Cloning and Characterization of the Novel Chimeric Gene p53/FXR2 in the Acute Megakaryoblastic Leukemia Cell Line CMK11-5

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KANEZAKI, R., TOKI, T., XU, G., NARAYANAN, R. and ITO, E. Cloning and Characterization of the Novel Chimeric Gene p53/FXR2 in the Acute Megakaryoblastic Leukemia Cell Line CMK11-5. Tohoku J. Exp. Med., 2006, 209 (3), 169-180 —— The loss of p53 function is a key event in tumorigenesis. Inactivation of p53 in primary tumors and cell lines is mediated by several molecular mechanisms, including deletions and rearrangements. However, generation of a p53 fusion gene has not yet been reported. Here we report a novel p53/an autosomal homolog of the fragile X mental retardation (FXR2) chimeric gene generated by an interstitial deletion. Western blot analyses have shown that the p53/FXR2 protein is indeed expressed in a Down syndrome-related acute megakaryoblastic leukemia cell line, CMK11-5 cells. To investigate the properties of the p53/FXR2 protein, we observed its subcellular localization. Flag-tagged expression vectors were transfected into COS-7 cells and the proteins were stained with an anti-Flag antibody. The p53/FXR2 protein was expressed at high levels in the cytoplasm, whereas wild-type p53 and FXR2 were localized primarily in the nucleus and in the periphery of the nucleus, respectively. Treatment with a topoisomerase II inhibitor, VP16, failed to induce expression of a p53 target gene, the cyclin-dependent kinase inhibitor p21WAF1/CIP1, in CMK11-5 cells, and transient transfection analysis showed that the p53/FXR2 protein failed to transactivate the p21WAF1/CIP1 promoter. These results suggest that the p53/FXR2 fusion protein lacks the ability of wild-type p53 to function as a transcription factor. The p53/FXR2 gene is the first reported p53 fusion gene. ——— p53; FXR2; acute megakaryoblastic leukemia; Down syndrome; fusion protein

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The p53 tumor suppressor gene plays a critical role in regulating cell proliferation, mainly through induction of growth arrest or apoptosis. p53 is a nuclear phosphoprotein induced in response to cellular stress such as DNA damage from radiation or alkylating agents. It binds DNA in a sequence-specific manner to activate transcription of a number of genes, including p21<sup>WAF-1/CIP1</sup>, mouse double mitute 2 homolog (<i>MDM2</i>), and BCL2-associated X protein (<i>BAX</i>)(Kirsch and Kastan 1998). The loss of p53 function is a key event in tumorigenesis and is associated with various characteristics of tumors, including deregulation of the cell cycle, genomic instability, and resistance to chemotherapy (Harris 1996; Kirsch and Kastan 1998). The p53 gene is frequently mutated in solid tumors (Harris 1996; Sakaguchi et al. 2005). p53 mutations are less frequent in hematological malignancies. Nevertheless, in these tumors a strong correlation was found associating p53 mutations with unfavorable prognostic factors and resistance to chemotherapy (Krug et al. 2002).

Inactivation of p53 in primary tumors and cell lines is mediated by several molecular mechanisms. The most frequent mechanisms by which the wild-type p53 is inactivated in primary human tumors are point mutations. It has been recently observed that p53 gene alterations often emerge in cell lines, even though the original tumor cells expressed wild-type p53. A relapse specimen carried an identical mutation to that of the derived cell line. It was suggested that a p53 alteration in a minor clone may confer a survival advantage to these malignant cells in vitro and presumably also in vivo (Drexler 2004). Another mechanism of p53 inactivation is through deletions and rearrangements in the p53 gene (Sugimoto et al. 1992). In cell lines, p53 gene expression was completely abrogated due to gross rearrangements and loss of normal p53 alleles. However, generation of a p53 fusion gene due to DNA rearrangements has not yet been reported.

Acute megakaryoblastic leukemia (AMKL) develops in approximately 20 to 30% of Down syndrome patients with transient myeloproliferative disorder (TMD) (Zipursky et al. 1992). Recently, acquired mutations of the <i>GATA1</i> gene have been detected in almost all cases of TMD and Down syndrome-related AMKL (DS-AMKL), suggesting that other genetic changes contribute to the development of AMKL in TMD (Wechsler et al. 2002; Mundschau et al. 2003; Groet et al. 2003; Hitzler et al. 2003; Rainis et al. 2003; Xu et al. 2003). It was reported that p53 mutations might be involved in the evolution from TMD to AMKL, because two out of three AMKL patients harbored p53 mutations, but none of the seven TMD harbored them (Malkin et al. 2000).

