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Late Cornified Envelope Group I, a Novel Target of p53, Regulates PRMT5 Activity[1]

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Abstract

p53 is one of the most important tumor suppressor genes involved in human carcinogenesis. Although downstream targets of p53 and their biologic functions in cancer cells have been extensively investigated, it is still far from the full understanding. Here, we demonstrate that *Late Cornified Envelope Group I* (*LCE1*) genes, which are located in the *LCE* gene clusters encoding multiple well-conserved stratum-corneum proteins, are novel downstream targets of p53. Exogenous p53 overexpression using an adenoviral vector system significantly enhanced the expression of *LCE1* cluster genes. We also observed induction of LCE1 expressions by DNA damage, which was caused by treatment with adriamycin or UV irradiation in a wild-type p53-dependent manner. Concordantly, the induction of *LCE1* by DNA damage was significantly attenuated by the knockdown of p53. Among predicted p53-binding sites within the *LCE1* gene cluster, we confirmed one site to be a p53-enhancer sequence by reporter assays. Furthermore, we identified LCE1 to interact with protein arginine methyltransferase 5 (PRMT5). Knockdown of LCE1 by specific small interfering RNAs significantly increased the symmetric dimethylation of histone H3 arginine 8, a substrate of PRMT5, and overexpression of LCE1F remarkably decreased its methylation level. Our data suggest that LCE1 is a novel p53 downstream target that can be directly transactivated by p53 and is likely to have tumor suppressor functions through modulation of the PRMT5 activity.

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Introduction

p53 is the most frequently mutated tumor suppressor gene involved in human cancers [1,2]. Its tetramer protein product can activate the transcription of a number of target downstream genes and mediate a variety of biologic functions through the transcriptional regulation of those targets [3]. To elucidate the critical roles of p53 in human carcinogenesis, we and others have attempted to identify p53 target genes through multiple approaches. We have mainly applied the expression profile analysis after the exogenous introduction of wild-type p53 into cancer cells using the adenovirus vector system and identified more than 50 p53 downstream candidate genes [4]. Among them, we have performed the functional analysis of more than a dozen of target genes including *p53R2*, *p53AIP1*, and *p53RDL1* [5–8]. Here, we report the characterization of the Late Cornified Envelope Group I (LCE1) as a novel downstream target of p53.

The *LCE* clusters contain multiple well-conserved genes encoding stratum-corneum proteins [9,10] and are located on chromosome 1q21 in a region called as the epidermal differentiation complex [11,12]. This region is enriched for genes, which are expressed during

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[1] This article refers to supplementary materials, which are designated by Tables W1 to W6 and Figures W1 to W6 and are available online at www.neoplasia.com.

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epidermal differentiation, including *loricrin*, *involucrin*, *filagrin*, the small proline-rich protein genes, and the *LCE* genes [9,13]. In mice, members in the LCE1 group are expressed in the relatively late stage of epithelial development and incorporated into the cornified envelope through cross-linking by transglutaminases [10]. In addition, real-time quantitative polymerase chain reaction (qPCR) analysis demonstrated that human *LCE1* and *LCE2* genes were primarily expressed in skin, whereas LCE4 and LCE5 gene expressions were undetectable in any human tissues examined [9]. In general, physiological functions of LCE proteins, especially their involvement in human cancer are still largely unknown.

Protein arginine methyltransferases (PRMTs) constitute of a large family of enzymes having the arginine methyltransferases activity responsible for catalyzing the formation of monomethyl arginine, asymmetric dimethyl arginine, and symmetric dimethyl arginine (SDMA) [14]. PRMT5 is one of the most well-characterized family members with SDMA activity and catalyzes formation of SDMA in proteins with a glycine and arginine-rich motif [15]. PRMT5 was reported to regulate various cellular functions including apoptosis, Golgi structure, pluripotency, cell growth, and snRNP biosynthesis [16–18]. One important key marker of the PRMT5 activity is the symmetrical dimethylation of histone 3 arginine 8 (H3R8me2s) level. Through hypermethylation of histone H3R8 around the promoter regions, PRMT5 could cause the transcriptional silencing of cell cycle regulator genes [19,20]. Since overexpression of PRMT5 has been reported in various types of human cancer, including melanoma, leukemia, lymphoma, glioma, as well as ovarian, breast, prostate, and lung cancers [16,21–23], this enzyme is considered as a good molecular target for development of novel cancer therapy [16].

In the present study, we demonstrate that LCE is a novel direct target of p53, can interact with PRMT5, and might modulate histone H3 methylation by PRMT5. This mechanism may be important for the interplay of two important cancer-related genes, *p53* and *PRMT5*, and our findings could indicate a possible role of LCE1 in human carcinogenesis.

Materials and Methods

Microarray Analysis

Replication-deficient recombinant adenovirus designed to express wild-type p53 (Ad-p53) or LacZ (Ad-LacZ) was generated and purified, as previously described [5,24]. Microarray analysis was then carried out as previously described [1,4,24]. In brief, poly(A)+ RNAs were isolated from U373MG cells at different time points after infection with Ad-p53 or Ad-LacZ. Each RNA sample was labeled and hybridized to a microarray consisting of 36,864 genes or Expressed Sequence Tags (ESTs) (http://www.ncbi.nlm.nih.gov/geo/index.cgi, Accession No. GSE14953).

