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Cancer Genes and Networks

Molecular Cancer Research

TP53 Mutation by CRISPR System Enhances the Malignant Potential of Colon Cancer

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Abstract

Tumor protein p53 (TP53) mutation is a well-known occurrence at the late phase of carcinogenesis during the adenoma–carcinoma sequence of a sporadic colon cancer. Although numerous reports about clinical information of the patients with colon cancer have suggested that *TP53* mutation might be related to various types of malignant potential, the direct effects of this mutation on the malignant potential of colon cancer remain unknown. Notably, no previous report has described a relationship between *TP53* mutation and cancer stemness. We therefore aimed to assess the function of a TP53 mutant induced by the CRISPR-Cas9 system in colon cancer cells. In this study, two *TP53* mutations, corresponding to exon 3 (TP53E3) and 10 (TP53E10), were generated in LS174T cells derived from a wild-type TP53 human colon cancer via a lentiviral CRISPR-Cas9 system. The loss of function of TP53 resulting from both mutations manifested as resistance to Nutlin3a-induced apoptosis and the downregulation of target genes of TP53. TP53 mutants exhibited an enhanced malignant potential, characterized by accelerated cell growth, invasiveness, chemoresistance, and cancer stemness. Interestingly, TP53E10 but not TP53E3 cells exhibited aberrant transcriptional activity of regenerating family member 1-α (*REG1A*) and expression of REG1A, resulting in the acquisition of enhanced malignant potential. In conclusion, we demonstrated for the first time that *TP53* genomic mutation into human colon cancer cells affects the malignant potential.

Implications: These findings suggest that both a loss of function and an aberrant gain of function of TP53 might promote high malignant potentials at the late phase of carcinogenesis in colon cancer.

Introduction

Colorectal cancer is the third most common cancer in Japan and worldwide (1, 2). Although the adenoma–carcinoma sequence is often used to describe the multistage carcinogenesis of sporadic colon cancer (3), the precise molecular pathogenic mechanisms underlying each stage have not been fully elucidated. Particularly, the mechanism by which a mutation in tumor protein p53 (TP53) enhances malignant potential at the late phase of carcinogenesis remains to be determined. Nevertheless, considerable clinical evidence suggests that TP53-mutated colon cancer exhibits several characteristics of a high malignant potential, including vascular invasion, chemoresistance, and a poor prognosis (4, 5). The diversity of mutation sites in TP53 may make it difficult to discern the associated molecular mechanisms. For example, a mutation in the N-terminal region might affect DNA binding and transcriptional activity (6). In contrast, a mutation in the C-terminal region might affect nuclear extra function and protein stability (7), suggesting that a stably expressed TP53 mutant with a preserved DNA binding site might have various functions.

In addition to the above issues, a system of assessing the functions of human TP53 mutants has not yet been established. As siRNA specific for *TP53* mRNA suppresses the expression of the entire TP53 protein, this manipulation cannot reflect the actual effects of a mutant TP53 protein. In addition, the overexpression of a mutant TP53 protein cannot exclude the potential effects of wild-type TP53 (TP53WT), and thus the effects of the latter might be interrupted by the function of the former. We therefore aimed to assess the function of TP53 mutants in colon cancer cells after using the CRISPR/Cas9 system to conduct *TP53* mutagenesis.

Materials and Methods

Cell culture and chemicals

The human colon adenocarcinoma–derived cell lines LS174T and HCT116, which expresses TP53WT, were cultured in DMEM (Life Technologies) and supplemented with 10% FBS and 1% Penicillin-Streptomycin. Lentiviral infection was performed according to the manufacturer's protocols and previous reports (8). Cell lines used in this study were purchased from the ATCC. All cells were free of *Mycoplasma* contamination and not authenticated after purchase from ATCC. The infected cell lines were supplemented with puromycin (5 μg/mL; Invitrogen) or Nutlin3a (5 ng/mL; Adooq Bioscience) during maintenance.

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5-Fluorouracil (5-FU; 20 μmol/L; Tocris Bioscience) was used to evaluate chemoresistance.

CRISPR-Cas9–mediated mutagenesis

Lenti-CRISPR v2 (plasmid no. 52961; Addgene), which includes *S. pyogenes* Cas9 and sgRNA in an improved one-vector system, were ligated with the target array according to the provided instructions. The target array for exon 3 of *TP53* as DNA binding site deletion model was determined according to a genome-scale CRISPR knockout (GeCKO) sgRNA library (9), because GeCKO library showed target array with minimized off-target effects only in exon 3 among upstream of DNA binding region of *TP53*. The target array for exon 10 of *TP53* as DNA-binding site preserving model was determined according to a previous study (10), in which it has been used to human normal colonic organoids for carcinogenesis model. The target array sequence is summarized in Supplementary Table S1.

The lentivirus generated from Lenti-CRISPR v2 vector with target array were infected to LS174T cells as described previously (11). As a negative control, LS174T TP53WT cells were generated by infection with the Lenti-CRISPR v2 vector lacking the target array such that these cells would also constitutively express Cas9. RT-PCR was used to confirm the expression of Cas9 (Supplementary Fig. S1). After single-cell cloning, target sequence mutation was confirmed by direct sequencing.

qRT-PCR

Total RNA was isolated using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed as described previously (11). One microliter of cDNA was amplified using a QuantiTect SYBR Green PCR Kit (Qiagen) in a reaction volume of 20 μL. The primer sequences are summarized in Supplementary Table S1. In all experiments except the work for REG1 expression, the level of expression in LS174T TP53WT cells was used as the standard. The level of REG1 expression in LS174T TP53E10 cells was used as the standard for REG1 expression analysis. The amounts of mRNA were normalized to the expression of β-actin mRNA.

