— **Regular Article** —

Promotion of Anchorage-independent Growth by Cytoplasmic and Nuclear Histone Deacetylase 9

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(Received May 23, 2010; Accepted July 14, 2010; Published online July 23, 2010)

Genetic mutation is a trigger for the generation of malignant cells and an aberrant epigenetic status contributes to the maintenance of mutations and proliferation of mutated cells. Along with DNA methylation, histone modifications such as acetylation and methylation are significant to biological processes. Histone deacetylases (HDACs) are important epigenetic regulators of chromatin modifications and gene expression. Though several HDAC inhibitors are currently being tested in clinical trials, the roles of HDACs in malignant transformation remain unknown. Here, we showed that the expression of two forms of *Hdac9*, a full-length version (*Hdac9*FL) and a splicing variant lacking exon 7 (*Hdac9*Δ7), both class IIa HDACs, was up-regulated during chemically induced hepatocarcinogenesis. In addition, we found that HDAC9FL and HDAC9Δ7 are located in the nucleus and cytoplasm, respectively. We also found their nuclear localization and nuclear export signals to be encoded in exon 7 and exon 25, respectively. Though the two isoforms could not transform mouse NIH-3T3 fibroblasts, they promoted tumor cell anchorage-independent growth on soft agarose. The HDAC9 variants do not seem to cause cell transformation, but cytoplasmic and nuclear HDAC9 may contribute to the survival of malignant cells in the early stages of hepatocarcinogenesis.

Key words —— histone deacetylase, epigenetics, anchorage-independent growth, hepatocarcinogenesis, nuclear localization signal, nuclear export signal

INTRODUCTION

In eukaryotes, DNA is packaged into chromatin, which regulates gene functions including gene expression, DNA replication, and DNA repair. Conformational changes of chromatin are controlled by the chromatin remodeling complex and enzymes posttranslationally modifying histones.[1,2] One of the most important modifications of histones is acetylation. Lysine acetylation status is controlled by histone acetyltransferase (HAT) and histone deacetylase (HDAC), the activities of which regulate gene expression. The aberrant expression and dysfunction of these enzymes can lead to disease including cancer.[3,4] Several HDAC inhibitors are currently in clinical trials for some cancers.[5,6] HDACs are divided into the Rpd3/Hdac1 (classical) and sirtuin families based on sequence homology. The Rpd3/Hdac1 family consists of 11 members and is grouped into three classes, class I (*Hdac1*, *Hdac2*, *Hdac3*, and *Hdac8*), class II, and class IV (*Hdac11*). Class II is further divided into class IIa (*Hdac4*, *Hdac5*, *Hdac7*, and *Hdac9*) and class IIb (*Hdac6* and *Hdac10*).[7] Though HDACs are thought to repress tumor suppressor genes and regulate the expression of several genes involved in the cell cycle and apoptosis,[8] their role in the early stages of carcinogenesis has not been clarified yet.

To identify factors involved in transcription and epigenetic regulation early in hepatocarcinogenesis, we previously performed a carcinogenic experiment with the Solt-Farber protocol.[9] Hyperplastic nodules were introduced into livers and a tumor marker, glutathione transferase placental form (GST-P), was induced to express. During experiment, we observed the expression of HATs in the nucleus and found that cAMP-response-element binding protein

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(CREB)-binding protein/KAT3A and p300/KAT3B, which function as co-activators for several transcription factors, were decreased and monocytic leukemia zinc finger protein (MOZ/KAT6A) was increased.[10] Further, we demonstrated that MOZ induced GST-P expression.

In this study, we investigated the expression profiles of HDACs at the early stages of hepatocarcinogenesis and revealed that nuclear and cytoplasmic HDAC9, which were induced to express at this stage, promoted anchorage-independent growth of cancer cells.

MATERIALS AND METHODS

Chemical Hepatocarcinogenesis of Rats —— Carcinogenic experiments were performed according to the Solt-Farber protocol as described previously.[9,11] 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. After the animals had been fed basal diets for 2 weeks, they were changed to basal diets containing 0.02% 2-acetylaminofluorene (Nacalai Tesque, Kyoto, Japan). Three weeks after diethylnitrosamine injection, partial hepatectomy was performed and livers were extirpated 8 weeks after diethylnitrosamine injection. At this stage, approximately 80% 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.

