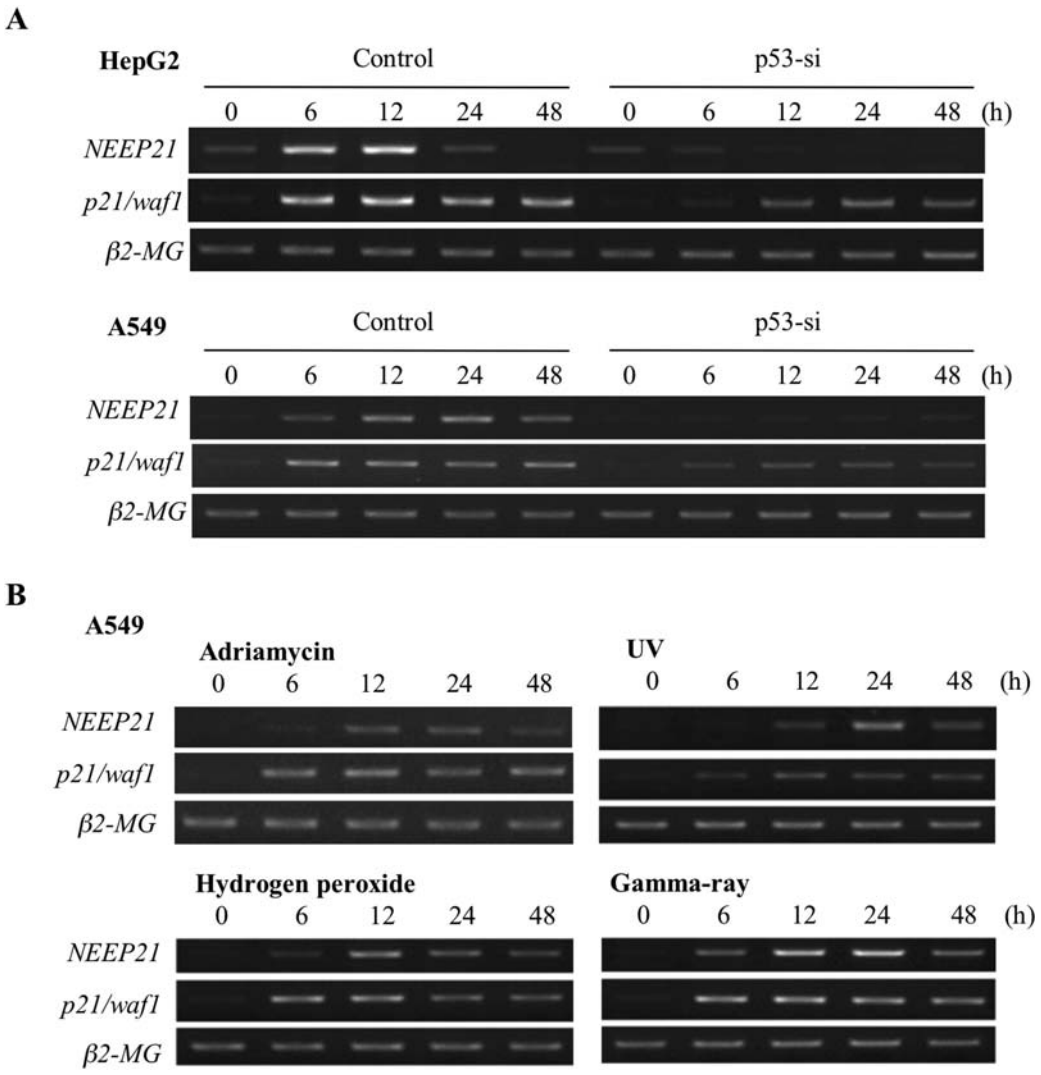

Figure 2. p53-dependent induction of *NEEP21* after DNA damage. (A) p53-dependency of *NEEP21* expression. HepG2/control cells (p53 wild-type), HepG2/p53-si cells (p53-knockdown), A549/control cells (p53 wild-type) or A549/p53-si cells (p53-knockdown) were treated with adriamycin 1 μg/ml for 2 h. *NEEP21* expression at the indicated times was examined by RT-PCR. (B) *NEEP21* induction in response to various genotoxic stresses. *NEEP21* expression at the indicated times was examined by RT-PCR in A549 cells treated by adriamycin (1 μg/ml, 2 h), hydrogen peroxide (100 μM, 1 h), UV irradiation (30 J/m^2), γ-irradiation (50 Gy). *p21/waf1* and *β2-MG* were used as the positive and quantity controls, respectively (A and B).

the spleen, colon, thymus and brain were isolated at indicated time points after irradiation. Total RNAs were purified from the tissues, from which cDNAs were synthesized, and were subjected to RT-PCR analysis. All mouse procedures were carried out according to the recommendations of the Institutional Animal Care and Use Committee of the National Cancer Center at Tsukiji, Japan.