Immunofluorescence analysis

The cells were fixed as described previously (12). Antibodies specific for the N-terminus of TP53 (Pab1801; Santa Cruz Biotechnology; generated by immunization via N-terminal epitope mapping of amino acids 32–79 of p53) and regenerating family member 1-α (REG1A; S-19, Santa Cruz Biotechnology) were used to label the cells. Anti-mouse IgG Alexa Fluor 594 (Invitrogen) and anti-goat IgG Alexa Fluor 488 (Invitrogen) were used as the secondary antibodies, respectively. The stained cells were mounted with VectaShield mounting medium containing DAPI (Vector Laboratories) and visualized under a confocal laser fluorescent microscope (BZ-X700; Keyence and FLUOVIEW FV10i; Olympus).

MTS assay

Cells were cultured in a 96-well tissue culture plate at a density of 1×10^4 per well. After a 24- or 96-hour incubation in the presence or absence of either Nutlin3a or 5-FU, Cell Titer96 Aqueous One Solution (20 μL/well; Promega) was added, and the cells were again incubated for 1 hour at 37°C and 5% CO_2. In each well, the absorbance at 490 nm was measured using a Glomax Discover Microplate Reader (Promega). Background absorbance was determined in wells containing medium alone and subtracted from that of the sample wells. The cell proliferation ratio was calculated as the ratio of absorbance at 96 hours to that at 24 hours. Relative viability (%) was calculated by dividing the cell proliferation ratio of the treated group by that of the untreated group.

Migration assay

The Oris Pro Cell Migration Assay kit (Platypus Technologies) was used as described previously (13). This assay was formatted for 96-well plates and used a nontoxic biocompatible gel to form a cell-free zone on cell culture surfaces. A total of 1×10^6 cells were seeded into each well and incubated for 1 hour. Premigration phase-contrast images were taken for reference. After a 12-hour incubation, additional phase-contrast images were captured. The ratio of the vacant area from pre- to postmigration was then analyzed.

In vitro sphere formation assay

LS174T cells expressing TP53WT or mutated TP53 were cultured at various densities (1,000, 500, 250, and 125 cells/well) in Matrigel GFR (Corning) with stem cell medium (SCM) in a 24-well tissue culture plate. The SCM was prepared using the following reagents: 500 mL of a 1:1 ratio of DMEM/F12 (Invitrogen), 1% Penicillin-Streptomycin, B27 supplement (Invitrogen), 4 μg/mL heparin, 1% (w/v) of nonessential amino acids, 1% (w/v) of sodium pyruvate, 1% (w/v) of L-glutamine, 10 ng/mL of FGF, and 20 ng/mL of EGF. After a 7-day culture, the number of spheroids was counted. The spheroid formation ability was calculated using a software application for extreme limiting dilution analysis (ELDA), as described previously (11).

Annexin V fluorescence analysis

Cells were cultured in a 12-well plate at a density of 1×10^4 per well either with or without 20 μmol/L 5-FU. After a 96-hour incubation, an Annexin V–Alexa Fluor594 conjugate (Invitrogen) and Hoechst 33342 (1:1,000, Lonza) were added according to the manufacturer's instructions. The cells were visualized using a confocal laser fluorescent microscope (BZ-X700; Keyence and FLUOVIEW FV10i; Olympus). ImageJ Software (NIH, Bethesda, MD; http://imagej.nih.gov/ij/) was used to assess the fluorescence intensity of 30 cells per group.

Microarray analysis

The SurePrint G3 Human GE 8 × 60K Microarray (Agilent Technologies) was used to perform a microarray analysis of LS174T TP53WT, TP53E3, and TP53E10 cells. This result was assigned the GEO accession number GSE124349. A gene set enrichment analysis (GSEA; Broad Institute) was also performed to detect specific activated cell signaling pathways, as described previously (14).

GFP reporter assay

MAT Inspector (Genomatix Software Suite; Genomatix AG) was used to identify a TP53 binding site (CATG) 869 bp upstream of ATG in the promoter region of the *REG1A* gene. Two STAT3 binding sites including the TTCC sequence were also identified at 1,552 bp and 117 bp upstream of ATG in the same promoter region. Accordingly, 1,900 bp of the *REG1A* promoter were extracted from LS174T cells by genomic PCR. This 1,900-bp fragment was then inserted into pLenti 6.4 (Invitrogen) with GFP

