

Identification of the p53-Responsive Element in the Promoter Region of the Human Decorin Gene

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Abstract

We have obtained some evidence that shows that the decorin gene is the target of p53 transactivation. Luciferase reporter plasmid, which contained the promoter region between positions -252 and -205, was activated by p53 dose-dependently up to 170-fold. The promoter region involved a sequence, 5'-AGGCAAGTAG-3', similar to p53-binding consensus sequence, 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3'. Chromatin immunoprecipitation assay using p53 antibodies revealed that the region between -413 and -232 of the promoter of the decorin gene was co-precipitated with p53. p53-binding to this region was further demonstrated by electrophoretic mobility shift assay, in which the complex between decorin promoter DNA and proteins decreased by pretreatment with anti-p53 antibodies. The mRNA expression levels of decorin increased after treatment with p53-activating nutlin-3 greatly and with genotoxic reagent, adriamycin, to some extent. Consequently, decorin promoter is useful to evaluate the p53 transactivation ability.

Keywords: Decorin; p53; Luciferase reporter assay

Abbreviations: ADM-Adriamycin; C/EBP-CCAAT/Enhancer Binding Protein; CPT-Camptothecin; CSPD-disodium 3-(4-methoxy Spiro[1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}] decan]-4-yl)phenyl phosphate; DMEM-Dulbecco's modified Eagle's minimum essential medium; DMSO-dimethyl sulfoxide; 5-FU-5-fluorouracil; ras-NIH3T3- Activated Ha-ras-transformed NIH3T3 mouse fibroblasts; RT-qPCR-reverse transcription-quantitative real-time polymerase chain reaction; VP-16-Etoposide.

Introduction

Mutations in the p53 gene are observed in many tumor cells most frequently [1]. p53 plays a main role in suppression of tumorigenesis because the deletion or mutation results in frequent oncogenesis [2,3]. p53 consists of N-terminal transactivation domain, DNA-binding domain and tetramerization domain. Wild-type p53 causes effects mainly as a transcription factor and regulates cell cycle, DNA repair, and apoptosis. For example, p53 induces cell cycle arrest by transactivating p21, GADD45, 14-3-3 σ , and so on [4], and induces apoptosis by direct binding to Bcl-2 and/or transactivating Bax, Noxa, Puma, and so on. [5-7]. A comprehensive search for target genes of p53 transcription factor has been attempted using p53-binding consensus sequence [8], ChIP-on-Chip [9, 10] and microarray [11]. However, despite such identification of many p53-target genes, the key molecule, which can explain the tumor suppression by p53, remains to be obscure.

In our previous study, the enforced expression of COUP-TFI in activated ras-transformed NIH3T3 mouse fibroblasts (ras-NIH3T3) resulted in growth inhibition as well as phenotypic reversion [12]. Microarray analysis suggested that the expression levels of decorin, cyclin G, lipocortin and LAMP-2 were elevated in COUP-TFI-expressing cells. Similarly, increased expression of decorin, biglycan, amyloid precursor protein and selenoprotein P was observed by enforced expression of C/EBP α and C/EBP β , which caused growth inhibition as well as phenotypic and morphological reversion [13]. Thus, decorin may play a key role not only in growth inhibition but also in tumor suppression. Similar growth-suppressive and tumor-suppressive roles were also reported for p53 [1-4]. Therefore, we examined whether p53 can transactivate the decorin gene, and found that the decorin promoter was activated by p53.

Materials and Methods

Chemicals

Adriamycin (ADM) was purchased from Merck Millipore

(Darmstadt, Germany). Camptothecin (CPT) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). 5-fluorouracil (5-FU) was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Etoposide (VP-16) was from Nippon Kayaku (Tokyo, Japan). Nutlin-3 was from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was from Wako Chemicals (Kyoto, Japan).