Reverse Transcriptase-coupled Polymerase Chain Reaction (RT-PCR) and Northern Blot Analysis —— Total RNA was prepared from three control livers (#50, #51, and #52) and from three livers with hyperplastic nodules (#43, #44, and #46) using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). cDNA was synthesized with ReverTra Ace (Toyobo, Osaka, Japan) and SuperScript II RNase H-Reverse Transcriptase (Invitrogen). The primer sets for PCR were selected from different exons (Table 1). The number of PCR cycles yielding products within the linear range was determined by analyzing serial 2-fold dilutions of the starting materials, and PCR products were separated on a 6% polyacrylamide gel, stained with ethidium bromide, and detected.

PolyA$^+$ RNA was prepared using Oligotex dT30 super (Takara Bio, Otsu, Japan) as specified by the manufacturer. Labeling was carried out with a BcaBEST labeling kit (Takara Bio). The Northern blot analysis was performed with standard methods.

Cloning of Rat *Hdac9* cDNA —— The 5′-untranslated region (UTR) of rat *Hdac9* cDNA was cloned by 5′-rapid amplification of cDNA ends (RACE) using Rat Liver Marathon-Ready cDNA (Takara Bio), and the open reading frame (ORF) and 3′-UTR were cloned by RT-PCR. Sequences of primers used in this study are listed in Table 1.

To construct pCMV-myc-HDAC9FL and pCMV-myc-HDAC9Δ7, the ORF of *Hdac9* amplified by PCR was subcloned into the blunt-ended *Eco*RI site of pCMV-myc (Clontech, Mountain View, CA, U.S.A.). All fragments generated by PCR were verified by sequencing. Mutations in the nuclear export signal (NES) were introduced using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) following the manufacturer's instructions, and confirmed by sequencing.

Subcellular Distribution of HDAC9 —— HeLa cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Myc-tagged HDAC9 expression plasmids were introduced into HeLa cells by the calcium phosphate co-precipitation method.[12] Cells were fixed with 3% paraformaldehyde, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ for 30 min at room temperature, and incubated with anti-Myc antibody (Roche, Indianapolis, IN, U.S.A.). After five washes with phosphate-buffered saline (PBS), secondary antibody conjugated with fluorescein isothiocyanate (FITC) was added for 1 hr. Then cells were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) and visualized under a fluorescence microscope (Olympus, Tokyo, Japan; BX51).

Focus Formation and Anchorage-independent Growth Assays and Western Blot Analysis —— For focus formation assay, 6×10^5 cells NIH-3T3 cells were plated on 10 cm dish and transfected with the HDAC9 or RAS^{val12} expression plasmid by the calcium phosphate co-precipitation method.[12] RAS^{val12} expression plasmid was kindly provided by Dr. Kaibuchi (Nagoya University). Transfected cells were incubated for 2 weeks for development of foci and Giemsa staining was performed.

To evaluate anchorage-independent growth on NIH-3T3 cells, the HDAC9 expression plasmid was introduced into cells by the cal-

