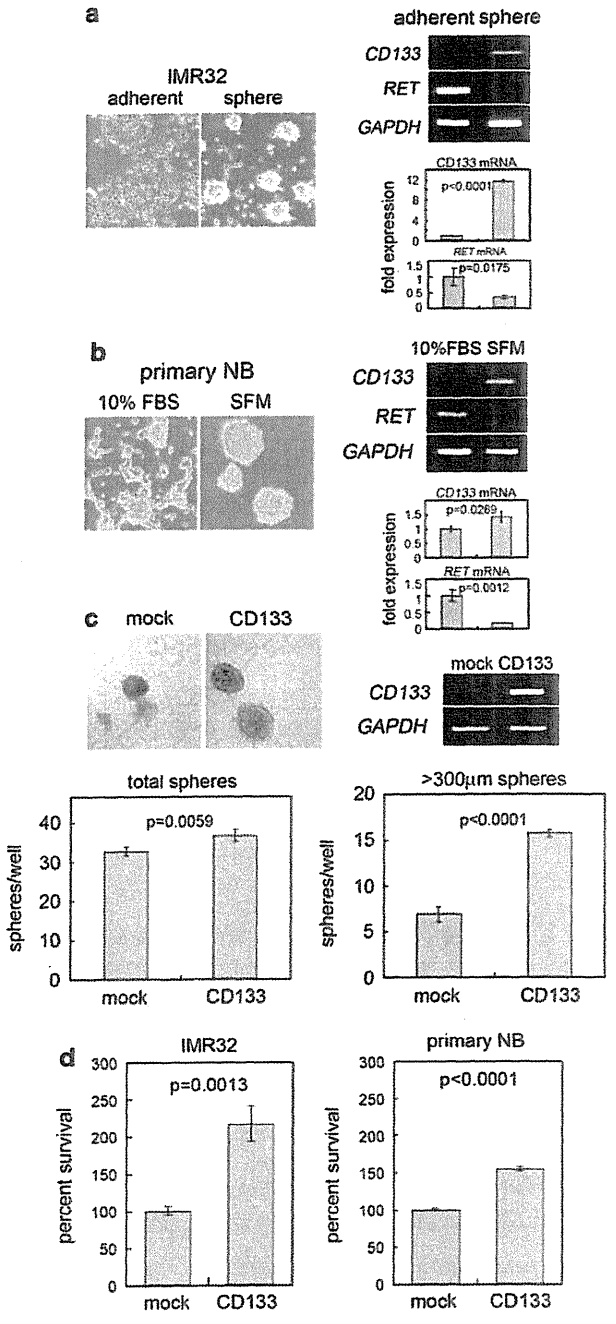

Figure 5 CD133 enhances cellular survival of NB cells in neurospheres. IMR32 cells (**a**) and primary NB cells (**b**) were cultured in 10% fetal bovine serum containing medium (adherent) or SFM (sphere) for a week. Semi-quantitative RT–PCR and qPCR analyses were performed with adherent or sphere cell RNAs using specific primers for *CD133* and *RET*. *GAPDH* was used as a loading control. Primary NB cell results are representative of three tumor samples. (**c**) IMR32 cells were stably infected with mock or CD133-expressing lentivirus. The expression levels of *CD133* and *GAPDH* were determined by semi-quantitative RT–PCR. Cells were cultured in 96-well culture plates with SFM. After 5 weeks, spheres were measured and counted under a microscope with an eyepiece micrometer. (**d**) Enzymatically dissociated IMR32- or primary NB-sphere cells were stained with trypan blue and counted to determine the number of viable cells.

the upregulation of CD133 (1.4-fold induction) and downregulation of RET receptors (5.9-fold reduction) were found in SFM medium, but not in the medium with 10% fetal bovine serum (Figure 5b). Next, we introduced CD133 into IMR32 cells by lentivirus infection, and after 5-week culture in SFM, we found that CD133-expressing cells formed many more large spheres than mock cells (Figure 5c). Moreover, CD133-expressing spheres made from IMR32 and primary NB cells contained many more living cells than the mock control (Figure 5d), suggesting that CD133 promotes NB cell survival in tumor-sphere formation.

Discussion

Increasing evidence highlights the function of CD133 as a marker of CSCs in various human tumors; however, its function in tumorigenesis remains to be elucidated by molecular biology experiments. In this study, CD133-knockdown experiments indicated that CD133 represses differentiation in NB cells; CD133 was clearly decreased by differentiation-inducing stimulation, for example ATRA and TPA treatments. Brodeur *et al.* (2000) indicated that neurotrophic factors and their receptors have a significant function in NB behavior and the potential to send intracellular signals into the nucleus to produce neuronal differentiation in the normal sympathetic nerve system. Among the NB cell differentiation-related neurotrophic receptors, *RET* transcription was regulated by CD133 in NB cell lines. The expression of CD133 effectively inhibited NB cell differentiation (neurite extension and differentiation markers). Furthermore, RET expression partly rescued the CD133-related inhibition of differentiation. These findings suggest that CD133-mediated RET suppression has a considerable function in NB cell differentiation. Regarding the function of RET in NB differentiation, Peterson and Bogenmann (2004) suggested that RET receptor activation inhibits cell cycle progression and enhances responsiveness to NGF; thus, NB cell differentiation requires the collaboration of functional RET and TrkA signal pathways; they also reported that GDNF treatment induced *RET* transcription in NB cells. Intriguingly, our results indicate that CD133 expression effectively suppressed *RET* mRNA in NB cells and CD133 knockdown induced NB cell differentiation, suggesting that suppression of CD133 by small-interference RNA administration will increase *RET* transcription in CD133-expressing NB tumors and may be useful in differentiation induction therapy for resistant- and relapsed-NB tumors. In addition, transcriptional suppression of *CD133* could be useful to induce differentiation in NB cells; however, the exact mechanism of transcriptional regulation of CD133 has not been clarified. Although seven CD133 mRNA isoforms controlled by five alternative promoters were reported previously (Shmelkov *et al.*, 2004), the promoter activities of these isoforms were studied only by pGL3-enhancer vector, suggesting the existence of other *cis*-elements in the CD133 locus.

Several studies have been reported to elucidate the molecular mechanism and signaling pathways that regulate the behavior of CD133-expressing cancer cells. Nikolova *et al.* (2007) reported that WNT-conditional media had effects on the proliferation and differentiation of cord blood-derived CD133-positive cells, and Fan *et al.* (2006) showed that Notch signal inhibition by GSI-18 reduced the CD133-positive fraction in brain tumor cells. Regarding the analysis of the intracellular signaling pathway related to the CD133 function, one report suggested the significance of the Akt/PKB pathway in the expression of survival proteins, phosphor-Bad and Bcl-2 in CD133-positive hepatocellular carcinoma cell survival (Ma *et al.*, 2007). In our study, CD133-knockdown experiments indicated that CD133-related RET repression and NB cell differentiation were caused by signal pathway activation, for example p38MAPK and PI3K/Akt pathways. To support this observation, treatment with kinase inhibitors showed a correlation between neurite elongation and RET induction in NB cells, and that differentiation marker protein induction was mainly dependent on the p38MAPK pathway. These findings suggest that CD133 prevents NB cell differentiation via signal transduction pathways. To the best of our knowledge, this is the first report of CD133-related signal pathway modification resulting in cell differentiation. As CD133 is a membranous protein on stem cells and cancer stem cells, it is possible that CD133 affects membranous receptor functions and the downstream signal pathways. In addition, Boivin *et al.* (2009) reported the phosphorylation of CD133-cytoplasmic tyrosine-828 and tyrosine-852 by Src and Fyn tyrosine kinases. Site-directed mutagenesis of these tyrosine residues in CD133 will provide important information for CD133 functions in our experimental system using NB cells.

Materials and methods

Cell culture and reagents

Human NB cell lines were obtained from official cell banks (RIKEN Cell Bank, Tsukuba, Japan and ATCC, Manassas, VA, USA) and cultured in high-glucose DMEM (Sigma-Aldrich, St Louis, MO, USA) or RPMI1640 (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 50 μg/ml penicillin/streptomycin (Sigma-Aldrich) in an incubator with humidified air at 37 °C with 5% CO_2. NB cell lines subjected to molecular biology and biochemistry experiments were MYCN single-copy SH-SY5Y cells and MYCN-amplified TGW, SK-N-DZ and IMR32 cells. GDNF was obtained from Invitrogen. ATRA was from Sigma-Aldrich. Phorbol-12-myristate-13-acetate (TPA) was from Nacalai Tesque (Kyoto, Japan). LY294002 was from Cell Signaling Technology (Beverly, MA, USA). PD98059 and SB203580 were from Calbiochem (San Diego, CA, USA).

Fluorescence-activated cell sorting analysis

NB cell lines growing in the log phase were enzymatically removed from 10 cm diameter culture dishes, washed with cold PBS and treated with biotinylated AC133 (CD133/1) monoclonal antibodies (Miltenyi Biotec, Auburn, CA, USA) or control IgG2A (eBioscience, San Diego, CA, USA) for 15 min at 4 °C. The primary antibody was removed, and then the cells were washed twice with ice-cold PBS containing 0.1% BSA, and a 1:200 dilution of phycoerythrin-labeled streptavidin (eBioscience) added for 15 min at 4 °C. After washing, flow cytometry was performed using a fluorescence-activated cell sorting Caliber (BD, San Jose, CA, USA).

