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Histone Deacetylase 6 Functions as a Tumor Suppressor by Activating c-Jun NH2-Terminal Kinase-Mediated Beclin 1-Dependent Autophagic Cell Death in Liver Cancer

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Ubiquitin-binding histone deacetylase 6 (HDAC6) is uniquely endowed with tubulin deacetylase activity and plays an important role in the clearance of misfolded protein by autophagy. In cancer, HDAC6 has become a target for drug development due to its major contribution to oncogenic cell transformation. In the present study we show that HDAC6 expression was down-regulated in a large cohort of human hepatocellular carcinoma (HCC) patients, and that low expression of HDAC6 was significantly associated with poor prognosis of HCC patients in 5-year overall, disease-free, and recurrence-free survival. Notably, we observed that ectopic overexpression of HDAC6 suppressed tumor cell growth and proliferation in various liver cancer cells, and elicited increased LC3B-II conversion and autophagic vacuole formation without causing apoptotic cell death or cell cycle inhibition. In addition, the sustained overexpression of HDAC6 reduced the *in vivo* tumor growth rate in a mouse xenograft model. It was also found that HDAC6 mediated autophagic cell death by way of Beclin 1 and activation of the LC3-II pathway in liver cancer cells, and that HDAC6 overexpression activated c-Jun NH2-terminal kinase (JNK) and increased the phosphorylation of c-Jun. In contrast, the induction of Beclin 1 expression was blocked by SP600125 (a specific inhibitor of JNK) or by small interfering RNA directed against HDAC6. *Conclusion:* Our findings suggest that loss of HDAC6 expression in human HCCs and tumor suppression by HDAC6 occur by way of activation of caspase-independent autophagic cell death through the JNK/Beclin 1 pathway in liver cancer and, thus, that a novel tumor suppressor function mechanism involving HDAC6 may be amenable to nonepigenetic regulation. (HEPATOLOGY 2012;56:644-657)

Hepatocellular carcinoma (HCC) is an aggressive form of cancer, the fifth most common cancer, and the third leading cause of cancer death worldwide.[1] Surgery with curative intent is feasible for only 15% to 25% of patients and most HCC patients die from locally advanced or metastatic disease in a relatively short period of time.[2] Hepatitis B virus, hepatitis C virus, and aflatoxin B1 are well-known major causes of HCC. However, the overall survival of patients with HCC has not improved significantly over the past two decades, and the mechanisms responsible for the development and progression of HCC remain poorly understood.[3] To date, molecular targeted therapy has shown promise for the treatment of advanced

Abbreviations: 3-MA, 3-methyladenine; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAT, histone acetyltransferase; HCC, hepatocellular carcinoma; HDAC, histone deacetylases; JNK, c-Jun NH2-terminal kinase; LC3, microtubule-associated protein 1 light chain 3; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; siRNA, small interfering RNA; TEM, transmission electron microscopy; TMA, tissue microarray.

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HCC,[4] but it is unclear how these genetic changes cause the clinical characteristics observed in individual HCC patients.

Histone deacetylases (HDACs) are often recruited by corepressors or multiprotein transcriptional complexes to gene promoters, whereby they regulate transcription by way of chromatin modification without directly binding to DNA.[5] There are 18 encoded human HDACs, which are classified as: class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 6, 7, 9, and 10), class III (SIRT1-7), and class IV (HDAC11) enzymes,[6] and evidence indicates that both histone acetyltransferases (HATs) and HDACs are involved in cell proliferation, differentiation, and cell cycle regulation.[7] In addition, it has been reported that the pathological activity and deregulation of HDACs can lead to several diseases, such as cancer, immunological disturbances, and muscular dystrophy.[8] However, despite the involvement of HDACs in the development of cancer, the specific roles fulfilled by individual HDACs in the regulation of cancer development remain unclear.

HDAC6 is a member of the class IIb family of HDACs and acts as a cytoplasmic deacetylase that associates with microtubules and deacetylates α-tubulin.[9] Microtubule-associated HDAC6 is a critical component of the lysosomal protein degradation pathway, and it has been recently suggested that HDAC6 plays an important role in the eventual clearance of aggresomes, which implies a functional connection between autophagic signaling and control of the fusion of autophagosomes and lysosomes associated with the control of autophagy by way of the recruitment of cortactin-dependent, actin-remodeling machinery to ubiquitinated protein aggregates.[10] On the other hand, HDAC6 has been shown to be involved in carcinogenic transformation and to modulate the epithelial-mesenchymal transition in several cancers by way of the regulations of several critical cellular functions,[11,12] and accumulating evidence indicates that the expression of HDAC6 is correlated with oncogenic transformation, anchorage-independent proliferation, and tumor aggressiveness. Furthermore, it has been shown that the inactivation of HDAC6 by genetic ablation or by specific short small interfering RNA (siRNA) increases resistance to oncogenic transformation and decreases the growth of human breast and ovarian cancer cell lines *in vitro* and *in vivo*.[13,14] Therefore, the up-regulation of HDAC6 in diverse tumors and cell lines suggests that HDAC6 plays an important role in cancer.

However, our previous transcriptome analysis on multistep hepatopathogenesis suggested the down-regulation of HDAC6 in overt HCC as compared with noncancerous tissues, and our initial analysis of HDAC6 in human HCC tissues indicated the loss of HDAC6 expression in HCCs. Therefore, in this study, to investigate the biological role of HDAC6 as a tumor suppressor during hepatocarcinogenesis, the expression of HDAC6 and its prognostic association were evaluated in a large cohort of HCC patients, and it was found that HDAC6 mediated autophagic cell death by way of the JNK/c-Jun activation signaling pathway to elicit the Beclin 1 / microtubule-associated protein 1 light chain 3 (LC3)-II-dependent autophagy process in liver cancer cells. Finally, the tumor suppressor activity of HDAC6 was experimentally investigated *in vivo* using cell lines stably overexpressing HDAC6.