Table 1. Primers Used in This Study

Primer name		Sequence
Hdac1	mm001	5′-AATTCTTGCGTTCTATTCGCCC-3′
Hdac1	mm002	5′-GCCAGCCCCAATATCCCG-3′
Hdac2	mm003	5′-CTCTCCTTCCCATCGAATTGG-3′
Hdac2	mm004	5′-ATCCGCTTGTCTGATGCTCG-3′
Hdac3	mm005	5′-GCGTATTTCTACGACCCCG-3′
Hdac3	mm006	5′-GTGGTACTTGAGCAGCTCC-3′
Hdac4	mm007	5′-AATTCTGTGGCAATTGCAGC-3′
Hdac4	mm008	5′-CATCACAGATGGCTGTCAGG-3′
Hdac5	mm009	5′-GACATCATGCTGAGGAGTCC-3′
Hdac5	mm010	5′-TATTCCACATCTCCAATGGG-3′
Hdac6	mm011	5′-CGCTGGATTTGATGCCCTCC-3′
Hdac6	mm012	5′-TCTCAGGATGATGATTGTCCC-3′
Hdac7	mm013	5′-AGCAGCAGCATCTTGCTGGG-3′
Hdac7	mm014	5′-CGTAGAGGAGCACGTGCC-3′
Hdac8	mm015	5′-ATGGAGATACCAGAGGAACC-3′
Hdac8	mm016	5′-TTCTTTGCATGATGCCACCC-3′
Hdac9	mm019	5′-GAGCTGGCTTCCAAAGTGGC-3′
Hdac9	mm020	5′-CTTCCAATGCATCAAATCCAGC-3′
Hdac10	mm021	5′-ATGTTCCACTTCTCCACTCC-3′
Hdac10	mm022	5′-GCCACCTGCAGCAACTTCC-3′
Hdac11	mm031	5′-CCTTTGATGCTGGGAAATG-3′
Hdac11	mm032	5′-ATCAATGATGGTAGCTCTGG-3′
Hdac9	ty015	5′-GCTCATTTCCTAGAAGGGCA-3′
Hdac9	ty017	5′-CTGCTGCTCTCTGCGATGCCTCTC-3′
Hdac9	ty020	5′-GACGTTCCACTGAGGGGTGGAGAG-3′
Hdac9	ty025	5′-ATGCACAGTATGATCAGCTCAG-3′
exon 5	hh003	5′-AATGAATTCCTATTGAGTAAATCAGCAACG-3′
exon 8	ty030	5′-GCCCATTGTTTGGTGAACTGG-3′

cium phosphate co-precipitation method.[12] After 24 hr, 2×10^5 cells/60 mm dish were plated in 0.55% agarose Dulbecco's modified Eagle medium (DMEM) onto a 0.5% agarose DMEM layer. After two weeks, plates were incubated for overnight at 4°C. Colonies (diameter of 100 μm or more) were counted from three plates. Results are shown as the mean ± standard deviation (S.D.).

In case of HeLa cells, plasmid was introduced into cells by the calcium phosphate co-precipitation method.[12] After 24 hr, 1×10^3 cells/60 mm dish were plated in 0.3% agarose MEM onto a 0.5% agarose MEM layer. After 10 days, 2 mg/ml of thiazolyl blue tetrazolium bromide was added to the upper layer of agarose and plates were incubated for 30 min at 37°C and then overnight at 4°C. Stained colonies (diameter of 100 μm or more) were counted from three plates. Results are shown as the mean ± S.D. Significant differences were identified with Student's *t* test.

For the Western blot analysis, transfected cells were seeded (6×10^5 cells/100 mm dish), incubated for 24 hr, harvested by centrifugation, and suspended in 1 x 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]. Samples were boiled, loaded onto a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and detected with the BCIP/NBT Western Blotting Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.). Anti-myc and β-actin antibodies were obtained from Roche and SIGMA (Saint Louis, MO, U.S.A.), respectively.

RESULTS

Expression of the *Hdac* Family during Hepatocarcinogenesis

We first investigated the expression profiles of classical HDACs during hepatocarcinogenesis. Total RNA was prepared from control livers and livers with hyperplastic nodules harboring approximately