To understand the role of p53 mutations in the evolution of DS-AMKL, we analyzed p53 mutations in the DS-AMKL cell lines. Previously, Sugimoto et al. (1992) reported the complete lack of p53 gene expression due to gross rearrangements and loss of normal p53 alleles in the DS-AMKL cell line CMK. However, in this study we report that a fusion gene containing p53 and an autosomal homolog of the fragile X mental retardation gene (<i>FXR2</i>) (Zhang et al. 1995) was expressed in CMK11-5 cells. The p53/FOX2 fusion protein lacks the ability of wild-type p53 to function as a transcription factor. The p53/FOX2 gene is the first reported p53 fusion gene.

**MATERIALS AND METHODS**

**Rapid amplification of cDNA 3′ ends (3′RACE)**

Poly A<sup>+</sup> RNA was extracted from CMK11-5 with a Quick prep micro mRNA purification kit (Amersham Biosciences, Piscataway, NJ, USA). A Marathon cDNA amplification kit (Clontech, Mountain View, CA, USA) was used for the cDNA synthesis. 3′RACE was performed with the following primers, p53 S1 (5′ GACACTTTGCGTTCGGGCTGGGAG 3′; for the first polymerase chain reaction [PCR]) and p53 S (5′ TCTGTCCCCCTTGCCGTCCCA 3′; for the nested PCR). 3′RACE products were ligated into pCR2.1 plasmids (Invitrogen, Carlsbad, CA, USA) and their inserts were sequenced with an ABI PRISM Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was isolated using the Isogen according to the manufacturer’s recommendations (Nippon gene, Tokyo). The first-strand cDNA was synthesized from 1
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μg of total RNA using M-MLV reverse transcriptase (GIBCO BRL, Rockville, MD, USA) with random primers. cDNAs were amplified with Ex Taq DNA polymerase (TaKaRa, Ohtsu). The study was approved by the Ethics Committee of Hirosaki University School of Medicine. All clinical samples were obtained with informed consent.

PCR with genomic DNA

Genomic DNA was extracted from CMK11-5. Amplification of the p53/FXR2 fusion gene was performed with the primers: p53S (5’-TCTGTCCCTCTTGGCTCCA-3’) and FXR2 AS1 (5’-CTCCCCATAGATGCCGAAATGCA-3’) and with an LA PCR kit (TaKaRa). PCR products were sequenced and the p53 break point was confirmed.

Southern blotting

Genomic DNA samples from CMK11-5, MGS, and normal healthy subjects were digested with BamHI or SacI. The DNA fragments were electrophoresed in a 0.8% Tris-borate-EDTA buffer (TBE) agarose gel and transferred to Hybond-N (Amersham Biosciences, Piscataway, NJ, USA). Hybridizations were performed with the 32P-labelled entire p53 coding region or with a 0.4 kb fragment of the FXR2 gene. The FXR2 fragment was prepared as follows. The amplification products of the p53/FXR2 genomic DNA (see PCR with genomic DNA) were digested with HhaI, and the 0.4 kb fragment containing FXR2 exon 8 and its upstream was extracted from gels and purified.

Construction of plasmids

Flag-tagged expression vectors (Flag p53, Flag FXR2 and Flag p53/FXR2) were constructed as follows. To amplify their entire coding regions, the PCR primers p53 5´-EcoRI (5´-AGGAATTCCATGGAGGAGCCGCCCTTGGAAGTCAG-3´), p53 3´-SalI (5´-TAGTCGACGTCAGTCAGTGTCAGGCCCT-3´), FXR2 5´-EcoRI (5´-TAGAATTCCATGGGGCGGCCTGGCTCTG-3´), and FXR2 3´-SalI (5´-CCGTCGACTTTTATGAAACCCATCATC-3´) were synthesized. PCR reactions were carried out using CMK11-5 or normal human leukocyte cDNA as a template. The combinations of primers were as follows: p53 5´-EcoRI and p53 3´-SalI were for Flag p53, FXR2 5´-EcoRI and FXR2 3´-SalI were for Flag FXR2, and p53 5´-EcoRI and FXR2 3´-SalI were for Flag p53/FXR2. PCR products were digested with EcoRI and SalI, then cloned into the EcoRI-SalI site of pFLAG-CMV2 (Sigma, St. Louis, MO, USA). Obtained clones were confirmed by sequencing.