Knockdown of CD133

For RNAi experiments, predesigned, double-stranded SMART-pool small-interference RNA targeting human *CD133* (*prominin-1*) was purchased from Dharmacon (Lafayette, CO, USA) and Silencer Negative Control small-interference RNA #1 was purchased from Ambion (Austin, TX, USA).

Lentivirus-mediated gene transduction and knockdown

The packaging cell line HEK 293T (4×10^6) was plated and transfected the following day. Then, 1.5 μg transducing vectors containing the gene [pHR-SIN-CMV-G-DL1 or CSII-CMV-MCS-IRES2-Bsd vector (RIKEN Bioresource Center, Ibaraki, Japan)] or shRNA [pLKO.1 (Sigma-Aldrich)] and 2.0 μg packaging vectors (Sigma-Aldrich) were co-transfected with Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocols. The medium was changed the following day, and cells were cultured for another 24 h. Conditioned medium was collected and cleared of debris by filtering through a 0.45 μm filter (Millipore, Bedford, MA, USA). Then, 1×10^5 NB cells were seeded in each well of a six-well plate, and transduced by lentiviral-conditioned media. Transduced cells were analyzed by western blotting and RT–PCR.

Cloning of human CD133 cDNA

The human *CD133* cDNA (RefSeq NM_006017) was cloned from human colon cancer cell line Caco-2 mRNA by RT–PCR using specific primer sets described in Supplementary Table 1S. *CD133* cDNA fragment was sub-cloned into a lentiviral-based vector (pHR-SIN-CSGW) (Hasegawa *et al.*, 2006).

Western blot analysis

The cells were lysed in buffer containing 5 mM EDTA, 2 mM Tris–HCl (pH 7.5), 10 mM β-glycerophosphate, 5 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, a protease inhibitor cocktail (Nacalai Tesque) and 1% SDS. Western blot analysis was performed as reported previously (Kurata *et al.*, 2008). For CD133 detection, we used AC133 monoclonal antibody. Anti-RET (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho- and total Akt, p38, ERK (Cell Signaling Technology) and anti-tubulin antibody from Lab Vision (Fremont, CA, USA) were also used.

Semi-quantitative RT–PCR

Semi-quantitative RT–PCR analysis was as described previously (Kurata *et al.*, 2008). Total cellular RNA for preparing RT–PCR templates was extracted using ISOGEN (Nippon Gene KK, Tokyo, Japan). The cDNA was synthesized from 1 μg total RNA and then subjected to PCR. Primer sequences are described in Supplementary Table 1S. RT–PCR results are representative of at least three independent experiments.

qPCR analysis

The qPCR analysis was performed as described previously (Ochiai *et al.*, 2010). The primers for qPCR were designed and synthesized to produce 50–150 bp products. The primer sequence is listed in Supplementary Table 1S. The results were representative of at least three independent experiments.

Cell proliferation and soft agar assay
Cells were seeded into 96-well plates (750 per well) in culture medium containing 10% fetal bovine serum. Every 24 h, cell viability was determined by water-soluble tetrazolium salt (WST-8) assay using Counting kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. For soft agar assay, 2×10^3 cells of stable infectants TGW or SH-SY5Y cells were seeded in soft agar as described previously (Aoyama *et al.*, 2005). Viable colonies were stained with 0.05 mg/ml MTT.

Tumor formation in nude mice
For tumor formation, 6-week-old female athymic BALB/c AJcl *nu/nu* mice (CLEA Japan, Shizuoka, Japan) were injected into the femur with 1×10^7 TGW cells as described previously (Aoyama *et al.*, 2005). The handling of animals was in accordance with the guidelines of Chiba Cancer Center Research Institute.

Patients and tumor specimens
The 12 tumor specimens used in this study were kindly provided by various institutions and hospitals in Japan. Informed consent was obtained at each institution or hospital. All tumors were diagnosed clinically as well as pathologically as NB and staged according to the International NB Staging System criteria. The patients were treated by standard chemotherapy protocols as described previously (Kaneko *et al.*, 2002; Iehara *et al.*, 2006). *MYCN* copy number, *TrkA* mRNA expression levels and DNA index were measured as reported previously (Ohira *et al.*, 2003). This study was approved by the Institutional Review Board of Chiba Cancer Center.

Subcloning of human RET (RET9)
Human *RET9* (Crowder *et al.*, 2004) full-length cDNA was a kind gift from Dr Hideki Enomoto (RIKEN Center for Developmental Biology, Hyogo, Japan). *RET9* cDNA fragment (3.4 kb) was sub-cloned into the *Not*I site of CSII-CMV-MCS-IRES2-Bsd vector, which had been altered to accept the *Xba*I and *Hin*dIII ends.

Cloning of human RET promoter
Human *RET* promoter 1.5 kb (−919 to +550, position +1 is the transcription start site determined in a previous report (Itoh *et al.*, 1992)) was amplified from human genomic DNA using Platinum *Pfx* polymerase (Invitrogen) with primers (described in Supplementary Table 1S) by PCR amplification and sub-cloned into pGEM-T easy vector (Promega, Southampton, UK). The *RET* 5′-flanking sequence from −453 to +227 was sub-cloned into the *Eco*RV site of pGL4.17 reporter vector (Promega).

Sphere culture of NB cells
The preparation of primary NB cells from stage 4 patients' bone marrow was described previously (Nakanishi *et al.*, 2007). Dissociated primary NB cells or IMR32 cells were cultured in SFM (DMEM-F12, 1:1 (Wako), 50 μg/ml penicillin/streptomycin, 2% B27 supplement (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich) and 20 ng/ml fibroblast growth factor basic (Invitrogen)). Half of the medium was replaced with fresh culture medium every 7 days. IMR32 cells and primary NB cells were seeded in 96-well (400 per well) or six-well (1×10^5 per well) and six-well (1.7×10^5 per well) plates, respectively. Spheres were counted and measured under a microscope with an eyepiece micrometer. Five-week cultured IMR32 or 2-month cultured primary NB spheres were dissociated by Accumax (Innovative Cell Technologies, San Diego, CA, USA) according to the manufacturer's protocol. Living cells were counted based on morphological criteria and trypan blue staining.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank K Sakurai and S Matsushita for technical assistance, Dr Hiroyuki Miyoshi (BioResource Center, RIKEN) for the gift of CSII-CMV-MCS-IRES2-Bsd plasmid and Daniel Mrozek, Medical English Service, for editorial assistance. This work was supported in part by a grant-in-aid from JSPS for Young Scientists (B) (number: 19790274), a grant-in-aid from the Ministry of Health, Labor, and Welfare for Third Term Comprehensive Control Research for Cancer, a grant-in-aid for Cancer Research (20–13) from the Ministry of Health, Labor, and Welfare of Japan and a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (number: 21591377).

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 603925, 13 pages
doi:10.1155/2011/603925

Review Article

p53: The Attractive Tumor Suppressor in the Cancer Research Field

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Received 16 August 2010; Accepted 7 October 2010

Academic Editor: Celina Janion

p53 is one of the most studied tumor suppressors in the cancer research field. Of note, over 50% of human tumors carry loss of function mutations, and thus p53 has been considered to be a classical Knudson-type tumor suppressor. From the functional point of view, p53 is a nuclear transcription factor to transactivate a variety of its target genes implicated in the induction of cell cycle arrest, DNA repair, and apoptotic cell death. In response to cellular stresses such as DNA damage, p53 is activated and promotes cell cycle arrest followed by the replacement of DNA lesions and/or apoptotic cell death. Therefore, p53 is able to maintain the genomic integrity to prevent the accumulation of genetic alterations, and thus stands at a crossroad between cell survival and cell death. In this paper, we describe a variety of molecular mechanisms behind the regulation of p53.

1. Introduction

p53 has been initially identified in crude cell lysates prepared from cells transformed by simian virus 40 (SV40) [1–5]. Subsequent studies demonstrated that p53 forms a stable complex in SV40-transfromed cells with SV40 large T antigen which has an oncogenic potential, and p53 had an ability to promote tumor growth. In support with these results, p53 was detectable in a variety of tumor-derived cell lines [1, 3, 4]. Based on these observations, *p53* came to be classified as an oncogene [6]. However, this classical point of view has been challenged by the findings showing that the initially discovered p53 is a mutant form of p53 [7]. In a sharp contrast to mutant forms of p53, subsequent studies revealed that wild-type p53 is capable to suppress the malignant growth of transformed cells as well as tumors, suggesting that p53 acts as a tumor suppressor [8–12]. Intriguingly, *p53* gene locates on the short arm of human chromosome 17 (17p13), where loss of heterozygosity (LOH) was detectable in a wide variety of tumor tissues. It is worth noting that the remaining nondeleting *p53* allele is mutated in some cases [13–18]. Extensive mutation searches demonstrated that over 50% of human tumors carry *p53* mutations. Indeed, *p53*-deficient mice developed spontaneous tumors at a relatively young age [19].

