— Regular Article —

Histone Methyltransferase PR-Set7 and Histone Variant H2A.Z, Induced during Hepatocarcinogenesis, Repress the Promoter Activity of the Tumor Marker Gene and the Ras-Induced Colony Formation Activity

Hiroshi Hashizume, Urara Gomita, Masayoshi Imagawa, and Shigehiro Osada*

Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3–1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467–8603, Japan

(Received January 8, 2011; Accepted February 22, 2011; Published online February 28, 2011)

Genetic and epigenetic studies are required to understand molecular mechanisms of carcinogenesis and tumorigenesis. Although alterations of DNA methylation and histone modification profiles are observed in cancer cells, the knowledge of the epigenetic regulatory factors involved in carcinogenesis is insufficient. In this study, we showed that the histone variant, *H2a.z*, and the histone methyltransferase, *Pr-set7*/*Set8*/*KMT5a*, were up-regulated during chemically induced hepatocarcinogenesis. During this process, the glutathione *S*-transferase placental form (GST-P), which is completely repressed in normal liver, is strongly induced and is therefore an excellent tumor marker. Reporter analysis performed using the regulatory region of the *Gst-p* gene revealed that H2A.Z and PR-SET7 repressed *Gst-p* promoter activity. The enhancer element responsible for hepatocarcinogenic-specific gene expression was required for repression by H2A.Z, and the negative regulation by PR-SET7 mediated the regulatory element but not the enhancer. Furthermore, we examined the effects of overexpression of H2A.Z and PR-SET7 on the colony formation activity of mouse NIH-3T3 cells grown on a soft agarose medium. Colony formation based on anchoring independent cell growth is a feature of malignant transformed cells. These factors did not exhibit colony formation activity but repression of the *ras* oncogene induced this activity. PR-SET7 suppressed RAS^{val12}-mediated colony formation through methyltransferase activity. These results suggest that *H2a.z* and *Pr-set7* that are induced during hepatocarcinogenesis may function as carcinogenesis suppressors.

Key words —— histone variant, histone methyltransferase, epigenetics, anchorage-independent growth, tumor marker gene, hepatocarcinogenesis

INTRODUCTION

Genetic mutations generate mutated proteins and disturb the regulation of gene expression. The dysregulation of epigenetic programming also alters gene expression. Genetic and epigenetic mutations may lead to the disruption of homeostatic maintenance and ultimately result in malignant transformation.[1,2] Epigenetic regulation involves DNA methylation, covalent modification of core histone, chromatin remodeling, and deposition of histone variants.

The best-studied epigenetic modification in cancer is DNA methylation within the CpG dinucleotide. Global genomic DNA hypomethylation in tumors disturbs genomic stability and hypermethylation of the regulatory region in tumor suppressor genes inactivates their expression. These changes found in many cancers initiate carcinogenesis and tumor development.[2,3] Histone modifications such as acetylation, methylation, and phosphorylation regulate gene function, while their dysregulation leads to cancer. These findings promote epigenetic therapies and develop inhibitors for DNA methyltransferase and histone deacetylase (HDAC) that are currently being evaluated in clinical trials.[4]

Although epigenetic aberrations are observed in cancer, epigenetic regulators, which lead to ma-

*To whom correspondence should be addressed: Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3–1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467–8603, Japan. Tel. & Fax: +81-52-836-3456; E-mail: osada@phar.nagoya-cu.ac.jp

lignant transformation in the early stages of carcinogenesis, are not fully understood. To identify the regulators of epigenetic status involved in malignant transformation and tumor marker expression, we followed the Solt-Farber protocol, and hepatic preneoplastic lesions were chemically induced in rats.[5–8] During hepatocarcinogenesis, the glutathione *S*-transferase placental form (GST-P) is up-regulated in hepatic preneoplastic lesions and can be used as an excellent tumor marker.[9] In this process, we found that histone acetyltransferases (HATs) p300 and cAMP-response-element-binding protein-binding protein were down-regulated, whereas the expression of the monocytic leukemia zinc-finger protein (MOZ) was induced. Furthermore, we found that MOZ induced GST-P expression.[7] In addition to HAT, we observed the expression profiles of histone deacetylase (*Hdac*) family members and found that *Hdac9* was induced during hepatocarcinogenesis and promoted anchorage-independent growth.[8]

As described above, we investigated the regulators of the histone acetylation status. Next, to gain insights about epigenetic regulatory factors, except for histone acetylation, involved in the early stages of carcinogenesis, we focused on genes up-regulated in GST-P positive foci compared with control liver by cDNA microarray analysis.[6] Genes induced in GST-P positive foci include negative and positive regulators of carcinogenesis. Genes involved in transcription induced in GST-P positive foci contained DNA-binding proteins, chromatin components, and chromatin modification enzymes.[6]

In this study, we focused on the histone variant, H2A.Z, and the histone methyltransferase, PR-SET7/SET8/KMT5a. *H2a.z* is a variant of *H2a* and is more conserved than other H2A proteins of the species.[10, 11] The specific localization of H2A.Z in chromatin contributes to the positive and negative regulation of gene expression.[12–15] PR-SET7 is a histone methyltransferase that specifically monomethylates histone H4 lysine 20 (H4K20me1).[16, 17] It is involved in DNA damage response, cell cycle regulation, chromatin condensation, and chromatin segregation; a lack of *Pr-set7* leads to embryonic lethal mutations.[18–24] H4K20me1 is related to both transcriptionally repressed chromatin and activated genes.[12, 18, 25–27] In this study, we demonstrated that these factors repressed the promoter activity of the *Gst-p* gene and the *ras*-induced colony formation activity. These results suggest that *H2a.z* and *Pr-set7* induced during hepatocarcinogenesis may possess protective effects against malignant transformation.