Cell Culture and Transfection

Human cell lines, U373MG (glioblastoma), H1299 (lung carcinoma), and Human Embryonic Kidney 293T (HEK293T), were purchased from American Type Culture Collection (ATCC, Manassas, VA). HCT116 $p53^{-/-}$ and HCT116 $p53^{+/+}$ cell lines were obtained from Dr Bert Vogelstein (Johns Hopkins University, Baltimore, MD). U373MG glioblastoma cells and H1299 lung cancer cells were infected with Ad-p53 or Ad-LacZ at various multiplicity of infection (MOI) conditions and incubated at 37°C until the time of harvest. HEK293T cells were transfected with HA-Mock (empty vector of pCAGGSnHC) or HA-LCE1F (pCAGGSnHC-LCE1F) using FuGENE6. For protein-protein interaction experiments, HEK293T cells were transfected with HA-Mock or HA-LCE1F and FLAG-Mock or FLAG-PRMT5 using FuGENE6. To examine the co-localization, HCT116 $p53^{+/+}$ cells were transfected by HA-Mock or HA-LCE1F using FuGENE6. For the gene reporter assay, U373MG (mutated p53) and H1299 (p53 null) cells were transfected with a reporter plasmid and either a Mock empty vector of pcDNA3.1+ or a wild-type p53 expression pcDNA3.1+ vector in combination with a pRL-CMV vector using FuGENE6 [25,26]. Small interfering RNAs (siRNAs) that were commercially synthesized by Sigma-Aldrich (St Louis, MO) were transfected with Lipofectamine RNAiMAX reagent (Life Technologies, Carlsbad, CA). Two siRNA oligonucleotides of *LCE1A-F* were designed to target the region commonly conserved among *LCE1A-F*. Sequences of oligonucleotides are shown in Table W1. Western blot or real-time qPCR was applied to validate the efficiency of overexpression or knockdown experiments.

DNA-Damaging Treatments

Cells were seeded 24 hours before treatment. When cells reached 60% to 70% confluency, cells were incubated with 1 μg/ml adriamycin for 2 hours followed by further incubation of the drug-free medium. Then, the cells were harvested at different time points as indicated in the figure legends. For UV irradiation (UVR) experiments, the cells were washed twice with phosphate-buffered saline (PBS) and exposed to UV rays at different doses using XL-1500 Spectrolinker (Spectronics, Westbury, NY; peak emission, 254 nm). Cells were harvested 36 hours after UVR treatment.

Real-Time qPCR

Total RNA was isolated from cultured cells using RNeasy mini-spin column kits (Qiagen, Hilden, Germany) according to the manufacturer's procedure. cDNAs were synthesized with the SuperScript Pre-amplification System (Life Technologies). Real-time qPCR was conducted using the SYBR Green I Master on the LightCycler 480 (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The primer sequences used in this manuscript are shown in Tables W1 and W2. Primers for *LCE1A*, *LCE1C*, *LCE1E*, and *LCE1F* were described previously [9]. Primers for *LCE1B*, *LCE1D LCE3A*, *LCE3B*, *LCE3C*, *LCE4A*, *XP33*, and *C1orf45* genes were designed by us. Except *LCE1D*, the specificity of all primers was confirmed by DNA sequencing of amplicons. For graphic representation of transcript data, all expression of target genes was shown relative to the housekeeping gene β_2-microglobulin expression in the same sample.

Prediction of Putative p53-Binding Sites

DNA sequences of an entire genomic region of *LCE1* including 10 kb of the 5′ upstream sequence were downloaded from the University of California Santa Cruz (UCSC) website (http://genome.ucsc.edu/), and the putative p53-binding sites (p53BSs) were screened according to the following criteria; at least 80% matched with the 20 nucleotides of consensus sequence RRRCWWGYYY__RRRCWWGYYY (R, purine; W, A, or T; Y, pyrimidine); we started the screening of 11 consensus sequences without any spacers between the two halves of p53BSs (Figure W1) and confirmed one site that was likely to be a direct p53-binding sequence.

Gene Reporter Assay

DNA fragments including each of the potential p53BSs of the *LCE1* gene cluster were amplified by KOD-Plus-DNA polymerase

(Toyobo, Osaka, Japan) and subcloned into the pGL3-promoter (pGL3-pro) vector. The primers for amplification are indicated in Table W1. To make a series of mutant vectors, a point mutation "T" was inserted into the site of the fourth and the fourteenth nucleotide "C" and into the seventh and the seventeenth nucleotide "G" of the consensus p53-BS using the KOD-Plus-Mutagenesis Kit (Toyobo). Since a functional p53BS is known in the *Fas* promoter region, a wild-type Fas promoter construct, pGL3-Fas, was used as a positive control. U373MG (mutated p53) and H1299 (p53 null) cells were plated in 12-well culture plates (5×10^4 cells per well) 24 hours before co-transfection of 125 ng of a reporter plasmid and either 125 ng of a Mock vector or a wild-type p53 expression vector in combination with 25 ng of a pRL-CMV vector. Cells were rinsed with PBS 36 hours after transfection and lysed in 250 μl of lysis buffer. Twenty microliters and 5 μl of lysates from U373MG and H1299, respectively, were sequentially measured using the PGD-S Dual Luciferase assay system following the manufacturer's procedure (Toyo Ink, Tokyo, Japan). The firefly luciferase value was normalized by the Renilla luciferase activity.

Expression Plasmid Construction

An entire coding sequence of PRMT5 was amplified using cDNA generated from mRNA of HEK293T cells and cloned into pCAGGSn3FC (PRMT5) vector. The entire coding sequences of *LCE1A-F* were amplified by the use of cDNA generated from mRNA that was extracted from HCT116 $p53^{+/+}$ cells after 70 J/m^2 of UVR treatment. Due to unknown reasons, we have been unsuccessful in cloning the *LCE1D* cDNA. An HA-epitope tag was placed at the C-terminus of the pCAGGSnHC vector and three-tandem FLAG epitope tags were placed at the C-terminus of the pCAGGSn3FC vector. The DNA sequences of expression constructs for LCE1 (pCAGGSnHC-LCE1) and PRMT5 (pCAGGSn3FC-PRMT5) were confirmed by DNA sequencing using ABI PRISM 3730XL Genetic Analyzer (Life Technologies).