Results

Identification of NEEP21 as a p53-inducible gene. In order to identify additional transcriptional targets of p53 in the human genome, we screened for p53-inducible transcripts as previously described (16). For the experiments reported here, we isolated mRNAs from the HepG2 cell line (human hepatocellular carcinoma) at the indicated time points after infection with adenovirus vectors designed to express either wild-type p53 (Ad-*p53*-WT), p53-46F (Ad-*p53*-46F), or EGFP (Ad-*EGFP*). p53-46F has previously been demonstrated to be an activated form of p53 with an enhanced ability to induce p53-dependent apoptosis (11). The obtained RNAs were hybridized to a cDNA microarray. From nearly 100 genes that appeared to be activated by p53, we chose one gene, *D4S234E* (UniGene accession number Hs.518595), whose mRNA level was especially elevated in a time-dependent manner (Fig. 1A). The *D4S234E* gene has also been designated as *NEEP21* in the mouse, and it was previously reported to be specifically expressed in neurons but not in non-neuronal cells (7). In the present paper, we refer to this gene as *NEEP21*.

We analyzed the mRNA expression in cancer cell lines HepG2 and H1299 (a lung cancer cell line) following p53 expression. These cells were infected with Ad-*p53*-WT, Ad-*p53*-46F, or the control vector Ad-*EGFP* at a multiplicity of infection (MOI) of 30, and mRNAs were isolated at the indicated times. The expression of *NEEP21* increased in a

