Identification and characterization of early growth response 2, a zinc-finger transcription factor, as a p53-regulated proapoptotic gene

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Abstract. Tumor suppressor p53 is a transcription factor that induces growth arrest and/or apoptosis in response to cellular stress. In recent years, many genes have been identified as p53-regulated genes; however, no single target gene has been shown to be required for the apoptotic effect. Using microarray analysis, we have identified the transcription factor early growth response 2 (EGR2) as a target of the p53 family, specifically p53, p63 and p73. EGR2 expression was up-regulated by DNA damage-induced p53 activity, as well as by overexpression of p53 family genes. Furthermore, we identified a responsive element to p53, TAp63, and TAp73 within the *EGR2* gene. This response element is highly conserved between human and rodents. We also found that overexpression of EGR2 induced apoptosis when combined with anticancer agents. Conversely, inactivation of EGR2 attenuated p53-mediated apoptosis. The results presented here suggest that EGR2 is a direct transcriptional target of p53 family that can in part mediate the p53-dependent apoptotic pathway.

Introduction

Alterations of the *p53* gene are among the most common and important events in human carcinogenesis. In response to inappropriate growth signals and various types of cellular stress, p53 is activated and inhibits cell growth through activation of both cell cycle arrest and apoptosis, maintaining genome stability and preventing cancer development (1). p53 achieves these functions mainly through transactivation of its downstream target genes. Unlike those for other tumor suppressor genes, most *p53* mutations in tumors are of the missense type and lead to single amino acid changes that predominantly affect residues in the DNA binding domain of the protein, strongly suggesting that the critical biochemical event for the p53 transactivation activity is its sequence-specific binding to DNA (2-4). All p53 targets contain p53-binding sites within the gene or promoter region, and each site can contribute to the p53 response.

The most important pathways involved in tumor suppression that are activated by p53 lead to growth arrest and apoptosis, and the balance of these two cellular events can determine the fate of individual cells. Among the p53-target genes, induction of the cyclin-dependent kinase inhibitor p21 by p53 is central to the activation of cell cycle arrest (5). However, activation of p21 is not required for the induction of apoptosis. Candidates for p53-regulated apoptotic targets include mitochrondrial proteins that are likely to contribute to the release of cytochrome c from the mitochondria, such as BAX (6), NOXA (7), p53AIP1 (8), PUMA (9,10) and also proteins that play a role in death receptor-mediated apoptosis, such as Fas (11) and KILLER/DR5 (12). There appears not to be a single gene that is the principal mediator of the p53 apoptotic signal; instead, it is likely that the activation of multiple apoptosis-related genes is involved.

The two *p53*-relatives, *p63* and *p73*, share high homology to *p53*, especially in the central DNA binding domain. Many studies have revealed p53-like functions of p63 and p73, such as their ability to induce apoptosis, suggesting that they may play a role in tumor suppression, yet initial studies indicated that *p63* and *p73* were not frequently mutated in human cancers (13,14). Flores *et al* recently reported that $p63^{+/-}$ and $p73^{+/-}$ animals die prematurely from tumors, suggesting a broader range of tumor suppressor functions for the p53 family than previously detected (15). Moreover, *p63* and *p73* are expressed in different isoforms as a result of differential promoter usage and alternative splicing. The isoforms containing a transactivation domain at their N-terminus can also bind to p53-responsive elements to transactivate the p53-target genes and are termed TAp73 and TAp63. In contrast, the ΔN isoforms are thought to play a role in blocking transactivation of p53-targets and their respective TA isoforms. In general, therefore, the TA isoforms might be expected to

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have a role in tumor suppression, while increased expression of the ΔN isoforms might be oncogenic.

In an attempt to identify novel genes responsive to p53-induced growth inhibition, we profiled Saos-2 human osteosarcoma cells with the Affymatrix U133 plus 2.0 GeneChip (16) and identified EGR2 as a direct target of the p53 family. We found that EGR2 is induced by p53 family proteins and DNA damage in a p53-dependent manner. The human *EGR2* gene contains a response element specific for the p53 family, which is highly conserved between human and rodents. Moreover, we found that knockdown of EGR2 attenuates DNA damage-induced apoptosis, and overexpression of EGR2 inhibits cancer cell growth. The *EGR2* gene was originally identified as a serum response immediate-early gene encoding a protein with three Cys2-His2-type zinc-finger motifs (17). Previous reports included findings that expression of endogenous EGR2 was significantly low in primary cancers (18) as well as in various cancer cell lines (19). Taken together, our data strongly indicate that EGR2 is one of the mediators downstream of p53 promoting apoptosis.

Materials and methods

Cell culture, recombinant adenovirus and plasmids. Human oral cancer cell lines (SAS, HSC2, HO-1-N1, OSC70 and Ca9-22), osteosarcoma cell lines (Saos-2 and MG-63), colorectal cancer cell line SW480, cervical cancer cell line HeLa and lung cancer cell line H1299 cells were obtained from either American Type Culture Collection or the Japanese Collection of Research Bioresources (Osaka, Japan). HCT116 (*p53* wild-type) and its derivative HCT116-p53$^{-/-}$ cells lacking *p53* were kindly provided by Dr Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University). Mouse embryonic fibroblasts (MEFs) from *p53*$^{+/+}$ mice and *p53*$^{-/-}$ mice were provided by Dr Motoya Katsuki (National Institute for Basic Biology, Japan). A rat embryo fibroblast cell line, REF-p53val135, was established by transforming with a murine temperature-sensitive mutant *p53val135* and *ras* (20).

The replication-deficient recombinant adenoviral vectors expressing human *p53* (Ad-p53), *TAp73α* (Ad-p73α), *TAp73ß* (Ad-p73ß), *TAp63γ* (Ad-p63γ) and TAp63α (Ad-p63α), or the bacterial *lacZ* gene (Ad-lacZ), were constructed using a replication-deficient, E1-deleted Ad5 vector pJM17 (Microbix Biosystem) as a backbone (21,22). To construct an EGR2-expressing plasmid, the entire coding region of the human EGR2 cDNA was inserted into a Halo-tagged vector, pFN21A (Promega), in-frame to allow Halo epitope tapping at the N-terminus. The plasmid construct was sequenced and named pFN21A-EGR2.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR). For semiquantitative RT-PCR analysis, cDNAs were synthesized from 5 μg of total RNA with the SuperScript Preamplification System (Invitrogen). The RT-PCR exponential phase was determined within 25-37 cycles to allow semiquantitative comparison among cDNA from identical reactions. Oligonucleotide primer sequences were as follows: human *EGR2* sense 5'-GAACTTCAGTTAGTGG TTTTGCG-3', human *EGR2* antisense 5'-CTAGTTGTCTCA ACCCTTGAAGC-3'; mouse and rat *Egr2* sense 5'-GGTTG TGCGAGGAGCAAATGTGA-3', mouse and rat *Egr2* antisense 5'-GCTGTGGTTGAAGCTGGAGGG-3'; human *survivin* sense 5'-CAAGGACCACCGCATCTCTAC-3', human *survivin* antisense 5'-ACCAGGGAATAAACCCT GGAAG-3'; human *p21* sense 5'-GTTCCTTGTGGAGCCG GAGC-3', human *p21* antisense 5'-GGTACAAGACAGTGA CAGGTC-3'; mouse and rat *p21* sense 5'-GCCCGAGAAC GGTGGAACTTT-3', mouse and rat *p21* antisense 5'-CCTC CTGACCCACAGCAGAA-3'; and *GAPDH* sense 5'-ACCA CAGTCCATGCCATCAC-3', *GAPDH* antisense 5'-TCCAC CACCCTGTTGCTGTA-3'.