Ninety-five percent of the mutations were detected within the central sequence-specific DNA-binding region of p53 [20–22]. These mutations disrupted the whole conformation of the sequence-specific DNA-binding domain of p53 and resulted in the loss of its sequence-specific DNA-binding ability [23]. Since p53 was a nuclear sequence-specific transcription factor which transactivated a set of its target genes involved in the induction of cell cycle arrest and/or apoptotic cell death, mutant forms of p53 lacked their critical function to maintain the genomic integrity. Furthermore, mutant forms of p53 has acquired a much longer half-life as compared with that of wild-type p53 [24, 25] and displayed a dominant-negative behavior toward wild-type p53 [26, 27]. This dominant-negative effect of mutant p53 on wild-type p53 might be mediated by the hetero-oligomerization through their oligomerization domains [7, 28–30]. In this connection, *p53* mutation conferred the resistance of tumor cells to anticancer drugs by inhibiting p53-dependent proapoptotic pathway [31–33].

Thus, the development of the novel devices to remove or suppress the dominant-negative effect of mutant forms of p53 on wild-type p53 will serve as a basis for providing new therapeutic strategies to treat tumors bearing *p53* mutations.

As described above, p53 had a strong proapoptotic activity. Under normal conditions, the expression level of this dangerous protein is kept at extremely low level. In response to multiple cellular stresses including DNA damage, oncogene activation, hypoxia, nucleotide imbalance, and oxidative damage, p53 was rapidly accumulated in cell nucleus through chemical modifications such as phosphorylation and acetylation and exerted its proapoptotic function to remove cells with seriously damaged DNA in which DNA damage was severe and repair was impossible [22, 34–36]. In this case, p53 transactivated proapoptotic target genes including *BAX*, *PUMA*, *NOXA*, and *p53AIP1*, and the collaboration of these gene products contributed to the disruption of mitochondrial membrane potential, which was a critical step in p53-dependent proapoptotic pathway [37]. On the other hand, p53 promoted G1 cell cycle arrest in the early stage of DNA damage response through the transactivation of $p21^{WAF1}$, *p53R2*, and *GADD45* implicated in the induction of cell cycle arrest and DNA repair [37]. After DNA repair had been completed, cells reentered into normal cell cycle. Upon DNA damage, cells underwent either cell cycle arrest or apoptotic cell death to allow DNA repair or suicide of cells, which was dependent on the degree and/or the nature of DNA damage.

For a long time, p53 has been considered to be a solitary gene product. Due to the improvement of cloning technologies, Kaghad et al. identified the first p53 homolog termed p73 [38]. Additionally, Yang et al. discovered the second p53 homolog termed p63 [39]. Cell-based studies demonstrated that p73 and p63 act as nuclear sequence-specific transcription factors which transactivate the overlapping set of p53-target genes and also have an ability to induce cell cycle arrest and/or apoptotic cell death in cancerous cells [40, 41]. Like p53, p73, and p63 were induced in response to a certain subset of DNA-damaging agents [42, 43]. Thus, p53 becomes a founding member of p53 tumor suppressor family composed of p53, p73 and p63.

Based on the above-mentioned brief background of p53, we focus primarily on a variety of regulatory mechanisms of p53 in the present paper.

2. General Feature of p53

p53 locates at a short arm of human chromosome 17p13 containing 11 exons spanning 20 kb. p53 acts as a nuclear sequence-specific transcription factor composed of NH_2-terminal transactivation domain (TA, amino acid residues 1–45), central sequence-specific DNA-binding domain (DB, amino acid residues 102–292), and COOH-terminal oligomerization domain (OD, amino acid residues 319–359). In addition to these representative functional domains, p53 contains three nuclear localization signals (NLS, amino acid residues 305–322, 369–375, and 379–384) recognized by importin α/β complex [44], a Leu-rich nuclear export signal (NES, amino acid residues 339–352) recognized by CRM1 (chromosomal region maintenance 1) [44], and a Pro-rich domain (amino acid residues 63–97). Cytoplasmic retention of p53 was observed in certain breast cancer-derived cells expressing the truncated form of importin α, indicating that importin α plays an essential role in nuclear import of p53 [45]. Cytoplasmic p53 is nonfunctional. Pro-rich domain has been shown to be associated with proapoptotic activity of p53 [46, 47]. Deletion of this Pro-rich region resulted in a complete loss of proapoptotic activity of p53. Active form of nuclear p53, which functions as a tetramer, recognizes and binds to a consensus sequence motif made of tandem 10 bp elements (RRRCWWGYYY: R, G/A; W, A/T; Y, C/T) separated by 1–13 bp found within the promoter regions of p53-target genes. p53 exerts its proapoptotic function through the transactivation of its target genes [48, 49]. Genome-wide analysis revealed that there exist over 4,000 putative p53-responsive elements [22]. Although all of these canonical p53-responsive elements might not always be functional, identification and functional analysis of new p53-target genes provide novel insights into understanding the precise molecular mechanisms behind p53-dependent proapoptotic pathway.

Since the sequence-specific DNA-binding ability of p53 is tightly linked to its proapoptotic activity [48, 49], the genomic integrity of *p53* gene encoding the sequence-specific DNA-binding domain (exons 5–8) is particularly important. Extensive mutation search revealed that over 50% of human tumors carry *p53* mutations [20, 21]. Among these mutations, 95% of them occurred within the genomic region encoding the sequence-specific DNA-binding domain of p53. These mutations disrupted the proper conformation of the sequence-specific DNA-binding domain of p53, and thus mutant forms of p53 lacked the sequence-specific transactivation ability. In contrast to the short-lived wild-type p53, mutant forms of p53 had a longer half-life [24, 25]. Moreover, mutant forms of p53 exhibited an oncogenic potential [50] and displayed the dominant-negative behavior toward wild-type p53 [26, 27], suggesting that mutant forms of p53 attenuate p53-dependent proapoptotic pathway (Figure 1).

Since the previous mutation search for *p53* gene focused on the genomic region encoding the central core sequence-specific DNA-binding domain of p53, it is likely that there could exist the unidentified mutations outside the central core sequence-specific DNA-binding domain. Indeed, Lomax et al. found point mutations (L344P and R337C) within the COOH-terminal oligomerization domain [51, 52]. Similarly, DiGiammarino et al. reported the presence of a point mutation (R337H) within the COOH-terminal oligomerization domain [53]. In addition to these mutations, we have found p53ΔC lacking a part of the COOH-terminal oligomerization domain and nuclear localization signals in human neuroblastoma-derived cell lines [54]. According to our results, p53ΔC was largely expressed in cytoplasm and had a significantly lower proapoptotic ability as compared with wild-type p53. Therefore, p53 mutations detected

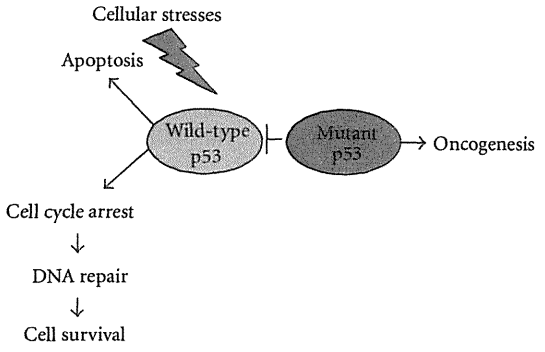

FIGURE 1: Dominant-negative effect of mutant p53 on wild-type p53. In response to cellular stresses, wild-type p53 is activated and induces cell cycle arrest and/or apoptotic cell death. Mutant p53 binds to wild-type p53 and inhibits its tumor suppressor function, thereby promoting tumor formation.

outside central core DNA-binding domain caused loss of function of p53.

From the clinical point of view, mutational inactivation of p53 was a frequent and serious molecular event in most human tumors. Since mutant forms of p53 had a longer half-life with oncogenic potential, exhibited the dominant-negative effect on wild-type p53, and thus led to chemoresistance, it is required to develop novel anticancer therapeutic approaches to suppress mutant forms of p53 or rescue wild-type p53 activity from mutant p53. In this regard, a small compound termed PRIMA-1, which has an ability to reactivate mutant p53, might be one of the promising and efficient anticancer drugs [55].

3. p53-Target Gene Products

To date, numerous genes have been identified as p53-target genes [22, 34–36]. In this section, we would like to describe the functional significances of several representative p53-target gene products. Since the inductions of cell cycle arrest and apoptotic cell death are the major roles of p53, p53-target gene products are closely involved in these cellular processes.

El-Deiry et al. identified p53-target gene termed $p21^{WAF1}$ by using a subtractive hybridization approach [56]. $p21^{WAF1}$ gene promoter contained a p53-responsive element, and its gene product had an ability to suppress cell growth. Alternatively, Harper et al. discovered $p21^{CIP1}$ as a Cdk2-(cyclin-dependent kinase 2-) binding partner by employing a yeast-based two-hybrid procedure [57]. $p21^{CIP1}$ tightly bound to Cdk2 and inhibited its protein kinase activity to block the phosphorylation of pRB. Noda et al. identified $p21^{SDI}$ by using an expression screening from senescent human diploid fibroblasts [58]. $p21^{SDI}$ blocked DNA synthesis and maintained the senescent phenotype. Surprisingly, these gene products were identical. Now, we call it $p21^{WAF1}$. In response to cellular stresses, p53 induces G1 cell cycle arrest through the upregulation of $p21^{WAF1}$.