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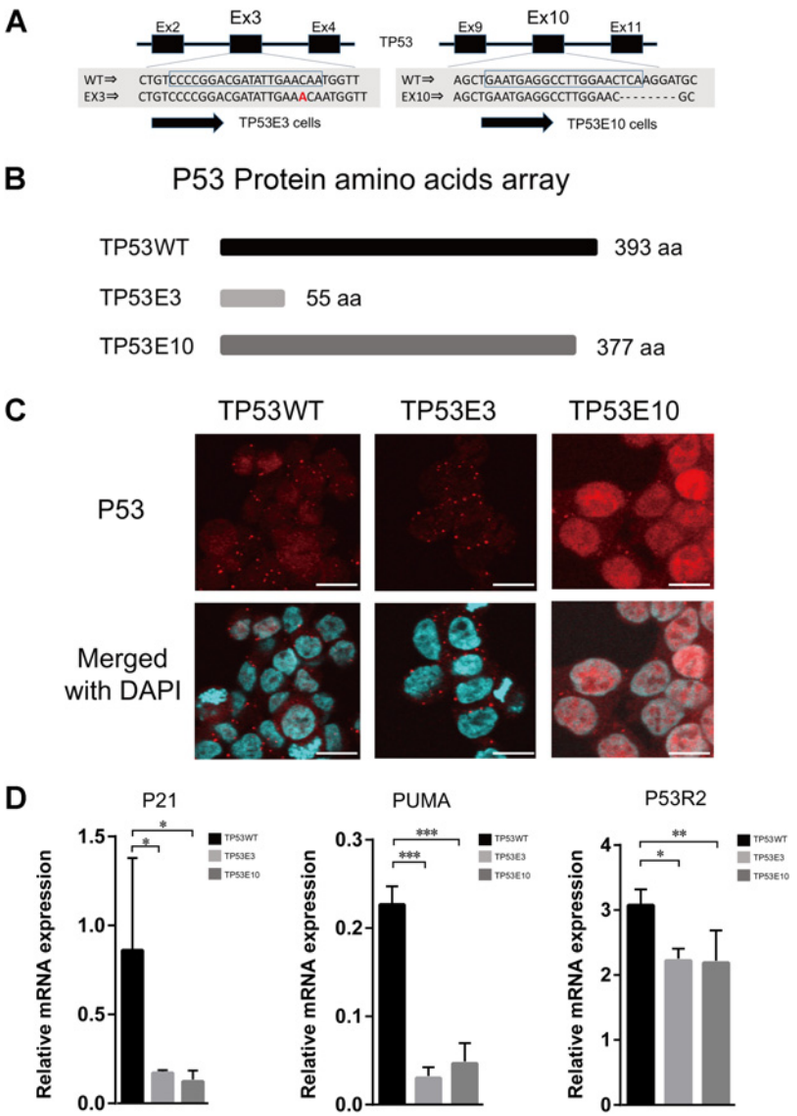

Figure 1.

Induction of a *TP53* mutation in LS174T cells using the CRISPR/CAS9 system. **A,** Schematic representation of the CRISPR-mediated mutations of exons 3 and 10 in *TP53*. The sequences of the guide RNAs in exons 3 and 10 are surrounded by squares in the top line. The mutated sequence is shown in the bottom line. A single insertion at 152 bp and 8-base deletion at 1,049 bp are indicated in TP53E3 and TP53E10, respectively. **B,** A schematic representation of the amino acids in the mutated forms of TP53. TP53E3 lacks the DNA binding domain encoded by exon 5. TP53E10 lacks the C-terminal nuclear export signal but preserves the DNA binding domain. **C,** Immunofluorescence staining reveals the nuclear localization of TP53E10 but not TP53E3 in LS174T cells. Slight TP53WT expression is shown in the nuclei. Scale bar, 25 μm. **D,** Expression of the TP53WT target genes *p21, PUMA*, and *P53R2* was significantly downregulated in both mutated TP53 cell lines as determined by RT-PCR (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $n = 3$).

(REG1Ap). A mutant *REG1A* promoter (REG1AΔp) was constructed by using PCR-mediated mutagenesis to delete the TP53 binding site as described previously. Lentivirus particles were generated by transfecting each lentivirus vector and packaging vectors into 293FT cells as described previously (11). The resulting particles were used to infect LS174T TP53WT or TP53E10 cells. One week after blasticidin selection, Hoechst 33342 (1:1,000; Lonza) was added to the cells according to the manufacturer's instructions. The cells were then visualized using a confocal laser fluorescent microscope (BZ-X700; Keyence and FLUOVIEW FV10i; Olympus). ImageJ was used to assess the intensity of GFP expression in 30 cells per well. The intensity of triple wells in each group was assessed following the average of the intensity in a well was calculated.

Statistical analysis

Student *t* test was used to statistically analyze qRT-PCR, MTS assay, migration assay, and annexin V fluorescence data. $P < 0.05$ was considered to indicate statistical significance.

Results

TP53 mutation was induced in LS174T cells using CRISPR/CAS9 system

To assess the contribution of the mutated TP53 protein to colon cancer malignancy, we selected the TP53WT-expressing human colon cancer line LS174T and HCT-116 cells. Guide RNA sequences in exons 3 and 10 of *TP53* were designed to assess the function of TP53 mutants with and without the DNA binding site (Fig. 1A). The mutated form of *TP53* was successfully introduced into LS174T cells using the CRISPR-Cas9 lentiviral vector. Following single-cell cloning, a sequence analysis revealed that the expected *TP53* mutations in exons 3 (TP53E3) and 10 (TP53E10) generated short forms of the TP53 protein (Fig. 1B; Supplementary Fig. S1A–S1D). Identical mutation in exon 3 and similar mutation in exon 10 were also generated in HCT-116 cells (Supplementary Fig. S2A and S2B). Only TP53E10 cells stably expressed the mutated TP53 protein in the nuclei, whereas TP53WT was detected at a low level in TP53WT cells. TP53E3 protein was not detected because of lack of a site recognized by a