Immunoblot analysis. The primary antibodies used for immunoblotting in this study are as follows: goat anti-human EGR2 polyclonal antibody (pAb) (Imgenex); mouse anti-human p53 monoclonal antibody (mAb) (DO-1, Santa Cruz Biotechnology); mouse anti-human p21 mAb (Calbiochem); rabbit anti-human survivin polyclonal antibody (Sigma); mouse anti-human PARP mAb (BD Pharmingen); rabbit anti-Halo-Tag pAb (Promega); mouse anti-ß-actin mAb (C4, Chemicon). Proteins were separated by electrophoresis on an SDS/acrylamide gel and transferred to Immobilon P membranes (Millipore). After non-specific binding was blocked with 5% milk and 0.1% Tween-20 in PBS, membranes were incubated with primary antibodies as recommended by the supplier. After incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), immunodetection was performed by enhanced chemiluminescence using the ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and a CCD camera (LAS3000, Fuji film).

Chromatin immunoprecipitation assay (ChIP). ChIP assay was performed using the ChIP assay kit (Upstate Biotechnology) and mouse anti-human p53 monoclonal antibody (mAb) (DO-1). An amount of $2x10^6$ cells were cross-linked with a 1% formaldehyde solution for 15 min at 37˚C. The cells were then lysed in 200 ml of SDS lysis buffer and sonicated to generate 300-800 bp DNA fragments. After centrifugation, the cleared supernatant was diluted ten-fold with ChIP dilution buffer and split into two equal portions; one portion was incubated with anti-p53 antibody (5 μg) at 4˚C for 16 h, and the other portion was used as a negative control (no antibody). A 1/50 volume of total extract was used for PCR amplification as the input control. Immune complexes were precipitated, washed and eluted, and DNA-protein cross-links were reversed by heating at 65˚C for 4 h. DNA fragments were purified and dissolved in 40 μl of TE. A 2-μl amount of each sample was used as a template for PCR amplification. PCR amplification of the consensus p53-binding sequence upstream of the human *EGR2* gene (EGR2-RE2, -1428) was performed on immunoprecipitated chromatin using the specific primers, 5'-GGAGTGGAAGACAAAAGAAGGT CAGAA-3' (forward) and 5'-AACAGTTCTGATTGGCGT AGCC-3' (reverse). Amplifications were also performed on immunoprecipitated chromatin using the specific primers, 5'-CTAATCACTTTCAAGACGCCATCCC-3' and 5'-GCAA CCTGCACAAACGACCAT-3' (EGR2-RE1, intron2), 5'-AGG GCTGTTGTGGTGCCAGTGTG-3' and 5'-GTAACAGG

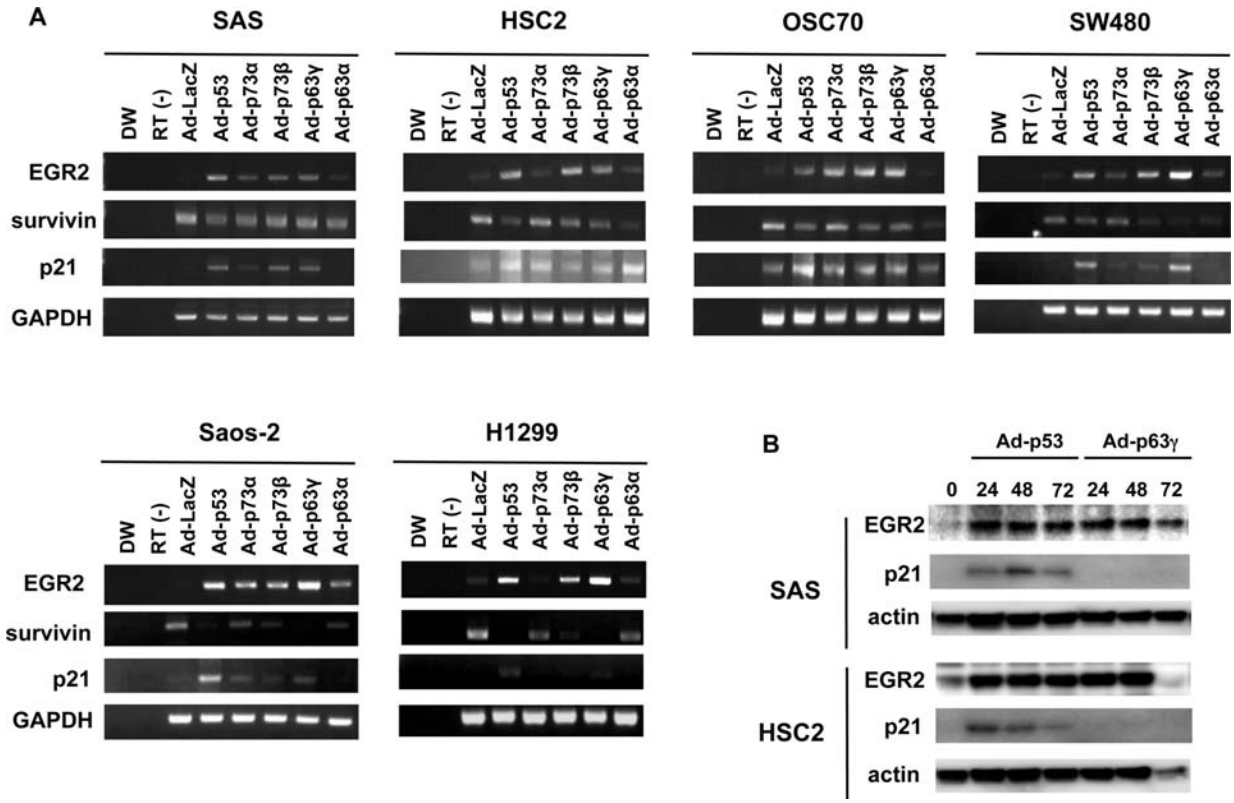

Figure 1. Overexpression of the p53 family in human cancer cells results in an increase in EGR2 mRNA and protein levels. (A) Semiquantitative RT-PCR analysis shows up-regulation of *EGR2* mRNA following adenovirus-mediated transfection of the p53 family gene into the indicated human cancer cell lines. Human cancer cell lines were infected with adenoviruses, and the cells were harvested at 24 h following infection. Semiquantitative RT-PCR analysis of *EGR2* was performed as described in Materials and methods. We also performed semiquantitative RT-PCR analysis of known p53 target genes such as *p21* and *survivin*. The *GAPDH* mRNA level was examined as a control for the integrity of the cDNA. Distilled water (DW) and total RNA untreated with reverse transcription (RT) were used as negative controls. (B) SAS or HSC2 cells were infected with adenovirus at a MOI of 25 and were harvested at 24, 48 and 72 h following infection. Immunoblot analysis was performed using an anti-EGR2 Ab.