For the purpose of constructing expression vectors without Flag tags, the full coding regions were cut out from the Flag tagged expression vectors with EcoRI and XbaI, and inserted into the EcoRI-XbaI site of pcDNA3.1 (Invitrogen), resulting in p53 pcDNA3.1, FXR2 pcDNA3.1, and p53/FXR2 pcDNA3.1. Expression vectors carrying the human T24 or mutated Ha-ras (pHO6T1 and pH06N1, respectively) were described previously (Spandidos and Wilkie 1984).

For promoter assays, the luciferase reporter plasmid PICA p21–2.3k was constructed as follows. A fragment containing 2.3 kb of the p21WAF-1/CIP1 promoter region was amplified from CMK11-5 genomic DNA by PCR using the primers 5´-AGGGTACCAGGAACATGCTTGGGAGC-3´ and 5´-TGAAGCTTCCGGCTCCACAAGGA-3´. The PCR products were digested with KpnI and HindIII, then subcloned into the PICA gene basic vector (Toyo Ink., Tokyo).

Cell culture

The human megakaryoblastic cell lines CMK11-5 (Sato et al. 1989; Adachi et al. 1991) and MGS were established from Down syndrome-related acute myeloid leukemia (AML) patients. CMK11-5 was obtained from the Institute for Fermentation (Osaka). MGS was a gift from Dr. Mitsui (Yamagata University School of Medicine). The human myeloblastic leukemia cell line ML-1 was obtained from Hayashibara Biochemical Laboratories (Okayama). These lines were maintained in RPMI1640 with 10% fetal bovine serum (FBS). COS-7, a green monkey renal epithelial cell line (Japanese Cancer Research Resources Bank, Tokyo) was grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS. NIH3T3-3-4, a mouse fibroblast cell line (Riken Bio Source Center, Tsukuba), was maintained with DMEM 10% bovine serum. All cells were cultured at 37°C in 5% CO2.

Western blotting

Whole cell lysates were extracted by a triple detergent lysis buffer, as described previously (Sambrook et al. 1989). In addition, nuclear extracts were prepared from ML-1 by Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Rockford, IL, USA). Cell extracts
were separated on SDS-polyacrylamide gels and transferred to Hybond-P membranes (Amersham Biosciences). Immunodetections were carried out with anti-p53 (clone PA1801: Zymed, Carlsbad, CA, USA), anti-FXR2 (Transduction Laboratories, San Jose, CA, USA) and anti-p21 (clone F-5: Santa Cruz, CA, USA) antibodies. Dilutions used were 1:2,000, 1:250, and 1:2,000, respectively. The bands were visualized with anti-mouse HRP conjugates (Santa Cruz, CA, USA) and ECL Western blotting detection reagents (Amersham Biosciences).

**Immunofluorescence staining**

COS-7 cells were seeded at a density of 1.5 × 10^5 cells/well (in six-well plates). Following overnight culture, cells were transfected with 2 μg DNA using DMRIE-C reagent (GIBCO BRL) according to the manufacturer’s procedure. After 24 hrs, transfected cells were fixed with a 10% formaldehyde neutral buffer solution (Nacalai tesque) for 15 min. In addition, they were permeabilized with 0.1% Triton-X 100 in phosphate-buffered saline (PBS) for 10 min. They were then incubated with an anti-FLAG antibody, M2 (Sigma) at 1 μg/ml, and then anti-mouse IgG fluorescein isothiocyanate (FITC) (Dako) 1:20 at 37˚C for 30 min. The nuclei were then stained with 10 mM Hoechst 33342.

**Focus forming assay**

NIH3T3 cells were seeded at a density of 3 × 10^5 cells/60 mm dish. Following overnight culture, cells were transfected with 4 μg DNA using Lipofectamine reagent (GIBCO BRL). The medium was changed 24 hrs later. After an additional 24 hrs the transfected cells were diluted 1:20 and split into two aliquots that were added to 100 mm dishes. These cells were cultured for 14 days, changing the medium twice per week. The cells were then fixed with 10% acetic acid and colonies were stained with 0.4% crystal violet in 10% ethanol. To observe the efficiency of transfection, G418 selection was carried out at the same time.