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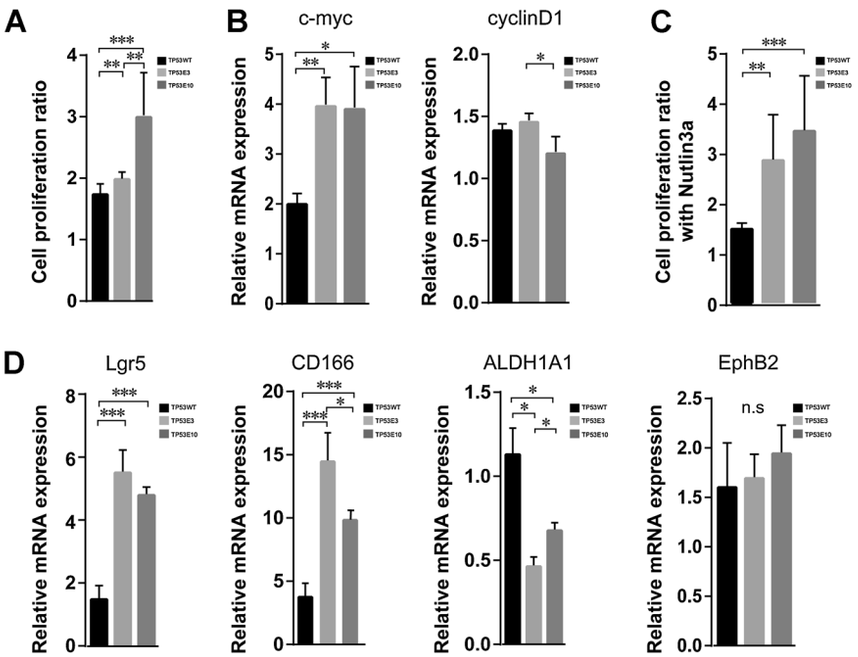

Figure 2.

TP53 mutation induces colon cancer cell proliferation and stemness. **A,** The MTS assay revealed a significant increase in cell proliferation in both TP53-mutant cell lines. The cell proliferation ratio was calculated by dividing the titer of cells cultured for 96 hours by that of cells cultured for 24 hours. **, $P < 0.01$; ***, $P < 0.001$; $n = 6$. **B,** The expression of c-myc but not cyclinD1 was significantly upregulated in both TP53-mutant cell lines (RT-PCR). *, $P < 0.05$; **, $P < 0.01$; $n = 3$. **C,** The MTS assay revealed the resistance to Nutlin3-induced apoptosis in both TP53-mutant cell lines. **, $P < 0.01$; ***, $P < 0.001$; $n = 6$. **D,** RT-PCR revealed significantly upregulated expression of *Lgr5* and *CD166* in both TP53-mutant cell lines. *EphB2* expression did not vary among all cell lines, whereas *ALDH1A1* expression was significantly downregulated in both TP53-mutant cell lines (*, $P < 0.05$; ***, $P < 0.001$; n.s, nonsignificant; $n = 3$).

TP53-specific antibody (Fig. 1C; Supplementary Figs. S1D and S2C). Both TP53E3 and TP53E10 cells exhibited reduced expression of target genes for TP53 that contribute to cell-cycle arrest (15) and apoptosis (16), thus indicating that the mutations led to TP53 protein dysfunction (Fig. 1D; Supplementary Fig. S2D).

TP53 mutation induces the proliferation and stemness of colon cancer cells

We first assessed the effects of mutated TP53 on the proliferation of LS174T cells. A MTS assay showed a significant increase in cell proliferation in TP53E10 cells relative to TP53WT cells, whereas only a slight increase was observed in TP53E3 cells (Fig. 2A; Supplementary Fig. S2E). The expression of c-myc but not cyclinD1 was induced in both TP53-mutant cell lines (Fig. 2B) whereas the expression of c-myc was not changed in HCT-116 cells (Supplementary Fig. S2F). Minichromosome maintenance complex component 7 (MCM7), which is one of the pivotal DNA replication licensing factors (17), was significantly induced in both TP53-mutated cells (Supplementary Fig. S3A). Moreover, the promotion of cell cycle was also shown in both TP53-mutated cells (Supplementary Fig. S3B), whereas apoptosis was not detected in all types of LS174T cells (Supplementary Fig. S3C), suggesting that TP53-mutated cells might be highly proliferative. To assess the effects of *TP53* mutation on apoptosis, LS174T cells were treated with Nutlin-3a, which induces MDM2-mediated apoptosis. Cell viability was maintained in both TP53E10 cells and TP53E3 cells after treatment with Nutlin-3a (Fig. 2C), suggesting that the TP53-mutant cells might also be resistant to apoptosis. To evaluate the malignant potential, we next assessed the effect of mutated TP53 on cancer stemness in LS174T cells. In a spheroid forming assay, both TP53E3 and TP53E10 cells yielded larger numbers of spheroids generated from single cells, compared with TP53WT cells. An ELDA also indicated the enrichment of cancer stem cells in both the TP53E3 and TP53E10 cell lines (Table 1; Supplementary Fig. S4A). Furthermore, the expression of leucine-rich repeat-containing G-protein–coupled receptor 5 (Lgr5) and CD166, both of which are cancer stem markers (18), was significantly induced in both TP53E3 and TP53E10 cells (Fig. 2D; Supplementary Fig. S4B).

TP53 mutation promotes various malignant potentials of colon cancer cells

We next assessed the effect of *TP53* mutation on invasiveness, a type of malignant potential, in LS174T cells. Both TP53-mutant cell lines exhibited enhanced migration when compared with TP53WT cells (Fig. 3A and B; Supplementary Fig. S4C). Expression of the human zinc transcription factor (ZEB1), a major regulator of the transforming growth factor-β–induced epithelial–mesenchymal transition (19), was significantly upregulated in both TP53-mutant cell lines (Fig. 3C; Supplementary Fig. S4D). We next evaluated chemoresistance to further confirm the malignant potential of the mutated forms of TP53 in colon cancer cells. Notably, 5-FU–induced apoptosis was suppressed in both TP53-mutant cell lines (Fig. 3D and E; Supplementary Fig. S4E). Consequently, these cell lines were more resistant to 5-FU, compared with TP53WT cells (Fig. 3F).