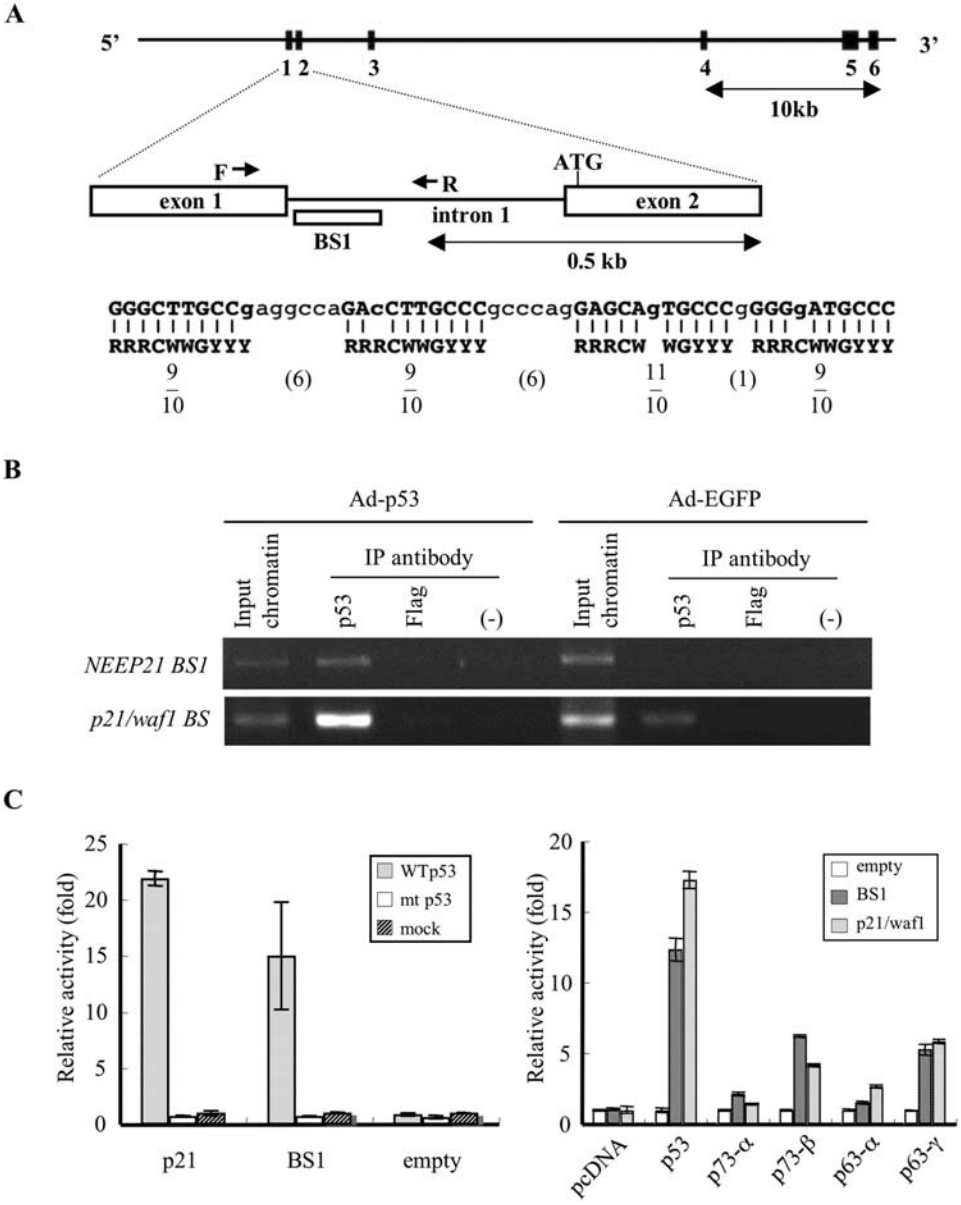

Figure 3. *NEEP21* is a direct target gene of p53. (A) Genomic structure of *NEEP21*. Locations and relative sizes of the 6 exons are indicated. The potential p53-binding sequence (BS1) in intron 1 is shown with a comparison to the consensus p53-binding sequence below it. R, purin; W, A or T; Y, pyrimidine. The figure also shows the location of the primers (F and R) used for PCR experiments to amplify a DNA fragment for preparing pGL-BS1 and for ChIP assay. (B) p53 interacts with BS1 of *NEEP21*. The ChIP assay was performed on genomic DNA fragments containing BS1. The cell lysates were isolated from Ad-*p53*-infected (lanes 1-4) or Ad-*EGFP*-infected (lanes 5-8) HepG2 cells. A protein complex including p53 and the genomic DNA was precipitated with the anti-p53 antibody (lanes 2 and 6) before PCR amplification. The input chromatin represents a portion of the sonicated chromatin before immunoprecipitation (lanes 1 and 5). The immunoprecipitates with the anti-FLAG antibody (lanes 3 and 7) or in the absence of an antibody (lanes 4 and 8) were used as negative controls. *p21/waf1* BS was used as the positive control. The BS1 sequence was amplified only in the samples precipitated with p53 antibody. The *p21/waf1* BS sequence was also amplified in the samples precipitated with anti-p53 antibody. (C) Luciferase assay. The plasmid construct containing the p53BS (pGL-BS1) was cloned into a luciferase reporter vector containing SV40 minimal promoter. pGL-BS1, pGL-WAF, or pGL-empty vector was co-transfected into H1299 cells with either of wild-type p53, mutant-p53, p73-α, p73-ß, p63-α or p63-γ expression construct, or pcDNA3.1, as well as a control reporter vector.

time-dependent manner after infection with Ad-*p53*-WT or Ad-*p53*-46F but not with Ad-*EGFP* (Fig. 1B), suggesting that exogenous p53 activates the transcription of *NEEP21*.

Transcriptional activation of NEEP21 by endogenous p53 after genotoxic stresses. In order to investigate whether *NEEP21* expression is inducible by endogenous p53, the stable cell lines HepG2 and A549 expressing p53 siRNA or control siRNA were treated with 1 μg/ml of adriamycin for 2 h, and mRNAs were isolated at the indicated times. Adriamycin treatment increased *NEEP21* expression in the control cells (p53 wild-type cells) but not in p53-si cells (p53-knockdown cells) (Fig. 2A), suggesting that the induction of *NEEP21* after DNA damage is dependent on endogenous p53. Similar results were also obtained using the other stable cell lines MCF7 (control/p53-si) and LS174T (control/p53-si) treated with adriamycin (1 μg/ml, 2 h) (data not shown).

In order to examine whether *NEEP21* expression is inducible in response to various genotoxic stresses, A549 cells were subjected to UV irradiation (30 J/m^2), γ irradiation (50 Gy), adriamycin (1 μg/ml, 2 h), or hydrogen peroxide (100 μM, 1 h). As indicated in Fig. 2B, all of these genotoxic