TGAGAGGACGAAAATGG-3' (EGR2 RE3, -6248) as other candidate sequences, or 5'-GTTCAGTGGGCAGGTTG ACT-3' and 5'-GCTACAAGCAAGTCGGTGCT-3' for *MDM2* promoter containing a p-53-binding sequence. To ensure that PCR amplification was performed in the linear range, template DNA was amplified for a maximum of 35 cycles. PCR products generated from the ChIP template were directly sequenced to verify the identity of the amplified DNA.

Luciferase assay. Fragments (26 bp) of the response element for p53 family RE-EGR2 (5'-GCACATGTTCTATGCC ATTCAAGTTT-3') and its mutant form RE-EGR2-mut (5'-GCAAATTTTCTATGCCATTAAATTTT-3') were synthesized and inserted upstream of a minimal promoter in the pGL3-promoter vector (Promega), and the resulting constructs were designated pGL3-RE-EGR2 and pGL3-RE-EGR2-mut, respectively. Subconfluent cells in 24-well plates were transferred with 2 ng of phRG-TK reporter (*Renilla* luciferase for internal control) and 100 ng of pGL3 reporter (*firefly* luciferase, experimental reporter), together with 100 ng of a pcDNA3.1 control vector (Invitrogen) or a vector that expresses p53, TA73ß, or TA63γ. Forty-eight hours after transfection, the reporter gene activities were measured by Dual-Luciferase reporter assay (Promega). Additionally, HCT116 and its derivative HCT116-p53$^{-/-}$ cells in 24-well plates were also transfected in cells with 100 ng of pGL3 reporter and 2 ng of phRG-TK reporter. Cells were treated with 0.5 or 1.0 μg/ml adriamycin (ADR) and incubated for 24 h. After 24 h incubation, reporter gene activity was measured by a Dual-Luciferase reporter assay.

Knockdown of EGR2 by RNA interference. We designed two small interfering RNA (siRNA) vectors against human EGR2. iLenti-siRNA vector (Applied Biological Materials, Canada) containing H1 promoter was used for the expression of siRNA. The target sequences are iLen-si-EGR2-1: 5'-GGA GATGGCATGATCAACATTGACATGAC-3'; iLenti-si-EGR2-2: 5'-ACCCACCACCTCCTTCCTATCCATCC CCC-3'. As controls, we used siRNA vector without the insert synthesized by Applied Biological Materials.

Cell viability assay and determination of caspase-3 activity. Proliferation of transfected cells was analyzed by measuring the uptake of tritium thymidine using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in each well of 96-well plates at 3x10^3 cells per well and, after 24 h, subsequently treated with CDDP (*cis*-diamminedichloride platinum) and ADR, respectively. After a 48-h incubation, the MTT assay was performed in triplicate with Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's instructions. For the colony formation assay, 5x10^3 cells were separately transfected with 2 μg of the EGR2 siRNA plasmid (iLenti-si-EGR2-1 and -2) and a control vector (iLenti-si-cont), using a

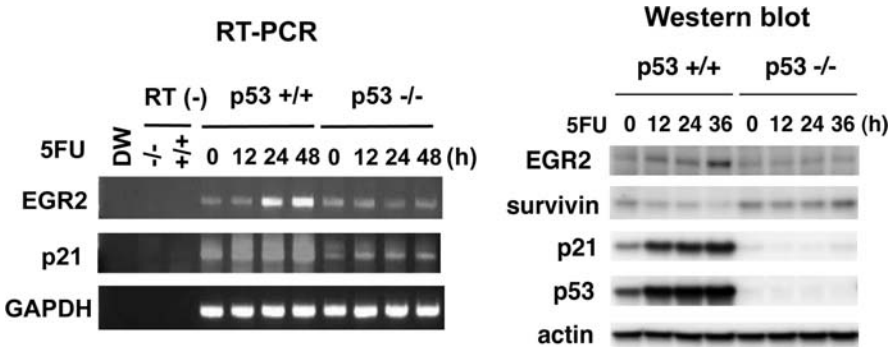

Figure 2. Endogenous p53 up-regulates EGR2 mRNA and protein levels. Up-regulation of EGR2 mRNA and protein by DNA damage in HCT116-p53$^{+/+}$ but not in HCT116-p53$^{-/-}$ cells. Both semiquantitative RT-PCR analysis and immunoblot analysis of EGR2 in HCT116-p53$^{+/+}$ and HCT116-p53$^{-/-}$ cells after treatment with 25 μg/ml 5FU for the indicated time (hours).

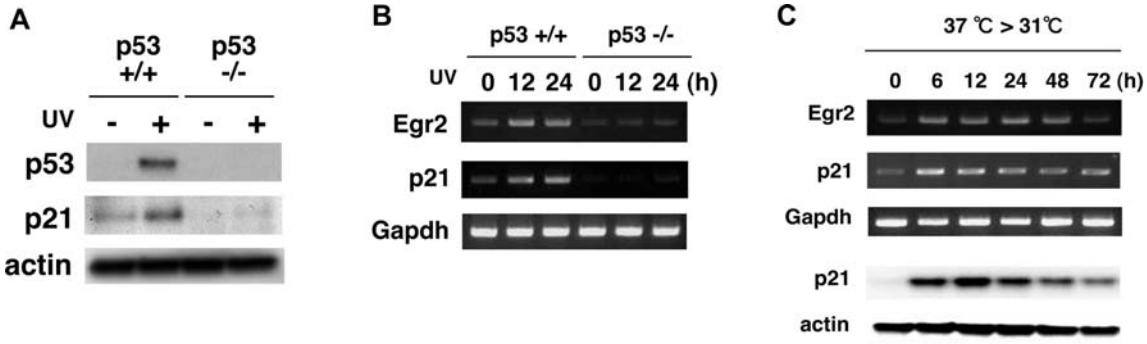

Figure 3. Expression of *Egr2* mRNA is induced in mouse and rat fibroblasts in a wild-type p53-dependent manner. (A) Immunoblot analysis of p53 protein in p53$^{+/+}$ and p53$^{-/-}$ MEFs following UV radiation. Cells lysates were prepared at 24 h after the UV treatment (50 J/m^2). Immunoblot analysis was performed on mouse p53, p21 and ß-actin. (B) Semiquantitative RT-PCR analysis of *Egr2* mRNA following UV irradiation in MEFs derived from p53$^{+/+}$ and p53$^{-/-}$ mice. Total RNA was prepared from the cells at 0, 12 and 24 h following the irradiation and subjected to semiquantitative RT-PCR analysis of *Egr2*. The mRNA levels of the *Gapdh* and *p21* genes were also examined as a control for the integrity of the cDNA or a known p53-target gene. (C) Semiquantitative RT-PCR analysis of *Egr2* mRNA in REF-p53val135 cells following thermoshift. Total RNA was prepared from the cells at 0, 6, 12, 24, 48 and 72 h following the thermoshift (37-31˚C), and subjected to RT-PCR analysis of *Egr2*. In addition, immunoblot analysis was performed on a known p53-target, p21 and actin.