Table 1. Cancer stem cells are enriched by *TP53* mutation

	Number of spheroids		
Number of cells	**TP53WT**	**TP53E3**	**TP53E10**
1,000	165	221	330
500	80	139	113
250	30	51	45
125	11	17	16
Spheroid-forming frequency (95% confidence interval)	1/4,258 (1/4,785–1/3,788)	1/2,728 (1/3,004–1/2,478)	1/2,212 (1/2,417–1/2,025)
P (vs. TP53WT)	—	4.18-E09	2.07E-19
P (vs. TP53E10)	2.07E-19	1.46E-03	—

NOTE: LS174T cells were cultured at various densities (1,000, 500, 250, and 125 cells/well). The numbers of spheroids were counted after 7 days. The 95% confidence intervals for the spheroid-forming frequencies were calculated using ELDA software application. $n = 5$.

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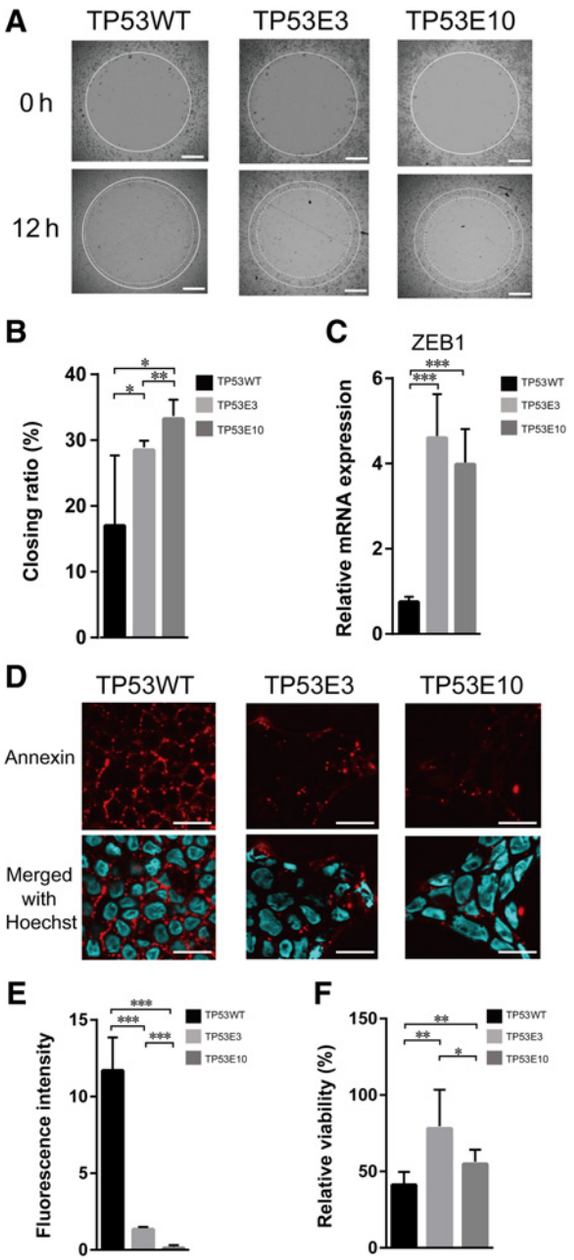

Figure 3.

TP53 mutation promotes malignant potentials. **A,** A migration assay revealed that both types of TP53-mutant cells occupied a larger proportion of the vacant area, compared with TP53WT cells. Scale bar, 500 μm. **B,** The ratios of the remaining vacant areas are shown. The vacant areas of the mutated TP53 cells were smaller than that of TP53WT cells at 12 hours after the cells were seeded. *, $P < 0.05$; **, $P < 0.01$; $n = 4$. **C,** *ZEB1* expression in LS174T cells was analyzed by RT-PCR. *ZEB1* expression was significantly upregulated in both TP53-mutant cell lines. ***, $P < 0.001$; $n = 3$. **D,** LS174T cells were treated with 5-FU (20 μmol/L) or DMSO for 96 hours to evaluate chemoresistance. After treatment with 5-FU, apoptosis was assessed using Annexin V fluorescence. Scale bar, 25 μm. **E,** Quantification of the Annexin V fluorescence intensity revealed that the mutated TP53 increased resistance to apoptosis. ***, $P < 0.001$; $n = 3$. **F,** The MTS assay revealed that treatment with 5-FU led to a less severe reduction of TP53-mutant cells, compared with TP53WT cells (*, $P < 0.05$; **, $P < 0.01$; $n = 6$).

A DNA binding site preserving *TP53* mutation induces *REG1* expression

We next assessed differences between TP53E3 and TP53E10, as our previous results had suggested some differences (Figs. 2A, 3B, and 3E; Table 1). We therefore performed a comprehensive microarray and GSEA of the TP53-mutant cells. Notably, the genes downregulated in TP53E3 and TP53E10 cells relative to TP53WT cells were nearly identical (Supplementary Table S2). The GSEA revealed similarities between the gene set related to the apoptotic signaling pathway and the downregulated genes in both TP53E3 and TP53E10 cells (Supplementary Table S3), suggesting that the latter might reflect the inactivation of an essential function of TP53WT. In contrast, the upregulated genes detected in TP53-mutant cells differed from those in TP53WT cells. Specifically, the TP53E10 cells expressed genes that were upregulated by 5-fold relative to the levels in TP53WT cells, whereas no upregulated genes were detected in TP53E3 cells because of the lack of a DNA binding site (Table 2). Therefore, the upregulation of genes in TP53E10 cells might have been induced by an irregular transcriptional function of TP53E10 mediated by nuclear DNA binding. Subsequently, we observed a strong induction of both *REG1A* and *REG1B*, which are poor prognostic markers in colon cancer (20), in TP53E10 cells relative to both TP53WT and TP53E3 cells (Table 2). We then confirmed the expression of both *REG1A* and *REG1B* only in TP53E10 cells (Fig. 4A). The expression of *REG1A* was also confirmed in HCT-116 cells (Supplementary Fig. S4F). *TP53* knockdown in TP53WT cells did not suppress the expression of both *REG1A* and *REG1B* (Supplementary Fig. S5A). We further confirmed the expression of REG1A protein only in TP53E10 cells (Fig. 4B; Supplementary Fig. S5B). Moreover, we observed increased *REG1A* promoter activity via the TP53 binding site only in TP53E10 cells. We constructed a lentiviral vector that would reflect the promoter activity of *REG1A* using a GFP reporter system (Fig. 4C). Notably, when TP53WT cells and TP53E10 cells were infected with the lentiviral REG1A-GFP reporter, only the latter cells expressed the fluorescent protein. In contrast, a mutant REG1A-GFP reporter from which the TP53 binding site had been deleted did not fluoresce, even in TP53E10 cells (Fig. 4D). A quantitative analysis of the GFP fluorescence strength yielded the same result (Fig. 4E), indicating the transcriptional activity of *REG1A* via the TP53 binding site.