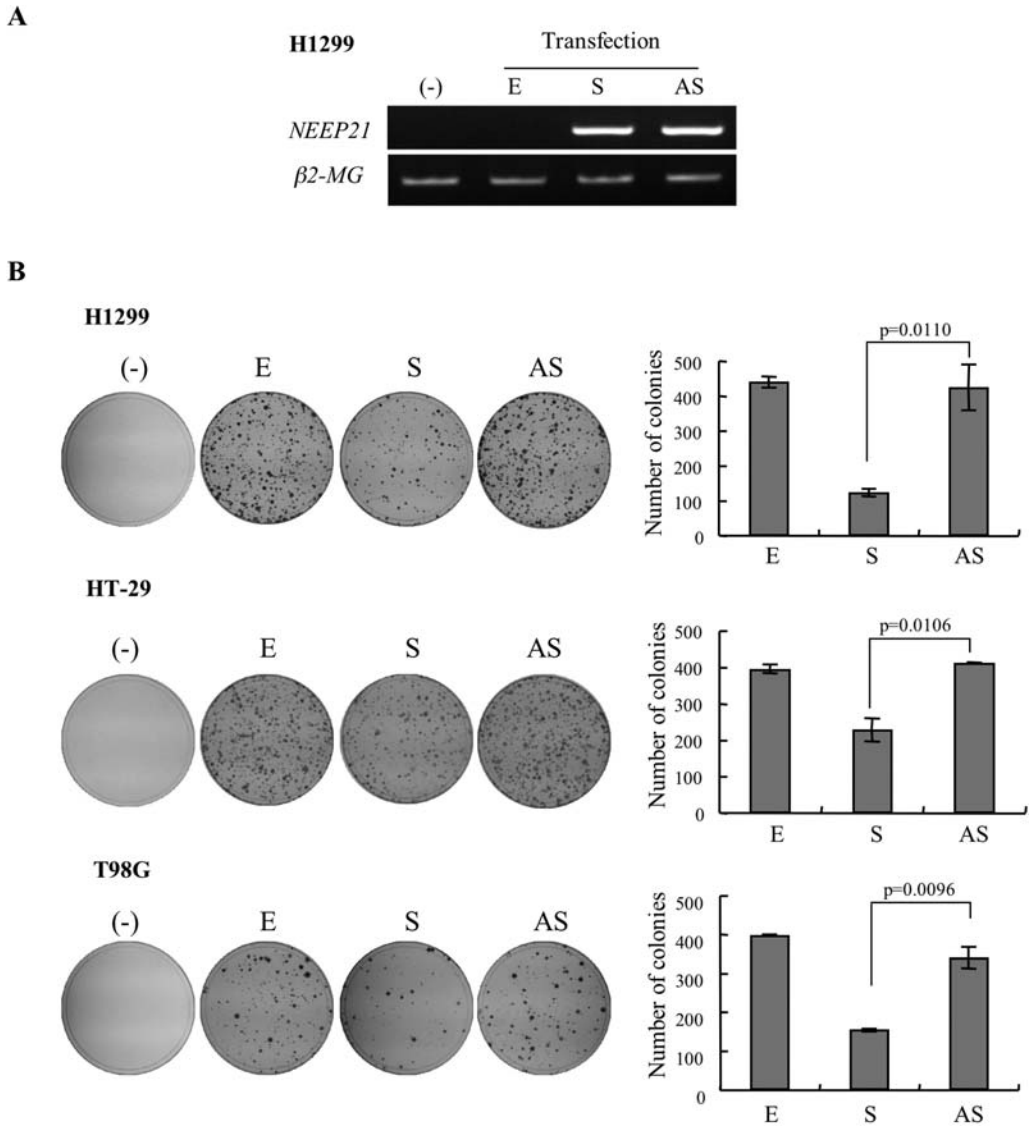

Figure 4. Suppression of cancer colony formation by NEEP21. (A) The expression level of *NEEP21* mRNA in the transfected H1299 cells. RT-PCR results indicates the expression levels of *NEEP21* mRNA in H1299 cells 24 h after transfection with the expression vectors, E, empty/pcDNA3.1(+); S, sense-*NEEP21*/pcDNA3.1(+); AS, antisense-*NEEP21*/pcDNA3.1(+). (-) indicates no transfection. (B) Colony formation assay. Representative G418-resistant colonies from H1299, HT-29 and T98G cells transfected with each plasmids are shown. The average numbers of colonies in triplicate samples are indicated with error bars indicating 1 standard deviation (SD) on the histograms.

stresses activated *NEEP21* transcription. In contrast, in the neuroblastoma cell line SH-SY5Y, genotoxic stresses did not increase the expression level of *NEEP21* mRNA because the basal expression level of *NEEP21* mRNA was already high without genotoxic stress (data not shown).

NEEP21 is a direct transcriptional target of the p53 family proteins. The genomic region of *NEEP21* contains a putative p53-binding site with multiple copies of the candidate p53 consensus motif. The putative p53-binding site (BS1) has four copies of the p53 consensus motif of sequence 5'-PuPu PuC(A/T)(A/T)GPyPyPy-3' separated by 0-13 base pair long fragments of any sequence; BS1 is located in intron 1 at 0.4 kb upstream of the *NEEP21*-coding region (Fig. 3A). In order to evaluate the *in vivo* binding of p53 with BS1, we performed a chromatin immunoprecipitation (ChIP) assay. A protein-DNA complex containing the p53 protein and its associated genomic DNAs were immunoprecipitated with an anti-p53 antibody; this was followed by PCR analysis for BS1. The fragment containing BS1 was present in the genomic DNAs immunoprecipitated with the anti-p53 antibody from the cells infected with Ad-p53, suggesting that BS1 interacts with p53 (Fig. 3B).

In order to determine whether p53 directly regulates *NEEP21* transcription, we performed a luciferase assay using a 'pGL-BS1' luciferase vector containing BS1 (pGL-BS1). The luciferase activity was increased by the co-transfection of the pGL-BS1 vector with the wild-type p53 expression vector but not with the mutant p53-175H vector (Fig. 3C, left panel). In addition, a point mutation within BS1 completely prevented the p53-dependent activation of the luciferase activity (data not shown). The p53 family proteins, p73-ß and