Antibodies

The following primary antibodies were deployed: rabbit anti-HA (Y-11; Santa Cruz Biotechnology, Santa Cruz, CA; dilution used in WB: 1:1000), rabbit anti-PRMT5 (07-415; Millipore, Billerica, MA; dilution used in WB: 1:1000, ICC: 1:400), rabbit anti-p53 (sc6243, Santa Cruz; dilution used in WB: 1:1000), mouse anti-p53 [Ab-1, Calbiochem, San Diego, CA, dilution used in chromatin immunoprecipitation (ChIP): 1:100], rabbit anti-H3R8me2s (ab130740, Abcam, Cambridge, United Kingdom; dilution used in Western blot (WB): 1:1000), mouse anti–α-tubulin (clone DM1A, Millipore; dilution used in WB: 1:1000), rat anti-HA (3F10, Roche; dilution used in Immunocytochemistry (ICC): 1:800), and anti-FLAG (F7425, Sigma-Aldrich; dilution used in ICC: 1:1000).

Immunocytochemistry

Forty-eight hours after transfection with HA-Mock (empty vector of pCAGGSnHC) or pCAGGSnHC-HA-LCE1F into HEK293T or HCT116 $p53^{+/+}$ cells in four-well chambers, the cells were fixed by 1.7% formaldehyde or 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS. After covering with blocking solution (3% BSA in 0.2% Triton X-100) for 1 hour at room temperature, cells were incubated with a rat anti-HA antibody and a rabbit PRMT5 antibody overnight under humidified atmosphere at 4°C. Further, the cells were stained with fluorescence-conjugated secondary antibodies and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in VECTASHIELD Mounting Media (HT1200; Vector Laboratories, Burlingame, CA).

Immunoprecipitation and Mass-Spectrometric Analysis

Forty-eight hours after transfection with HA-Mock or HA-LCE1F, HEK293T cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 0.4% NP-40, and 150 mM NaCl] containing Protease Inhibitor Cocktail Set III (Calbiochem). Whole-cell lysates were pre-cleared by incubation with normal mouse IgG (sc2025, Santa Cruz Biotechnology) and recombinant protein G-sepharose 4B (Life Technologies) at 4°C for 1 hour, followed by incubation with anti-HA agarose (A2095, Sigma-Aldrich) overnight. The proteins were separated in Mini-PROTEAN 5% to 20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis precast gels (Bio-Rad Laboratories, Hercules, CA) and stained with the silver-staining kit (Life Technologies). Protein bands that were specifically observed in the cell extracts transfected with HA-LCE1F compared with Mock were excised and analyzed by liquid chromatography–tandem mass spectrometry as previously described [27]. For the co-immunoprecipitation experiments, HEK293T cells were co-transfected with HA-LCE1F and FLAG-PRMT5 or FLAG-Mock as a control. An HA pull-down experiment is exactly the same as mentioned above. As for FLAG pull-down experiment, whole-cell lysates were pre-cleared by incubation with normal mouse IgG (sc2025, Santa Cruz Biotechnology) and recombinant protein G-sepharose 4B (Life Technologies) at 4°C for 1 hour, followed by incubation with mouse anti-FLAG M2 agarose (F3165, Sigma-Aldrich) overnight. Rat anti-HA (3 F10, Roche) and rabbit anti-Flag (F7425, Sigma-Aldrich) antibodies were used for Western blot. For the histone methylation analysis, histones were extracted by the histone purification mini kit (40026, Active Motif, Carlsbad, CA) following the manufacturer's procedure strictly.

ChIP Assay

ChIP assays were performed using ChIP Assay kit (17-295; Millipore) according to the manufacturer's protocol [28]. Briefly, HCT116 $p53^{+/+}$ cells were treated with 1 μg/ml adriamycin for 2 hours, and cells were cultured in the drug free-medium for 24 hours. Then, cells were harvested and the fragment of p53 and chromatin complexes was immnoprecipitated with an anti-p53 antibody (Ab-1, Calbiochem). After DNA fragments bound to p53 were eluted out, an aliquot was subjected to real-time qPCRs. Protein A agarose/Salmon Sperm DNA (16-157; Millipore) was used as a negative control. Primers were designed to amplify the region containing the p53BS2-binding site, and their sequence information is shown in Table W1.

Results

Identification of a Novel p53 Downstream Target

We had previously performed expression profile analysis to compare U373MG glioblastoma cells infected with wild-type p53 (Ad-p53) with those infected with LacZ (Ad-LacZ) to screen possible downstream genes that are regulated by p53 and identified more than 50 genes that were likely to be induced by wild-type p53. Among them, we confirmed that the transcriptional levels of LCE1B and LCE1C were elevated more than seven-fold higher in the cells infected with wild-type p53 than those with LacZ (data not shown). *LCE1B* and *LCE1C* belong to the *LCE* gene cluster containing multiple well-conserved genes that encode stratum-corneum proteins.