Plasmids

pCMV-p53WT [14] was provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute). pGL3-basic and SV40-Rluc reporter plasmids were purchased from Promega (Madison, WI). The promoter region from -1338 to -205 or from -204 to +42 of human decorin gene was amplified by PCR using genomic DNA isolated from human esophageal carcinoma cell line, T.Tn [15], as a template. After subcloning by TA cloning, the promoter regions were inserted into Xho I/Hind III site of pGL3-Basic vector (Promega) as described previously [12]. Deletion mutants such as Deco(-1237:-205)-Luc, Deco(-1037:-205)-Luc, Deco(-736:-205)-Luc, Deco(-599:-205)-Luc, Deco(-414:-205)-Luc, Deco(-287:-205)-Luc, Deco(-252:-205)-Luc and Deco(-252:-205)m-Luc were constructed by reverse PCR using KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan). All constructs were confirmed by base sequencing.

Cell lines and culture

ras-NIH3T3 [16] were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 5% bovine serum and 100 μ g/ml of kanamycin. Human glioblastoma U-87 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 μ g/ml of kanamycin [17].

Luciferase assay

The cells (1×10^5 cells/well) seeded on 24-well plates were transfected

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with p53 expression plasmids together with firefly luciferase and control Renilla luciferase reporter plasmid, SV40-Rluc, by using LipofectAMINE-Plus (Thermo Fisher Scientific, Waltham, MA). Two days after the transfection, firefly and Renilla luciferase activities were determined with a Dual Luciferase Assay System (Promega) and a luminescencer (Atto, Tokyo, Japan) as reported previously [17,18]. Firefly luciferase activities were normalized with the Renilla luciferase control activities.

Chromatin immunoprecipitation (ChIP) assay

U-87 glioblastoma cells were treated with adriamycin at a concentration of 10 μ M for 24 h, and the chromatin fraction was prepared. ChIP assay was carried out using ChIP Assay Kit (Active Motif) according to the manufacturer's instruction. Briefly, chromatin preparation was treated with DNase I, and immunoprecipitated with anti-p53 antibodies such as DO-1 and Bp53-12 (Santa Cruz Biotechnology). Using the precipitated DNA as a template, the decorin promoter region between positions -413 and -232 was amplified by PCR using primers of 5'-AACTGGTGGACAGGGAGAAAG-3' and 5'-TCGGATTCTACTTGCCTTGG-3'. The PCR products were separated by agarose gel electrophoresis.

Electrophoretic mobility shift assay (EMSA)

The DNA probe of the sequence between -256 and -232 of the decorin promoter was labeled with digoxigenin, mixed with nuclear extract of adriamycin (ADM)-treated human glioblastoma U-87 cells in the presence or absence of anti-p53 antibodies (N-19 and M-19, Santa Cruz Biotechnology), anti-NF κ B p65 antibody or unlabeled excess probes, and subjected to polyacrylamide gel electrophoresis. The DNA was transferred onto the membrane and visualized by using alkaline phosphatase-conjugated anti-digoxigenin antibody and CSPD [disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate].

Reverse transcription-quantitative real-time PCR (RT-qPCR)

U-87 human glioblastoma cells were treated with nutlin-3 (10 μ M),

5-FU (1 μ g/ml), VP-16 (500 ng/ml), CPT (10 nM), ADM (1 μ g/ml) or solvent DMSO (0.1%) for 2 h, and total cellular RNA was isolated from using the AquaPure RNA Isolation kit (Bio-Rad, Hercules, CA). Reverse transcription was performed with an oligo(dT)₂₀ primer using the ThermoScript RT-PCR System (Thermo Fisher Scientific). The expression levels of decorin transcripts were quantitated by real-time RT-PCR with the standard of GAPDH using the following primers and probes (Universal Probe Library; Roche, Basel, Switzerland).

decorin sense: 5'-GGAGACTTTAAGAACCTGAAGAACC-3'

decorin antisense: 5'-CGTTCCAACCTCACCAAAGG-3'

decorin probe: #1

GAPDH sense: 5'-AGCCACATCGCTCAGACA-3'

GAPDH antisense: 5'-GCCCAATACGACCAAATCC-3'

GAPDH probe: #60

PCR was performed using Fast Start Taq Man probe Master (Roche) and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems LLC) as follows: an initial denaturation step at 95°C for 2 min, followed by 55 cycles of denaturation at 95°C for 15 sec and annealing/ extension at 60°C for 30 sec. The mean and SD for the $\Delta\Delta$ CT were calculated and normalized to the GAPDH value as described [19].