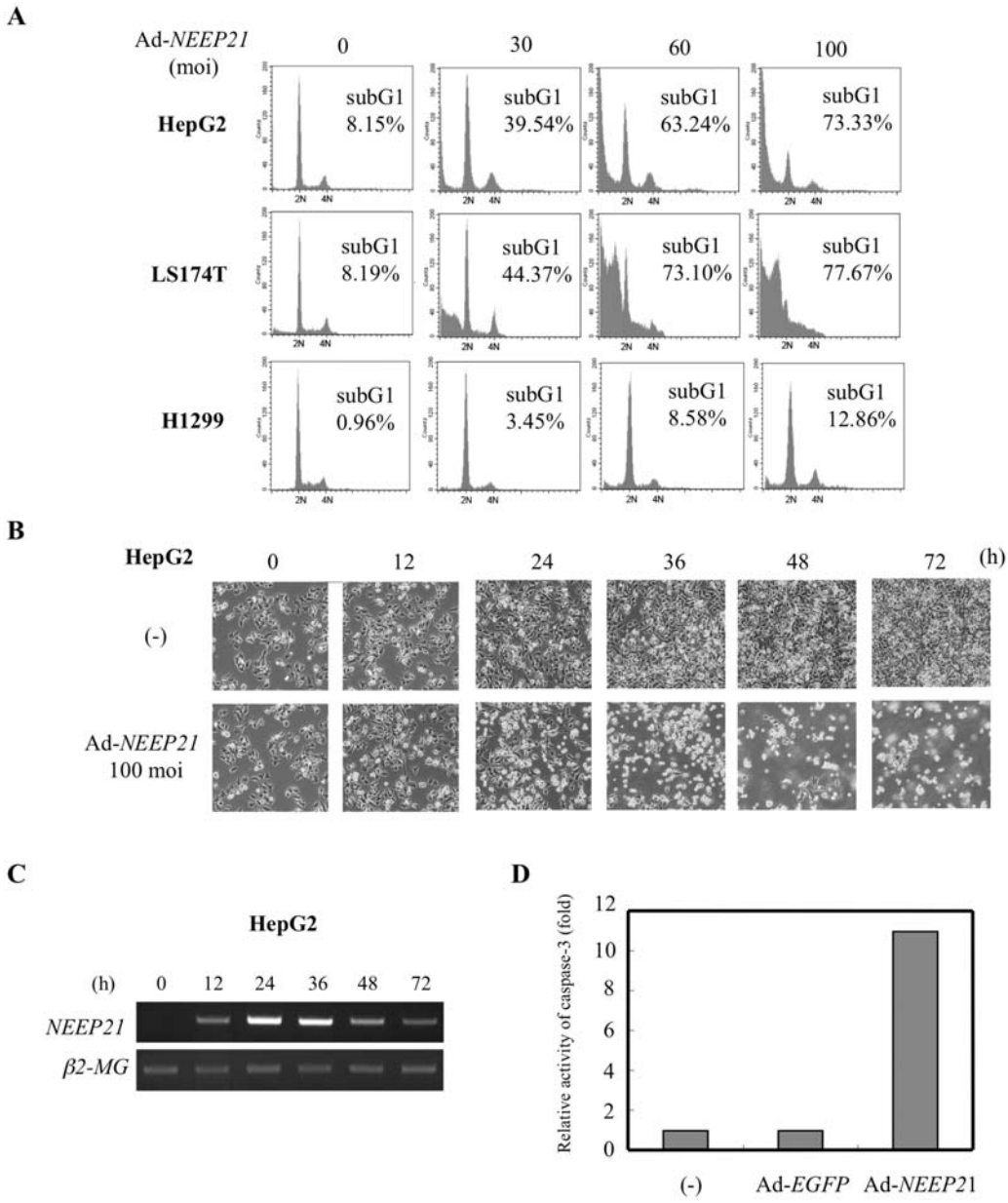

Figure 5. Apoptotic cell death induced by NEEP21. (A) Induction of apoptosis by Ad-*NEEP21*. HepG2, LS174T and H1299 cells were infected with Ad-*NEEP21* (at a MOI of 0, 30, 60, 100), and 72 h after infection, apoptosis was analyzed by FACS scan. (B) Light microscopic analysis of apoptosis. HepG2 cells infected with Ad-*NEEP21* at a MOI of 100 are shown at the indicated times after infection. Original magnification is x100. (C) RT-PCR. Expression levels of *NEEP21* mRNA in HepG2 cells infected with Ad-*NEEP21* at an MOI of 100 are shown at the indicated times. (D) Activation of caspase-3 by NEEP21. The caspase-3 activity in HepG2 cells infected with Ad-*NEEP21* or Ad-*EGFP* at an MOI of 100 was analyzed 36 h after infection. (-) indicates no infection.

p63-γ also activated the transcription of pGL-BS1 though their activities were lower than that of p53 (Fig. 3C, right panel). The effects of p73-α and p63-α were negligible.

Overexpression of NEEP21 suppresses colony formation of cancer cells. In order to explore the potential effect of NEEP21 on cell death and proliferation, we carried out colony formation assay. We prepared the plasmid vectors sense-*NEEP21*/pcDNA3.1(+) (S) and antisense-*NEEP21*/pcDNA3.1(+) (AS), and we checked the expression levels of *NEEP21* mRNA in H1299 cells after transfection with each expression vector (Fig. 4A). The expression (S and AS) vectors and empty plasmids (E) were transfected into the following cancer cell lines: H1299 (lung, p53 mutant), T98G (brain, p53 mutant), and HT-29 (colon, p53 mutant) cells; after 2 weeks, the G418-resistant colonies were scored. As shown in Fig. 4B, transfection with the plasmid expressing sense-*NEEP21* (S) reduced the number of cancer cell colonies as compared to transfection with the plasmid expressing antisense *NEEP21* (AS) or empty vector (E) in all three cancer cell lines.