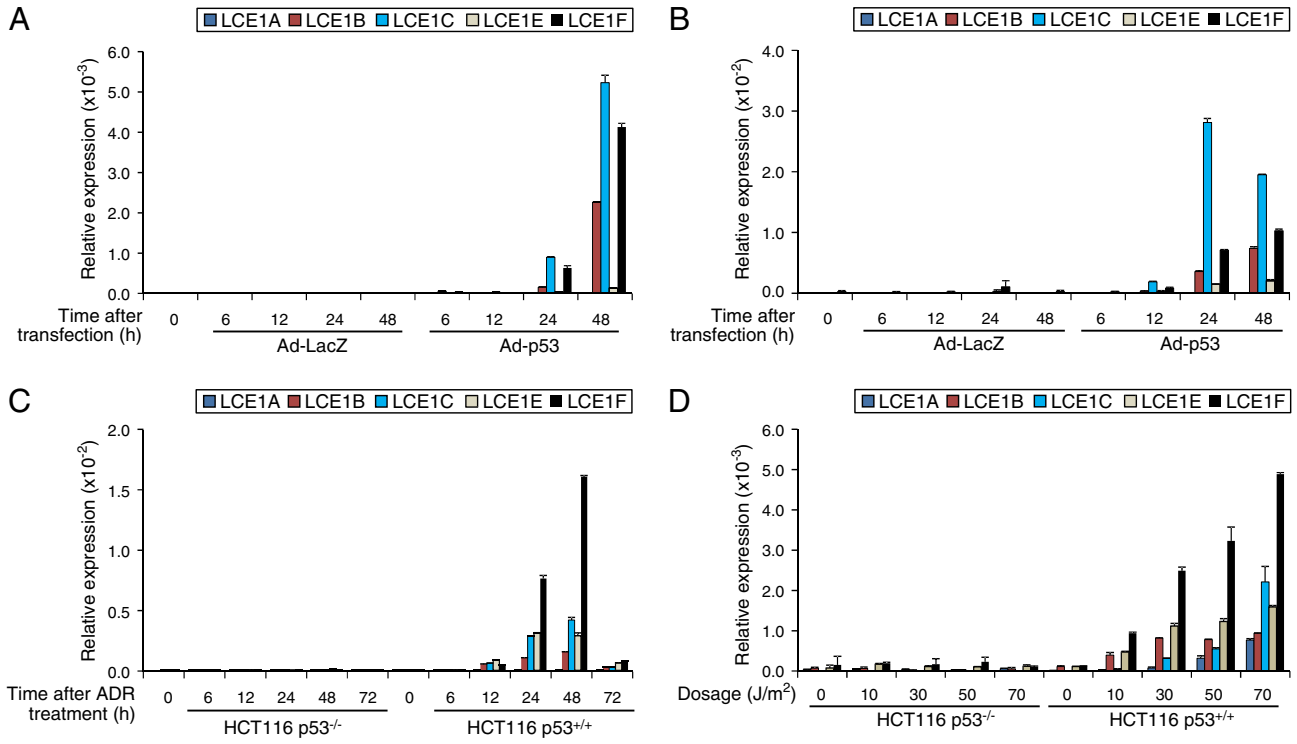

Figure 1. *LCE1* is upregulated by *p53*. Real-time qPCR analysis of *LCE1* mRNA expression in U373MG (*p53 mutant*) (A) and H1299 (*p53 null*) (B) cells at indicated time points after infection with Ad-p53 or Ad-LacZ at 8 MOIs. (C) Endogenous p53-dependent induction of *LCE1* mRNA transcription in $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells after treatment with adriamycin (1 μg/ml) at various time points. (D) Cells were treated with UVR at different doses and harvested 36 hours after UVR. β_2-Microglobulin was used for the normalization of expression levels.

Within the *LCE* cluster, multiple genes form "groups" at chromosome 1q21 and are known to respond "group-wise" to various environmental stimuli like calcium and UV light [9], suggesting that other *LCE* members that were not included in our microarray might also be regulated by p53. We first compared the sequence similarity of the *LCE1B* and *LCE1C* transcripts with other members in the *LCE* cluster (Table W3) and selected genes showing high similarity (>80%) for further validation by real-time qPCR because the microarray results might reflect the cross-hybridization of other LCE members that were possibly induced by p53. Interestingly, expression levels of *LCE1* group genes were significantly increased in both U373MG (Figure 1*A*) and H1299 (Figure 1*B*) cells after 8 MOIs of Ad-p53 infection (Table W4), although the induction levels are very different. However, other genes in the LCE cluster were not induced by wild-type p53 introduction. In addition, endogenous p53, which was activated by adriamycin and UVR, could also induce expressions of the *LCE1* group genes in HCT116 $p53^{+/+}$ cancer cells but not in HCT116 $p53^{-/-}$ cancer cells (Figure 1, *C* and *D*), indicating that the LCE1 group genes can respond group-wise to the genotoxic stress condition in a p53-dependent manner [9].

LCE1 Is a Direct Target of p53

We then attempted to clarify whether *LCE1* is directly or indirectly regulated by p53. First, 11 putative p53BSs of 20 nucleotides with at least 80% match to the consensus p53-binding sequence (see Materials and Methods section) were selected from the *LCE1* cluster region (Figure W1*A*). We then constructed serial reporter vectors containing each of the 11 predicted p53BSs. Luciferase activity of p53BS2, p53BS6, and p53BS7 was likely to be enhanced by co-transfection of each luciferase vector and a p53 wild-type expression vector into U373MG (Figure W1*B*) and H1299 cells (Figure W1*C*) in comparison with those with the mock vector. Hence, these three p53BSs were further investigated by replacement of critical nucleotides in the core consensus sequence with other nucleotides (Figure 2*A*). As a result, we observed that substitutions in either of the p53BS2, p53BS6, and p53BS7 sequences significantly diminished the enhancement of the luciferase activity in both U373MG and H1299 cells (Figure 2, *B* and *C*).These results imply that p53 directly binds to these p53BSs in the *LCE1* cluster and regulates their transcriptions.