Results

Activation of decorin promoter by p53

We examined the effects of a typical tumor suppressor p53 on the expression of the decorin gene. The reporter plasmid, decorin-Luc, was constructed by insertion of the PCR product of decorin promoter from -1338 to -205 or from -204 to +42 into the XhoI/HindIII site of pGL3-basic (Promega). These reporter plasmids as well as the deletion constructs were co-transfected into ras-NIH3T3 cells with the control Renilla luciferase reporter, SV40-Rluc, and the p53 expression

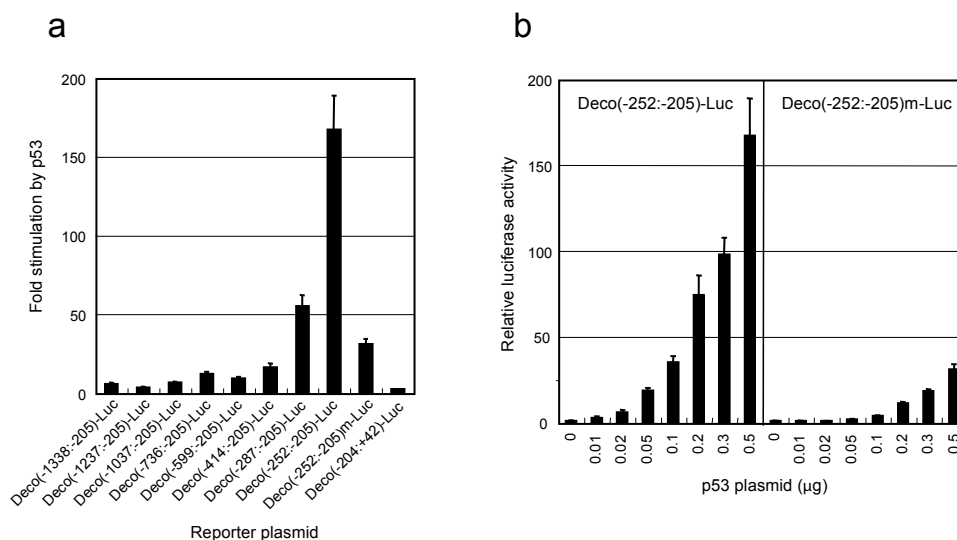


Figure 1: Activation of decorin promoter by p53. a, Comparison of various decorin luciferase reporters in activation by p53. ras-NIH3T3 cells were transfected with 0.1 μ g of firefly reporter plasmids containing decorin promoter such as Deco(-1338:-205)-Luc, Deco(-1237:-205)-Luc, Deco(-1037:-205)-Luc, Deco(-736:-205)-Luc, Deco(-599:-205)-Luc, Deco(-414:-205)-Luc, Deco(-287:-205)-Luc, Deco(-252:-205)-Luc, Deco(-252:-205)m-Luc or Deco(-204:+42)-Luc together with 0.01 μ g of SV40-Rluc Renilla reporter plasmid and 0.5 μ g of p53 expression plasmid or control empty vector, pcDNA3.1 by lipofection method. After two days of culture at 37°C, firefly and Renilla luciferase activities in cell lysates were sequentially measured. Firefly luciferase activities were normalized with the Renilla luciferase control activities, and the ratios of p53-activated levels versus empty vector-transfected levels are shown. b, Dose-response activation of decorin reporter plasmids by p53. Deco (-252:-205)-Luc or Deco(-252:-205)m-Luc was co-transfected with various amounts of p53 expression plasmid or control empty vector as described above. Total amount of p53 expression plasmid and the empty vector was adjusted to 0.5 μ g. Error bars represent SD of three independent experiments.

plasmid. The luciferase activity was increased by co-transfection with the p53 expression plasmid as compared with that of the control empty expression plasmid (Figure 1a). Most prominent activation was observed for Deco (-252:-205)-Luc, which was activated by p53 dose-dependently up to 170-fold (Figure 1b). Similar results were observed by transfection into U-87 glioblastoma cells (Figure 2). These suggest that the inhibitory sequences against p53 responsiveness exist in the region between -1338 and -252 of the promoter region of the decorin gene.