Tanaka et al. employed a differential display approach to isolate a novel p53-inducible gene termed *p53R2* whose gene product was highly homologous to ribonucleotide reductase small subunit (R2) [59]. *p53R2* was significantly induced in response to DNA damage in a p53-dependent manner. Intriguingly, p53R2 induced G2/M arrest and was directly involved in repair of damaged DNA.

Under normal condition, p53 was kept at an extremely low level. MDM2 (murine double minute 2), which has an intrinsic E3 ubiquitin protein ligase activity, controlled the expression level of p53 by targeting it to ubiquitin-/proteasome-dependent degradation [60–62]. Barak et al. described that *MDM2* is a direct transcriptional target of p53 [63]. Thus, MDM2 participates in a negative autoregulatory feedback loop which controls p53 expression level (see below).

Among p53-target genes, those that encode mitochondrial proteins are particularly attractive, because p53-dependent apoptosis appears to proceed through mitochondrial dysfunction. Selvakumaran et al. reported that *BAX* (Bcl2-associated X protein) is an immediate early p53-responsive gene [64]. BAX contains two highly conserved Bcl2 homology 1 and 2 (BH1 and BH2) domains and displayed a dominant-negative effect over prosurvival Bcl2. Upon apoptotic stimuli, BAX, which resides on the mitochondrial outer membrane, dysregulated the mitochondrial outer membrane permeability and induced the release of cytochrome c from the mitochondrial intermembrane space to cytosol [65].

p53AIP1 (p53-regulated apoptosis-inducing protein 1), NOXA (Latin for damage), and PUMA (p53 upregulated modulator of apoptosis), which were included in BH-3 domain-containing mitochondrial Bcl2 family, were direct transcriptional target gene products of p53 [22, 34–36]. For p53AIP1, DNA damage-mediated induction of p53AIP1 was tightly associated with p53-dependent apoptotic cell death and phosphorylation of p53 at Ser-46. Matsuda et al. described that p53AIP1 promotes downregulation of the mitochondrial membrane potential through the direct interaction with Bcl2 and induces the release of cytochrome c [66].

NOXA has been rediscovered in a differential display approach [67]. Forced expression of NOXA induced cytochrome c release from mitochondria followed by subsequent caspase activation, and cells underwent apoptotic cell death. The intact BH 3 domain of NOXA was required for the induction of apoptotic cell death.

PUMA has been identified by using a microarray analysis [68, 69]. PUMA was induced in response to ADR (adriamycin), and p53-responsive element was found within intron 1 of *PUMA* gene. Indeed, *PUMA* was one of the transcriptional target genes of p53. PUMA was localized to mitochondria and induced apoptotic cell death. Like p53AIP1, PUMA interacted with Bcl2 and functioned to induce cytochrome c release, thereby activating caspase 9 and 3. Jeffers found that BAX is required for PUMA-mediated apoptotic cell death, placing BAX downstream of PUMA [70].

4. Stability Control of p53

Steady-state expression level of the endogenous p53 is maintained at extremely low level, keeping this dangerous protein in an inactive state. The expression level of p53 is dependent on a balance between protein production and degradation. It has been well-documented that p53 is rapidly induced at protein level in response to a variety of cellular stresses such as DNA damage [22, 34–36]. This accumulation is largely due to a significant increase in its protein stability. The proteolytic degradation of p53 was mediated by the physical interaction between p53 and oncogenic MDM2 [60–62]. MDM2, which is a RING-finger type E3 ubiquitin protein ligase, bound to NH_2-terminal transactivation domain of p53, ubiquitylated COOH-terminal 6 Lys residues (Lys-370, Lys-372, Lys-373, Lys-381, Lys-382, and Lys-386), and thereby targeting p53 for proteasome-dependent degradation [71]. MDM2 masked NH_2-terminal transactivation domain of p53 and thereby inhibiting its transcriptional activity [72]. A small compound termed Nutlin, which bound to p53-binding pocket of MDM2, inhibited the interaction between p53 and MDM2, and thereby stabilizing p53 followed by activation of p53-dependent proapoptotic pathway [73]. Thus, this small compound might provide a novel strategy for cancer therapy.

In general, polyubiquitin chains target proteins to proteasome and initiate the process of proteolytic degradation. Recently, Kulikov et al. found that MDM2 associated with several subunits of proteasome, suggesting that MDM2 might promote not only the ubiquitylation of p53 but also recruit the ubiquitylated forms of p53 into proteasome [74]. In addition to MDM2, RING-finger type E3 ubiquitin protein ligases Pirh2 (p53-induced RING H2 domain protein) [75] and COP1 (constitutive photomorphogenic 1) [76] also interacted with p53 and mediated the ubiquitin-/proteasome-dependent degradation of p53. As expected, all of them inhibited transcriptional as well as proapoptotic function of p53. Since MDM2, Pirh2, and COP1 were p53-induced target gene products, they participated in a negative autoregulatory feedback loop which controls p53. Alternatively, p53-interacting protein termed HAUSP (herpes virus-associated ubiquitin-specific protease) had an intrinsic enzymatic activity to deubiquitylate p53 and thereby increasing its stability [77].

5. Posttranslational Modification

The induction and activation of p53 in response to cellular stresses have been shown to be largely regulated at posttranslational level through multiple mechanisms. Upon cellular stresses, p53 is phosphorylated at Ser-15, Ser-20, and Ser-46 [22, 34–36]. NH_2-terminal phosphorylation of p53 converted p53 from latent form to active and stable one. On the other hand, protein phosphatases PP-1 and PP2A had an ability to dephosphorylate p53 and negatively modulated its activity [78, 79]. Ser-15 was phosphorylated by ATM (ataxia-telangiectasia mutated) [80], ATR (ataxia-telangiectasia mutated and Rad3-related) [81], Chk1 (checkpoint kinase 1) [82], and DNA-PK (DNA-dependent protein kinase) [83]. Ser-20 was phosphorylated by Chk2 (checkpoint kinase 2) [84] and Plk3 (polo-like kinase 3) [85]. In addition, HIPK2 (homeodomain interacting protein kinase 2) and PKCdelta (protein kinase C delta) have been considered to be involved in phosphorylation of p53 at Ser-46 [86, 87]. As mentioned above, MDM2 bound to NH_2-terminal transactivation domain of p53 to destabilize and also inactivate p53. Stress-induced NH_2-terminal phosphorylation of p53 promoted the dissociation of MDM2 from MDM2/p53 complex and led to the stabilization and activation of p53 [22, 34–36]. Alternatively, COOH-terminal region of p53 was also phosphorylated. For example, CKII (Casein kinase II) phosphorylated p53 at Ser-392 [88], and PKC phosphorylated p53 at Ser-371, Ser-376, and Ser-378 [89]. These COOH-terminal phosphorylations enhanced the sequence-specific DNA-binding activity of p53 [22, 34–36]. It has been shown that COOH-terminal region of p53 acts as a negative regulator and might mask its sequence-specific DNA-binding domain in a latent conformation [90, 91]. It is likely that phosphorylation of COOH-terminal region of p53 might lead to a conformational shift that enables p53 to bind more efficiently to its target motif, and thereby enhancing its sequence-specific transactivation function. In contrast, Plk11 (polo-like kinase 1) inhibited transcriptional as well as proapoptotic function through physical interaction and phosphorylation [92]. Therefore, phosphorylation of p53 does not always act as an activation signal.

In addition to stress-induced phosphorylation of p53, p53 was subjected to the extensive acetylation mediated by p300 with intrinsic histone acetyltransferase activity [93]. p300 which acts as a ubiquitous transcriptional coactivator, bound to NH_2-terminal region of p53 and promoted its acetylation of a cluster of COOH-terminal Lys residues (Lys-370, Lys-372, Lys-373, Lys-392, and Lys-381) [37]. PCAF (p300/CBP-associated factor), another histone acetyltransferase, had an ability to acetylate Lys-320 of p53 [94]. Intriguingly, theses COOH-terminal Lys residues were the sites for ubiquitin ligation. It is likely that p53 acetylation catalyzed by p300 reduces its ubiquitylation levels by competition between acetylation and ubiquitylation. Thus, p300-mediated acetylation of p53 increased the stability of p53 and enhanced the transcriptional as well as proapoptotic activity of p53 [95]. Consistent with this notion, SIRT1 (Silent mating type information regulation 2 homolog 1), which has an intrinsic deacetylase activity, interacted with p53 and attenuated p53-dependent cell cycle arrest as well as apoptotic cell death in response to DNA damage through deacetylation of Lys-382 [96].

Kawai et al. described that p300 has a dual role in the regulation of p53 stability [97]. According to their results, p300 acted as a positive regulator to increase p53 stability in the presence of lower level of MDM2, whereas p300 became a negative regulator for p53 to induce MDM2-dependent degradation in the presence of higher level of MDM2. Surprisingly, Grossman et al. found that, in addition to histone acetyltransferase activity, p300 has an E4 ubiquitin protein ligase activity which catalyzes polyubiquitylation of monoubiquitylated precursor p53 [98].