Nucleofector Electroporation System (Amaxa). They were then plated in 60-mm culture dishes, selected for 14 days in medium containing G418, and stained with Giemsa.

Caspase activity was determined by colorimetric assay using a caspase-3 assay kit (Biovision) according to the manufacturer's protocol. The kit utilizes synthetic tetrapeptides labeled with *p*-nitroanilide. Briefly, the cells were lysed in the supplied lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and substrates at 37˚C. The caspase activity was determined by measuring the change in absorbance at 405 nm using a microplate reader.

Results

The p53 family up-regulates expression of the EGR2 mRNA and protein. To express the human p53 family genes, we generated replication-deficient recombinant adenoviral vectors Ad-p53, Ad-p73α, Ad-73ß, Ad-63γ and Ad-63α for the *p53*, *TAp73α*, *TAp73β*, *TAp63γ* and *TAp63α* genes, respectively. After infection with Ad-p53, Ad-p73α, Ad-p73ß, Ad-p63γ and Ad-p63α, expression of endogenous p53, TAp73α, TAp73ß, TAp63γ and TAp63α proteins was observed, respectively (16,21,22). In an effort to identify specific targets regulated by the p53 family, we performed Affymetrix GeneChip analysis and compared expression patterns in a human osteosarcoma cell line Saos-2 transfected separately with Ad-p53 and Ad-p63α. Microarray data were deposited into NCBI Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) and are accessible through GEO series accession number GSE13504. Using this approach, we found that the *EGR2* gene was reproducibly up-regulated at least ten-fold in *p53*- or *p63*-transfected Saos-2 cells as compared to *lacZ*-transfected cells.

The results from the microarray were confirmed by semiquantitative RT-PCR with six human cancer cell lines, SAS, HSC2, OSC70, SW480, Saos-2 and H1299. As shown in Fig. 1, *EGR2* mRNA was clearly increased by p53, p73ß and p63γ in all cell lines tested. *EGR2* was induced in response to p73α in four of the six cell lines tested (SAS, OSC70, SW480 and Saos-2). As negative and positive controls, we also performed semiquantitative RT-PCR analysis of known p53-target genes, *survivin* and *p21*, respectively (Fig. 1, second and third rows).

To determine whether the increase in *EGR2* mRNA was accomplished by an increase in protein expression, we then examined the level of EGR2 protein by immunoblot analysis using an antibody against EGR2. Fig. 2 shows that total cellular EGR2 protein was increased in *p53*- or *p63γ*-transfected SAS and HSC2 cells, consistent with the RT-PCR analysis.

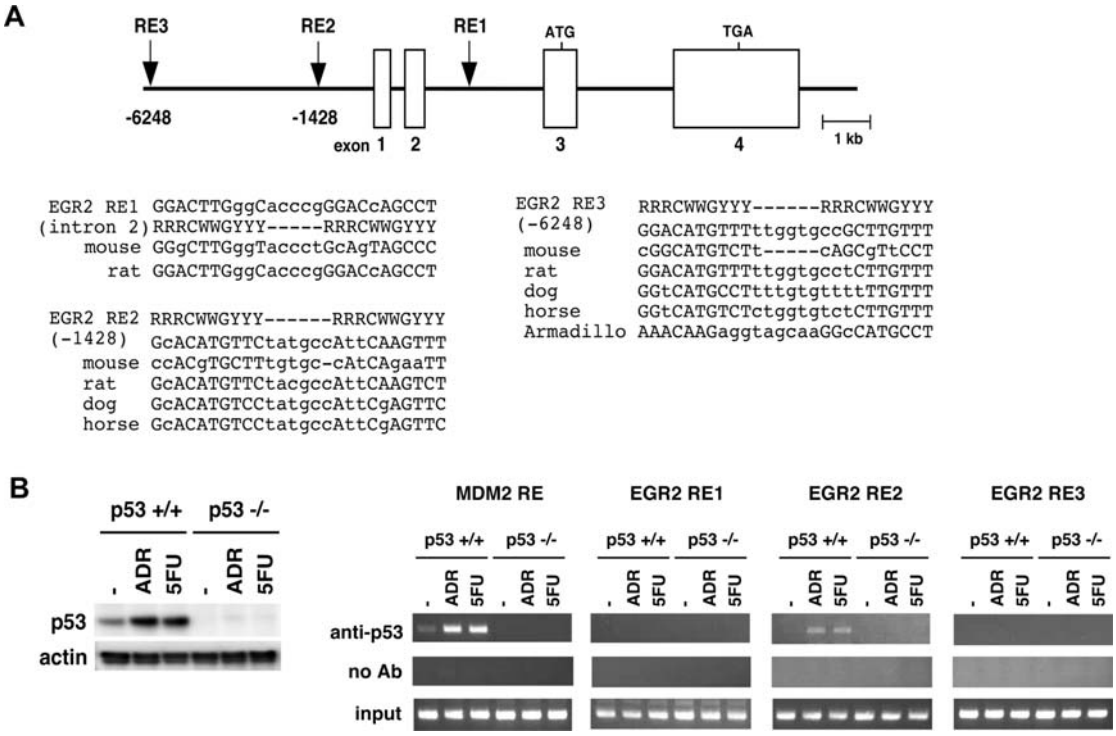

Figure 4. The response element for the p53 family in the human *EGR2* gene. (A) The position and nucleotide sequence of a response element for the p53 family in the *EGR2* gene, EGR2-RE2 and other candidates, EGR2-RE1 and EGR2-RE3. Open boxes represent exons. EGR2-RE2 is located at 1428-bp upstream of the first exon and consists of two copies of the consensus 10-bp motif of the p53-binding sequence. The consensus sequences are indicated by upper-case letters, and the spacer sequences between 10-bp motifs are indicated by -. Lower-case letters identify mismatches with the consensus sequence. Alignment of the conserved binding sites in mouse and rat sequences from the orthologous gene, are shown at the bottom. R, purine; Y, pyrimidine; W, adenine or thymine. (B) HCT116-p53$^{+/+}$ and HCT116-p53$^{-/-}$ cells were treated with 0.5 μg/ml ADR or 20 μg/ml 5FU for 24 h and subjected to immunoblot analysis with an anti-p53 Ab (left). ChIP assay for the presence of p53 protein at three candidate binding sites and the *MDM2* promoter was performed (right).