In the region between -252 to -205 of the promoter of the decorin gene, a possible p53-binding site, 5'-AGGCAAGTAG-3'; similar to the consensus sequence, 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' [20] was observed (Figure 3). We then constructed Deco(-252:-205)m-Luc of which the core CAAG sequence was mutated to AAAT. Compared to 170-fold stimulation by p53 using the wild-type Deco (-252:-205)-Luc, only 30-fold increase was observed for Deco(-252:-205)m-Luc (Figure 1a). p53 stimulated the mutant reporter much less effectively at any dose (Figure 1b).

ChIP assay verified p53 binding to the decorin promoter

U-87 cells were treated with adriamycin, and the chromatin was digested with DNase I and immunoprecipitated with anti-p53 antibody (DO-1, Santa Cruz Biotechnology). PCR amplification using co-precipitated DNA as a template revealed the DNA band of the size similar to the expected 182 bp (Figure 4). A similar result was observed using another p53 antibody (Bp53-12, Santa Cruz Biotechnology), but not without an antibody. This suggests that p53 binds to the region between -413 and -232 of the promoter of the decorin gene.

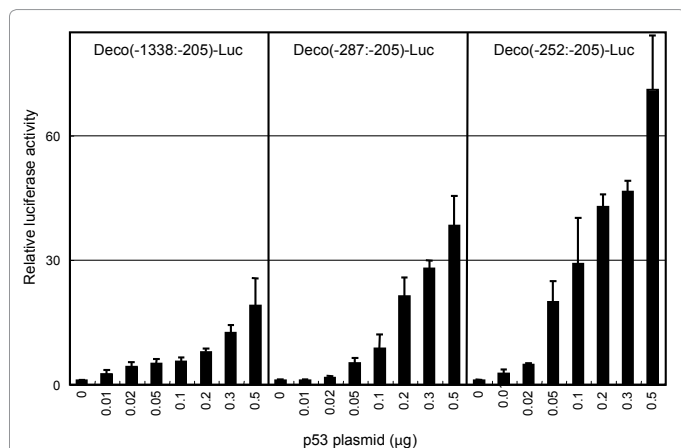


Figure 2: Dose-responsive activation of decorin promoter by p53 in U-87 glioblastoma cells. U-87 human glioblastoma cells were co-transfected with Deco(-1338:-205)-Luc, Deco(-287:-205)-Luc or Deco(-252:-205)-Luc with Renilla reporter plasmid together with various amounts of p53 expression plasmid or control vector, pcDNA3.1 as described in Figure 2. Relative activation of luciferase reporters by p53 is shown. Error bars represent SD of three independent experiments.

-287 CTCCACA GCATAGTGCT TGAATTTACA CAAACATT
 -252 CC AAGGCAAGTA GGAATCCGAA TGAGTAGTTT CAC
 -217 ATAAGGC TACTCA -205

Figure 3: Base sequence of Decorin promoter region. Base sequences of the decorin promoter region between positions -287 and -205 are shown. The sequences similar to p53-binding consensus sequence are underlined.