Sumoylation is the other type of posttranslational modification. SUMO (SUMO-1, SUMO-2, and SUMO-3) was a ubiquitin-related small protein which covalently binds to substrates through a mechanism similar to ubiquitylation. PIAS (protein inhibitor of activated STAT) family acted as a SUMO E3 ligase for p53 [99]. Unlike ubiquitylation, the modification by SUMO-1 did not target proteins for proteolytic degradation. Previous studies suggest that sumoylation targets p53 to the nucleoli, and sumoylated p53 tightly binds to chromatin structure. Sumoylation of p53 at Lys-386 resulted in a loss of a sequence-specific DNA-binding ability and thus inhibited its transcriptional activity, although sumoylated p53 bound to coactivator p300 [100].

Previously, Jackson and Tjian demonstrated that *O*-linked glycosylation enhances the sequence-specific transcriptional activity of Sp1 [101]. Close inspection of the amino acid residues of wild-type p53 revealed that there exists a putative *O*-linked glycosylation site within the COOH-terminal basic region. Shaw et al. described that *O*-linked glycosylation enhances sequence-specific transcriptional activity of p53, which might be due to the disruption of intramolecular interaction between the COOH-terminal inhibitory domain and sequence-specific DNA-binding domain [102].

6. Transcriptional Regulation

As described above, p53 is largely regulated at protein level through chemical modifications such as phosphorylation and acetylation. On the other hand, p53 expression is also regulated at transcriptional level in some cases. Previously, Reich and Levine found that p53 is transcriptionally regulated in response to mitogen stimulation and serum starvation [103]. Bruno et al. reported that *p53* is transactivated in response to anticancer drug ADR [104]. Raman et al. described that homeobox protein HOXA5 acts as a transcriptional activator for *p53* [105]. In addition, Noda et al. identified the *cis*-acting element termed PE21 at a nucleotide position from −79 to −60 (relative to the first transcriptional initiation site) within the *p53* promoter region responsible for *p53* gene basal as well as inducible expression in response to UV [106].

Recently, we have found that AMPK (AMP-activated protein kinase), which acts as an intracellular energy sensor by monitoring cellular energy levels, plays an important role in the regulation of apoptotic cell death in response to glucose deprivation [107]. According to our results, the activated form of AMPK was closely involved in the transcriptional activation of p53 under low glucose conditions. It has been shown that the activation of AMPK affects the gene expression, suggesting that AMPK itself and/or AMPK-containing cellular complex might have a transactivation potential [108]. Subsequent studies demonstrated that there exists a putative AMPK-responsive element within the genomic sequence between −531 and −239 relative to the transcriptional initiation site of *p53*. Within this region, we have found out canonical CREB- (cAMP-responsive element-binding protein-) binding site (5′-ATTACGGAA-3′). Finally, we have shown that AMPK collaborates with CREB through CREB-binding site to transactivate *p53* in response to energetic stress [109]. Therefore, in addition to posttranslational modification, p53 is transcriptionally regulated in response to a certain subset of cellular stresses.

7. Subcellular Localization

Appropriate subcellular localization is critical for regulating function of p53. p53 acts as a sequence-specific transcription factor in cell nucleus. In addition to the mutational inactivation of p53, the abnormal cytoplasmic localization of p53 resulted in loss of function of p53. In contrast to the other human tumors, *p53* was rarely mutated in human neuroblastoma [110]. Moll et al. found that wild-type p53 is detectable in cytoplasm of the majority of undifferentiated neuroblastoma, suggesting that the inability of nuclear access of p53 attenuates its tumor suppressor activity [111]. Subsequent study demonstrated that this abnormal cytoplasmic localization of wild-type p53 is due to the hyperactive nuclear export of p53 through the exposure of highly conserved COOH-terminal NES [112]. Intriguingly, Becker et al. described that the hyperubiquitylation of p53 contributes to its aberrant cytoplasmic retention in neuroblastoma in association with the impaired interaction between p53 and HAUSP which catalyzes the deubiquitylation of p53 [113]. In addition, MDM2 had an ability to significantly enhance nuclear export of p53 through its COOH-terminal NES [114].

By using an affinity purification strategy, Nikolaev et al. discovered a large cytoplasmic protein termed Parc (p53-associated, Parlin-like cytoplasmic protein), which associated with cytoplasmic p53 [115]. According to their results, NH_2-terminal region of Parc interacted with the COOH-terminal region of p53. Parc had an intrinsic E3 ubiquitin ligase activity; however, Parc had an undetectable effect on the steady-state expression level of p53. Importantly, Parc was associated with the majority of cytoplasmic p53 and acted as a cytoplasmic anchor protein for p53. Indeed, depletion of Parc promoted nuclear localization of p53 and induced p53-dependent apoptotic cell death in neuroblastoma cells. Recently, it has been shown that the introduction of the COOH-terminal peptide of p53 containing Parc-interacting region disrupts the interaction between p53 and Parc in cytoplasm and results in the nuclear relocalization of p53 [116]. The treatment of this small peptide in cancerous cells increased the sensitivity of cancerous cells to anticancer drug and enhanced p53-dependent proapoptotic pathway.

It is worth noting that, in response to genotoxic stress, a certain fraction of p53 translocates to mitochondria, where p53 collaborates with BclXL and Bcl2 to induce permeabilization of the outer mitochondrial membrane, and thereby releasing cytochrome c [117]. Further study revealed that MDM2-mediated monoubiquitylation of p53 promotes the recruitment of p53 to mitochondria in which p53

undergoes a rapid deubiquitylation catalyzed by mitochondrial HAUSP, generating nonubiquitylated proapoptotic p53 [118]. Thus, targeting p53 to mitochondria, which resulted in the dysfunction of mitochondria, might be one of the transcription-independent proapoptotic pathways mediated by p53.

8. Variant Forms of p53

Yin et al. detected full-length wild-type p53 and another p53 with a relative molecular mass of 47 KDa termed p53/47 [119]. p53/47 was detectable by the 421 monoclonal antibody which recognized COOH-terminal portion of p53 and also detectable by the 1801 antibody which recognized an epitope between amino acid residues 46 and 55. On the other hand, the DO-1 (amino acid residues 20–25) and the DO-13 (amino acid residues 26–35) failed to recognize p53/47. Subsequent study demonstrated that p53/47 is generated from the NH_2-terminal alternative initiation site (Met-40). Since p53/47 lacked an NH_2-terminal MDM2-binding domain, it was not targeted for proteasome-dependent degradation by MDM2. Previous studies indicate that NH_2-terminal transactivation domain of p53 is divided into two independent domains such as TA I (amino acid residues 1–40) and TA II (amino acid residues 43–63) [120, 121]. p53/47 lacked TA I domain but contained TA II domain. Of note, p53/47 failed to transactivate $p21^{WAF1}$ but was able to induce the transcription of *MDM2*, *GADD45*, and *BAX*. These observations suggest that TA I and TA II domains contribute to enhance the specificity of p53-target promoter usage [119].

In addition to the alternative translation product of p53, Bourdon et al. found the presence of multiple variant forms of p53 arising from alternative promoter usage and alternative splicing events [122]. Based on their results, they identified the alternative promoter located within intron 4, and mRNA transcribed from this internal promoter generated NH_2-terminally truncated form of p53 initiated at codon 133 (Δ133p53), which lacked NH_2-terminal transactivation domain and Pro-rich domain. Further studies demonstrated that the alternative splicing of intron 9 results in the generation of p53β and p53γ, which deleted the COOH-terminal oligomerization domain. Thus, p53 is expressed as multiple variants including p53, p53β, p53γ, Δ133p53, Δ133p53β, Δ133p53γ, Δ40p53, Δ40p53β, and Δ40p53γ. Δ40p53 corresponds to p53/47 (Figure 2).

Immunostaining experiments revealed that most of these p53 variants were localized largely in cell nucleus, whereas p53γ was detectable both in cell nucleus and cytoplasm. Additionally, Δ133p53γ was localized exclusively in cytoplasm. p53 variants had an ability to bind differentially to p53-responsive promoters and modulated p53-target gene expression. For example, p53β bound preferentially to *BAX* and $p21^{WAF1}$ promoters rather than *MDM2* promoter, whereas p53 bound preferentially to $p21^{WAF1}$ and *MDM2* promoters than to *BAX* promoter [122].

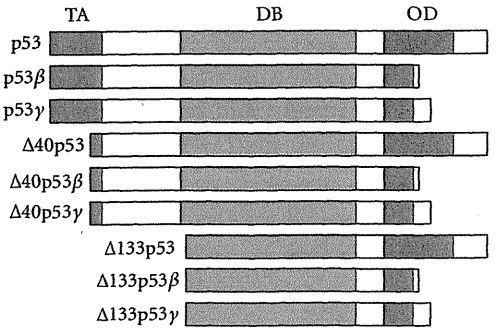

FIGURE 2: Schematic diagram of p53 variants. TA: transactivation domain; DB: sequence-specific DNA-binding domain; OD: oligomerization domain.