MATERIALS AND METHODS

Chemical Hepatocarcinogenesis of Rats —— Hepatocarcinogenic experiments were performed according to the Solt-Farber protocol as described previously.[5, 6] Experiments were initiated by intraperitoneal injection of diethylnitrosamine (200 mg/kg, Wako Pure Chemical Industries, Ltd., Osaka, Japan) into 5-week-old Sprague-Dawley rats. They were fed basal diets containing 0.02% 2-acetylaminofluorene (Nacalai Tesque, Kyoto, Japan) and a partial hepatectomy was performed at 2 and 3 weeks after diethylnitrosamine injection. Livers were resected 8 weeks after diethylnitrosamine injection. At this stage, approximately 80% of the areas were GST-P positive. Control rats were injected with saline and fed basal diets. Animal experiments were performed at Osaka University. All animal care and handling procedures were approved by the animal care and use committee of Osaka University.

Cloning of Rat *Pr-set7* cDNA —— To generate primers for the cDNA cloning of rat *Pr-set7* open reading frame (ORF) by reverse-transcriptase-coupled polymerase chain reaction (RT-PCR), we referred to sequences of rat genomic DNA and expressed sequence tag (EST; accession no. CK366036). Primer sequences such as the predicted translation initiation and termination codon were as follows: 5′-ATAGCTAGCGGAGATGGTGGAGCGGAGG-3′ and 5′-ATAGGATCCTTAGTGCTTCAGCCAAGGGTAG-3′. RT-PCR using rat liver RNA and ReverTra Ace (Toyobo, Osaka, Japan) was performed, and the amplified DNA was sequenced and cloned. The resultant sequence encoding rat *Pr-set7* cDNA was registered with the DNA Data Bank of Japan (accession number AB606422).

Northern Blot Analysis —— Total RNA was prepared from 3 control livers (#50, #51, and #52) as well as 3 livers with hyperplastic nodules (#43, #44, and #46) using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Twenty-five microgram of total RNA was separated on 1% agarose gel containing 2% formaldehyde and transferred to a Hybond-N^+ nylon membrane (GE Healthcare, Piscataway, NJ, U.S.A.). Specific probes for *H2a.z*

and *Pr-set7* were amplified by RT-PCR using rat liver RNA as a template with primers corresponding to *H2a.z* (position 368–785; accession number M37584) and *Pr-set7* (position 159–620; accession number AB606422). Labeling was performed with a BcaBEST labeling kit (Takara Bio, Shiga, Japan) and [α-^{32}P] deoxycytidine 5′-triphosphate (dCTP), and hybridizations were performed using standard methods.

Plasmid Construction —— Reporter plasmids were constructed by subcloning the regulatory region of the *Gst-p* gene, −2.5 kb to +59 bp and −2.15 kb to +59 bp, into the *Kpn*I-*Hin*dIII and *Xho*I-*Hin*dIII sites of pGL4.17 (Promega, Madison, WI, U.S.A.), respectively.

Expression plasmids for H2A.Z and PR-SET7 were constructed by subcloning *H2a.z* and *Pr-set7* ORFs into the *Eco*RI-*Bam*HI site of p3xFLAG-CMV-10 (Sigma, St. Louis, MO, U.S.A.) and the *Eco*RI-*Not*I site of pCMV-Myc (Clontech Mountain View, CA, U.S.A.), respectively.

RAS^{val12} expression plasmid (pCEV4-*ras^{val12}*) was kindly provided by Kaibuchi (Nagoya University) for colony formation assay.[28] To synthesize the construct of pCEV4-Myc-PR-SET7, the Myc-tagged *Pr-set7* ORF from pCMV-Myc-PR-SET7 was subcloned into the *Bam*HI site of pCEV4. pCEV4-Myc-PR-SET7ΔC lacking the carboxy terminal region was constructed by inserting the Myc-tagged fragment corresponding to amino acid residues 1–186 to the *Bam*HI site of pCEV4. A mutation was introduced in the catalytic region of PR-SET7 using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) following the manufacturer's instructions, and confirmed by sequencing. All fragments generated by PCR were also verified by sequencing.

Cell Culture —— Rat hepatoma H4IIE cells, purchased from Dainippon Sumitomo Pharma, Osaka, Japan, and mouse fibroblast NIH-3T3 cells, obtained from the Japanese Collection of Research Bioresources, were maintained in an α-medium supplemented with 10% (v/v) fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) calf serum.

Reporter Assay —— Transfection of H4IIE cells in 24-well plates was performed using HilyMax (Dojindo, Kumamoto, Japan) in accordance with the manufacture's instructions. All transfections included 50 ng of the reporter plasmid and 350 ng of the expression plasmids for H2A.Z and PR-SET7. The amount of plasmid during transfection was kept constant by using an empty vector. The β-galactosidase expression plasmid, pCMV-β-gal, kindly provided by Hayashi (Nagoya City University), was used as the internal control.[29] Transfectants were harvested 40 hr after transfection. A luciferase assay was performed as described previously.[30] Protein concentrations were determined by the Bradford method. In case of experiments using H2A.Z and PR-SET7, luciferase activities were normalized to the protein amount and β-galactosidase activity, respectively.[30]

Anchorage-independent Growth Assays and Western Blot Analysis —— To evaluate anchorage-independent growth of NIH-3T3 cells, 5 μg of the expression plasmid or empty vector was introduced with 5 μg of the RAS^{val12} expression plasmid into them by the calcium phosphate co-precipitation method.[31] As a negative control, 10 μg of the empty vector was transected. After 24 hr, 2×10^5 cells in 2 ml of DMEM containing 0.55% agarose were poured over a layer of 0.5% agarose/DMEM in a 60-mm dish. After 2 weeks, the plates were incubated overnight at 4°C. Colonies (diameter greater than or equal to 100 μm) were counted on 3 plates. Results are shown as the mean ± standard deviation (S.D.). Significant differences were identified using Student's *t*-test.