We subsequently transfected siRNA targeting p53 or that commonly targeting LCE1A-F into HCT116 $p53^{+/+}$ cells and then the cells were treated with adriamycin. As shown in Figure 2*D*, induction of *LCE1* genes by adriamycin treatment was significantly attenuated in the cells transfected with si-p53, also supporting that *LCE1* family genes are p53-direct targets. Additionally, ChIP analysis showed the direct binding of p53 to the *LCE1* cluster region in HCT116 $p53^{+/+}$ cells after adriamycin treatment (Figure W2). We then examined the expression of *LCE1F* in 82 cancer cell lines by real-time qPCR (Table W5) and confirmed that *LCE1F* was significantly downregulated in p53 mutant cells compared with p53 wild-type cells in colon and lung cancer cell lines (Figure W3). This is consistent with the induction of *LCE1F* by p53 in HCT116 colon and H1299 lung cancer cells as shown in Figure 1.

LCE1F Interacts with PRMT5

To further analyze possible biologic functions of LCE1F in cancer cells, we constructed an expression vector designed to express full-

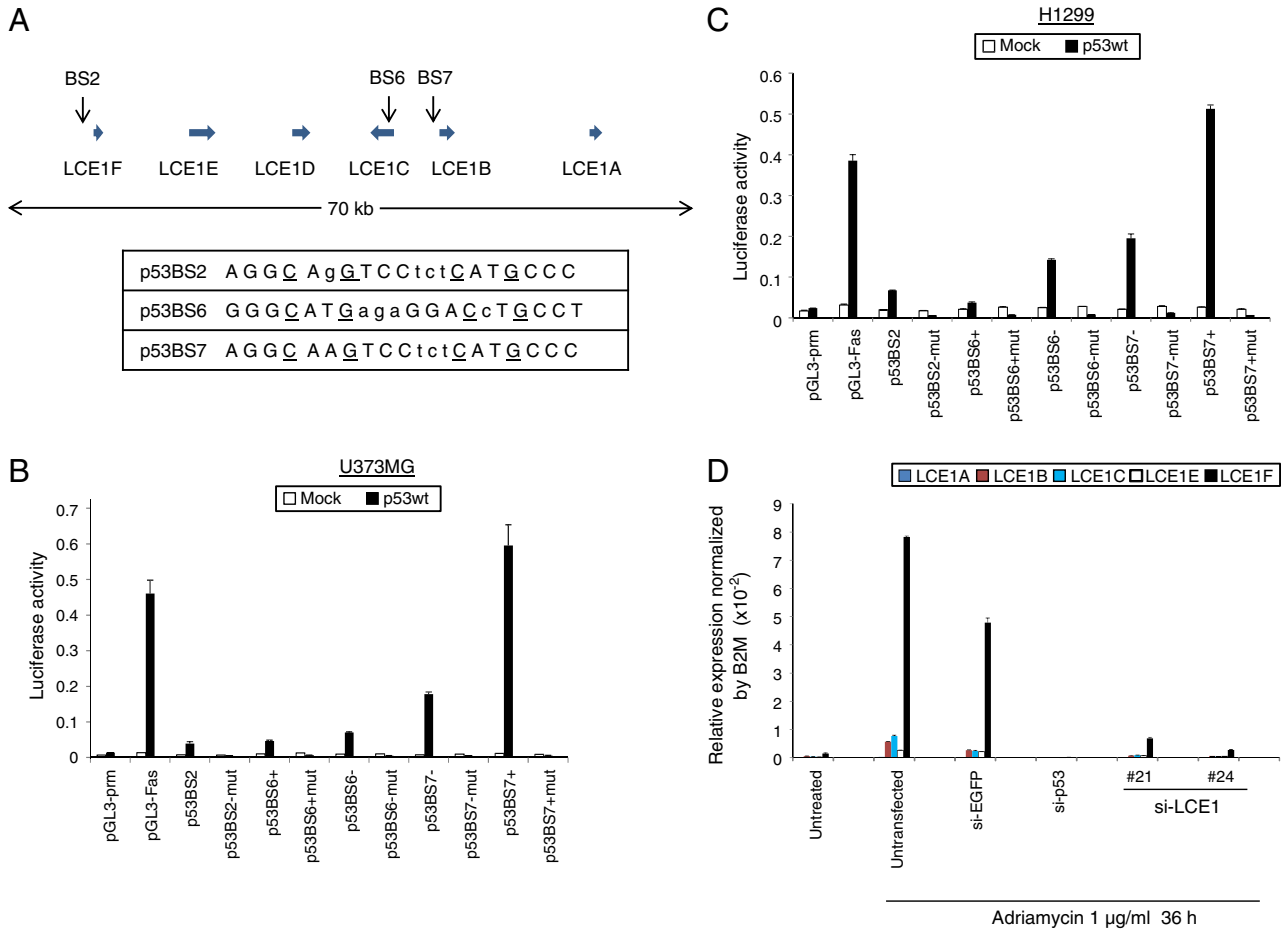

Figure 2. Identification of p53BSs in the *LCE1* cluster genes. (A) In the *LCE1* cluster genes, we identified 11 potential p53BSs that have at least 80% match to the consensus p53-binding sequence without any spacer nucleotides between the two halves of p53BSs. Among them, three possible p53 enhancer sequences are shown in the figure. Bold horizontal arrows indicate the locations and relative sizes of each *LCE1* gene. Vertical arrows show the potential p53BS locations. Identical nucleotides to the p53-binding sequence are written in capital letters. The underlined cytosine and guanine were substituted with thymine to introduce a mutant-type reporter vector at each p53BS. (B, C) Reporter assays of wild-type p53 and mutant-type p53 in U373MG (B) and H1299 (C) cells. Cells were co-transfected with a Mock (empty pcDNA3.1 +) or a wild-type p53 vector and each reporter plasmid, and luciferase assays were conducted 36 hours after transfection. Results are shown as the firefly luciferase activity normalized by the Renilla luciferase activity with 1 SD. (D) Real-time qPCR of *LCE1* family genes. HCT116 $p53^{+/+}$ cells were transfected with siRNAs targeting EGFP, p53, or LCE1 for 24 hours and then treated with 1 μg/ml adriamycin. Total RNA was extracted from the cells 36 hours after adriamycin treatment.

length LCE1F protein (pCAGGS-nHC-LCE1F) and transfected it into HEK293T cells. After confirmation of the LCE1F protein expression by Western blot analysis (Figure W4*A*), we performed immunocytochemical analysis to examine the subcellular localization of LCE1F. As shown in Figure W4*B*, LCE1F was strongly stained in both nucleus and cytoplasm.