EGR2 is up-regulated after DNA damage in a p53-dependent manner. The positive regulation of p53 family members on EGR2 expression shown above was based on their over-expression. Treatment of cells with chemotherapeutic drugs promotes activation of endogenous p53 and increases the p53 protein level as a physiological response. To assess if endogenous p53 could induce EGR2 expression, HCT116 cells expressing wild-type *p53* (HCT116-p53$^{+/+}$) and isogenic mutant cells lacking *p53* (HCT116-p53$^{-/-}$) were exposed to 5FU. Treatment of HCT116-p53$^{+/+}$ cells with 5FU resulted in up-regulation of p53 and its target p21 with an increase in EGR2 mRNA and protein (Fig. 2). However, the increase of EGR2 expression is not observed in HCT116-p53$^{-/-}$ cells following the same 5FU treatment. Similarly, when we treated various cancer cells with ADR, we found an increase of EGR2 expression in A172, U2-OS, LoVo and HEK293 cells with wild-type p53, but not in Saos-2 or H1299 cells that had no wild-type p53 (data not shown). These results indicate that activation of endogenous p53 following DNA damage mediates induction of EGR2 mRNA and protein.

EGR2 expression is induced in mouse and rat fibroblasts in a p53-dependent manner. If the *EGR2* gene has an important role downstream of p53, we might expect that EGR2 induction by p53 would be conserved among species. This prediction was tested in the following series of experiments. First, we compared the effects of UV-induced DNA damage on the expression of the mouse *Egr2* in isogenic *p53*$^{+/+}$ MEFs and *p53*$^{-/-}$ MEFs. UV treatment caused, as expected, p53 activation with an induction of p21 in *p53*$^{+/+}$ MEFs, but not in *p53*$^{-/-}$ MEFs (Fig. 3A). Similar to *p21* induction, the *Egr2* expression was significantly induced in *p53*$^{+/+}$ MEFs (Fig. 3B). These results suggested that *Egr2* transcription is mainly regulated by p53 in mouse embryo fibroblasts. Second, REF-p53val135 containing a stably integrated murine temperature-sensitive mutant p53 was used to examine whether rat *Egr2* transcription was induced by wild-type p53. Total RNA was prepared from REF-p53val135 cells incubated at either 37˚C (mutant p53 conformation) or 31˚C (wild-type p53 conformation). RT-PCR analysis showed that *Egr2* expression in the rat fibroblasts was increased after shifting the treatment from 37 to 31˚C (Fig. 3C). Increased p21 levels were also observed in cells grown at 31˚C (Fig. 3C). These findings indicated that transcription of the *Egr2* gene is regulated by p53 in mouse and rat normal cells as well as human cancer cells.

Identification of a responsive element for the p53 family in the EGR2 gene. To investigate whether the *EGR2* gene is a direct target of transcriptional activation by the p53 family, we searched for the consensus p53-binding sequence in the *EGR2* gene. We found three putative p53-binding sites within 10 kb in and around exon 1 of the *EGR2* gene. These three candidate sequences were identified at nucleotide position +2822 (EGR2-RE1, intron2), -1428 (EGR2-RE2, within the promoter) and -6248 (EGR2-RE3, distal region of the core promoter), where +1 represents the transcription start site (Fig. 4A). To determine whether the endogenous