For the Western blot analysis, transfected cells were seeded (1.25×10^5 cells/12-well plate or 6×10^5 cells/60-mm dish), incubated for 24 hr, harvested by centrifugation, and suspended in 1x sodium dodecyl sulfate (SDS) loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.01% bromophenol blue, and 10% glycerol] or lysed with the lysis buffer [25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 137 mM NaCl, 10% Glycerol, 1% Nonidet P-40] or RIPA buffer [50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM dithiothreitol]. Samples were boiled, loaded onto a 10% or 12% SDS-polyacrylamide gel, transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare) or a Immobilon-P transfer membrane (Millipore, Billerica, MA, U.S.A.), and detected using the ECL Western blotting analysis detection reagents (GE Healthcare) and a BCIP/NBT Western Blotting Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.). The following antibodies were obtained commercially: anti-myc (Roche, Indianapolis, IN, U.S.A.), anti-FLAG (Sigma), anti-β-actin (Sigma), and anti-H-RAS (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.).

RESULTS

Molecular Cloning of Rat *Pr-set7* cDNAs —— Nucleotide sequences for human and mouse, but not rat, *Pr-set7* cDNA are publicly available. We cloned rat cDNA encoding *Pr-set7* by RT-PCR using rat liver RNA and primers based on rat genomic DNA and EST sequences. The rat *Pr-set7* ORF included 969 nucleotides and encoded 322 amino acids. Mammalian PR-SET7 proteins were highly conserved in the whole region. In particular, the sequences for the su(var)3-9, enhancer-of-zeste, and trithorax (SET) domains, required for histone methyltransferase activity, were almost identical to each other (Fig. 1). PR-SET7 was evolutionarily conserved in several species; the predicted PR-SET7 in yeast has been also registered with the database.

Expression of *H2a.z* and *Pr-set7* during Hepatocarcinogenesis —— The cDNA microarray, utilized previously, uses 3′-untranslated regions as probes. To certify the expression of *H2a.z* and *Pr-set7* during chemical hepatocarcinogenesis, we performed Northern blot analysis using probes that included coding regions and RNA prepared from control livers and livers with hyperplastic nodules, harboring approximately 80% GST-P positive foci, from each of the 3 rats (Fig. 2). No *Gst-p* was detected in control rats, but it was drastically induced during hepatocarcinogenesis. In this condition, both *H2a.z* and *Pr-set7* in livers with hyperplastic nodules were up-regulated compared with the control liver.

Effects of Overexpression of H2A.Z and PR-SET7 on the Promoter Activity of Tumor Marker Gene —— GST-P is a reliable tumor maker for chemically induced hepatocarcinogenesis, and the regulation of its expression is well understood.[32] A typical promoter region includes a TATA-box and GC-box, and the antioxidant responsive element/phorbol 12-*O*-tetradecanoate 13-acetate (TPA) responsive element (ARE/TRE) is located at 61 bp upstream of transcription start site (TSS) (Fig. 3A). These elements are critical for the basal expression.[33] For specific activation of *Gst-p* during hepatocarcinogenesis, a strong enhancer element (*Gst-p* enhancer, GPE), located 2.5 kb upstream from the TSS, is required.[34] In addition to the positive regulatory elements, silencer elements recognized by nuclear factor 1 and

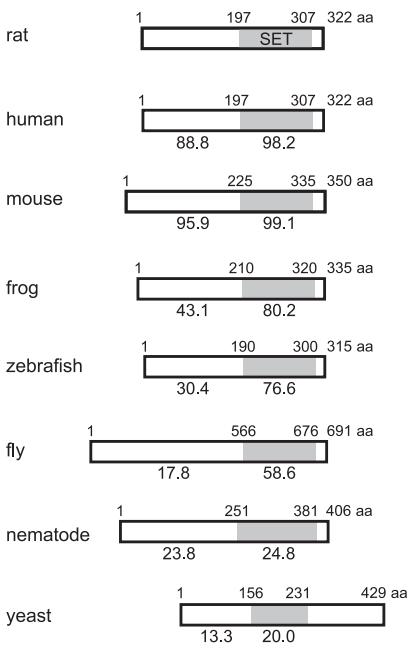

Fig. 1. Schematic Diagram of PR-SET7 of Several Species

The SET domain required for histone methyltransferase activity is indicated by a gray box. Numbers below SET domain and the amino-terminal side of the SET domain indicate the percentage identity compared with the rat sequence. Accession numbers of the sequences compared are as follows: human (*Homo sapiens*, AAL40879), mouse (*Mus musculus*, NP_084517), frog (*Xenopus laevis*, NP_001121300), zebrafish (*Danio rerio*, NP_001093559), fly (*Drosophila melanogaster*, NP_650354), nematode (*Caenorhabditis elegans*, NP_510241), yeast [*Schizosaccharomyces pombe,* NP_593610 (predicted)]. The number of amino acid positions is presented above the schematic diagram.

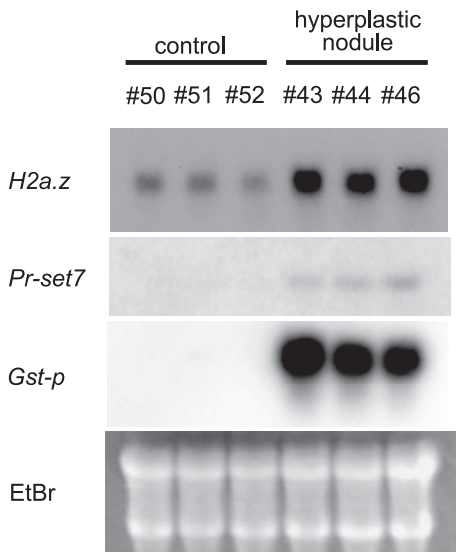

Fig. 2. Expression of *H2a.z* and *Pr-set7* during Hepatocarcinogenesis

Northern blot analysis of *H2a.z* and *Pr-set7* using total RNA prepared from control livers (#50, #51, and #52) and livers with hyperplastic nodules (#43, #44, and #46). Hyperplasic nodules were induced as described in the Materials and methods. *Gst-p* is control for a drastic induction during hepatocarcinogenesis. Ethidium bromide (EtBr) staining showed that equal amount of RNA was loaded for Northern blot analysis.