**Treatment with a DNA damaging agent**

ML-1 and CMK11-5 cells were prepared in fresh medium at a density of 2 × 10^5/ml and cultured overnight. The following day, cells were treated with 0-1 mM VP-16 (Myers Squibb, Santa Cruz, CA, USA) for 24 hrs. The cells were then harvested and their proteins were extracted (see Western blotting).

**Reporter assay**

MG-63 cells were seeded at a density of 4 × 10^4 cells/well (12 well plate). Following overnight culture, the cells were transfected using Lipofectamine reagent (GIBCO BRL) with a total of 0.8 μg DNA per well: 0.3 μg of luciferase reporter, 0.3 μg of each expression vector, and 0.2 μg of pcDNA lacZ β-galactosidase expression plasmid. Luciferase activities were measured by the Picagene luciferase assay kit (Toyo Ink.) at 24 hrs post transfection. The results were standardized with β-gal assays (Tropix, Bedford, MA, USA).

**RESULTS**

**Isolation of cDNA for p53/FXR2 fusion transcripts**

To find the p53 gene mutations in MGS and CMK11-5 cells, we attempted to amplify the entire coding regions by RT-PCR. In the case of MGS cells, the amplification was successful, and sequencing analysis showed one mutation causing an amino acid change, C176Y (data not shown). However, none of the RT-PCR products were obtained from CMK11-5 cells. Next, we attempted to amplify five short fragments of p53. Only one fragment, which contained exons 1 through 4, was successfully amplified. We hypothesized that in CMK11-5 cells, one of the p53 gene alleles had been lost completely and the other was deleted after exon 4. We then performed 3’RACE in order to obtain the 3’ sequence of the transcript. As a result, we unexpectedly found that the FXR2 gene was fused in-frame to exon 4 of p53 (Fig. 1A). In addition, we found that codon 49 was mutated from GAT to CAT, giving rise to the amino acid change D to H (data not shown). The same alteration was previously reported in several leukemia cases (Ahuja et al. 1991; Kawamura et al. 1995).

To confirm expression of the p53 chimeric transcript, we performed RT-PCR with the p53 sense primer (p53 S) and the FXR2 anti-sense primer (FXR2 AS1 or FXR2AS2) using CMK11-5 cDNA. A PCR product having the expected size was successfully amplified, and the p53/FXR2 mRNA product was confirmed by sequencing. The p53/FXR2 mRNA was in-frame, and thus we predicted that it encoded a protein...
Fig. 1. Detection of the p53/FXR2 fusion gene in CMK11-5 cells.  
(A) The p53/FXR2 chimeric transcript detected by 3’ RACE in CMK11-5 cells. The arrow indicates the fusion point. Amino acids derived from p53 are shown in black and those derived from FXR2 are in blue.  
(B) Genomic structure of the p53/FXR2 fusion gene. The sequence surrounding the fusion point is shown. Red arrows indicate the fusion point between p53 and FXR2. The sequences derived from p53 and FXR2 are shown in black and blue, respectively.  
(C) The p53/FXR2 gene was generated by an interstitial deletion on chromosome 17. About 80 kb of the sequences between p53 exon 5 and FXR2 intron 7 was deleted. Arrows indicate the 5’ to 3’ direction of each gene.
consisting of 578 amino acids: the N-terminal amino acids 1 to 125 of p53 followed by 453 amino acids, from 221 to 673, of the FXR2 C-terminal region. The same fusion gene was detected in CMK86, the parent cell line of CMK11-5 (data not shown).

We next examined other leukemic cells for expression of p53/FXR2. RT-PCR analysis was carried out for human hematopoietic cell lines: HL-60 (myeloid), U937 (monocytic), Raji (B-lymphoid), CEM (T-lymphoid), K562, HEL (erythro-megakaryocytic) and some patient samples: eight patients with transient myeloproliferative disorder (TMD), two AML patients, and three Down syndrome-related AML patients. However, we could not detect the p53/FXR2 fusion gene (data not shown).

Genomic structure of p53/FXR2

Normally, FXR2 is approximately 50 kb downstream of p53 on chromosome 17p13.1 (Fig. 1C). We confirmed the points where p53 joined with FXR2 by means of PCR with CMK11-5 genomic DNA as template, followed by sequencing. In comparing our PCR results with the genomic sequence of chromosome 17 (accession number NT 010718), we calculated that about 80 kb between exon 5 of p53 and intron 7 of FXR2 was deleted. Furthermore, we found an insertion of the sequence AA at the fusion point (Fig. 1B). The fusion gene contains part of exon 5 from p53, but it is spliced out after being transcribed to mRNA.