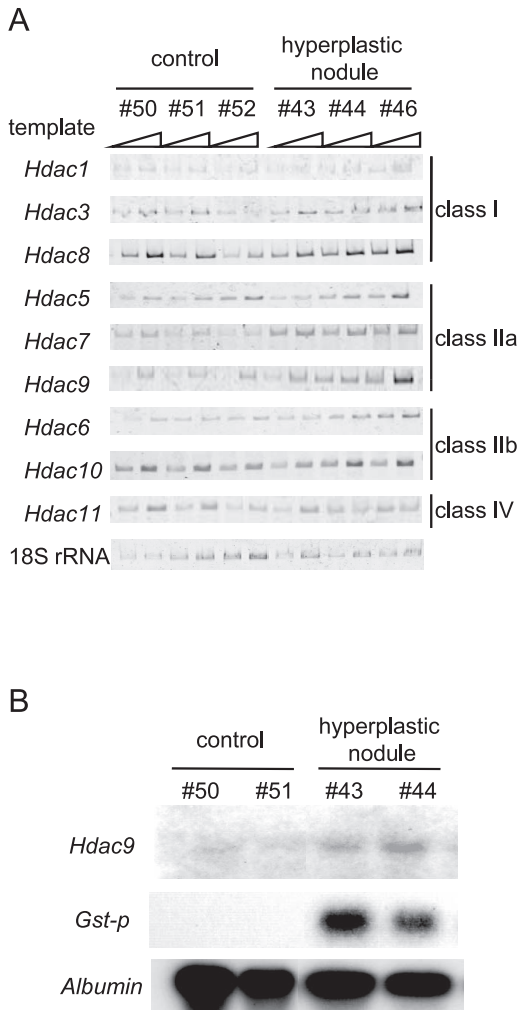


Fig. 1. Expression of the *Hdac* Family during Hepatocarcinogenesis

(A) The expression of class I, IIa, IIb and IV *Hdac*s detected by semi-quantitative RT-PCR. 18S rRNA was used as an internal control. Total RNA prepared from control livers (#50, #51, and #52) and livers with hyperplastic nodules (#43, #44, and #46) was used for the reverse transcription reaction. A two-fold dilution of the reverse transcription reaction solution was used as a template for PCR. (B) Northern blot analysis of *Hdac9* using polyA$^+$ RNA derived from control livers (#50 and #51) and livers with hyperplastic nodules (#43 and #44). *Gst-p* and *Albumin* are controls for a drastic induction and slight reduction, respectively.

80% GST-P positive foci. RT-PCR was performed with primers specific to each *Hdac* (Fig. 1A). The number of PCR cycles yielding products within the linear range was determined by analyzing serial 2-fold dilutions of the starting materials. *Hdac9* was expressed during hepatocarcinogenesis, and *Hdac7* and *Hdac6* were slightly overexpressed. Though *Hdac2* and *Hdac4* were not detected, the levels of other *Hdac*s were indistinguishable. To confirm the change in *Hdac9* expression, we prepared polyA$^+$ RNA from total RNA and performed a Northern blot analysis (Fig. 1B). Previous reports showed that the tumor marker GST-P was markedly expressed and albumin was slightly decreased during hepatocarcinogenesis.[13,14] Under our conditions, these changes were reproduced and the induction of *Hdac9* expression was also observed. These results indicated that *Hdac9* was expressed during hepatocarcinogenesis.