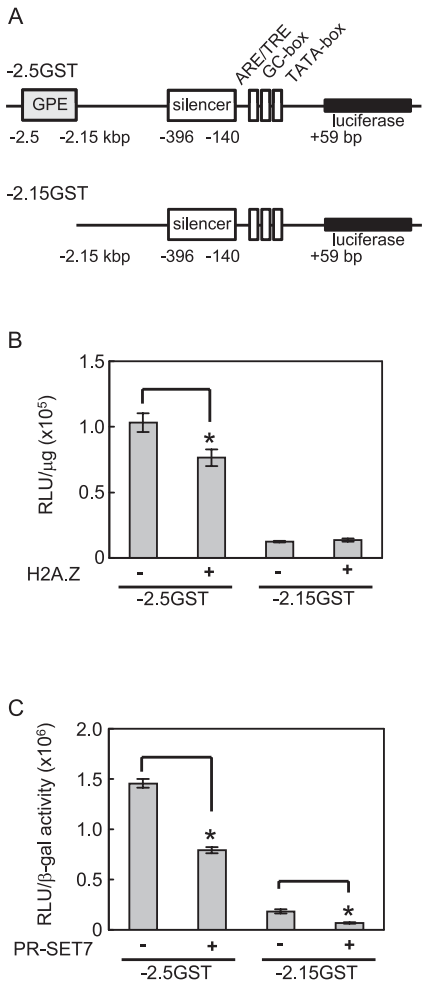

Fig. 3. Effects of Overexpression of H2A.Z and PR-SET7 on *Gst-p* Promoter Activity in Rat Hepatoma H4IIE Cells

(A) Schematic diagram of luciferase reporter plasmids. GPE, the enhancer element required for the specific activation of *Gst-p* during hepatocarcinogenesis, and silencer elements are located at 2.5 kb and 396 bp upstream from the TSS, respectively. ARE/TRE, a GC-box, and a TATA-box are present near the TSS. −2.5 GST and not −2.15 GST possesses the enhancer element, GPE. (B) Effects of overexpression of H2A.Z on *Gst-p* promoter activity. Each reporter plasmid was transfected with the empty vector or the H2A.Z expression plasmid. Luciferase activity was normalized to the protein amount. (C) Effects of overexpression of PR-SET7 on *Gst-p* promoter activity. Luciferase activity was normalized to β-galactosidase activity. All transfection experiments were performed in triplicates or quadruplicates. A typical result from 2 or 4 independent experiments is shown. Values are presented as the mean ± S.D. Significant differences identified with Student's *t*-test at $p < 0.01$ versus the results from the empty vector are indicated by ∗.

CCAAT/enhancer binding protein families are located between the −396 bp and −140 bp region.[30, 35–37]

To analyze the effects of H2A.Z and PR-SET7 on the specific expression of *Gst-p* during hepatocarcinogenesis, we performed a luciferase reporter assay using rat hepatoma cells H4IIE and reporter plasmids including the entire regulatory regions of the *Gst-p* gene (−2.5 GST) and −2.15 GST lacking GPE. Although the β-galactosidase expression plasmid was used as an internal control, β-galactosidase expression controlled by a promoter of cytomegalovirus and Rous sarcoma virus was affected by H2A.Z (data not shown). Therefore, the transfection was carefully done, and it was confirmed that the transfection efficiency was constant. Luciferase activities were normalized to the protein amount (Fig. 3B). In contrast, as PR-SET7 did not affect the β-galactosidase activity, luciferase activities were normalized to the β-galactosidase activity (Fig. 3C).

H2A.Z repressed the luciferase activity from −2.5 GST, but this repression was not observed when −2.15 GST was used (Fig. 3B). These results suggest that H2A.Z repressed *Gst-p* promoter activity through the enhancer element, GPE. PR-SET7 repressed the luciferase activity derived from −2.5 GST and −2.15 GST (Fig. 3C). This indicates that PR-SET7 represses *Gst-p* promoter activity in a GPE-independent manner.

Effects of Overexpression of H2A.Z and PR-SET7 on Anchorage-independent Growth —— One of the characteristics of the transformed cells is acquisition of anchorage-independent growth. Mouse NIH-3T3 fibroblasts possess a normal cellular phenotype and are often used to estimate the colony formation activity descended from anchorage-independent growth.

To investigate whether H2A.Z and PR-SET7 induce anchorage-independent growth, we introduced expression plasmids for H2A.Z and PR-SET7 in NIH-3T3 cells, and these cells were disseminated on soft agarose medium. As a positive control for colony formation, the oncogenic mutant of RAS, RAS^{val12}, was used. Two weeks after the introduction of genes, the RAS^{val12} transfected cells produced numerous colonies, unlike the empty vector and the expression plasmids for H2A.Z and PR-SET7 (data not shown). These results indicate that *H2a.z* and *Pr-set7* were unable to transform mouse NIH-3T3 fibroblasts.

Next, we focused on the protective effect of H2A.Z on transformation (Fig. 4). For this purpose, the empty vector or expression plasmid for H2A.Z was co-transfected with the RAS^{val12} expression plasmid into NIH-3T3 cells. Although the RAS^{val12} expression plasmid produced approximately 80 colonies in the absence of H2A.Z, the number of colonies was halved when H2A.Z was cotransfected. Western blot analysis indicated that

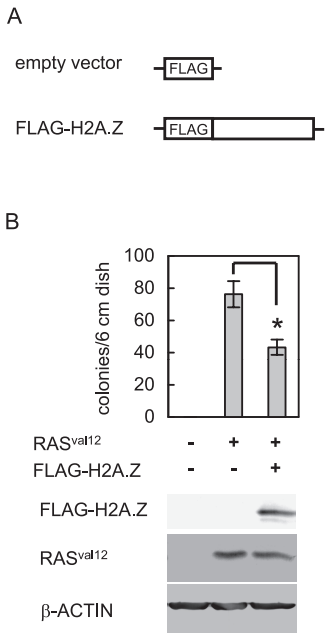

Fig. 4. Effects of Ectopic Expression of H2A.Z on RAS^{val12}-Induced Colony Formation in NIH-3T3 Cells

(A) Schematic diagram of the empty vector and H2A.Z expression plasmid. (B) RAS^{val12} expression plasmid or empty vector was transfected with the H2A.Z expression plasmid or empty vector into NIH-3T3 cells and incubated for 2 weeks. The resultant colonies were counted. Values are presented as the mean ± S.D. A typical result from 3 independent experiments is shown. Significant differences identified with Student's *t*-test at $p < 0.01$ versus the control are indicated by ∗. Ectopically expressed H2A.Z and RAS^{val12} proteins were detected by Western blotting with β-actin used as an internal control.