9. DNA Damage Response

During the early phase of DNA damage response, the activated forms of ATM (phospho-ATM at Ser-1981) phosphorylated histone variant H2AX at Ser-139 (γH2AX). This phosphorylation event took place at a large chromatin region surrounding DNA lesions and formed the nuclear foci, suggesting that γH2AX acts as a sensitive marker for the presence of DNA damage [123–125]. The disruption of H2AX resulted in an induction of genome instability and DNA double-strand break repair defects [126]. Then, NFBD1/MDC1 (nuclear factor with BRCT domain 1/mediator of DNA damage checkpoint protein 1), which had an antiapoptotic potential [127], interacted directly with γH2AX through its COOH-terminal BRCT domains and recruited MRN (MRE11, Rad50, and NBS1) complex onto the sites of DNA damage to facilitate the efficient DNA repair [128–130]. γH2AX then served as a platform for the recruitment of DNA checkpoint signaling factors as well as multifunctional MRN complex. Therefore, NFBD1-mediated local accumulation of DNA repair machinery such as MRN complex at the chromatin regions flanking the sites of DNA damage contributed to an increase in the fidelity of genomic integrity in response to DNA damage. Indeed, *NFBD1*-deficient mice exhibited chromosome instability, DNA repair defects, and radiation sensitivity [131]. In response to DNA damage, cell cycle checkpoint was activated to arrest cells at G1 phase, giving them time to repair damaged DNA. Recently, we have found that, during the early phase of DNA damage response, NFBD1 binds to NH_2-terminal region of p53 to inhibit ATM-mediated p53 phosphorylation at Ser-15, and thereby blocking its proapoptotic activity [132]. During the late phase of DNA damage response, the expression level of NFBD1 is sharply downregulated, and then the activated forms of ATM phosphorylate free p53 at Ser-15 to enhance its proapoptotic activity [132].

Runt-related (*RUNX*) gene family is composed of three members including *RUNX1*, *RUNX2*, and *RUNX3* [133]. Among them, RUNX3 has been considered to be a candidate tumor suppressor for human gastric cancer [134]; however, it remains unclear how RUNX3 exerts its tumor suppressor function. Recently, we have found that, in response to ADR

treatment, RUNX3 is induced to accumulate in cell nucleus and binds to p53 [135]. Based on our results, RUNX3 acted as a coactivator for p53 to enhance its transcriptional and proapoptotic activities. Of note, knocking down of the endogenous RUNX3 significantly repressed ADR-mediated phosphorylation of p53 at Ser-15. Since RUNX3 had an ability to interact with activated forms of ATM, it is likely that RUNX3 recruits the activated forms of ATM to latent form of p53 and thereby assisting ATM-dependent phosphorylation of p53 at Ser-15 in response to ADR. Thus, RUNX3 is closely involved at least in part in the regulation of DNA damage-mediated activation of p53 (Figure 3).

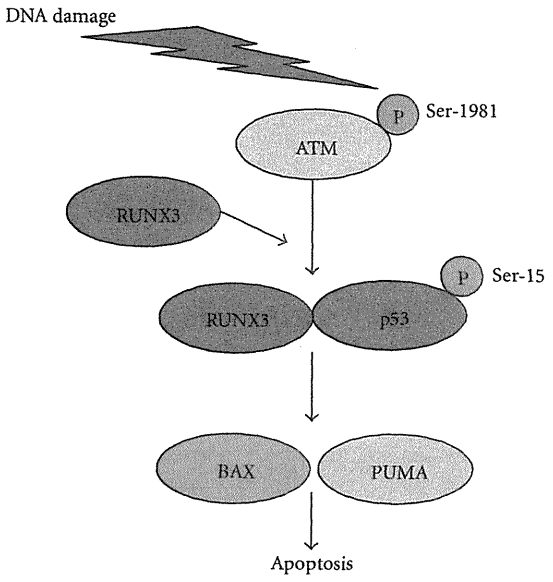

FIGURE 3: RUNX3 acts as a coactivator for p53 in response to DNA damage. In response to DNA damage, RUNX3 collaborates with activated form of ATM and induces phosphorylation of p53 at Ser-15. Phosphorylated form of p53 transactivates proapoptotic *BAX* and *PUMA*.

10. p53 Family

p53 tumor suppressor family is composed of three members including p53, p73 and p63. Like p53, p73, and p63 act as sequence-specific nuclear transcription factors and induce cell cycle arrest and/or apoptotic cell death in response to a certain subset of cellular stresses. p73 is expressed as multiple splicing variants with different COOH-terminal structures arising from the alternative splicing of the primary transcript such as p73α, p73β, p73γ, p73δ, p73ε, and p73ζ (37, 40, 135). Since all of them contain an intact NH_2-terminal transactivation domain, they have an ability to transactivate the overlapping set of p53-target gene (TA variants). Intriguingly, *p73* encodes the NH_2-terminally truncated variants termed ΔNp73 [136]. Since ΔNp73 lacked the intact NH_2-terminal transactivation domain, ΔNp73 was the transactivation defective and acted as the dominant-negative inhibitor toward TAp73 and wild-type p53 [136]. In this connection, ΔNp73 had an oncogenic potential [137]. Additionally, mutant forms of p53 inhibited the transcriptional and proapoptotic activities of TAp73 [138]. Of note, we and others demonstrated that TAp73 induces the transcription of its own inhibitor ΔNp73, creating a dominant-negative feedback loop which regulates the proapoptotic activities of both TAp73 and wild-type p53 [139–141]. Similarly, *p63* encodes TAp63 and ΔNp63 [39]. Therefore, relative ratio of TA and ΔN variants might be an important determinant of the cell fate.

Since p73 and p63 induce apoptotic cell death in cancerous cells, extensive mutation searches were performed. In a sharp contrast to *p53*, *p73* and *p63* were rarely mutated in various human tumor tissues [142], suggesting that p73 and p63 might not be classical Knudson-type tumor suppressors. Initial genetic studies revealed that *p73*-deficient mice and also *p63*-deficient mice do not develop spontaneous tumors [143–145]. Instead, *p73*-deficient mice displayed severe developmental defects including hydrocephalus, hippocampal dysgenesis, and abnormal pheromone sensory pathways. In *p63*-deficient mice, differentiation of apical ectodermal ridge failed, and generation of skin was not observed. These results suggest that p73 as well as p63 plays an important role in normal development. Strikingly, it has been shown that p73 and p63 heterozygous mice develop malignant tumors at high frequency. Tumors derived from these mice exhibited loss of the remaining wild-type allele at high frequency.

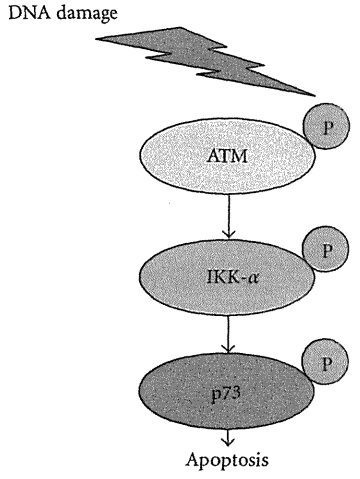

FIGURE 4: Upon DNA damage, activated form of ATM phosphorylates IKK-α and promotes nuclear accumulation of IKK-α. IKK-α enhances transactivation as well as proapoptotic function of p73 in a p53-independent manner.

These observations strongly suggest that loss of p73 and/or p63 function causes tumor development [146].

p73 and p63 are regulated at transcription as well as posttranslation levels. It has been shown that there exist several E2F1-binding sites within *p73* promoter region, and E2F1 actually acts as a transcriptional activator for *p73* [147–149]. Recently, Logotheti et al. demonstrated that Sp1 binds to the external promoter of *p73* gene and induces the expression of p73 [150]. On the other hand, Fontemaggi et al.

found that ZEB (zinc finger/homeodomain repressor) acts as a transcriptional repressor for p73 [151]. Of note, we have described that TATA-binding protein- (TBP-) like protein (TLP) has an ability to directly transactivate p63 [43].

Like p53, the activity and stability of p73 and p63 is regulated by chemical modifications such as phosphorylation. c-Abl-dependent phosphorylation of p73 at Tyr-99 resulted in an increase in its stability [152–154]. PKCδ phosphorylated p73 at Ser-289 to enhance its transcriptional activity [155]. Chk1-mediated phosphorylation of p73 at Ser-47 resulted in an enhancement of its transcriptional activity [156]. Recently, we have found that, in response to cisplatin (CDDP), IKK-α (IκB kinase-α) is induced to be accumulated in cell nucleus and interacts with p73 to increase its stability, thereby enhancing its proapoptotic activity in a p53-independent manner [157, 158]. IKK-α had an ability to phosphorylate the NH_2-terminal portion of p73 (Figure 4). On the other hand, CDK-mediated phosphorylation of p73 led to a significant inhibition of its transcriptional activity [159]. In addition, we have demonstrated that p73 is strongly inhibited by Plk1 through physical interaction and phosphorylation at Thr-27 [160]. Therefore, phosphorylation of p73 might not always act as an activation signal. Similarly, we have found that p63 is inhibited by Plk1-mediated phosphorylation at Ser-52 [161].

Since p73 and p63 were rarely mutated in human tumors, elucidation of the precise regulatory mechanisms behind DNA damage-induced activation of p73 and p63 might provide a clue to develop a novel strategy for the treatment of malignant tumors bearing nonfunctional p53.