These results were confirmed by Southern blot analysis using parts of the p53 and FXR2 genes as probes. Abnormal bands were detected only in CMK11-5 cells using both the FXR2 probe as well as the p53 probe (Fig. 2). In addition, the bands detected by both the p53 and the FXR2 probes in CMK11-5 cells migrated to the same position, indicating they had an identical size (Fig. 2 lanes 1 and 7, or lanes 4 and 10). These results strongly suggest there is a p53/FXR2 fusion gene in CMK11-5 cells.

Expression of the p53/FXR2 protein in CMK11-5 cells

To examine whether the p53/FXR2 protein is indeed expressed in CMK11-5 cells, we next carried out a Western blot analysis. Wild-type p53 was not expressed in CMK11-5 cells (Fig. 3 lane 3, and Fig. 5 upper panel), and FXR2 protein having wild-type sizes (95 kDa) was not detected (Fig. 3 lane 7). However, bands having identical sizes (approximately 80 kDa) were detected in CMK11-5 cells using antibodies against both p53 and FXR2 (Fig. 3 lanes 3 and 7). These results
Fig. 3. Expression of the p53/FXR2 fusion protein in CMK11-5 cells.

Western blot analysis was performed with an anti-p53 antibody (clone PAb1801: Zymed) or an anti-FXR2 antibody (Transduction Laboratories). Note that the epitope recognized by the p53 antibody is located in the N-terminal region (between amino acids 32-79 of p53). The FXR2 antibody recognizes the C-terminal amino acids 520-639 of FXR2. As controls, COS-7 cells transfected with pcDNA3.1 (Invitrogen) (lanes 1 and 5) or with the p53/FXR2 expression vector (lanes 2 and 6) were used. As additional controls, MGS cells expressing wild-type p53 and FXR2 proteins (lanes 4 and 8) were used. * Indicates non-specific band.

Fig. 4. Subcellular localization of the p53/FXR2 fusion protein.

Flag-tagged expression vectors were transfected into COS-7 cells. The Flag-tagged proteins were stained with an anti-Flag antibody, M2 (Sigma), and then with anti-mouse IgG FITC (Dako). The nuclei were stained with 10 mM Hoechst 33342. The p53/FXR2 protein was extensively distributed throughout the cytoplasm, while wild-type p53 and FXR2 were mainly localized to the nucleus and the periphery of the nucleus, respectively.
clearly demonstrate that the p53/FXR2 fusion protein was expressed in CMK11-5 cells.

Subcellular localization of p53/FXR2 protein

The p53/FXR2 protein lacks the DNA binding and C-terminal domains of p53. To investigate the function of the p53/FXR2 protein, we next examined its subcellular localization. Flag-tagged p53, FXR2, and p53/FXR2 were expressed in COS-7 cells, and the proteins were stained with an anti-Flag antibody (M2). p53 is diffusely distributed in the cytoplasm of normal, unstressed cells. However, p53 is localized in the nuclear compartment of transformed cells (Rotter et al. 1983). In COS-7 cells, which express the SV40 large T antigen, p53 was localized exclusively in the nucleus, as previously reported (Fig. 4) (Shaulsky et al. 1990). Wild-type FXR2 was localized mainly at the periphery of the nucleus, forming a punctuated pattern. However, p53/FXR2 was extensively distributed throughout the cytoplasm, suggesting functional differences distinguishing the p53/FXR2 fusion protein from p53 and FXR2.

Treatment with a topoisomerase II inhibitor, VP16, failed to induce expression of a p53 target gene, p21\textsuperscript{WAF-1/CIP1} in CMK11-5 cells