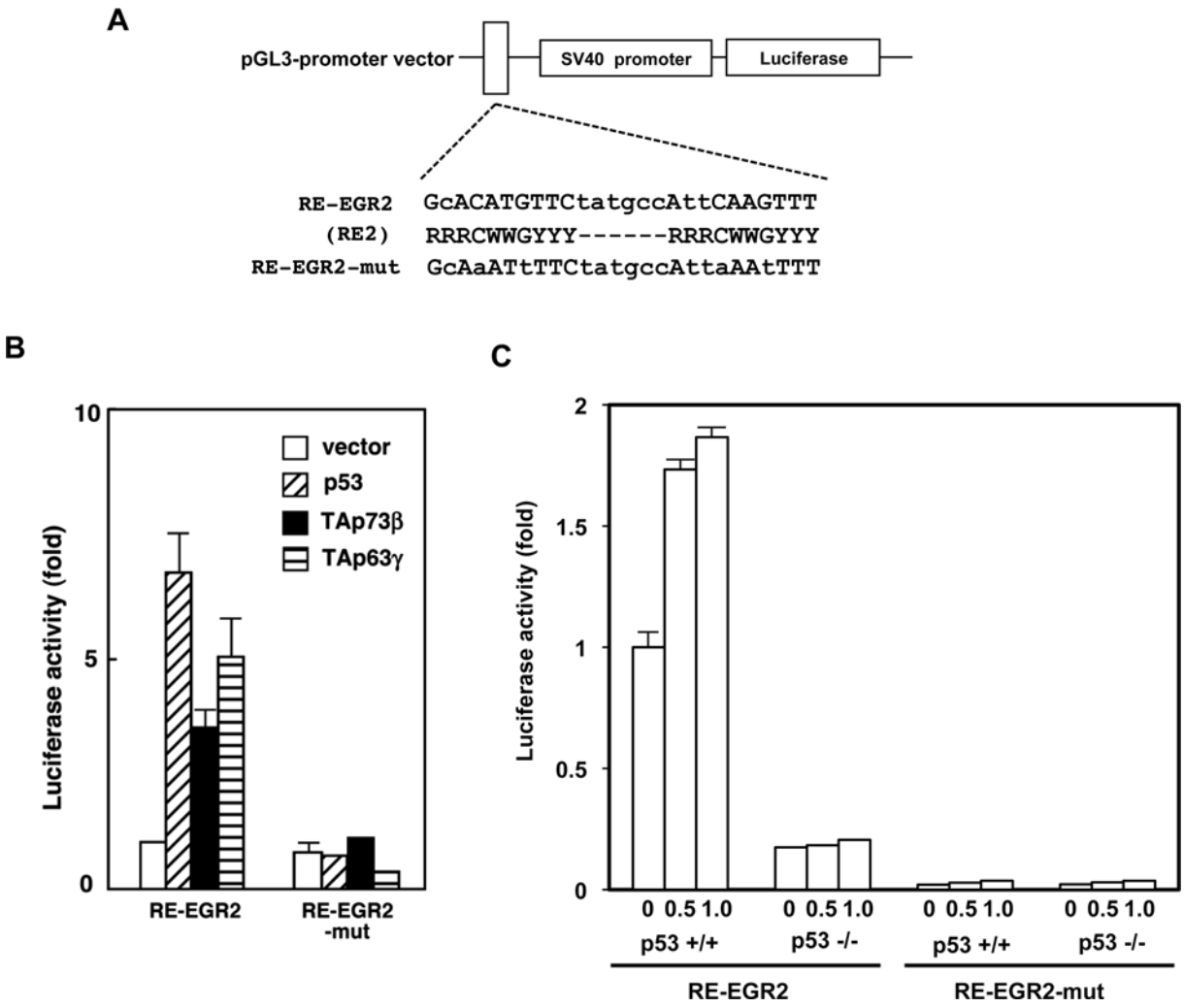

Figure 5. The RE-EGR2 sequence is responsive to the p53 family proteins. (A) The RE-EGR2 sequence (formerly named EGR2-RE2) and its mutated sequence corresponding to potentially critical nucleotides used in the luciferase assay are indicated. (B) H1299 cells were transiently transfected with the pGL3-promoter vector containing RE-EGR2 (pGL3-RE-EGR2) or its mutant (pGL3-RE-EGR2-mut) along with a transfection-control plasmid expressing *Renilla* luciferase, PhRG-TK. Cells were co-transfected with a control pcDNA3.1 vector or a vector that expresses p53 family members 48 h prior to performing the luciferase assay. Luciferase activity was measured using the Dual-luciferase reporter assay system with the *Renilla* luciferase activity as an internal control. All experiments were performed in quadruplicate, and the mean and standard deviation (SD) are indicated by the bars, respectively. (C) HCT116-p53$^{+/+}$ and HCT116-p53$^{-/-}$ cells were co-transfected with pGL3-RE-EGR2 or pGL3-RE-EGR2-mut together with PhRG-TK. At 4 h after transfection, cells were treated with 0, 0.5, or 1.0 μg/ml ADR for 24 h and subjected to the dual-luciferase assay. *Firefly* luciferase activity relative to the *Renilla* luciferase control is given. Activity in the control HCT116-p53$^{+/+}$ cells was set to 100%.

p53 protein interacts with the chromatin region containing these candidate binding sites *in vivo*, we performed a ChIP assay using HCT116-p53$^{+/+}$ and HCT116-p53$^{-/-}$ cells. ADR or 5FU treatment caused, as expected, p53 activation in HCT116-p53$^{+/+}$ but not in HCT116-p53$^{-/-}$ cells (Fig. 4B, left). Cross-linked chromatin complex from these cells was immunoprecipitated with an anti-p53 antibody. We then measured the abundance of candidate sequences within the immunoprecipitated complexes by PCR amplification. The ChIP assay revealed that endogenous p53 protein interacts with the chromatin region containing EGR2-RE2 in HCT116-p53$^{+/+}$ cells treated with DNA damaging agent (Fig. 4B, right). We designated this binding sequence as RE-EGR2 (for response element in EGR2). Other candidates were amplified in the input-positive control for PCR but not in the immunoprecipitated samples with an antibody against p53 (Fig. 4B). The *MDM2* promoters served as positive controls for p53 binding. If the *EGR2* gene has an important role in the downstream effect of p53 family members, the response elements within the *EGR2* gene would be conserved among species. Nucleotide sequence comparison revealed that the RE-EGR2 sequence is well conserved among mouse, rat, dog and horse at a nearly identical position within their genome (Fig. 4A), suggesting that EGR2 is an evolutionarily conserved transcriptional target of the p53 family member genes.

To determine whether the RE-EGR2 sequence that confers transcriptional activity depends on the p53 family members, we performed a heterologous promoter-reporter assay using a luciferase vector pGL3-RE-EGR2 prepared by cloning the oligonucleotide corresponding to RE-EGR2 upstream of a minimal promoter (Fig. 5A). H1299 cells were transiently co-transfected with pGL3-RE-EGR2 together with a p53-, p73ß-, or p63γ-expressing plasmid. Fig. 5B shows an induction