11. Future Perspective

p53 plays a pivotal role in the regulation of cell fate determination in response to a variety of cellular stresses. Dysfunction of p53 such as mutational inactivation permits the abnormal cell growth and finally results in the malignant tumor development. In addition, loss of function of p53 contributes to the significant decrease in the sensitivity of tumor cells to anticancer drugs. Therefore, the elucidation of the precise molecular mechanisms behind stress-induced activation of p53 might provide a clue(s) to find out the attractive therapeutic target(s) for cancer treatment. In contrast to *p73* and *p63*, *p53* is frequently mutated in human tumors. Mutant forms of p53 lack their proapoptotic function and display a dominant-negative behavior toward wild-type p53 family. Further efforts should be required to develop the novel strategies and/or the chemical compounds which could convert the abnormal conformations of mutant p53 to normal ones. In this connection, one of the remaining questions is that why *p53* is frequently mutated in tumor tissues. Although it could be due to the serious defects in the DNA repair machinery in these tumors, further studies should be required to address this issue.

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Oncogenic LMO3 Collaborates with HEN2 to Enhance Neuroblastoma Cell Growth through Transactivation of Mash1

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Abstract

Expression of *Mash1* is dysregulated in human neuroblastoma. We have also reported that LMO3 (LIM-only protein 3) has an oncogenic potential in collaboration with neuronal transcription factor HEN2 in neuroblastoma. However, the precise molecular mechanisms of its transcriptional regulation remain elusive. Here we found that LMO3 forms a complex with HEN2 and acts as an upstream mediator for transcription of *Mash1* in neuroblastoma. The high levels of *LMO3* or *Mash1* mRNA expression were significantly associated with poor prognosis in 100 primary neuroblastomas. The up-regulation of *Mash1* remarkably accelerated the proliferation of SH-SY5Y neuroblastoma cells, while siRNA-mediated knockdown of *LMO3* induced inhibition of growth of SH-SY5Y cells in association with a significant down-regulation of *Mash1*. Additionally, overexpression of both LMO3 and HEN2 induced expression of *Mash1*, suggesting that they might function as a transcriptional activator for *Mash1*. Luciferase reporter assay demonstrated that the co-expression of LMO3 and HEN2 attenuates HES1 (a negative regulator for *Mash1*)-dependent reduction of luciferase activity driven by the *Mash1* promoter. Chromatin immunoprecipitation assay revealed that LMO3 and HEN2 reduce the amount of HES1 recruited onto putative HES1-binding sites and E-box within the *Mash1* promoter. Furthermore, both LMO3 and HEN2 are physically associated with HES1 by immunoprecipitation assay. Thus, our present results suggest that a transcriptional complex of LMO3 and HEN2 may contribute to the genesis and malignant phenotype of neuroblastoma by inhibiting HES1 which suppresses the transactivation of *Mash1*.

Citation: Isogai E, Ohira M, Ozaki T, Oba S, Nakamura Y, et al. (2011) Oncogenic LMO3 Collaborates with HEN2 to Enhance Neuroblastoma Cell Growth through Transactivation of *Mash1*. PLoS ONE 6(5): e19297. doi:10.1371/journal.pone.0019297

Editor: Laszlo Tora, Institute of Genetics and Molecular and Cellular Biology, France

Received December 21, 2010; **Accepted** March 31, 2011; **Published** May 5, 2011

Funding: This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labour and Welfare for the Third Term Comprehensive Control Research for Cancer; a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan; and a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Neuroblastoma is one of the typical childhood cancers and is originated from sympathetic cell lineage of the neural crest [1,2]. Since the tumor never occurs from the other lineages of the neural crest, the oncogenic events to cause neuroblastoma might be strictly regulated in a lineage-specific manner [1,2].

LIM-only protein (LMO) family is composed of four members, LMO1, LMO2, LMO3 and LMO4. Although LMO proteins lack a DNA-binding activity, accumulating evidence suggest that LMO proteins are involved in transcriptional regulation of specific target genes in collaboration with other transcription factors [3]. Genetic analyses demonstrated that LMO1 and LMO2 contribute to the genesis of immature and aggressive T-cell leukemia [4], whereas LMO4 was implicated in development of breast cancer [5,6]. Previously, we reported that *LMO3* is expressed at significantly high levels in human unfavorable neuroblastomas relative to favorable ones, and has an oncogenic potential in neuroblastoma [7]. LMO3 formed a complex with neuronal-specific basic helix-loop-helix (bHLH) transcription factor HEN2, which was also expressed at higher levels in unfavorable neuroblastoma than favorable one, raising a possibility that LMO3 may form a complex with HEN2 and play an important role in genesis and development of neuroblastoma through transcriptional regulation of as yet unidentified target gene(s).

A proneural bHLH transcription factor termed Mash1 plays a critical role in development of sympathetic neuron and is highly expressed in neuroblastoma [8,9]. However, its possible contribution to development of neuroblastoma remains elusive. A bHLH protein termed HES1 acts as a negative regulator for *Mash1* [10]. Intriguingly, studies in *Drosophila* demonstrated that expression levels of *achaete-scute*, a *Drosophila* homolog of *Mash1*, are remarkably induced by a transcriptional complex composed of *Drosophila* homolog of LMO (dLMO) and bHLH proteins [11,12].

In this study, we examined whether there could exist functional relationship between LMO3/HEN2 and Mash1 in neuroblastoma, and found that LMO3/HEN2 attenuates HES1 function and enhances transactivation of *Mash1*, leading to aggressive phenotype of neuroblastoma.

Results

High levels of *Mash1* expression is associated with poor outcome of neuroblastoma

Mash1 is constitutively expressed at high levels in neuroblastoma cell lines and primary neuroblastoma tumors [9,13], however, its prognostic significance remained elusive. On the other hand, expression of *LMO3* was significantly associated with poor outcome of the patients [7]. To verify whether a significant relationship could be observed between expression of *LMO3* and that of *Mash1* in primary neuroblastomas, we quantitatively measured the expression levels of *LMO3* and *Mash1* mRNA in 100 primary tumors by using a quantitative real-time RT-PCR. The student's t-test showed that high expression of *LMO3* was significantly associated with ≥1 year of age ($p = 0.036$), low expression of *TrkA* ($p = 0.003$) and *MYCN* amplification ($p = 0.04$), but not with the tumor stage ($p = 0.17$), tumor origin ($p = 0.083$) and Shimada classification ($p = 0.082$). High expression of *Mash1* was significantly associated with advanced tumor stage ($p = 0.004$) but not with age ($p = 0.81$), *TrkA* expression ($p = 0.4$), *MYCN* copy number ($p = 0.11$), tumor origin ($p = 0.2$) and Shimada classification ($p = 0.45$) (Table S1). No significant relationship was observed between *LMO3* and *Mash1* mRNA expression levels (the Pearson correlation coefficient was 0.27). Kaplan-Meier survival curves indicated that high expression of *LMO3* as well as that of *Mash1* were significantly associated with poor prognosis (log-rank test, $p = 0.006$ and $p = 0.037$, respectively; Figure 1). The univariate analysis according to the Cox proportional hazard model also indicated that the expression levels of *Mash1* and those of *LMO3* were significantly associated with poor outcome of the patients ($p = 0.048$ and $p = 0.012$, respectively; Table S2). The multivariate Cox proportional hazard model analysis showed that the expression of *Mash1* was significantly independent prognostic factor from *LMO3* expression and age, marginally from *MYCN* copy number and origin, but not from the disease stage, and that the expression of *LMO3* was significantly independent prognostic factor from *Mash1* expression, age, the disease stage and origin, but not from *MYCN* copy number (Table S2). Thus, the results obtained from the primary neuroblastomas suggested that both high mRNA expression of *LMO3* and *Mash1* were strongly associated with poor prognoses of the patients with neuroblastoma but the way of contribution of those seemed to be rather independent.

Mash1 mediates growth promotion of neuroblastoma cells

Since *Mash1* is highly expressed in primary neuroblastoma [9] and its higher expression was significantly correlated with poor prognosis of the patient with neuroblastoma, we then investigated a possible contribution of Mash1 to neuroblastoma cell growth. For this purpose, we established three stable *Mash1* infectants derived from the parental SH-SY5Y neuroblastoma cells expressing exogenous Mash1 (M-1, M-2 and M-3) and two control vector alone infectants (V-1 and V-2) by retrovirus-mediated gene transfer (Figure 2A). As shown in Figure 2B, constitutive expression of *Mash1* in SH-SY5Y cells resulted in a remarkable increase in their growth rate as compared with the control infectants, suggesting that Mash1 is involved in regulation of neuroblastoma cell growth.

As described previously [7], LMO3 has an oncogenic potential in collaboration with HEN2 in neuroblastoma cells. We then asked whether or not LMO3 is involved in the Mash1-mediated enhancement of cell growth. As shown in Figure 2C, siRNA-mediated knockdown of *LMO3* in SH-SY5Y cells was significantly associated with a down-regulation of *Mash1*. Additionally, LMO3-knocked down SH-SY5Y cells showed a slower growth rate than the control SH-SY5Y cells (Figure 2D), which might be at least in part due to reduction of *Mash1*. We conducted the same experiments by using another cell line SK-N-BE and obtained the similar results (Figure S1A and B). We then hypothesized that *Mash1* could be one of transcriptional targets of LMO3/HEN2 complex.