In addition, we attempted to identify the interacting protein(s) of LCE1F. An HA-tagged LCE1F expression vector or an empty mock vector was transfected into HEK293T cells, and cell lysates were immunoprecipitated with anti–HA-conjugated agarose beads. Protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the gel was silver stained. We found a 70-kDa protein, which was strongly stained only in HA-LCE1F–overexpressing cells, conducted liquid chromatography–tandem mass spectrometry analysis of this protein, and identified it to be an arginine methyltransferase, PRMT5. This result was confirmed using an anti–PRMT5-specific antibody (Figures 3*A* and W5). Reversibly, cell lysates of HA-LCE1F–overexpressed cells were immunoprecipitated with an anti-PRMT5 antibody, and the immunoprecipitates were blotted with an anti-HA antibody, which also validated the interaction between LCE1F and PRMT5 (Figure 3*B*). Moreover, we co-transfected either FLAG-PRMT5 or FLAG-Mock with HA-LCE1F or HA-Mock, and cell lysates were immunoprecipitated with an anti-HA antibody (Figure 3*C*) or an anti-FLAG antibody (Figure 3*D*). Subsequent Western blot analysis clearly indicated the interaction between PRMT5 and LCE1F. These results imply that LCE1F interacts with PRMT5 in the cells. Interestingly, we confirmed that all LCE1 family proteins examined could interact with PRMT5 (Figure W6), suggesting that the interaction with PRMT5 is likely to be a common characteristic among LCE1 family members.

LCE1F Suppresses PRMT5 Activity

Since PRMT5 was reported to regulate the transcription of various genes through the methylation of arginine 8 on histone H3 (H3R8), which is considered to play an important role in human carcinogenesis [16,20,21], we further investigated the biologic

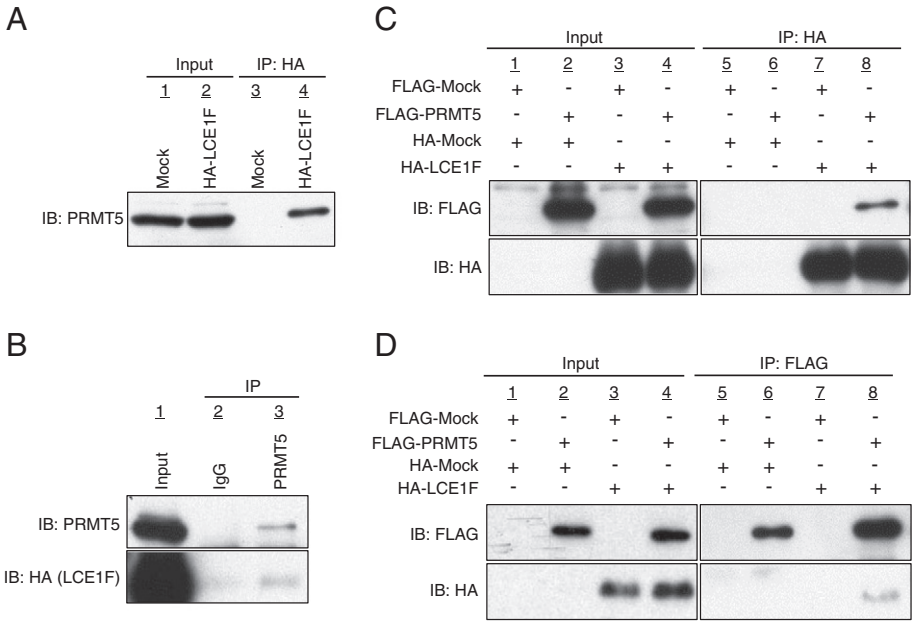

Figure 3. Interaction between LCE1F and PRMT5. (A) HEK293T cells were transfected with either a Mock (empty) or an HA-LCE1F expression vector and immunoprecipitated with anti-HA agarose. Samples were immunoblotted with an anti-PRMT5 antibody (Millipore). (B) HA-LCF1F–transfected HEK293T cells were immunoprecipitated with an anti-PRMT5 antibody or normal rabbit IgG (negative control). Samples were immunoblotted with an anti-PRMT5 or anti-HA antibody. (C, D) HEK293T cells were co-transfected with either FLAG-Mock or FLAG-PRMT5 and either HA-Mock or HA-LCE1F. Cells were immunoprecipitated with anti-HA agarose (C) or anti-FLAG antibody (D), and samples were immunoblotted with anti-FLAG or anti-HA antibodies.

significance of the interaction of PRMT5 and LCE1 members. We first performed immunocytochemical analysis and detected co-localization of LCE1F and PRMT5 in both the nucleus and the cytoplasm of the HCT116 $p53^{+/+}$ cells as shown in Figure 4. We then examined the methylation status of H3R8 after standardizing the quantity of PRMT5 using the anti-PRMT5 antibody. Using an antibody for symmetric dimethylation of H3R8 (H3R8me2s), we conducted Western blot analysis after transfection with siRNAs