DNA-damaging agents trigger an increase in p53 expression leading to activation of particular target genes, most notably the cyclin-dependent kinase inhibitor gene, p21\textsuperscript{WAF-1/CIP1} (El-Deiry et al. 1993, 1994). This transcriptional activation of p21\textsuperscript{WAF-1/CIP1} expression is mediated by the interaction of p53 with two response elements located in the p21\textsuperscript{WAF-1/CIP1} promoter (El-Deiry et al. 1995). To study the function of the p53/FXR2 fusion protein, p21\textsuperscript{WAF-1/CIP1} expression was examined after VP16 treatment of CMK11-5 cells. We used nuclear extracts from ML-1 cells and whole cell extracts from CMK11-5 cells to detect p53, because p53 was not clearly detected when we used whole cell extracts of ML-1 cells. In ML-1 cells, which express normal p53 (Kastan et al. 1991), p21 was induced in coordination with p53 after VP16 treatment. However, no induction of p21 expression was observed in CMK11-5 cells, although the p53/FXR2 protein was expressed at a relatively high level before VP16 treatment, and

![Western blot analysis of p53 target gene, p21\textsuperscript{WAF-1/CIP1}, in CMK11-5 cells after treatment with VP16.](image)

Two cell lines, p53/FXR2 expressing CMK11-5 and normal p53 expressing ML-1, were treated with various concentrations of VP16 for 24 hours. Extracts were prepared from ML-1 cells (30 μg) and CMK11-5 (50 μg), and then separated by 10% SDS-PAGE. After electrophoresis, samples were incubated with an anti-p53 antibody (clone PAb1801: Zymed) and with an anti-p21\textsuperscript{WAF-1/CIP1} antibody (clone F-5: Santa Cruz). Western blot analysis was performed using an anti-β-actin antibody as a control for the amount of protein loaded in each lane. Note that treatment with VP16 failed to induce the expression of p21\textsuperscript{WAF-1/CIP1} in CMK11-5 cells, whereas p21\textsuperscript{WAF-1/CIP1} was induced in ML-1 cells after VP16 treatment.
Expression of p53/FXR2 Fusion Protein in Leukemic Cells

was induced after VP16 treatment of CMK11-5 cells (Fig. 5). These results suggest that the p53/FXR2 fusion protein lacks the ability of wild-type p53 to function as a transcription factor. To test this notion directly, p53 negative MG-63 cells (Masuda et al. 1987) were transfected with a luciferase reporter construct containing a 2.3 kb fragment that included the transcription initiation site for the \( p21^{\text{WAF-1/CIP1}} \) gene was transiently co-transfected with a p53, FXR2, or p53/FXR2 expression vector into p53-negative MG-63 cells by Lipofectamine. The assays were repeated three times and representative data (mean ± s.d.) are shown. Each activity was standardized by reference to the \( \beta \)-galactosidase activity from the co-transfected expression plasmid, which was driven by the CMV promoter.

\[ \text{Relative luciferase activity (\%)} \]

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<tr>
<th>Control</th>
<th>pcDNA3.1</th>
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Fig. 6. A p53/FXR2 expression plasmid failed to induce \( p21^{\text{WAF-1/CIP1}} \) promoter activity.

A luciferase reporter construct containing a 2.3 kb fragment that included the transcription initiation site for the \( p21^{\text{WAF-1/CIP1}} \) gene was transiently co-transfected with a p53, FXR2, or p53/FXR2 expression vector into p53-negative MG-63 cells by Lipofectamine. The assays were repeated three times and representative data (mean ± s.d.) are shown. Each activity was standardized by reference to the \( \beta \)-galactosidase activity from the co-transfected expression plasmid, which was driven by the CMV promoter.

was induced after VP16 treatment of CMK11-5 cells (Fig. 5).

These results suggest that the p53/FXR2 fusion protein lacks the ability of wild-type p53 to function as a transcription factor. To test this notion directly, p53 negative MG-63 cells (Masuda et al. 1987) were transfected with a luciferase reporter construct containing a 2.3 kb of the human \( p21^{\text{WAF-1/CIP1}} \) promoter and with a p53, FXR2, or p53/FXR2 expression vector. As expected, the p53 expression plasmid induced the reporter gene activity driven by the \( p21^{\text{WAF-1/CIP1}} \) promoter. However, p53/FXR2 as well as the FXR2 expression plasmid failed to induce this promoter activity (Fig. 6). These results suggest that the p53/FXR2 protein is an inactive form of the p53 transcription factor.

\( p53/FXR2 \) failed to transform NIH3T3 cells

Multiple experiments have shown that p53 mutations have gain-of-function or dominant-negative properties. To examine whether p53/FXR2 had a dominant-acting transformation activity, focus formation assays using NIH3T3 cells were performed. The efficiencies of transfection did not differ among the expression plasmids, since the number of G418 resistant colonies was almost the same for each expression vector. As expected, NIH3T3-3-4 cells were highly transformed by mutated Ras. However, no significant focus formation was observed after transfection with p53/FXR2 expression plasmids, as was seen with pcDNA3.1, p53, FXR2, and normal Ras expression plasmids (Fig. 7). These results suggest that the p53/FXR2 protein has no dominant-acting oncogenetic activity.