Discussion

The results of this study reveal that *TP53* mutation in addition to the other oncogenes leads to the acquisition of additional malignant potential in colon cancer cells. Two types of mutated *TP53* generated using a CRISPR/Cas9 system revealed not only a dysfunction of TP53WT but also the irregular transcriptional function of mutated TP53 (Fig. 5). Although numerous studies have reported functional analyses of *TP53* mutations in human cells, nearly all have involved the knockdown of TP53WT using siRNA (21) or the overexpression of a TP53 mutant (22), both of which yield artificial effects. In a recent study, a naturally occurring *TP53* mutation in a human colon cancer cell line was knocked out by the stable expression of a TP53 short hairpin RNA using the CRISPR system to assess the function of a mutant TP53 (23). This system allowed the authors to detect differences in function between the mutant TP53 and null TP53 cell lines. Nevertheless,

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Table 2. A microarray analysis–based comparison of TP53-mutant and TP53WT cells

		Fold change ≧ 5.0	
WT vs. E10	**TP53WT**	**TP53E10**	**TP53E10/WT**
Gene name	**Normalized**	**Normalized**	**Fold change**
REG1B	0.01	10.46	774.54
REG1A	1.63	716.66	439.75
ENST00000565901	0.01	1.6	158.85
CCR2	0.61	10.19	16.83
DACH1	0.61	6.91	11.35
VCAN	0.24	1.8	7.51
CCR5	0.46	3.23	6.99
CDH11	0.64	3.47	5.43
HOXA3	0.23	1.18	5.03
		Fold change ≧ 5.0	
WT vs. E3	**TP53WT**	**TP53E3**	**TP53E3/WT**
Gene name	**Normalized**	**Normalized**	**Fold change**
	None		
		Fold change ≧ 5.0	
E3 vs. E10	**TP53E3**	**TP53E10**	**TP53E10/E3**
Gene name	**Normalized**	**Normalized**	**Fold change**
REG1B	0.03	10.46	318.39
REG1A	3.95	716.66	181.22
ENST00000565901	0.01	1.6	139.47
HOXB8	0.04	2.33	62.23
CD70	0.22	9.16	41.19
CLDN1	0.08	1.29	17.07
PDZD2	0.33	4.59	13.82
HOXB9	1.29	17.37	13.49
UGT2B15	0.14	1.61	11.3
ANGPT1	0.24	2.22	9.15
LOC101927497	0.17	1.46	8.71
HOXA3	0.14	1.18	8.25
TP53	0.29	2.21	7.53
DACH1	0.33	2.43	7.36
CDH11	0.54	3.47	6.45
PRDM8	0.55	3.48	6.31
DACH1	1.2	6.91	5.74
THBS4	0.7	3.7	5.28

NOTE: A microarray analysis revealed genes upregulated by more than 5-fold in TP53E10 cells relative to TP53WT cells (top table), no such genes were detected in TP53E3 cells relative to TP53WT cells (middle table). We also assessed genes upregulated by more than 5-fold in TP53E10 cells relative to TP53E3 cells (bottom table). Only the top three genes in TP53E10 were upregulated relative to both TP53E3 and TP53WT.

the function of a mutant TP53 relative to that of TP53WT remained unknown in human colon cancer.

We therefore used CRISPR/Cas9-mediated mutagenesis of *TP53* in human colon cancer cells, which express TP53WT, to assess the function of mutated TP53. Two forms of mutated TP53 with (TP53E10) or without (TP53E3) the DNA binding site revealed both losses and gains of function of TP53 in human colon cancer cells. TP53E3 cells expressed a very short form of TP53 and suppressed the expression of TP53WT target genes such as *p21* and *PUMA*, suggesting that the function of this mutant might be equal to that of a *TP53* knockdown. TP53E10 similarly suppressed TP53WT target genes, suggesting that the function of this mutant includes a loss of TP53WT function. In addition, the function of TP53E10 might include a gain of function, exemplified by irregular transcriptional activity via the DNA binding site.