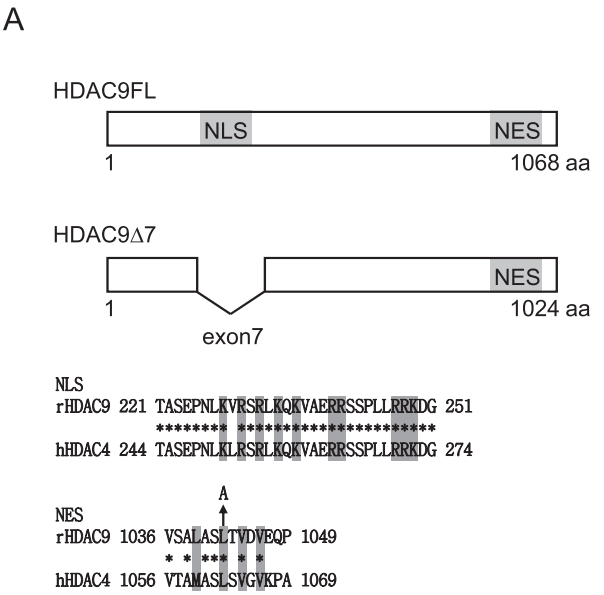
Cloning of Rat *Hdac9* cDNA

Human *Hdac9* cDNA has been cloned and several splicing variants isolated.[15] When we started cloning *Hdac9* cDNA, two predicted rat *Hdac9* cDNA sequences (XM_576017 and XM_234063) were registered in the database, but they did not overlap. To isolate the full-length ORF, we performed 5′-RACE and RT-PCR. Cloning of the rat cDNA revealed that the rat *Hdac9* ORF (accession no. AB558551) included 3204 nucleotides and encoded 1068 amino acids. Comparison of the rat *Hdac9* cDNA with the genomic sequence revealed that the rat *Hdac9* gene comprised at least 26 exons spanning 561 kb of genomic sequence on chromosome 6. The intact form of HDAC9 is referred to as HDAC9FL. During the isolation of the *Hdac9* cDNA, a splicing variant, lacking exon7 (*Hdac9*Δ7), was also identified (accession no. AB558552). This type of variant has also been cloned in humans.[15]

Subcellular Distribution of HDAC9 Splicing Variants

The nuclear localization signal (NLS) and nuclear export signal (NES) of HDAC are well characterized in human HDAC4.[16] These sequences were encoded by exon 7 and exon 25 in rat *Hdac9*, respectively. All of the basic amino acid residues, which are crucial for nuclear localization, were conserved in human HDAC4 and rat HDAC9 (Fig. 2A). Further, three of the four amino acid residues critical for nuclear export were also conserved.

To investigate the subcellular distribution of HDAC9, Myc-tagged HDAC9FL and HDAC9Δ7 expression plasmids were introduced into HeLa cells and immunostained with anti-myc antibody (Fig. 2B). Though almost all HDAC9FL was distributed within the nucleus and only part was located in the cytoplasm, HDAC9Δ7 was completely absent from the nucleus. Further, substitution of a leucine residue (1042) which may be critical for nuclear export in the putative NES, with alanine led to nuclear accumulation in HDAC9(L1042A)-



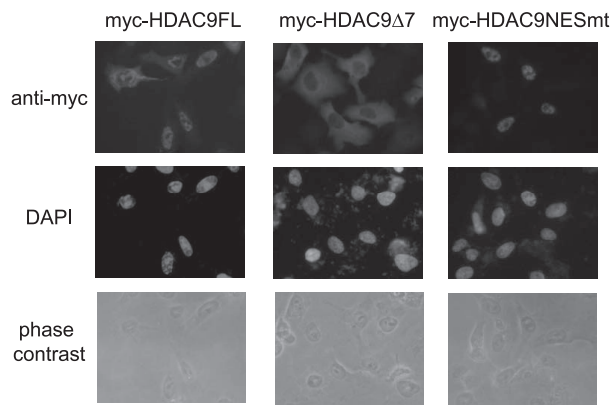


Fig. 2. Structure of the HDAC9 Variants and Subcellular Distribution of HDAC9

(A) Schematic representation of the intact HDAC9 and the splicing variant lacking exon 7. Conservation of the NLS and NES in rat HDAC9 and human HDAC4 is indicated by an asterisk and critical residues are shaded gray. The substitution of a leucine residue (1042) in the putative NES with alanine is shown. (B) HeLa cells were transfected with the myc-tagged HDAC9 expression plasmids indicated at the top of panels. The cells were fixed and immunostained with the anti-myc antibody (upper panels). DAPI staining and phase contrast views are also shown (middle and lower panels, respectively).

transfected HeLa cells. These results and the conservation of the amino acid sequence in HDACs suggest that the NLS and NES of HDAC9 are located in exon 7 and exon 25, respectively.

Expression of *Hdac9* Splicing Variants during Hepatocarcinogenesis

As described above, the subcellular distribution of HDAC9FL differs from that of HDAC9Δ7. Next,

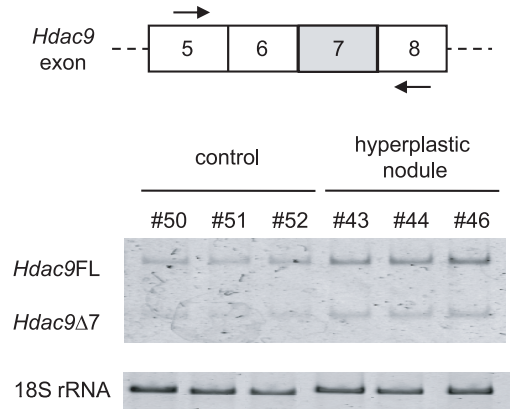


Fig. 3. Expression of *Hdac9* Splicing Variants during Hepatocarcinogenesis

Semi-quantitative RT-PCR was performed using primer pairs located in exon 5 and exon 8. PCR products were separated in polyacrylamide gel and stained with ethidium bromide. The positions of PCR products derived from *Hdac9*FL and *Hdac9*Δ7 are indicated. 18S rRNA was used as an internal control.