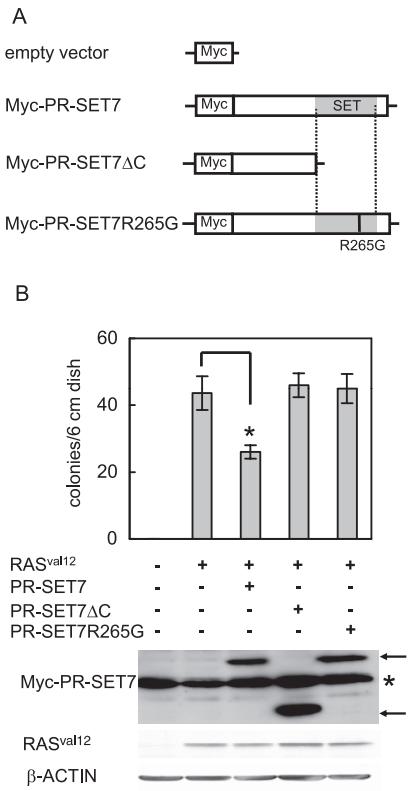

Fig. 5. Effects of Overexpression of PR-SET7 and Their Mutants on RAS^{val12}-Induced Colony Formation on NIH-3T3 Cells

(A) Schematic diagram of the empty vector and PR-SET7 expression plasmids. (B) Colony formation assay was performed, and the results are shown in Fig. 4. Significant differences identified with Student's *t*-test at $p < 0.01$ versus the control are indicated by ∗. Ectopically expressed PR-SET7 and RAS^{val12} proteins were detected by Western blotting with β-actin used as an internal control. Wild-type and mutated Myc-tagged PR-SET7 are indicated by arrows, and the asterisk denotes the non-specific signal.

endogenous RAS was low or undetectable levels and confirmed that the protein level of RAS^{val12} was indistinguishable in both types of transfected cells. These results show that *H2a.z* is a negative regulator of RAS mediated transformation of NIH-3T3 cells.

In addition, we observed the effects of PR-SET7 on RAS^{val12}-mediated colony formation (Fig. 5). The number of colonies produced by RAS^{val12} introduction was reduced by the cotransfection of PR-SET7. To investigate whether the methyltransferase activity of PR-SET7 is required to reduce the colony formation activity, the expression plasmids, Myc-PR-SET7ΔC and Myc-PR-SET7R265G, lacking the methyltransferase activity were constructed. Loss of methyltransferase activity by the deletion of the carboxy terminal region including the methyltransferase domain and the substitution of arginine residue (265) by glycine was already known in human PR-SET7.[18, 25] The number of colonies formed by expression plasmids transfected with mutated PR-SET7 was similar to that with RAS^{val12} alone. The levels of RAS^{val12} in NIH-3T3 cells transfected with PR-SET7 expression plasmids were indistinguishable from those of RAS^{val12} and empty vector transfected cells. These results imply that PR-SET7 suppresses RAS^{val12}-mediated colony formation activity by methyltransferase activity.

DISCUSSION

In this study, we demonstrated that the histone variant, *H2a.z*, and the histone methyltransferase, *Pr-set7*, were induced during hepatocarcinogenesis, and these factors repressed *Gst-p* promoter activity and *ras*-induced colony formation activity.

H2a.z is a variant of *H2a* and is more conserved than other H2A proteins of the species.[10, 11] H2A.Z is incorporated into the nucleosome by H2A.Z specific remodeling factor complexes.[38–41] Chromatin immunoprecipitation of H2A.Z coupled with DNA

microarray studies showed that H2A.Z containing nucleosomes are enriched at the TSSs in the active genes, and a few nucleosomes are positioned further downstream.[12,13] On the other hand, H2A.Z negatively regulates gene expression. H2A.Z locates at the p53-binding site in $p21^{WAF1/CIP1}$, and knockdown of H2A.Z expression increases *p21* expression.[14] Ectopic expression of c-Myc, a known negative regulator of *p21* expression, increased H2A.Z localization at the c-Myc recognition site in the promoter region of the *p21* gene.[14] The effects of H2A.Z deposition on gene expression are dependent on the target genes. Our results demonstrated that the repressive effect of H2A.Z on *Gst-p* promoter activity was dependent on the enhancer element, GPE. Positive regulators for GPE are nuclear factor erythroid 2-related factor 2/musculoaponeurotic fibrosarcoma K heterodimer and histone acetyltransferase MOZ: the presence of additional factors is also speculated.[7,42] Analysis of the effects of H2A.Z on the localization of positive regulators to GPE is required to understand of H2A.Z-mediated negative regulation of *Gst-p* promoter activity.

As PR-SET7 is the only known enzyme that monomethylates H4K20me1, PR-SET7 may both positively and negatively regulate gene expression mediated by H4K20me1. We showed that PR-SET7 repressed *Gst-p* promoter activity independently of GPE. Recently, Congdon *et al*. reported that PR-SET7-mediated H4K20me1 induces transcriptional repression at specific genomic regions, and H4K20me1 is required for the transcriptional repression of all H4K20me1-associated genes analyzed regardless of their basal expression status.[43] Silencer elements located between the −396 bp and −140 bp region in the *Gst-p* gene function in rat non-hepatoma and hepatoma cells and are thought to be general regulatory element for gene expression.[35] The repressive effect of PR-SET7 may be mediated by silencer elements and the proximal promoter, required for the basal transcription. Both H2A.Z and PR-SET7 repressed *Gst-p* promoter activity. As H2A.Z and PR-SET7 targeted to GPE and the regulatory region except for GPE, respectively, these factors repressed *Gst-p* promoter activity by different mechanisms.