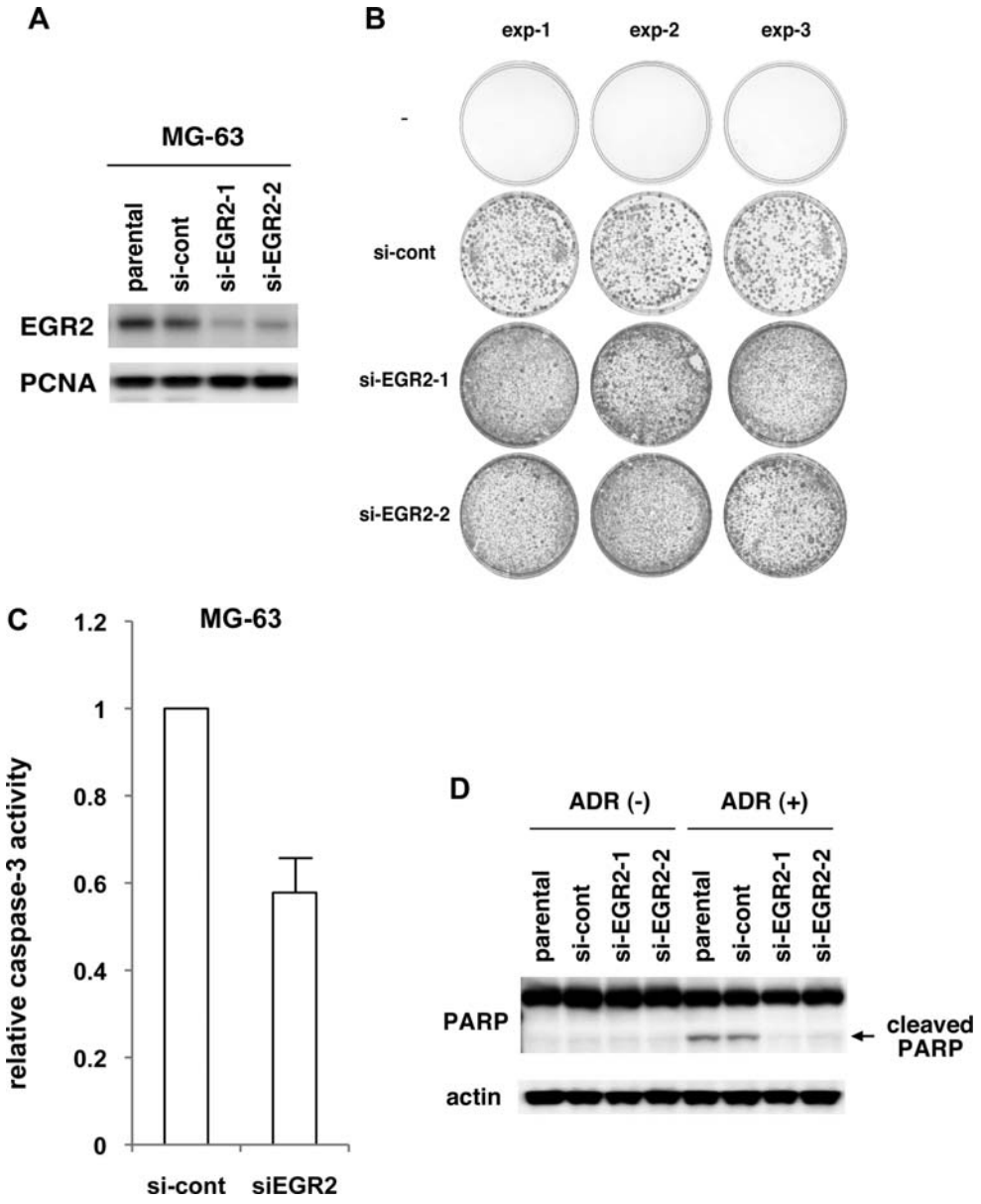

Figure 6. EGR2 siRNA protects cancer cells from DNA damage-induced apoptosis. (A) Stable transfectants of MG-63 human cancer cells were established with EGR2 siRNA plasmid (iLenti-si-EGR2-1 and -2) and empty vector (si-cont). Immunoblot analysis of the stable-transfected cells was performed with EGR2 and PCNA antibodies. (B) Colony formation assay of MG-63 cells transfected with EGR2 siRNA plasmid. MG-63 cells were transfected with EGR2 siRNA plasmid (iLenti-si-EGR2-1 and -2) or empty plasmid (si-cont) and then plated. Following 14 days of geneticin selection, cells were fixed and stained with Giemsa. The experiments were performed three times, and the representative results are shown. (C) Caspase-3 activity after ADR treatment. The stably transfected MG-63 cells were treated with or without 0.5 mg/ml ADR for 48 h, and the peptidase activity of caspase-3 was measured as the cleavage of the peptide substrate DEVD-pNA. Caspase-3 activity is indicated relative to the activity in the mock-transfected cells with ADR treatment. Data are the mean ± SD from three independent experiments. (D) Detection of PARP protein cleavage by immunoblotting. The stably transfected MG-63 cells were treated as described above. Immunoblot of PARP and control actin proteins are shown. The bands of caspase-cleaved products (89 kDa) are indicated by an arrow.

of luciferase activity from pGL3-RE-EGR2 by the p53 family, when compared to transfection of the empty vector. In contrast, the mismatches in the RE-EGR2-mut (pGL3-RE-EGR2-mut) significantly abolished the transactivation by the p53 family.

We next examined whether endogenous p53 could induce EGR2 transcription through RE-EGR2. HCT116 cells expressing wild-type *p53* (HCT116-p53$^{+/+}$) and isogenic mutant cells lacking *p53* (HCT116-p53$^{-/-}$) were transiently transfected with pGL3-RE-EGR2 or pGL3-RE-EGR2-mut and incubated with 0, 0.5, or 1.0 μg/ml ADR. The basal luciferase activity was higher in HCT116-p53$^{+/+}$ cells than in HCT116-p53$^{-/-}$ cells (Fig. 5C, compare lane 4 with lane 1). When pGL3-RE-EGR2 was transiently transfected into HCT116-p53$^{+/+}$ cells, treatment with ADR significantly induced luciferase expression from this construct (Fig. 5C, lanes 1-3). In contrast, ADR treatment did not substantially induce luciferase activity in HCT116-p53$^{-/-}$ cells. These data suggest that the RE-EGR2 sequence responds to DNA damage agents in a p53-dependent manner.

Silencing EGR2 by siRNA promotes cancer cell growth. To address the functional importance of EGR2 in human cancer cells, we introduced two siRNA vectors targeting EGR2

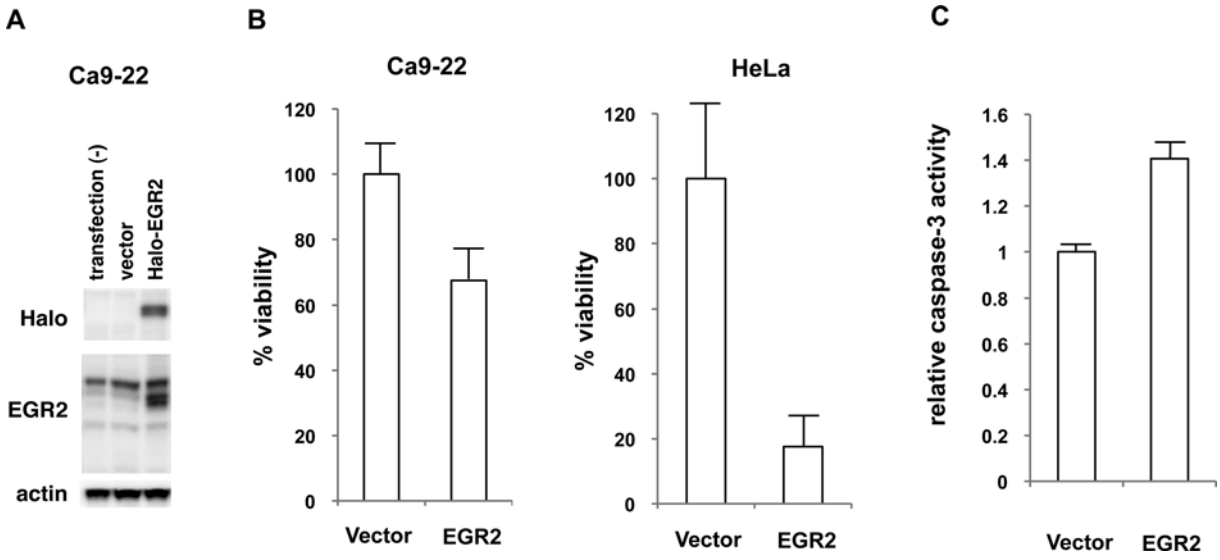

Figure 7. EGR2 suppresses the growth of human tumor cells. (A) Ca9-22 human oral cancer cells were transfected with EGR2 expression plasmid (pFN21A-EGR2) or empty vector (vector). At 24 h after transfection, cell lysates were prepared, and the expression of Halo-EGR2 protein was detected by immunoblot analysis using an anti-Halo-tag pAb. (B) Ca9-22 and HeLa cells were transfected with EGR2 expression plasmid or empty vector. After 48 h, the cell survival was subsequently determined by MTT assay. Data are the mean ± SD from three independent experiments. (C) Caspase-3 activity after 5FU treatment. The transfected Ca9-22 cells were exposed to 5FU (20 μg/ml) for 48 h, and induction of apoptosis by EGR2 was validated by caspase-3 activity. Caspase-3 activity is indicated relative to the activity in the mock-transfected cells with 5FU treatment.