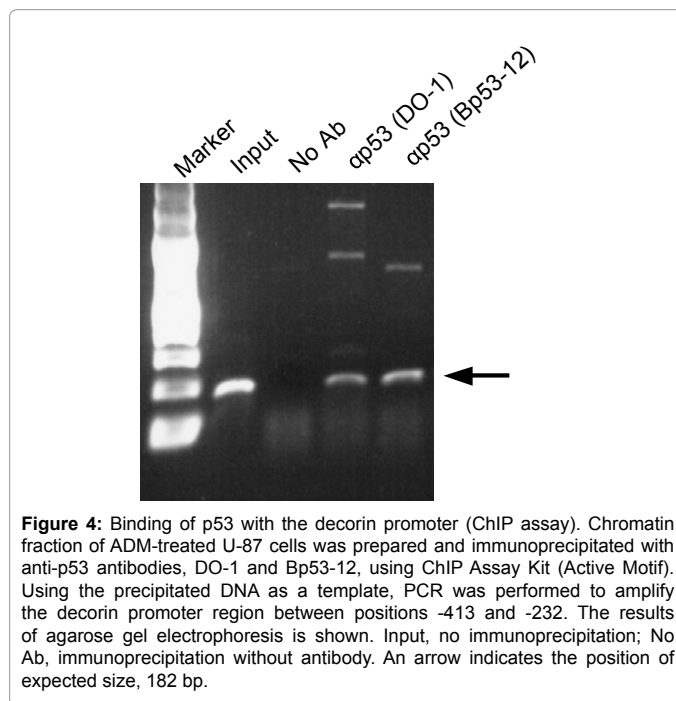


Figure 4: Binding of p53 with the decorin promoter (ChIP assay). Chromatin fraction of ADM-treated U-87 cells was prepared and immunoprecipitated with anti-p53 antibodies, DO-1 and Bp53-12, using ChIP Assay Kit (Active Motif). Using the precipitated DNA as a template, PCR was performed to amplify the decorin promoter region between positions -413 and -232. The results of agarose gel electrophoresis is shown. Input, no immunoprecipitation; No Ab, immunoprecipitation without antibody. An arrow indicates the position of expected size, 182 bp.

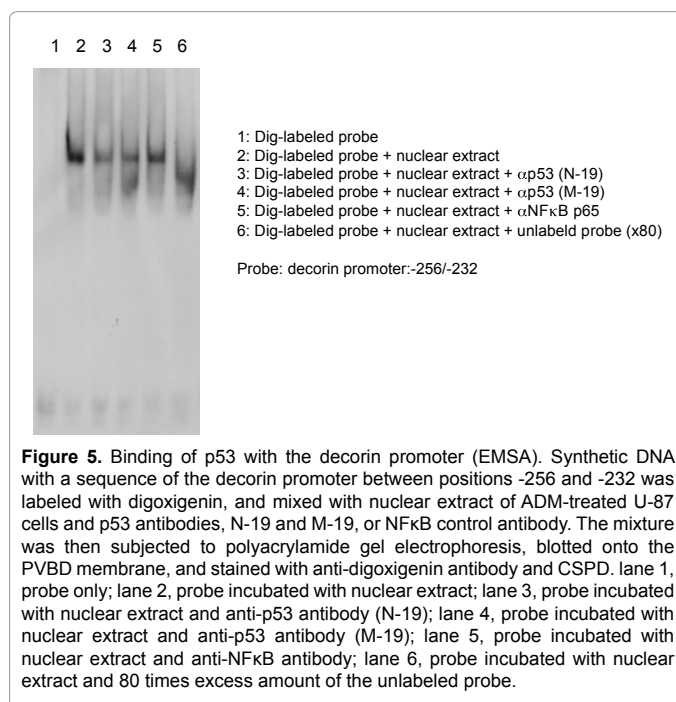


Figure 5: Binding of p53 with the decorin promoter (EMSA). Synthetic DNA with a sequence of the decorin promoter between positions -256 and -232 was labeled with digoxigenin, and mixed with nuclear extract of ADM-treated U-87 cells and p53 antibodies, N-19 and M-19, or NFκB control antibody. The mixture was then subjected to polyacrylamide gel electrophoresis, blotted onto the PVDF membrane, and stained with anti-digoxigenin antibody and CSPD. lane 1, probe only; lane 2, probe incubated with nuclear extract; lane 3, probe incubated with nuclear extract and anti-p53 antibody (N-19); lane 4, probe incubated with nuclear extract and anti-p53 antibody (M-19); lane 5, probe incubated with nuclear extract and anti-NFκB antibody; lane 6, probe incubated with nuclear extract and 80 times excess amount of the unlabeled probe.

EMSA showed the complex between p53 and the decorin promoter

The synthetic probe of decorin promoter containing -256 to -232 was labeled with digoxigenin and incubated with nuclear extract of ADM-treated U87 cells. The mixture was electrophoresed through polyacrylamide gel, and blotted onto the PVDF membrane [18], which was then treated with anti-digoxigenin antibody followed by staining with CSPD. The bands of low-mobility represent DNA-protein complexes (Figure 5, lane 2). The density decreased by pretreatment with anti-p53 antibodies, N-19 or M-19, but not with control anti-