NEEP21 induces apoptosis via the activation of caspase-3. We constructed an adenovirus vector expressing *NEEP21*, and examined the effect of overexpression of *NEEP21* on HepG2, LS174T, and H1299 cancer cell lines. These cell lines were infected with Ad-*NEEP21* (0, 30, 60, or 100 MOI)

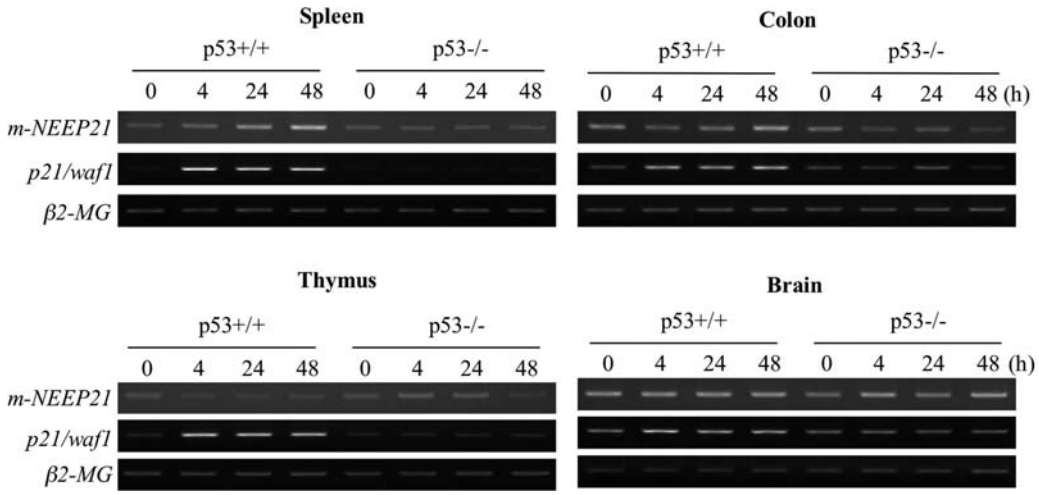

Figure 6. Potential *in vivo* role of *NEEP21* in p53-regulated response to DNA damage. RT-PCR results indicated expression levels of *NEEP21* in various tissues, including spleen, colon, thymus and brain tissues isolated from $p53^{+/+}$ or $p53^{-/-}$ mice at the indicated time points after irradiation with γ-rays (10 Gy).

and were harvested 72 h after infection. The cells were subjected to FACS analyses. Infection with Ad-*NEEP21* significantly increased the number of apoptotic cells in a dose-dependent manner in all cancer cell lines (Fig. 5A). In HepG2 cells, the proliferation was suppressed 24 h after infection with Ad-*NEEP21*, and after 36 h, cell death was significantly observed; the expression of *NEEP21* mRNA in these cells was confirmed by RT-PCR (Fig. 5B and 5C). We were able to confirm the activation of caspase-3 in the cells at 36 h after infection with Ad-*NEEP21* (Fig. 5D). These results suggest that NEEP21 induces apoptosis via the activation of caspase-3.

p53-dependent induction of NEEP21 in γ-irradiated mice. In order to investigate the role of NEEP21 *in vivo*, we examined the p53 dependency of mouse *NEEP21* expression. $p53^{+/+}$ and $p53^{-/-}$ mice were irradiated with 10 Gy of γ rays and the expression of mouse *NEEP21* mRNA was examined in various tissues at the indicated time points. In the spleen and colon, γ-irradiation increased *NEEP21* expression in the $p53^{+/+}$ mice but not in the $p53^{-/-}$ mice (Fig. 6), suggesting that NEEP21 plays an important role in the response to genotoxic damage *in vivo* in addition to the role it plays in neurons. We were not able to observe any induction of *NEEP21* or *p21/Waf1* by γ-irradiation in the brain regardless of the p53 status, suggesting that the brain may have higher resistance to γ-rays than the spleen, colon, or thymus.

Discussion

NEEP21 has been reported to be necessary for receptor recycling as the neuron-enriched endosomal protein (7,10), but the expression in non-neuronal cells or the regulatory mechanism has not been identified. In the present study, we showed that *NEEP21* transcription is directly upregulated by p53 in response to various genotoxic stresses in non-neuronal cancer cell lines (HepG2, H1299, and A549), in which the expression level of *NEEP21* is negligible under normal conditions. Furthermore, NEEP21 was shown to have a strong suppressive effect on the colony formation of several cancer cell lines. In addition, the overexpression of *NEEP21* using an adenovirus vector induced apoptosis via caspase-3 activation. These results suggest that NEEP21 is involved in DNA damage-induced apoptosis.