In this study, we showed that both H2A.Z and PR-SET7 repressed RAS^{val12}-mediated colony formation activity. RAS proteins are well-studied oncogene products, and the signaling cascade is diverse and complex.[44,45] RAS functions as guanosine triphosphatase, interacts with serine/threonine kinase RAF1, and connects to mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK), ERK1/2, and E26-transcription factor proteins (ETS). This signaling is sufficient and necessary for RAS-mediated transformation of NIH-3T3 cells. RAS signals are also involved in the phosphoinositide 3-kinases pathway, including the serine/threonine kinase AKT/protein kinase B and the transcription factor nuclear factor-κB (NF-κB). These signal transductions lead to anti-apoptotic effects. Analysis of the effects of H2A.Z and PR-SET7 on the expression of the target genes of ETS and NF-κB will reveal the molecular mechanism of repressive effects of H2A.Z and PR-SET7 on RAS-mediated transformation.

We found that *H2a.z* was induced during the early stages of hepatocarcinogenesis, and over expression of *H2a.z* was also observed in colorectal cancer and breast cancer.[46,47] Furthermore, overexpression of H2A.Z increases estrogen dependent breast cancer proliferation.[14] This study demonstrated that H2A.Z did not possess the colony formation activity and repressed RAS-mediated transformation of NIH-3T3. These observations and previous reports suggest that H2A.Z may exhibit protective effects against the establishment of transformed state of normal cells and the progression of cancer cells. H2A.Z may be a bifunctional epigenetic regulator possessing both promoting and suppressing activities for tumorigenesis. These bifunctional activities are known in macroH2A (mH2A), another member of H2A variants. mH2A is involved in epigenetic silencing of tumor suppressor gene p16, and knockdown of mH2A has a growth inhibitory effect.[48] On the other hand, mH2A suppresses melanoma progression.[49] These observations indicate that histone H2A variants may function as positive and negative regulators for tumorigenesis. The methylation status of H4K20 reflects various cellular processes including gene expression, cell cycle progression, and DNA damage repair.[18–24] The levels of H4K20me3 decreased in a mouse model of multistage skin carcinogenesis and liver and breast cancers.[50–52] Further research on the conversion of the methylation status of H4K20 in normal and *ras*-dependent transformed cells may enable us to understand carcinogenesis.

As H2A.Z and PR-SET7 repressed the promoter activity of the tumor marker and *ras*-mediated colony formation activity, these factors may possess protective effects against malignant transfor-

mation. Though the roles of the up-regulated *H2a.z* and *Pr-set7* in the livers with hyperplastic nodules have not been defined, these genes may be induced to mask the hepatocarcinogenic specific *Gst-p* induction. Ras mutation in GST-P positive foci during chemical hepatocarcinogenesis has not been observed. Further analyses of *H2a.z* and *Pr-set7* in GST-P positive foci are required for the understanding of mechanism of the malignant transformation in the early stages of hepatocarcinogenesis. We previously found that the over-expression of histone acetyltransferase MOZ and histone deacetylase HDAC9 activates GST-P expression and promotes anchorage-independent growth, respectively; these may function as positive regulators for carcinogenesis.[7, 8] Further investigation of the factors involved in epigenetic regulation induced during hepatocarcinogenesis will reveal the mechanism of malignant transformation based on aberrant epigenetic status.

Acknowledgements We thank Dr. Hidetoshi Hayashi (Nagoya City University) and Dr. Kozo Kaibuchi (Nagoya University) for providing the expression plasmid for β-galactosidase and RAS^{val12}, respectively. This research was supported in part by grants from Long-range Research Initiative (LRI) by Japan Chemical Industry Association (JCIA) and the Japan Society for the Promotion of Science (JSPS).

REFERENCES

1) Miremadi, A., Oestergaard, M. Z., Pharoah, P. D. and Caldas, C. (2007) Cancer genetics of epigenetic genes. *Hum. Mol. Genet.*, **16**, R28–R49.
2) Portela, A. and Esteller, M. (2010) Epigenetic modifications and human disease. *Nat. Biotechnol.*, **28**, 1057–1068.
3) Lopez, J., Percharde, M., Coley, H. M., Webb, A. and Crook, T. (2009) The context and potential of epigenetics in oncology. *Br. J. Cancer*, **100**, 571–577.
4) Kelly, T. K., De Carvalho, D. D. and Jones, P. A. (2010) Epigenetic modifications as therapeutic targets. *Nat. Biotechnol.*, **28**, 1069–1078.
5) Solt, D. and Farber, E. (1976) New principle for the analysis of chemical carcinogenesis. *Nature*, **263**, 701–703.
6) Osada, S., Naganawa, A., Misonou, M., Tsuchiya, S., Tamba, S., Okuno, Y., Nishikawa, J., Satoh, K., Imagawa, M., Tsujimoto, G., Sugimoto, Y. and Nishihara, T. (2006) Altered gene expression of transcriptional regulatory factors in tumor marker-positive cells during chemically induced hepatocarcinogenesis. *Toxicol. Lett.*, **167**, 106–113.
7) Ohta, K., Ohigashi, M., Naganawa, A., Ikeda, H., Sakai, M., Nishikawa, J., Imagawa, M., Osada, S. and Nishihara, T. (2007) Histone acetyltransferase MOZ acts as a co-activator of Nrf2-MafK and induces tumour marker gene expression during hepatocarcinogenesis. *Biochem. J.*, **402**, 559–566.
8) Yura, T., Hashizume, H., Suzuki, E., Imagawa, M. and Osada, S. (2010) Promotion of anchorage-independent growth by cytoplasmic and nuclear histone deacetylase 9. *J. Health Sci.*, **56**, 581–588.
9) Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M. and Ito, N. (1984) The placental form of glutathione S-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. *Gann*, **75**, 199–202.
10) Iouzalen, N., Moreau, J. and Mechali, M. (1996) H2A.ZI, a new variant histone expressed during Xenopus early development exhibits several distinct features from the core histone H2A. *Nucleic Acids Res.*, **24**, 3947–3952.
11) Jiang, W., Guo, X. and Bhavanandan, V. P. (1998) Histone H2A.F/Z subfamily: the smallest member and the signature sequence. *Biochem. Biophys. Res. Commun.*, **245**, 613–617.
12) Barski, A., Cuddapah, S., Cui, K., Roh, T. Y., Schones, D. E., Wang, Z., Wei, G., Chepelev, I. and Zhao, K. (2007) High-resolution profiling of histone methylations in the human genome. *Cell*, **129**, 823–837.
13) Zlatanova, J. and Thakar, A. (2008) H2A.Z: view from the top. *Structure*, **16**, 166–179.
14) Gevry, N., Chan, H. M., Laflamme, L., Livingston, D. M. and Gaudreau, L. (2007) p21 transcription is regulated by differential localization of histone H2A.Z. *Genes Dev.*, **21**, 1869–1881.
15) Svotelis, A., Gevry, N. and Gaudreau, L. (2009) Regulation of gene expression and cellular proliferation by histone H2A.Z. *Biochem. Cell Biol.*, **87**, 179–188.
16) Couture, J. F., Collazo, E., Brunzelle, J. S. and Trievel, R. C. (2005) Structural and functional analysis of SET8, a histone H4 Lys-20 methyltransferase. *Genes Dev.*, **19**, 1455–1465.
17) Xiao, B., Jing, C., Kelly, G., Walker, P. A., Muskett, F. W., Frenkiel, T. A., Martin, S. R., Sarma, K., Reinberg, D., Gamblin, S. J. and Wilson, J. R. (2005) Specificity and mechanism of the histone methyltransferase Pr-Set7. *Genes Dev.*, **19**, 1444–1454.