we investigated the expression of these variants during hepatocarcinogenesis by RT-PCR using primer pairs located in exon 5 and exon 8 and total RNA derived from control livers and livers with hyperplasic nodules (Fig. 3). In the linear range of amplification, the product obtained from *Hdac9*FL was more abundant than that from *Hdac9*Δ7 and both products increased during hepatocarcinogenesis. These results raise the possibility that both variants contribute to the malignant transformation of cells or proliferation of transformed cells.

Effects of Overexpression of the Variants on Anchorage-independent Growth

Malignant transformation is typically characterized by morphological changes, increased cell growth, loss of contact inhibition of growth, acquisition of anchorage-independent growth, and so on. Mouse NIH-3T3 fibroblasts possess a normal cellular phenotype and are often used to study the malignant transformation of genes. To investigate whether HDAC9 induces loss of contact inhibition of growth, we examined HDAC9-dependent focus formation in NIH-3T3 cells (Fig. 4A). Oncogenic mutant of RAS, RAS^{val12}, was used as a positive control for focus formation. Two weeks after transfection, the RAS^{val12} introduction produced numerous foci, while either empty vector or HDAC9 gave rise to none of foci. To evaluate anchorage-independent growth, colony formation assay was performed. RAS^{val12} yields numerous colonies, but neither HDAC9FL nor HAC9Δ7 produced colony (Fig. 4B). These results indicate that the two iso-

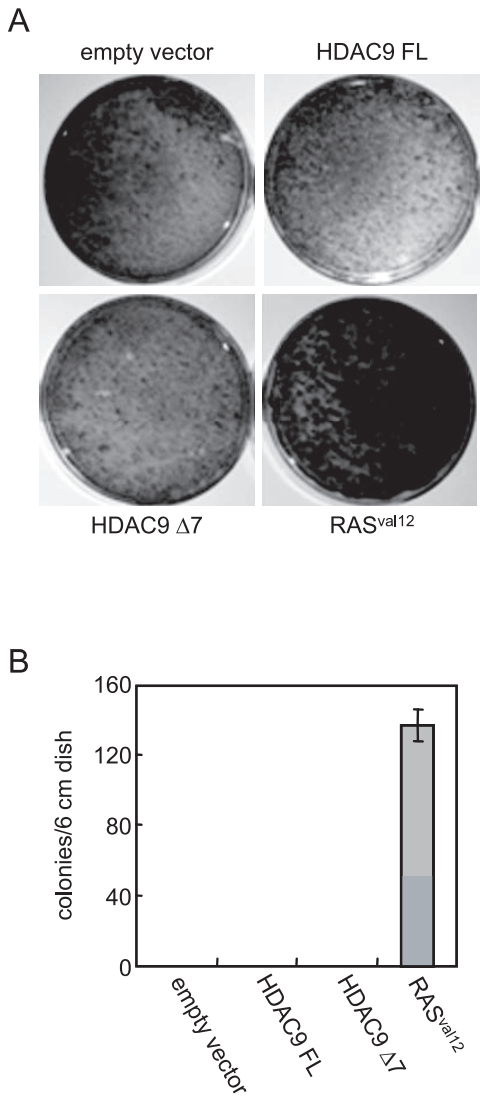


Fig. 4. Effects of Overexpression of HDAC9 Splicing Variants on Focus and Colony Formation on NIH-3T3 Cells

(A) NIH-3T3 cells were transfected with the HDAC9 expression plasmid or empty vector and incubated for 2 weeks. Pictures of Giemsa stained cells are shown. (B) NIH-3T3 cells were transfected with the plasmid and incubated for 2 weeks. Generated colonies were counted. Values are presented as the mean ± S.D. A typical result from three independent experiments is shown.

forms of HDAC9 could not transform mouse NIH-3T3 fibroblasts.