(iLenti-si-EGR2-1 and -2) into MG-63 osteosarcoma cells, which express relatively high levels of endogenous EGR2. Stable transfectants were selected by growth in G418-containing medium. The expression level of EGR2 protein in two-pooled stable clones (si-EGR2-1 and si-EGR2-2) and the empty vector control (si-cont) are shown in Fig. 6A. To assess the effect of EGR2 knockdown on cell growth, we carried out colony formation assays after transfecting MG-63 cells with the EGR2 siRNA or control vector. Following transfection, a markedly higher number of cells stably expressing EGR2 siRNA vector were grown as compared with cells expressing control siRNA (Fig. 6B). Finally, to determine whether EGR2 knockdown mediated DNA-damage-induced apoptosis, apoptosis was validated by caspase-3 activity in cells treated with ADR. As shown in Fig. 6C, knockdown of EGR2 reduced caspase-3 activity after ADR treatment compared with mock-transfected cells. PARP protein cleavage is mediated by activated caspases, an event commonly used as an apoptotic hallmark. We also found that PARP cleavage was detected in parental and mock-transfected MG-63 cells after 48 h of ADR treatment, whereas the knockdown of EGR2 by siRNA vector clearly blocked the effect on cleavage (Fig. 6D). These results suggest that EGR2 can potentially inhibit the sensitivity of solid tumor cells to DNA damage-mediated apoptosis.

Overexpression of EGR2 inhibits cancer cell growth. To further obtain information about the function of EGR2, we constructed a plasmid designed to express EGR2 protein containing Halo-tag at the N-terminus (pFN21A-EGR2) and examined the effect of its overexpression in cancer cells (Fig. 7A). EGR2 was localized in the nucleus, which is consistent with its DNA-binding domain and its role in gene regulation (data not shown). We then carried out an MTT assay after transfecting pFN21A-EGR2 or empty plasmid into Ca9-22 and HeLa cells, which express only low levels of endogenous EGR2. Following transfection, cells expressing EGR2 showed markedly lower viability than that of control cells (Fig. 7B). In addition, OSC70 oral cancer cells were treated with 20 μg/ml 5FU followed by EGR2 transfection. We observed the enhancing effect of EGR2 on 5UF-induced apoptosis, as determined by caspase-3 activity (Fig. 7C). These results further support the notion that high levels of EGR2 may confer sensitivity to chemotherapy.

Discussion

We have shown that EGR2 induction by the p53 family is conserved across multiple species. The endogenous EGR2 mRNA and protein were up-regulated in human cancer cells by the ectopic p53 family (Fig. 1). In response to exposure to anticancer reagents, EGR2 was induced in human cancer cells that possess wild-type p53 activity (Fig. 2). In addition, endogenous *Egr2* was induced in mouse and rat embryo fibroblasts in a p53-dependent manner (Fig. 3). We also identified a conserved p53-response element in the *EGR2* gene (Figs. 4 and 5). These results suggest that the *EGR2* gene is a direct target of transcriptional activation by p53. Moreover, we showed that overexpression of EGR2 inhibits cancer cell growth, and knockdown of EGR2 attenuates DNA damage-induced apoptosis. Taken together, we concluded that EGR2 is one of the mediators downstream of p53 to promote apoptosis. Our results also suggest that EGR2 transfer might permit a reduction in the dose of chemotherapeutic agents and thereby minimize harmful side-effects.

The EGR family is composed of four members: EGR1 (krox24), EGR2 (krox20), EGR3 (PILOT) and EGR4 (NGFI-C). All of these members share a highly conserved

DNA-binding domain composed of three zinc finger motifs that bind and transactivate transcription from the consensus sequence GCGGGGGCG (23). EGR-mediated transcription has important roles in multiple pathways including central nervous system function and development, cancer development, T cell development and hematopoietic cell fate determination (24-28). Moreover, EGR family members have been previously implicated in apoptosis regulation. EGR1 has both pro-apoptotic and pro-survival functions depending on the cell lineage. In support of a pro-apoptotic role, EGR1 is down-regulated in various cancer cell lines and ectopic expression of EGR1 promotes apoptosis in these cells (28,29). However, EGR1 may also promote cell survival (23,30). In contrast, EGR2 promotes apoptosis, not survival, through transactivation of Bak and BNIP3L in cancer cell lines of varying origins as well as FasL and TRAIL expression in intestinal epithelial cells (19,29). EGR2 is also involved in the PTEN signaling pathway (18). Our report is the first to identify a role of EGR2 in the p53 pathway, suggesting an additional tumor-suppressive function of EGR2.

The tumor suppressor p53 is critically important in the cellular DNA damage response. Two members of the mammalian p53 family, p63 and p73, encode proteins that share considerable structural homology with p53, suggesting that the p53 family genes have a potential for functional overlap. Indeed, several p53-regulated genes can also be induced by p63 and p73, although other studies showing a marked divergence in the developmental roles of p63 and p73 significantly distinguished these two p53 family members from p53. Thus, target genes of p53 family members can be divided into at least two categories. The first category includes genes that are activated by all the p53 family genes and broadly involved in growth arrest and apoptosis (5,31). This category includes *p21*, *MDM2*, *14-3-3s*, and *Bax*. The cell context, the type of stimulus, and the quantitative balance between the diverse p53 family members could be some of the key determinants in dictating the final biological output. In the second category, target genes are selectively activated by each of the p53 family members, comprising p53, p63, p73 and their related isoforms, and mediate a specific biological activity. The present study indicates that *EGR2* might fall into the first category of genes. We have found a specific binding site for p53 family proteins, RE-EGR2, within the promoter of *EGR2* by a ChIP assay (Fig. 4). This response element is highly conserved between human and rodents. A reporter assay demonstrated that RE-EGR2 is responsive to various p53 family proteins (Fig. 5). When the critical nucleotides are mutated, the mutant p53 response element is inert. We concluded that the *EGR2* gene is a direct and evolutionarily conserved transcriptional target of the p53 family.

Among p53 family members, p73 and p63 appear to play critical roles in normal development. *p73*-deficient mice have neurological, pheromonal and inflammatory defects (32). On the other hand, *p63*-deficient mice have major defects in their limbs and craniofacial development, as well as a striking absence of stratified epithelia (33). In humans, heterozygous germline mutations in the *p63* gene are the cause of ectrodactyly, ectodermal dysplasia and facial clefts syndrome (34), suggesting that p63 is required for limb and epidermal morphogenesis. Defects in the human *EGR2* gene are associated with some types of peripheral demyelinating neuropathies including Charcot-Marie-Tooth disease type 1D (CMT1D), Charcot-Marie-Tooth disease type 4E (CMT4E), and with Dejerine-Sottas syndrome (DSS) (35-38). Moreover, the *Egr2* knock-out mouse exhibits perinatal lethality and shows a deficiency in hindbrain and bone development (39). Our findings therefore raise the possibility that p53 family member genes play a role in normal development through modulation of EGR2 expression.

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