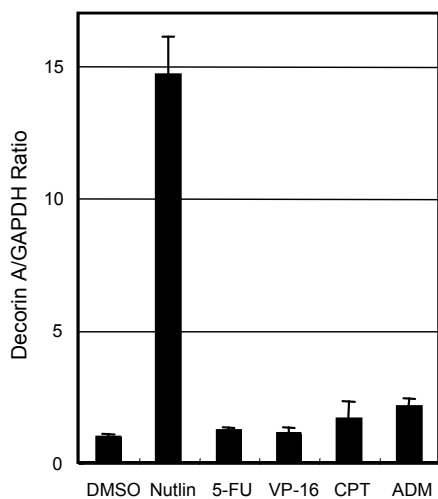


Figure 6. Elevated expression of decorin mRNA in cells treated with nutlin-3 and ADM. U-87 cells were treated with Nutlin-3, 5-FU, VP-60, CPT, ADM or the solvent DMSO for 2 h. Total RNA was isolated and RT-qPCR was performed. Relative amounts of decorin mRNA normalized with the levels of GAPDH are shown. Error bars represent SD of three independent results.

NF κ B p65 antibody (Figure 5, lane 3-5). Similar results were obtained by using different p53 antibodies such as Pab240, Bp53-12 and DO-1 (Supplementary Figure S1). This band disappeared after the addition of the excess amount of unlabelled probes (Figure 5, lane 6).

Decorin expression level was elevated by p53-activating nutlin-3

It has been well-known that p53 is activated after treatment with genotoxic anticancer drugs such as 5-FU, VP-16, CPT and ADM. p53-activating drug named Nutlin-3 has been developed as an inhibitor of MDM2 [21]. The mRNA expression levels of decorin were elevated by treatment with Nutlin-3 almost 15-fold (Figure 6). ADM also increased the level significantly but only 2.5-fold. None of 5-FU, VP-6 or CPT enhanced the decorin expression levels. Thus, certain signaling pathways leading to p53 but not by all of the p53-activating signals may induce the expression of decorin.

Activation of decorin promoter by COUP-TFI, C/EBP α , C/EBP β and C/EBP δ

Our previous microarray analysis revealed that the expression of decorin was elevated by enforced expression of COUP-TFI and C/EBP α [12, 13]. Thus, we examined the effects of COUP-TFI and C/EBP family