**DISCUSSION**

In the present study, we describe the isolation and characterization of a novel \( p53/FXR2 \) fusion gene, which is expressed in an acute megakaryoblastic cell line CMK11-5. The \( p53/FXR2 \) gene was generated by an interstitial deletion on chromosome 17. The \( p53/FXR2 \) gene encodes a protein, consisting of 578 amino acids, which lacks the DNA-binding domain of p53. In transfected cells, the p53/FXR2 fusion protein was localized mainly to the cytoplasm, whereas p53 accumulated in the nucleus. To our knowledge, the \( p53/FXR2 \) gene is the first reported p53 fusion gene.

Fragile X syndrome is caused by absence of the fragile X mental retardation protein (FMRP). FMRP and its structural homologues FXR1P and FXR2P form a family of RNA-binding proteins (FXR proteins) (Siomi et al. 1995; Zhang et al. 1995). The three proteins associate with polyribosomes as cytoplasmic mRNP particles. FXR2P, like the other members of the FXR family, contains two KH domains and an RGG box, which are common among RNA-binding proteins, and it
binds RNA with some degree of sequence specificity. In addition, FXR2P contains a functional nuclear localization signal (NLS), a nuclear export signal (NES), and a nucleolar-targeting signal (NoS) (Eberhart et al. 1996; Tamanini et al. 1999, 2000). Its subcellular localization may be determined by the net balance of NLS signals and NES activities. FXR2 is localized mainly in the periphery of the nucleus, while p53/FXR2 was found to be extensively distributed throughout the cytoplasm. Because the p53/FXR2 fusion protein lacks an NLS, the NES activity should be predominant.

The tumor suppressor gene p53 encodes a sequence-specific transcription factor that can mediate many downstream effects, such as growth arrest and apoptosis, through activation or repression of its target genes (Kirsch and Kastan 1998). The p53 protein directly transactivates a cell cycle control protein, the p21^{WAF-1/CIP1}, which is a specific inhibitor that blocks CDK2 kinase activity. DNA damaging agents such as UV irradiation and anticancer drugs were shown to increase cellular levels of p53 (Maltzman and Czyzyk 1984; Kastan et al. 1991; Fan et al. 1994). We have demonstrated here that VP16 treatment did not induce the expression of p21^{WAF-1/CIP1} in CMK11-5 cells that express only the p53/FXR2 protein, although p53/FXR2 protein expression itself was induced after VP16 treatment. Furthermore, transient transfection analysis demonstrated that the p53/FXR2 protein failed to activate the p21^{WAF-1/CIP1} promoter. Together with the findings that the p53/FXR2 protein lacks the DNA binding domain and is distributed extensively throughout the cytoplasm, these results suggest that the p53/FXR2 protein is an inactive form of the p53 transcription factor.

Although most of the effects of p53 are ascribed to its function as a transcription factor, recent reports suggest that activation of the pro-apoptotic Bcl-2 protein Bax by p53 occurs in a transcription-independent manner (Chipuk et al. 2003, 2004). p53 directly activates Bax in the absence of other proteins, causing it to permeabilize mitochondria and engage the apoptotic program from the cytoplasm in the absence of p53-induced transcription (Chipuk et al. 2004). Although the focus formation assay using NIH3T3 cells showed no oncogenic activity of p53/FXR2, it remains to be clarified whether there is a dominant negative effect of the p53/FXR2 protein on the apoptotic program in the cytoplasm. Further study will be required to deter-

![Fig. 7. Focus forming assay shows p53-FXR2 fails to transform NIH3T3 cells. NIH3T3 cells were transfected with 4 μg DNA using Lipofectamine reagent (GIBCO BRL). After an additional 48 hours of culture, transfected cells were diluted 1:20, and split into two aliquots that were added to 100 mm dishes. These cells were cultured for 14 days, changing the medium twice per week (upper and middle panels, respectively). To observe the efficiency of transfection, G418 selection was carried out at the same time (lower panel).](image)
mine if and how p53/FXR2 contributes to leukemogenesis.

Acknowledgments

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