18) Nishioka, K., Rice, J. C., Sarma, K., Erdjument-Bromage, H., Werner, J., Wang, Y., Chuikov, S., Valenzuela, P., Tempst, P., Steward, R., Lis, J. T., Allis, C. D. and Reinberg, D. (2002) PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin. *Mol. Cell*, **9**, 1201–1213.
19) Rice, J. C., Nishioka, K., Sarma, K., Steward, R., Reinberg, D. and Allis, C. D. (2002) Mitotic-specific methylation of histone H4 Lys 20 follows increased PR-Set7 expression and its localization to mitotic chromosomes. *Genes Dev.*, **16**, 2225–2230.
20) Sakaguchi, A. and Steward, R. (2007) Aberrant monomethylation of histone H4 lysine 20 activates the DNA damage checkpoint in Drosophila melanogaster. *J. Cell Biol.*, **176**, 155–162.
21) Houston, S. I., McManus, K. J., Adams, M. M., Sims, J. K., Carpenter, P. B., Hendzel, M. J. and Rice, J. C. (2008) Catalytic function of the PR-Set7 histone H4 lysine 20 monomethyltransferase is essential for mitotic entry and genomic stability. *J. Biol. Chem.*, **283**, 19478–19488.
22) Huen, M. S., Sy, S. M., van Deursen, J. M. and Chen, J. (2008) Direct interaction between SET8 and proliferating cell nuclear antigen couples H4-K20 methylation with DNA replication. *J. Biol. Chem.*, **283**, 11073–11077.
23) Oda, H., Okamoto, I., Murphy, N., Chu, J., Price, S. M., Shen, M. M., Torres-Padilla, M. E., Heard, E. and Reinberg, D. (2009) Monomethylation of histone H4-lysine 20 is involved in chromosome structure and stability and is essential for mouse development. *Mol. Cell. Biol.*, **29**, 2278–2295.
24) Wu, S., Wang, W., Kong, X., Congdon, L. M., Yokomori, K., Kirschner, M. W. and Rice, J. C. (2010) Dynamic regulation of the PR-Set7 histone methyltransferase is required for normal cell cycle progression. *Genes Dev.*, **24**, 2531–2542.
25) Fang, J., Feng, Q., Ketel, C. S., Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Tempst, P., Simon, J. A. and Zhang, Y. (2002) Purification and functional characterization of SET8, a nucleosomal histone H4-lysine 20-specific methyltransferase. *Curr. Biol.*, **12**, 1086–1099.
26) Kohlmaier, A., Savarese, F., Lachner, M., Martens, J., Jenuwein, T. and Wutz, A. (2004) A chromosomal memory triggered by Xist regulates histone methylation in X inactivation. *PLoS Biol.*, **2**, 991–1003.
27) Talasz, H., Lindner, H. H., Sarg, B. and Helliger, W. (2005) Histone H4-lysine 20 monomethylation is increased in promoter and coding regions of active genes and correlates with hyperacetylation. *J. Biol. Chem.*, **280**, 38814–38822.
28) Fujioka, H., Kaibuchi, K., Kishi, K., Yamamoto, T., Kawamura, M., Sakoda, T., Mizuno, T. and Takai, Y. (1992) Transforming and c-fos promoter/enhancer-stimulating activities of a stimulatory GDP/GTP exchange protein for small GTP-binding proteins. *J. Biol. Chem.*, **267**, 926–930.
29) Ohoka, N., Yoshii, S., Hattori, T., Onozaki, K. and Hayashi, H. (2005) TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J.*, **24**, 1243–1255.
30) Osada, S., Takano, K., Nishihara, T., Suzuki, T., Muramatsu, M. and Imagawa, M. (1995) CCAAT/enhancer-binding proteins alpha and beta interact with the silencer element in the promoter of glutathione S-transferase P gene during hepatocarcinogenesis. *J. Biol. Chem.*, **270**, 31288–31293.
31) Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.*, **7**, 2745–2752.
32) Sakai, M. and Muramatsu, M. (2007) Regulation of glutathione transferase P: a tumor marker of hepatocarcinogenesis. *Biochem. Biophys. Res. Commun.*, **357**, 575–578.
33) Sakai, M., Okuda, A. and Muramatsu, M. (1988) Multiple regulatory elements and phorbol 12-O-tetradecanoate 13-acetate responsiveness of the rat placental glutathione transferase gene. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9456–9460.
34) Morimura, S., Suzuki, T., Hochi, S., Yuki, A., Nomura, K., Kitagawa, T., Nagatsu, I., Imagawa, M. and Muramatsu, M. (1993) Trans-activation of glutathione transferase P gene during chemical hepatocarcinogenesis of the rat. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 2065–2068.
35) Imagawa, M., Osada, S., Okuda, A. and Muramatsu, M. (1991) Silencer binding proteins function on multiple cis-elements in the glutathione transferase P gene. *Nucleic Acids Res.*, **19**, 5–10.
36) Imagawa, M., Osada, S., Koyama, Y., Suzuki, T., Hirom, P. C., Diccianni, M. B., Morimura, S. and Muramatsu, M. (1991) SF-B that binds to a negative element in glutathione transferase P gene is similar or identical to trans-activator LAP/IL6-DBP. *Biochem. Biophys. Res. Commun.*, **179**, 293–300.
37) Osada, S., Daimon, S., Ikeda, T., Nishihara, T., Yano, K., Yamasaki, M. and Imagawa, M. (1997) Nuclear factor 1 family proteins bind to the silencer element in the rat glutathione transferase P gene. *J. Biochem.*, **121**, 355–363.
38) Kobor, M. S., Venkatasubrahmanyam, S., Meneghini, M. D., Gin, J. W., Jennings, J. L.,