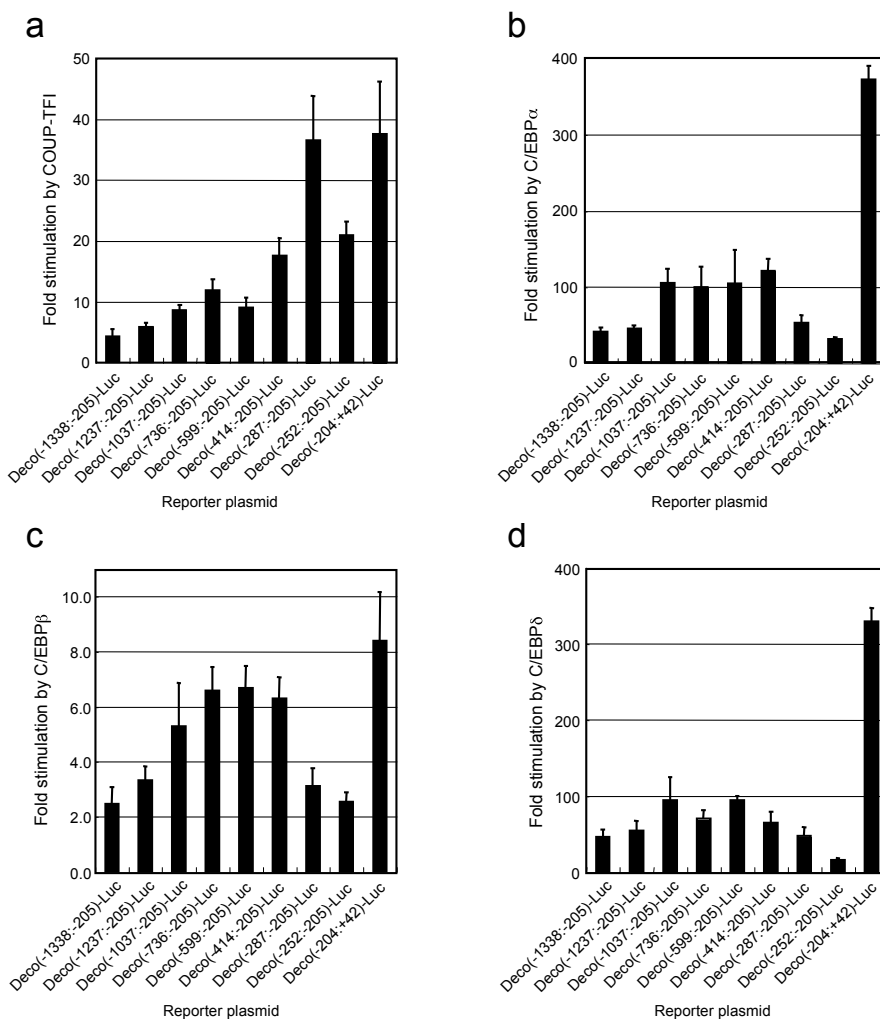


Figure 7. Activation of decorin promoter by COUP-TFI, C/EBP α , C/EBP β and C/EBP δ . ras-NIH3T3 cells were transfected with 0.1 μ g of firefly reporter plasmids containing various lengths of decorin promoter together with 0.01 μ g of SV40-Rluc and 0.5 μ g of expression plasmids of COUP-TFI (a), C/EBP α (b), C/EBP β (c) and C/EBP δ (d) or control empty vector as described in the legends of Figure 1. The ratios of co-transfected gene-activated levels versus empty vector-transfected levels are shown. Supplementary.

on the decorin promoter. The regions between -287 to -252 and between -204 and +42 of the promoter of the decorin gene were most activated by COUP-TFI (Figure 7a), whereas the regions between -414 and -287 and between -204 and +42 were responsible to C/EBP β (Figure 7c). C/EBP α and C/EBP δ activated mainly the region between -204 and +42 of the decorin promoter (Figure 7b and d). These regions were different from the region between -252 and -205 which was responsible to p53.

Discussion

We have demonstrated that the decorin gene is the target of p53 transactivation. Decorin is a member of leucine-rich proteoglycan family, and causes effects on proliferation, apoptosis, carcinogenesis and metastasis by binding to growth factor receptors such as EGFR, ErbB4 and IGF-IR [22,23]. Despite the comprehensive screening of p53 target genes [8-11], decorin has not yet been identified. The sequence, 5'-AGGCAAGTAG-3', similar to the consensus sequence, 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' [20] in the promoter region of the decorin gene may be responsible for p53 transactivation (Supplementary Figure S1) because the p53 transactivation ability was much reduced in the mutant with core CAAG replaced by AAAT. On the other hand, recent reports have shown that two repeated sequence of 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' was responsible to p53 [8]. Similar consensus sequence was also observed in the decorin promoter at positions between -275 and -266 (Supplementary Figure S1). However, Deco (-414:-205)-Luc containing this region was less responsible to p53 than Deco (-252:-205)-Luc (Figure 1a). The promoter region between positions -414 and -253 may involve binding sites for other regulatory molecules. Both basal and p53-activated expression levels of Deco (-204:+42)-Luc was very low (Figure 1a), suggesting that the region between positions -204 and +42 may not be involved in the regulation of gene expression.

Pleiotropic effects of p53 can be explained by the target molecules transactivated by p53; for example, growth-inhibition induced by p21 [4] and 14-3-3 σ [24], apoptosis induced by Bax [5], Noxa [6] and Puma [7], senescence induced by PAI-1 [25], DNA repair induced by p53R2 [26] and Ku70 [27]. We have carried out a microarray analysis of ras-NIH3T3 cells transfected with various transcription factors, and found that decorin expression was commonly elevated by enforced expression ether of COUP-TFI, C/EBP α or C/EBP β [12, 13]. Consistently, Deco (-1237:-205)-Luc was also activated by COUP-TFI, C/EBP α , C/EBP β and C/EBP δ , yet the different regions were responsible to each molecule (Figure 7). In addition to COUP-TFI [12], growth-inhibitory activity of C/EBP α and C/EBP β has been reported [28,29]. C/EBP α acts as a tumor suppressor in multiple tissues [30]. Thus, it is possible that decorin plays a key role not only in inhibition of proliferation but also in suppression of tumorigenesis. Interestingly, in the p53-binding site, AGGCAAGTAG, T and last G are SNP sites, rs13312819 and rs13312820, respectively. Further genome-wide analysis study in this region may clarify the tumor-suppressive role of decorin.

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