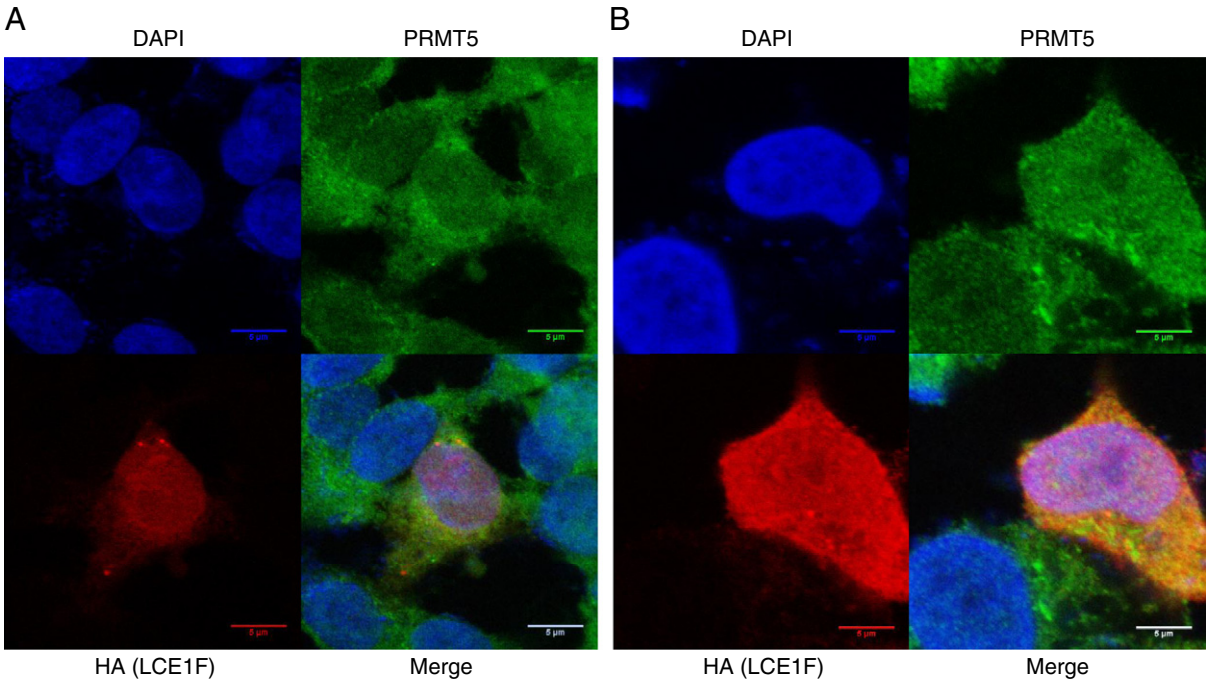

Figure 4. Co-localization of LCE1F and PRMT5. HCT116 $p53^{+/+}$ cells were transfected with HA-LCE1F. Cells were fixed by 1.7% formaldehyde and stained with an anti-HA antibody (red) and an anti-PRMT5 antibody (green).