LMO3/HEN2 mediate transcriptional induction of *Mash1*

To address whether *Mash1* transcription could be induced by LMO3/HEN2, SH-SY5Y cells were infected with the indicated combinations of recombinant adenoviruses encoding HA-LMO3 or FLAG-HEN2, and the expression levels of *Mash1* were examined by semi-quantitative RT-PCR. Time course experiments demonstrated that *Mash1* is readily detectable in cells expressing HA-LMO3 alone or in cells co-expressing with HA-LMO3 and FLAG-HEN2 at 48 h after infection

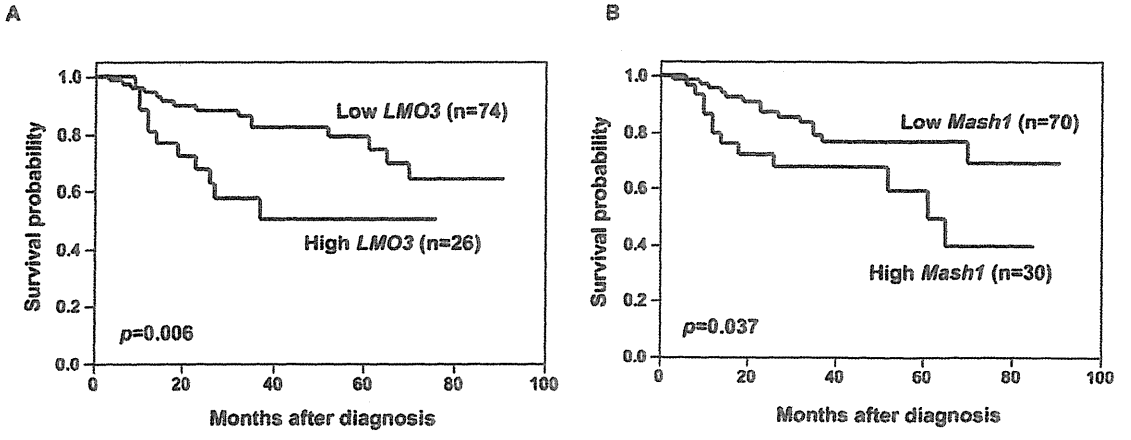

Figure 1. Kaplan-Meier survival curves of patients with neuroblastomas based on high or low expression of LMO3 (A) or Mash1 (B). Kaplan-Meier survival curves (n = 100) in relation to the expression levels of *LMO3* or *Mash1* (average cutoff). The patients with high expression of *LMO3* or *Mash1* represented significantly poor prognosis than those with its low expression.
doi:10.1371/journal.pone.0019297.g001

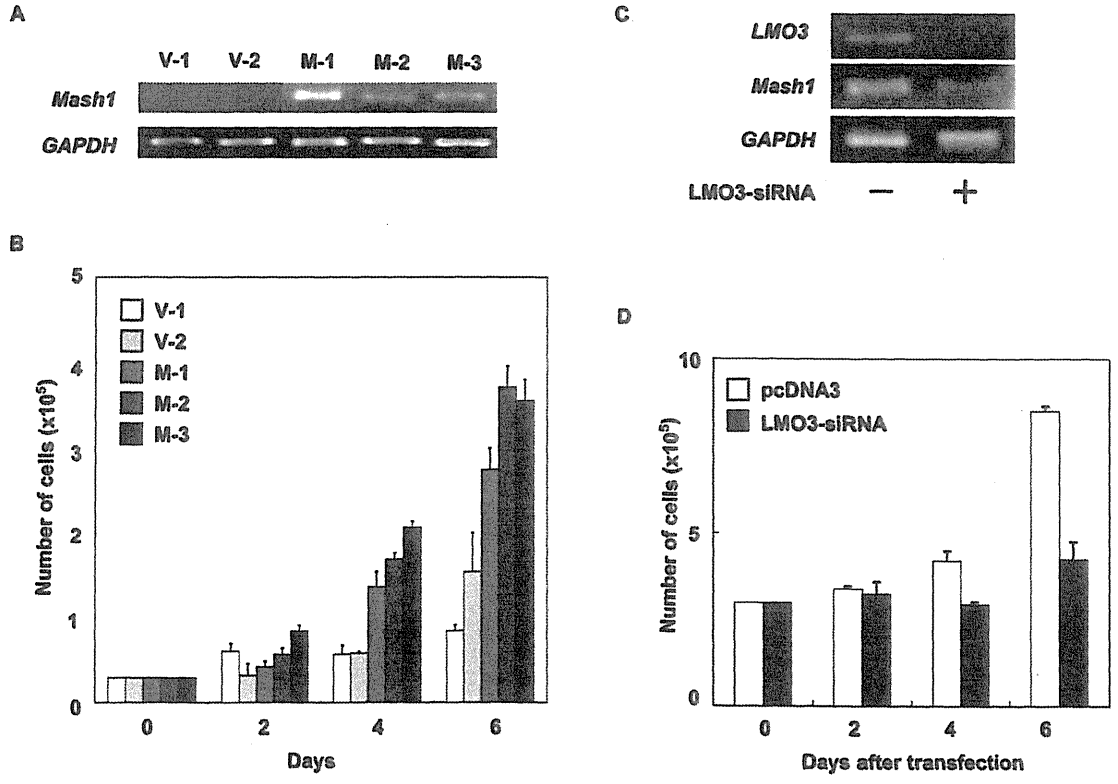

Figure 2. Mash1-mediated growth promotion of neuroblastoma cells. (A) Enforced expression of *Mash1*. Neuroblastoma SH-SY5Y cells were infected with empty retrovirus or with retrovirus encoding Mash1 and established two control infectants (V-1 and V-2) and three infectants expressing *Mash1* (M-1, M-2 and M-3). Total RNA was extracted from the indicated cell clones and subjected to RT-PCR to examine expression levels of *Mash1*. *GAPDH* was used as an internal control. (B) Mash1-mediated growth promotion. The indicated infectants were seeded at a density of 3×10^4/cell culture dish and allowed to attach overnight. At the indicated time periods, number of viable cells was measured. (C) siRNA-mediated knockdown of LMO3. SH-SY5Y cells were transfected with empty plasmid (4 μg) or with expression plasmid for siRNA targeting LMO3 (4 μg). Forty-eight hours after transfection, total RNA was prepared and analyzed for expression levels of *LMO3* and *Mash1* by RT-PCR. (D) Decreased growth rate in LMO3-knocked down cells. SH-SY5Y cells (3×10^5 cells/cell culture dish) were transfected as in (C). Forty-eight hours after transfection, cells were transferred into fresh medium. At the indicated time points, number of viable cells was measured.
doi:10.1371/journal.pone.0019297.g002

(Figure 3A). Seventy-two hours after infection, co-expression of HA-LMO3 and FLAG-HEN2 led to a significant induction of *Mash1*. The induction of *Mash1* was also observed in SK-N-BE cells transfected with expression vector HA-LMO3 alone or HA-LMO3 and FLAG-HEN2 at 72 h after transfection (Figure S1C). To further confirm these observations, we generated a luciferase reporter construct carrying human *Mash1* promoter (pluc-Mash1). As shown in Figure 3B, the 5′-upstream region of *Mash1* gene contains three putative HES1-binding sites and one E-box. In both SH-SY5Y cells and SK-N-BE cells, siRNA-mediated knockdown of human *LMO3* reduced promoter activity of *Mash1* in a dose-dependent manner (Figure 3C and Figure S1D). For luciferase reporter assay without siRNA for human LMO3, we used mouse neuroblastoma Neuro2a cells which displayed higher transfection efficiency than human neuroblastoma cells as examined by GFP staining (data not shown). Consistent with the above expression studies, LMO3 enhanced luciferase activity driven by *Mash1* promoter (Figure 3D). Furthermore, we examined the effect of HEN2 on *Mash1* promoter activity in Neuro2a cells, showing that HEN2 itself inhibited *Mash1* promoter activity (Figure 3E). Intriguingly, however, LMO3 interfered with HEN2 function, resulting in up-regulation of *Mash1* transcription (Figure 3F). Thus, it is likely that the LMO3 complex including HEN2 and HES1 regulates transcription of *Mash1*. The mRNA expression pattern of *LMO3*, *HEN2*, *Mash1* and *HES1*, a negative regulator of *Mash1* transcription, in neuroblastoma cell lines is shown in Figure S2.

LMO3/HEN2 attenuates HES1-dependent down-regulation of *Mash1*

As reported previously [10], HES1 is one of the negative regulators for *Mash1*. In accordance with the previous observations, enforced expression of HES1 dramatically reduced luciferase activity driven by *Mash1* promoter (Figure 4A). The inhibitory effect of HES1 on *Mash1* promoter was stronger than that of HEN2. To investigate the relationships between HES1 and LMO3/HEN2 in transcriptional regulation of *Mash1*, we examined effects of HEN2 and LMO3 on HES1-dependent down-regulation of *Mash1* (Figure 4B). The HES1-dependent inhibition of *Mash1* promoter activity was attenuated by co-expression with FLAG-HEN2 alone or with co-expression with FLAG-HEN2 plus HA-LMO3. Inhibitory effects of FLAG-HEN2 plus HA-LMO3 on HES1 were larger than that of FLAG-HEN2 alone, suggesting that LMO3/HEN2 complex plays a critical role in regulation of *Mash1* transcription by neutralizing the inhibitory effect of HES1.