Regarding the loss of TP53WT function, we focused on commonalities between the two mutant forms of TP53. We expected that *TP53* mutation would suppress apoptosis and promote proliferation because TP53WT induces the transcription of genes encoding PUMA and p21, which play roles in apoptosis and cell-cycle arrest, respectively. Interestingly, an increase of cancer stem cells with the induction of cancer stem cell markers was observed in both mutants, suggesting that cancer stemness might be induced by a loss of function of TP53WT but not a gain of function of the TP53 mutants. A recent report indicated the induction of Lgr5 in SW480 cells by *TP53* mutation as gain of function by comparing that of TP53 knockdown (23). However, Lgr5 expression has not been assessed in cells harboring TP53WT. Moreover, shRNA-mediated knockdown yielded an incomplete deletion of TP53, suggesting that the function of TP53WT regarding the suppression of Lgr5 expression might not have been completely eliminated. In this study, we found that a TP53 mutant lacking the DNA binding site significantly induced the expression of not only Lgr5 but also CD166 relative to TP53WT, which suggests a loss of function of TP53WT. In addition, we were the first to identify a relationship between CD166 and *TP53* mutation. CD166, a cancer stem cell marker, has been reported to correlate with a poor prognosis and advanced clinicopathologic characteristics in patients with colorectal cancer (24), suggesting that the expression of this marker might cause a loss of function of TP53 at a late stage of the adenoma–carcinoma sequence. Furthermore, a GSEA revealed the loss of function of TP53 in both TP53-mutant colon cancer cell lines, as indicated by the downregulation of common gene sets. Interestingly, two

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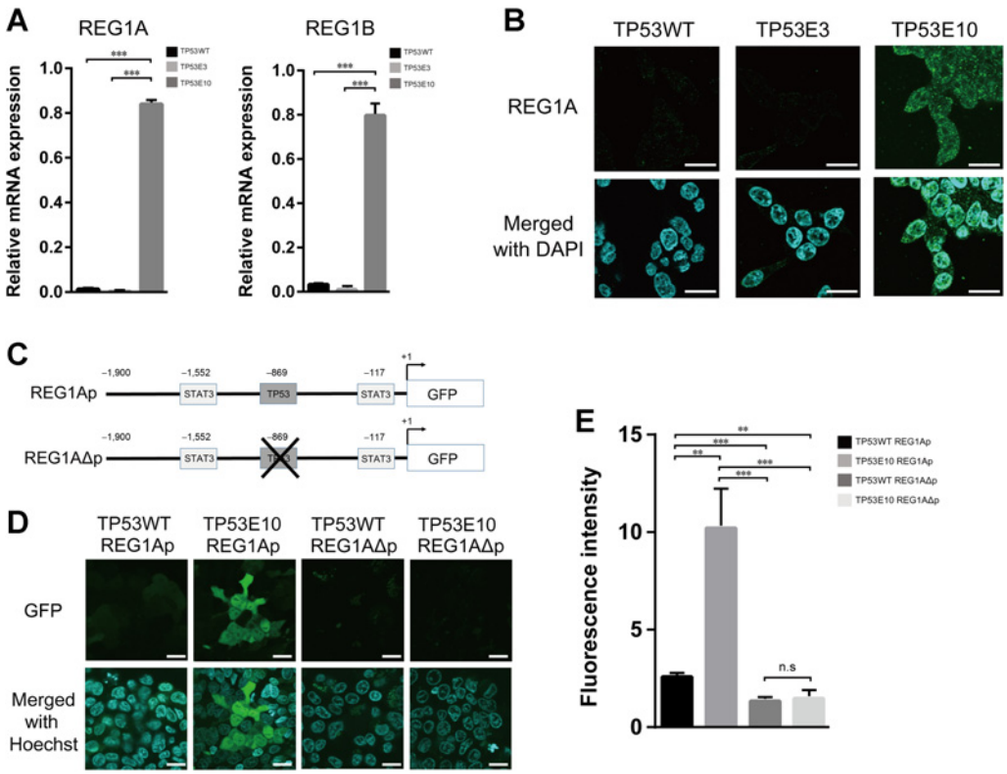

Figure 4.

TP53 mutation with a preserved DNA binding site induces *REG1* expression. **A,** The expression of *REG1A* and *REG1B* in LS174T cells was analyzed by RT-PCR. Both genes were upregulated significantly only in TP53E10 cells. ***, $P < 0.001$; $n = 3$. **B,** Immunofluorescence staining of REG1A protein revealed expression only in LS174T TP53E10 cells. Scale bar, 25 μm. **C,** Schematic representation of the construction of a *REG1A* reporter system. A 1,900-bp fragment of the *REG1A* promoter region, which contains two STAT3 binding sites and one TP53 binding site, was combined with GFP (REG1Ap). A mutant *REG1A* promoter was then generated by deleting the TP53 binding site by mutagenesis (REG1AΔp). **D,** The cells were infected with a lentivirus containing either REG1Ap or REG1AΔp. GFP fluorescence in living cells was detected using a fluorescence microscope. Scale bar, 25 μm. **E,** The GFP fluorescence intensity was quantitatively analyzed (**, $P < 0.01$; ***, $P < 0.001$; $n = 3$).

gene sets involved in intestinal epithelial cell differentiation–related genes were downregulated in both mutated TP53 cells, indicating that TP53WT might promote the differentiation of cancer cells. In normal cells, TP53WT has been suggested as a contributor to the process of switching off pluripotency upon differentiation (25), because TP53WT in the colon cancer cell might contribute to the transition of cancer stem cells to differentiated cancer cells. In Fig. 4, gene set for cell junction also confirmed invasiveness with ZEB1 expression by both *TP53* mutations. Taken together, TP53-mutant cancer cells might acquire malignant potentials such as cancer stemness, dedifferentiation, chemoresistance, and cell migration due to a loss of function of TP53WT.