Another important feature of malignant transformed cells is resistance to cell death including apoptosis and anoikis.[17,18] In spite being derived from human cervical cancer, HeLa cells are relatively sensitive to anoikis, which is apoptosis induced by incorrect cell/extracellular matrix attachment. Some populations of HeLa cells are not able to grow on soft agarose medium. We next transfected HDAC9FL and HDAC9Δ7 expression plasmids into HeLa cells and investigated anchorage-independent growth on agarose medium (Fig. 5).

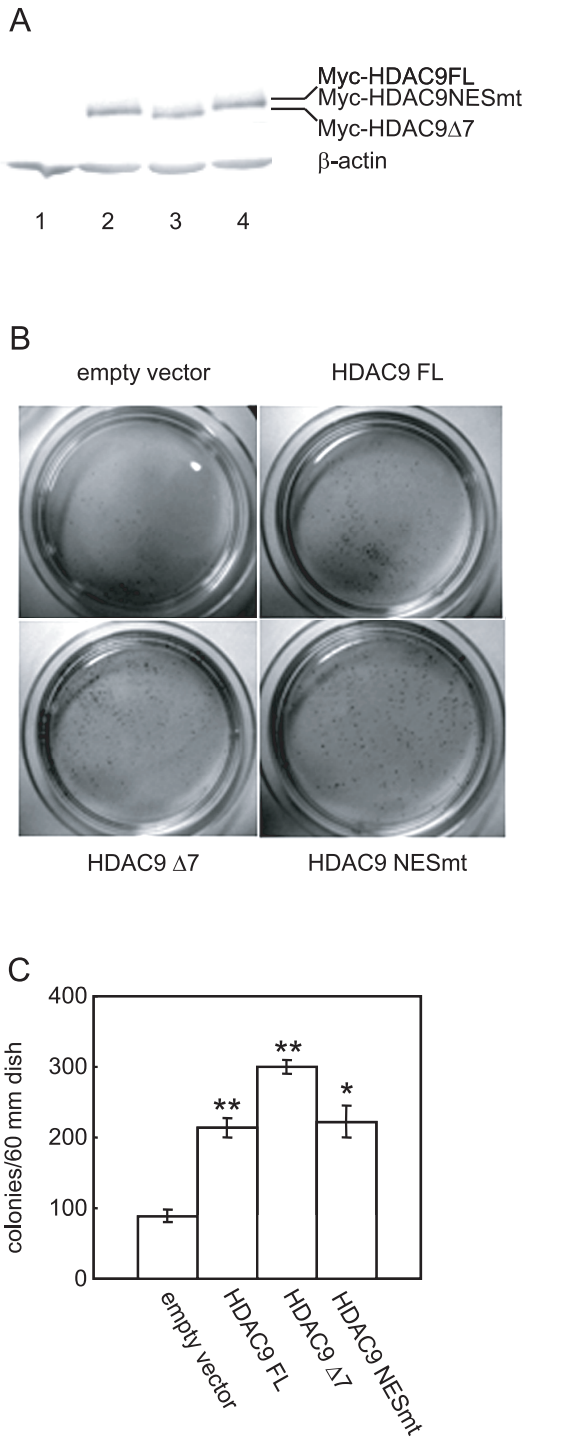


Fig. 5. Effects of Overexpression of HDAC9 Splicing Variants on Anchorage-independent Growth in HeLa Cells

(A) Ectopically expressed HDAC9 proteins were detected by Western blotting with β-actin used as an internal control. (B) HeLa cells were transfected with the HDAC9 expression plasmids and trypsinized 24 hr later. One thousand cells were seeded on the lower agarose medium in 60-mm plates and allowed to grow for ten days, and colonies were counted. Pictures of colonies are shown. (C) A typical result from three independent experiments is shown. Values are presented as the mean ± S.D. Significant differences identified with Student's t test at $p < 0.05$ and $p < 0.01$ versus the control are indicated by * and **, respectively.