NEEP21 has been reported to participate in recycling receptors. It was reported that the conversion of endogenous Ca^{2+}-permeable AMPA receptors to Ca^{2+}-impermeable receptors by adenoviral vector-mediated transfer of GluR2 cDNA inhibited migration and induced apoptosis in human glioblastoma cells (17), whereas NEEP21 suppression led to a strong retardation of GluR1 and GluR2 recycling (8). In another report, NEEP21 overexpression accelerated transferrin internalization and recycling, and its downregulation delayed transferrin recycling (7). An increase in transferrin recycling may cause abnormal iron uptake, which may cause oxidative DNA damage followed by apoptosis.

In the present study, we found that the p53-binding site of human *NEEP21* contains two pairs of oligomers in tandem with long spacers: GGGCTTGCCG (aggcca) GACCTTGCCC (gcccag) GAGCAGTGCCC (g) GGGGATGCCC. The sequence reacted with not only p53 but also p63 and p73. The canonical p53 responsive element contains a pair of oligomers, RRRCWWGYYY, which are linked by a spacer of 0-13 bp (R, purine; C, cytosine; W, adenine or thymidine; G, guanine and Y, pyrimidine) (18). It has also been reported that the spacers are predominantly 0 bp long; a few spacers are 1 bp long and longer spacers are rare (19). The p63 and p73 proteins seemed to recognize similar but slightly disordered sequences (20,21). The slight differences between the p53-binding sequences of *NEEP21* and the typical p53-responsive element and the rather long spacers can explain the reactivity to p63 and p73 in addition to p53.

The homology between the human and mouse sequences is preserved with 93% DNA sequence identity through the open reading frame (ORF) of *NEEP21*. The homology between the human and mouse sequences is not preserved in the p53-binding site of *NEEP21* (data not shown), although the cDNA sequences in the ORF are highly preserved. We observed the induction of mouse-*NEEP21* in γ-irradiated $p53^{+/+}$ mice, consistent with the p53-dependent induction of human *NEEP21*

in human cancer cell lines under genotoxic conditions. The result suggests that another unidentified p53-binding site might be used in mice.

On the other hand, the expression level of *NEEP21* was not increased by γ-irradiation in the brain tissue of $p53^{+/+}$ mice, in which the basal expression level of *NEEP21* was much higher than those in the other tissues. Similarly, *NEEP21* mRNA was not increased by genotoxic stresses in SH-SY5Y cells (neuroblastoma, p53 wild-type), which shows high level of *NEEP21* expression under normal conditions (data not shown). Therefore, the function of NEEP21 related to the regulation of synaptic transmission and plasticity in neuronal cells is probably regulated by a p53-independent mechanism. In other words, it is likely that the apoptotic function of NEEP21 in non-neuronal cells is specifically regulated by p53.

It has been reported that apoptosis is especially induced in the spleen and moderately in the colon of $p53^{+/+}$ mouse after γ-ray exposure, in contrast to weak induction in the liver, lung, and kidney (22). In the present study, *NEEP21* was significantly induced after γ-irradiation in the spleen of $p53^{+/+}$ mice but not in that of $p53^{-/-}$ mice. These results suggest that NEEP21 may play an important role in p53-induced apoptosis *in vivo*. Further investigation is needed to clarify the precise role of NEEP21 in p53-regulated apoptosis.

Very recently, a study reported that p53 regulates endocytosis, in which p53 deficiency causes delayed internalization of EGF and the sustained activation of EGF signaling (23). Moreover, another study reported that mutant p53 enhanced the recycling of EGFR and integrin, leading to constitutive activation of EGFR/integrin signaling (24). Therefore, we speculate that p53 might regulate endocytosis of EGFR and integrin via the transcriptional activation of NEEP21, resulting in the degradation of these oncogenic receptors. If this is true, NEEP21 is a novel p53 target that mediates p53-dependent endocytic degradation of oncogenic receptors, representing a new mechanism for p53-regulated tumor suppression. Further investigation on the role of NEEP21 in p53-regulated endocytosis is urgently required.