To evaluate potential gains of function of the mutated forms of TP53, we next focused on differences between TP53E3 and TP53E10. Although the malignant potentials of cells expressing these mutated forms were nearly identical, some data (Figs. 2A, 3B, and 3E; Table 1) indicated that TP53E10 might exhibit a relatively stronger malignant potential. The mutation of *TP53* in TP53E10 cells started on the domain for the tetramerization (Supplementary Fig. S1C), which is required for proper conformation and transcriptional activity of TP53 (26), suggesting that aberrant transcriptional activity of TP53E10 might be induced by the conformational change of TP53 complex (27). We then searched for genes upregulated in TP53E10 cells relative to both TP53WT and TP53E3 cells. In colon cancer cells, only *REG1A* and *REG1B* met the criterion of a gain of function in this context. REG1A has been reported as a prognostic marker of colorectal cancer and is associated with peritoneal carcinomatosis (20), suggesting that this protein might induce malignant potential via a gain of function of mutated TP53. Especially, it has been reported that the expression of REG1A was significantly associated with TP53 overexpression in the patients with ulcerative colitis (28, 29), indicating that REG1A might involve in the development of ulcerative colitis–associated carcinogenesis with TP53 mutation. In further support of this hypothesis, REG1A has also been reported as a prognostic marker in other primary malignancies (30–32).

Although this study has revealed the functions of typical *TP53* mutations involving the maintenance or loss of DNA binding ability in human cancer cells as representative model for *TP53* mutation with or without DNA binding site, there are some limitations of note. First, very few human colon cancer cell lines express TP53WT. To confirm our findings, we achieved similar results in another human colon cancer cell line, HCT-116 (Supplementary Figs. S2 and S4); nevertheless, our results should be confirmed in additional cancer cell lines and primary human TP53WT cancer cells to determine whether the observations

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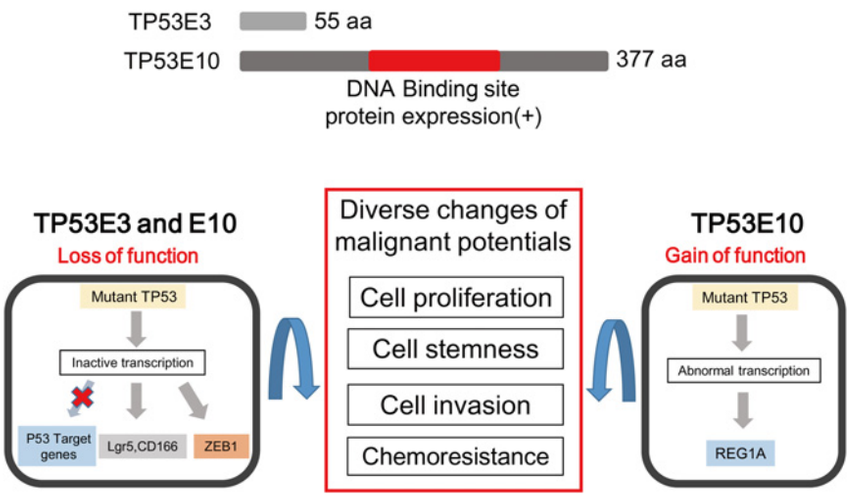

Figure 5.
Schematic representation for the function of *TP53* mutations in colon cancer cells. Two types of *TP53* mutation with or without DNA binding site were generated by the CRISPR-mediated mutation. As loss of function of mutated TP53, cancer stemness by the expression of Lgr5/CD166, and invasiveness by the expression of ZEB1 were newly identified in this study. Moreover, as gain of function of mutated TP53 with DNA binding site, the expression of REG1A, which promotes malignant potential, was newly identified by the comparison between two types of *TP53* mutation.

represent universal functions of *TP53* mutations. Especially, the irregular transcriptional activity should be confirmed as a gain of function of a TP53 mutant; also, whether the genes targeted by the TP53 mutants are specific to human colon cancer or are universally regulated among other primary malignancies should be confirmed. Moreover, it needs to be careful to mention whether the changes of expression by *TP53* mutation are due to loss or gain of function of TP53. Certainly, there are a lot of genes whose expression was suppressed by TP53WT (33, 34). We have found downregulated genes only in TP53E10 cells but not in TP53E3 cells (data not shown), suggesting that these genes might be suppressed as a consequence of gain of function of TP53. Although this model could classify the genes into whether a consequence of loss or gain of function of TP53, it should be individually confirmed whether each gene was directly changed because of loss or gain of function of TP53. Second, our *TP53* mutation models in Exon3 and Exon10 do not represent the majority of *TP53* genomic mutation, which is point mutation in the DNA binding domain (4). In addition, *TP53* mutation in clinical samples is quite variable and these mutations have not been well known about the ability of DNA binding and transcriptional activity individually. In future, TP53 mutants detected in clinical samples should be investigated using a CRISPR knock-in approach and multiple cancer cells to clarify the function of each TP53 mutant including the ability of DNA binding and transcriptional activity.

In conclusion, with this study, we are the first to generate a CRISPR-mediated *TP53* mutation in human colon cancer cells, which allowed us to evaluate both losses and gains of function of TP53 during the late phase of the adenoma–carcinoma sequence. Moreover, we identified CD166 and REG1 as novel targets of the loss and gain of function of TP53, respectively. A deeper analysis of these findings might be contributing to an understanding of the progression to advanced cancer, which is critical to determining the prognosis of patients with colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Watanabe, K. Tsuchiya
Development of methodology: S. Watanabe, K. Tsuchiya, T. Shirasaki
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Watanabe, K. Tsuchiya, R. Nishimura, N. Katsukura, S. Hibiya
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Watanabe, K. Tsuchiya, S. Hibiya
Writing, review, and/or revision of the manuscript: S. Watanabe, K. Tsuchiya
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Watanabe, K. Tsuchiya
Study supervision: R. Nishimura, T. Shirasaki, N. Katsukura, R. Okamoto, T. Nakamura, M. Watanabe

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