Link, A. J., Madhani, H. D. and Rine, J. (2004) A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. *PLoS. Biol.*, **2**, 587–599.

39) Krogan, N. J., Keogh, M. C., Datta, N., Sawa, C., Ryan, O. W., Ding, H., Haw, R. A., Pootoolal, J., Tong, A., Canadien, V., Richards, D. P., Wu, X., Emili, A., Hughes, T. R., Buratowski, S. and Greenblatt, J. F. (2003) A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. *Mol. Cell*, **12**, 1565–1576.
40) Mizuguchi, G., Shen, X., Landry, J., Wu, W. H., Sen, S. and Wu, C. (2004) ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science*, **303**, 343–348.
41) Luk, E., Ranjan, A., Fitzgerald, P. C., Mizuguchi, G., Huang, Y., Wei, D. and Wu, C. (2010) Stepwise histone replacement by SWR1 requires dual activation with histone H2A.Z and canonical nucleosome. *Cell*, **143**, 725–736.
42) Ikeda, H., Nishi, S. and Sakai, M. (2004) Transcription factor Nrf2/MafK regulates rat placental glutathione S-transferase gene during hepatocarcinogenesis. *Biochem. J.*, **380**, 515–521.
43) Congdon, L. M., Houston, S. I., Veerappan, C. S., Spektor, T. M. and Rice, J. C. (2010) PR-Set7-mediated monomethylation of histone H4 lysine 20 at specific genomic regions induces transcriptional repression. *J. Cell. Biochem.*, **110**, 609–619.
44) Karnoub, A. E. and Weinberg, R. A. (2008) Ras oncogenes: split personalities. *Nat. Rev. Mol. Cell Biol.*, **9**, 517–531.
45) Lau, K. S. and Haigis, K. M. (2009) Non-redundancy within the RAS oncogene family: insights into mutational disparities in cancer. *Mol. Cells*, **28**, 315–320.
46) Dunican, D. S., McWilliam, P., Tighe, O., Parle-McDermott, A. and Croke, D. T. (2002) Gene expression differences between the microsatellite instability (MIN) and chromosomal instability (CIN) phenotypes in colorectal cancer revealed by high-density cDNA array hybridization. *Oncogene*, **21**, 3253–3257.
47) Hua, S., Kallen, C. B., Dhar, R., Baquero, M. T., Mason, C. E., Russell, B. A., Shah, P. K., Liu, J., Khramtsov, A., Tretiakova, M. S., Krausz, T. N., Olopade, O. I., Rimm, D. L. and White, K. P. (2008) Genomic analysis of estrogen cascade reveals histone variant H2A.Z associated with breast cancer progression. *Mol. Syst. Biol.*, **4**, 188.
48) Barzily-Rokni, M., Friedman, N., Ron-Bigger, S., Isaac, S., Michlin, D. and Eden, A. (2011) Synergism between DNA methylation and macroH2A1 occupancy in epigenetic silencing of the tumor suppressor gene p16(CDKN2A). *Nucleic Acids Res.*, **39**, 1326–1335.
49) Kapoor, A., Goldberg, M. S., Cumberland, L. K., Ratnakumar, K., Segura, M. F., Emanuel, P. O., Menendez, S., Vardabasso, C., Leroy, G., Vidal, C. I., Polsky, D., Osman, I., Garcia, B. A., Hernando, E. and Bernstein, E. (2010) The histone variant macroH2A suppresses melanoma progression through regulation of CDK8. *Nature*, **468**, 1105–1109.
50) Fraga, M. F., Ballestar, E., Villar-Garea, A., Boix-Chornet, M., Espada, J., Schotta, G., Bonaldi, T., Haydon, C., Ropero, S., Petrie, K., Iyer, N. G., Perez-Rosado, A., Calvo, E., Lopez, J. A., Cano, A., Calasanz, M. J., Colomer, D., Piris, M. A., Ahn, N., Imhof, A., Caldas, C., Jenuwein, T. and Esteller, M. (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat. Genet.*, **37**, 391–400.
51) Pogribny, I. P., Ross, S. A., Tryndyak, V. P., Pogribna, M., Poirier, L. A. and Karpinets, T. V. (2006) Histone H3 lysine 9 and H4 lysine 20 trimethylation and the expression of Suv4-20h2 and Suv-39h1 histone methyltransferases in hepatocarcinogenesis induced by methyl deficiency in rats. *Carcinogenesis*, **27**, 1180–1186.
52) Tryndyak, V. P., Kovalchuk, O. and Pogribny, I. P. (2006) Loss of DNA methylation and histone H4 lysine 20 trimethylation in human breast cancer cells is associated with aberrant expression of DNA methyltransferase 1, Suv4-20h2 histone methyltransferase and methyl-binding proteins. *Cancer Biol. Ther.*, **5**, 65–70.