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References

1. Vogelstein B, Lane D and Levine AJ: Surfing the p53 network. Nature 408: 307-310, 2000.
2. Vousden KH and Prives C: p53 and prognosis: New insights and further complexity (mini-review). Cell 120: 7-10, 2004.
3. Vousden KH and Lu X: Live or let die: the cell's response to p53. Nat Rev Cancer 2: 594-604, 2002.
4. Nakamura Y: Isolation of p53-target genes and their functional analysis. Cancer Sci 95: 7-11, 2004.
5. Arakawa H: p53, apoptosis and axon-guidance molecules. Cell Death Differ 12: 1057-1065, 2005.
6. Carlock L, Vo T, Lorincz M, Walker PD, Bessert D, Wisniewski D and Dunbar JC: Variable subcellular localization of a neuron-specific protein during NTera 2 differentiation into post-mitotic human neurons. Brain Res Mol Brain Res 42: 202-212, 1996.
7. Steiner P, Sarria JC, Glauser L, Magnin S, Catsicas S and Hirling H: Modulation of receptor cycling by neuron-enriched endosomal protein of 21 kD. J Cell Biol 157: 1197-1209, 2002.
8. Steiner P, Alberi S, Kulangara K, Yersin A, Sarria JC, Regulier E, Kasas S, Dietler G, Muller D, Catsicas S and Hirling H: Interactions between NEEP21, GRIP1 and GluR2 regulate sorting and recycling of the glutamate receptor subunit GluR2. EMBO J 24: 2873-2884, 2005.
9. Alberi S, Boda B, Steiner P, Nikonenko I, Hirling H and Muller D: The endosomal protein NEEP21 regulates AMPA receptor-mediated synaptic transmission and plasticity in the hippocampus. Mol Cell Neurosci 29: 313-319, 2005.
10. Debaigt C, Hirling H, Steiner P, Vincent JP and Mazella J: Crucial role of neuron-enriched endosomal protein of 21 kDa in sorting between degradation and recycling of internalized G-protein-coupled receptors. J Biol Chem 279: 35687-35691, 2004.
11. Nakamura Y, Futamura M, Kamino H, Yoshida K, Nakamura Y and Arakawa H: Identification of p53-46F as a super p53 with an enhanced ability to induce p53-dependent apoptosis. Cancer Sci 97: 633-641, 2006.
12. Futamura M, Kamino H, Miyamoto Y, Kitamura N, Nakamura Y, Ohnishi S, Masuda Y and Arakawa H: Possible role of semaphorin 3F, a candidate tumor suppressor gene at 3p21.3, in p53-regulated tumor angiogenesis suppression. Cancer Res 67: 1451-1460, 2007.
13. Kamino H, Futamura M, Nakamura Y, Kitamura N, Kabu K and Arakawa H: B-cell linker protein prevents aneuploidy by inhibiting cytokinesis. Cancer Sci 99: 2444-2454, 2008.
14. Miyamoto Y, Futamura M, Kitamura N, Nakamura Y, Baba H and Arakawa H: Identification of UNC5A as a novel transcriptional target of tumor suppressor p53 and a regulator of apoptosis. Int J Oncol 36: 1253-1260, 2010.
15. Tsukada T, Tomooka Y, Takai S, Ueda Y, Nishikawa S, Yagi T, Tokunaga T, Takeda N, Suda Y, Abe S, Matsuo I, Ikawa Y and Aizawa S: Enhanced proliferative potential in culture of cells from p53-deficient mice. Oncogene 8: 3313-3322, 1993.
16. Masuda Y, Futamura M, Kamino H, Nakamura Y, Kitamura N, Ohnishi S, Miyamoto Y, Ishikawa H, Ohta T, Ohki M, Kiyono T, Egami H, Baba H and Arakawa H: The potential role of DFNA5, a hearing impairment gene, in p53-mediated cellular response to DNA damage. J Hum Genet 51: 652-664, 2006.
17. Ishiuchi S, Tsuzuki K, Yoshida Y, Yamada N, Hagimura Y, Tamura M, Sasaki T and Ozawa S: Blockage of Ca^{2+}-permeable AMPA receptors suppresses migration and induces apoptosis in human glioblastoma cells. Nat Med 8: 971-978, 2002.
18. El-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW and Vogelstein B: Definition of a consensus binding site for p53. Nat Genet 1: 45-49, 1992.
19. Wei CL, Wu Q, Vega VB, Chiu KP, Ng P, Zhang T, Shahab A, Yong HC, Fu YT, Weng Z, Liu JJ, Zhao XD, Chew JL, Lee YL, Kuznetsov VA, Sung WK, Miller LD, Lim B, Liu ET, Yu Q, Ng HH and Ruan Y: A global map of p53 transcription-factor binding sites in the human genome. Cell 124: 207-219, 2006.
20. Osada M, Park HL, Magakawa Y, Begum S, Yamashita K, Wu G, Kim MS, Trink B and Sidransky D: A novel response element confers p63- and p73-specific activation of the WNT4 promoter. Biochem Biophys Res Commun 339: 1120-1128, 2006.
21. Harms K, Nozell S and Chen X: The common and distinct target genes of the p53 family transcription factors. Cell Mol Life Sci 61: 822-842, 2004.
22. Masi AD, Antoccia A, Dimauro I, A-Storino A, Mosiello A, Mango R, Novelli G and Tanzarella C: Gene expression and apoptosis induction in p53-heterozygous irradiated mice. Mutat Res 594: 49-62, 2006.
23. Endo Y, Sugiyama A, Li SA, Ohmori K, Ohata H, Yoshida Y, Shibuya M, Takei K, Enari M and Taya Y: Regulation of clathrin-mediated endocytosis by p53. Genes Cells 13: 375-386, 2008.
24. Muller PA, Caswell PT, Doyle B, Iwanicki MP, Tan EH, Karim S, Lukashchuk N, Gillespie DA, Ludwig RL, Gosselin P, Cromer A, Brugge JS, Sansom OJ, Norman JC and Vousden KH: Mutant p53 drives invasion by promoting integrin recycling. Cell 139: 1327-1341, 2009.