Levels of exogenous HDAC9 protein were detected by Western blotting and indistinguishable between variants (Fig. 5A). Transfected cells were cultured on soft agarose medium for ten days and the colonies were generated. Pictures of colonies are shown in Fig. 5B. As shown in Fig. 5C, approximately 100 colonies were produced by the empty vector-transfected cells. The number of colonies increased two and three fold on introduction of the HDAC9FL and HDAC9Δ7 expression plasmids, respectively. These results indicate that both cytoplasmic and nuclear HDAC9 promote anchorage-independent growth of HeLa cells.

DISCUSSION

HDACs regulate histone acetylation status and have roles in chromatin conformational change and transcriptional regulation. Though many HDAC inhibitors are at various stages of clinical testing for cancer therapy,[6] the roles of HDACs in carcinogenesis remain unclear. In this study, we cloned rat *Hdac9* cDNA and showed that *Hdac9* was expressed during hepatocarcinogenesis. Human *Hdac9* cDNAs have been cloned (AY032737, AY032738, and AY197371) and their predicted amino acid sequences share 92% identity with rat HDAC9FL.[15,19] Isoforms lacking the region encoded by exon 7 have also been identified in humans and found to be located in the cytoplasm. We showed that rat HDAC9Δ7 too was selectively expressed in the cytoplasm, suggesting the function of the NLS encoded in exon 7 to be conserved in humans and rats.

Class IIa and class IIb HDACs are found in both the nucleus and cytoplasm, but their function in the cytoplasm remains unknown. The only exception is HDAC6.[20] The substrates of HDAC6 in the cytoplasm are tubulin and a heat shock protein, HSP90.[21,22] HDAC6 associates with microtubules and the actin cytoskeleton and is involved in cell migration and the degradation of misfolded proteins.[20] Lee *et al.* reported that HDAC6 is required for oncogene-induced transformation and *Hdac6*-deficient mice were resistant to carcinogen-induced skin tumors.[23] Further, they showed that HDAC6 was involved in resistance to anoikis in cancer cells. Such resistance is crucial to tumorigenesis and the metastatic spread of malignant transformed cells.[17,18] Though the function of human HDAC9Δ7 has not been investigated, we found that cytoplasmic HDAC9Δ7 enhanced anchorage-independent growth in HeLa cells. HDAC9Δ7, as well as HDAC9FL, did not transform mouse fibroblast-derived NIH-3T3 cells, but promoted anchorage-independent growth. HDAC9 expressed in the early stages of carcinogenesis may be involved in the promotion of hepatocarcinogenesis.

Class IIa HDACs seem not to possess their own activity, but to derive activity from class I HDACs.[24,25] These reports imply that the promotion of anchorage-independent growth mediated by nuclear HDAC9FL requires a class I HDAC. On the other hand, the activities of cytoplasmic HDAC9Δ7 may be independent of histones. Acetylated proteins, which regulate anoikis, have not been identified. Anoikis is essentially an apoptotic process and seems to occur in the disturbance of mitochondria function or the triggering of cell surface death receptors.[18] Mediators in these signals, for example B-cell CLL/lymphoma 2 (Bcl-2) family, may be target of HDAC9Δ7. Bax, structurally related to Bcl-2 family, is inactive in the cytoplasm by associating with Ku70, a DNA repair factor. Acetylation of Ku70 disrupts the BAX-Ku70 interaction, and dissociated Bax localizes to mitochondria and initiates apoptosis.[26] Though sirtuin 1 (SIRT1) deacetylase is involved in deacetylation of Ku70, HDAC9Δ7 may also deacetylate Ku70 or regulators for apoptosis. Identification and characterization of the substrates of, and the factors associating with, HDAC9Δ7 should improve our understanding of the molecular mechanism of cytoplasmic HDAC9-mediated carcinogenesis.

Acknowledgements Preliminary results were obtained at Osaka University and we would like to express our gratitude to Masashi Misonou, Jun-ichi Nishikawa, and Tsutomu Nishihara. We thank Dr. Kozo Kaibuchi for providing the expression plasmid for RAS^{val12}. 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).

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