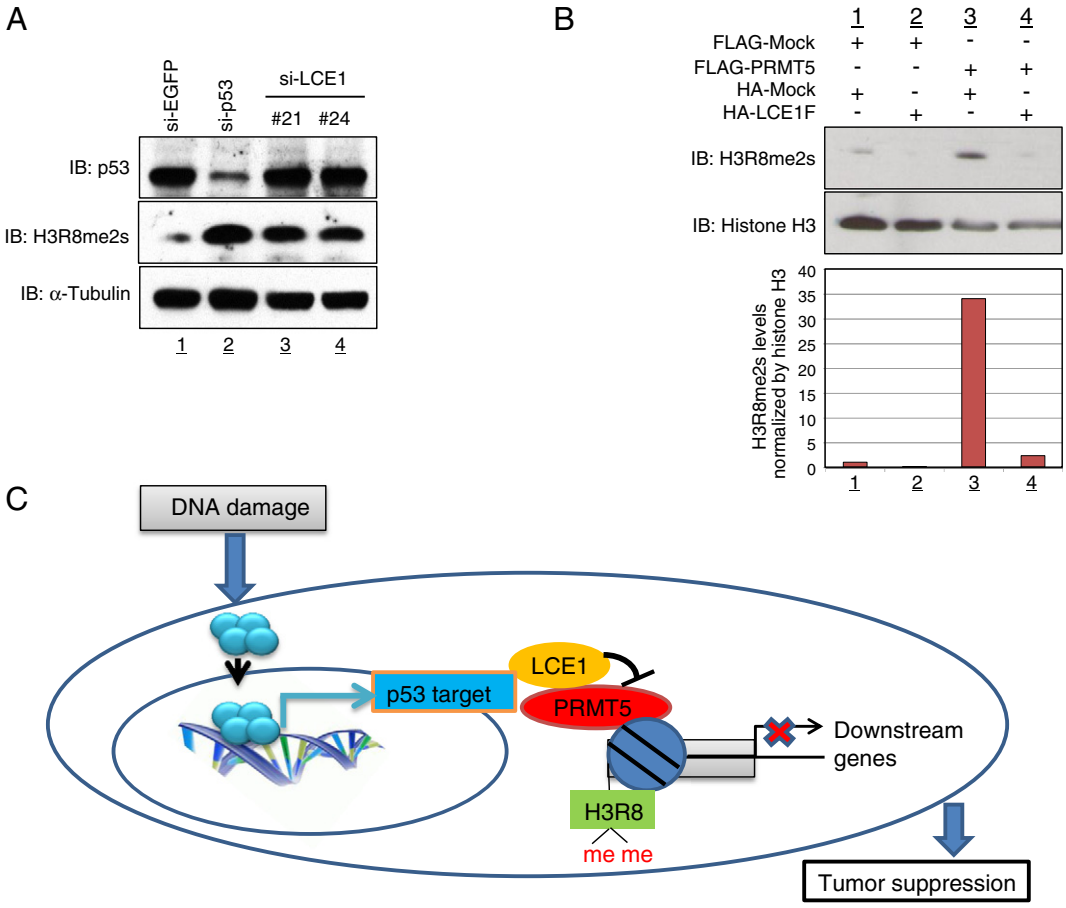

Figure 5. Possible mechanism of LCE1F on regulation of PRMT5 activity. (A) HCT116 p53$^{+/+}$ cells were transfected with siRNAs targeting EGFP, p53, or LCE1 for 24 hours and then treated with 1 μg/ml adriamycin. Proteins were extracted from the cells 36 hours after adriamycin treatment and immunoblotted with anti-p53, anti-H3R8 SDMA (H3R8me2s), or anti–α-tubulin (control) antibodies. (B) HEK293T cells were co-transfected with either FLAG-Mock or FLAG-PRMT5 and either HA-Mock or HA-LCE1F. Cells were harvested 48 hours after transfection and immunoblotted with anti-H3R8me2s or histone H3 (control) antibodies after histone purification. (C) A possible model of the network among p53, LCE1F, and PRMT5. p53 induced by DNA damage activates transcription of LCE1F, which suppresses the PRMT5 methyltransferase activity through their interaction.

targeting Enhanced green fluorescent protein (EGFP), p53, or LCE1 under treatment with adriamycin (Figure 5*A*) and found that knockdown of p53 and LCE1 significantly enhanced the methylation levels of H3R8 in HCT116 p53$^{+/+}$ cells, compared with the control cells, suggesting that LCE1 may modulate the PRMT5-dependent H3R8 methylation. To further validate this result, we co-transfected either FLAG-PRMT5 or FLAG-Mock and HA-LCE1F or HA-Mock into HEK293T cells and purified histone proteins from cell lysates. As shown in Figure 5*B*, in the presence of LCE1F, H3R8 methylation by PRMT5 protein was significantly suppressed. Taken together, our data indicate that LCE1F may negatively regulate PRMT5-dependent H3R8 methylation through the direct association to PRMT5.

Discussion

In this study, we showed that *LCE1* is a novel p53 target gene and that the LCE1F protein interacts with the arginine methyltransferase PRMT5. Through the interaction with PRMT5, LCE1F may suppress the PRMT5 methylation activity on arginine 8 of histone H3 (Figure 5*C*). Since PRMT5 is overexpressed in a wide range of human cancer and plays a critical role in tumorigenesis through the regulation of histone methylation, we unveiled a novel mechanism of tumor suppression mediated by p53.

The *LCE* gene cluster contains multiple conserved genes encoding stratum-corneum proteins [9,10,12,29]. Our study demonstrated that most of the members in the LCE1 group are transcriptionally regulated by the tumor suppressor p53 although the induction levels varied. Concordantly, *LCE1* genes were reported to be significantly upregulated in response to UVB irradiation of the skin cells [9]. UVR causes DNA damage, photoperoxidation of lipids, protein cross-linking, and isomerization of urocanic acid that lead to immunosuppression, photo-induced aging and cancer. p53 protein acts as a molecular sensor for the damages generated by UVR through mediating cell cycle arrest and apoptosis in damaged keratinocytes [30–34]. Taken together, cells may possess the function to express *LCE1* family genes through p53 activation to eliminate dangerous cells with DNA damages. Importantly, LCE1 family proteins show a high level of sequence similarity and LCE1F has more than 90% homology with other members (LCE1A-E; Table W6). In fact, as mentioned above, we confirmed the interaction between all of LCE1 proteins examined and PRMT5 (Figure W6). Since the expression of *LCE1* cluster genes is regulated by p53 as a whole (may not be all of the members), this protein family

members might play important roles to complementally or redundantly function as a tumor suppressor.

We identified PRMT5, a histone methyltransferase, as a key binding partner of the LCE1 proteins. Current progress of molecular medicine revealed that the enzymes relevant to histone methylation play critical roles in human carcinogenesis [28,35–45]. PRMT5 is one of the type II arginine methyltransferases, which catalyze the formation of symmetric dimethylation of arginine residues (SDMA) and regulates various cellular pathways [16,17,21]. A number of reports described the importance of this arginine methyltransferase in tumorigenesis [16,18,21,46–49]. Intriguingly, PRMT5 was reported to interact with BRG1- and hBRM-based hSWI/SNF chromatin remodelers and methylate arginine 8 on histone H3 [20]. The H3R8 methylation mediated by PRMT5-containing BRG1 and hBRM complexes directly repressed the expression of *suppressor of tumorigenicity 7* and *nonmetastatic 23*, tumor suppressor genes [20]. This line of pathway seems to be a key mechanism in the PRMT5-dependent tumorigenesis. Our data presented here have implied that LCE1, which is regulated by the tumor suppressor p53, negatively regulates H3R8 methylation mediated by PRMT5 (Figure 5, *A* and *B*), suggesting a new role of p53 in the regulation of histone modification. Since PRMT5 was reported to methylate p53 through the direct interaction and this methylation prevents p53-dependent apoptosis in cancer cells [50], we suggest an interesting feedback mechanism among p53, LCE1, and PRMT5.

Overall, our study elucidates a novel function of p53 as a tumor suppressor through the transcriptional regulation of the *LCE1* cluster genes. Further functional analysis may explore the importance of the LCE1 group proteins as tumor suppressors and the physiological relevance among p53 downstream genes.

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Appendix A. Supplementary Materials

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2014.07.008.

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