Materials and Methods

This study was approved by the Institutional Review of Board (IRB) of the Songeui Campus, College of Medicine, Catholic University of Korea (IRB approval number; CUMC09U117). All animal experiments were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Department of Laboratory Animals, College of Medicine, Catholic University of Korea. This animal study was also approved by the IRB for the care and use of animals at the Catholic University Medical Center (approval number; CUMC-2009-0050-03).

A full description of the Materials and Methods are given in the Supporting Information.

Results

Loss of HDAC6 Expression and Its Association With Poor Prognosis of HCC Patients. The overexpression of HDAC6 has been reported in a variety of cancer cell lines and has been found to be required to maintain the transformed phenotypes of a number of

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Additional Supporting Information may be found in the online version of this article.

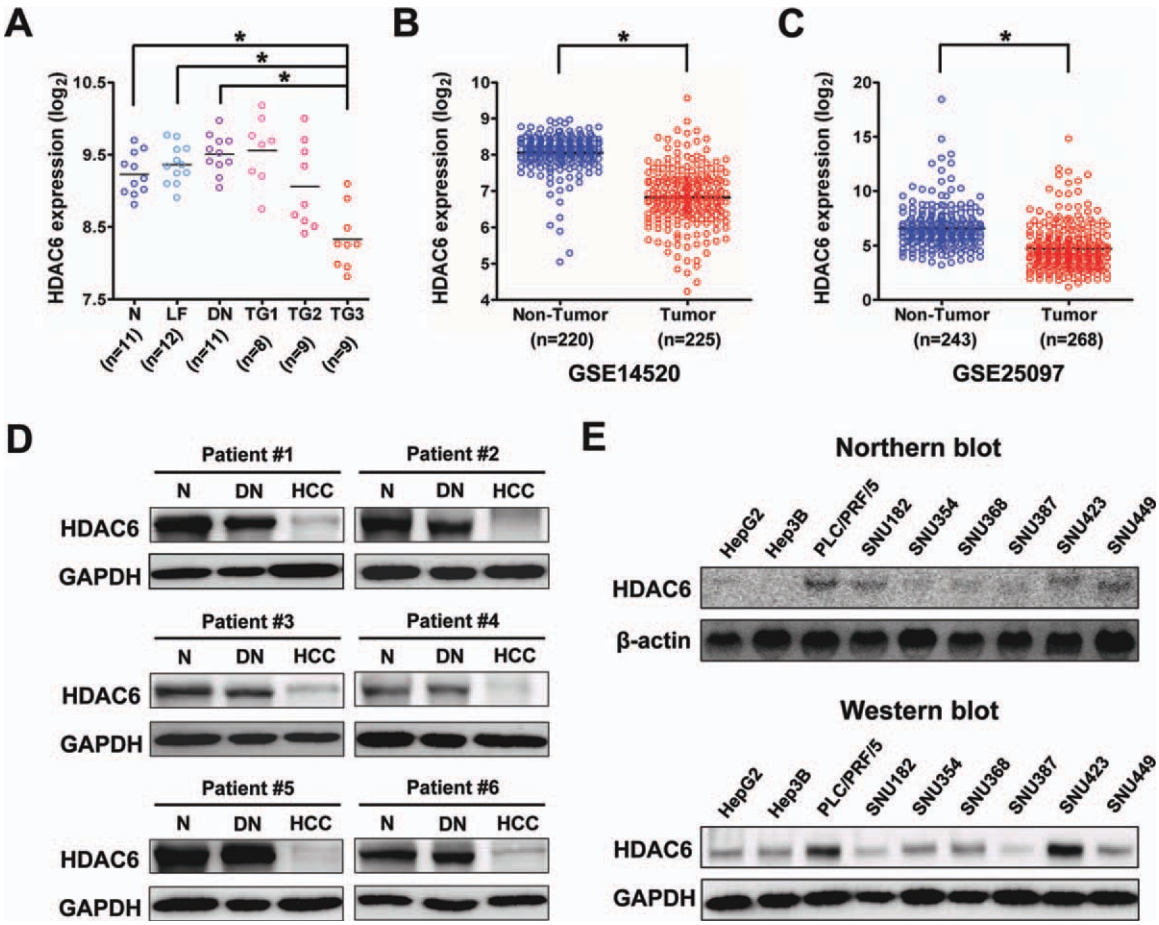

Fig. 1. Loss or down-regulation of HDAC6 expression in human HCC tissues and liver cancer cell lines. (A) Gene expression of HDAC6 mRNA based on microarray analysis in a subset of primary HCCs (Edmondson grade 1 [TG1, n = 8], grade 2 [TG2, n = 9], grade 3 [TG3, n = 9]) and premalignant lesions of HCC (DN; dysplatic nodule, n = 11) and liver fibrosis (LF, n = 12) and histologically normal liver tissue (N, n = 11). Gene expression levels (log_2 intensity) were assigned for these categories and represented as scatterplots and their difference was determined using the unpaired *t* test (*$P < 0.0001$; two-tailed). (B,C) Recapitulated *HDAC6* gene expression levels of the large cohort of HCC patients. Two sets of microarray data were obtained from the NCBI GEO database (accession numbers GSE14520 and GSE25097). The relative expression of HDAC6 mRNA in noncancerous tissues (Non-Tumor) and patients with HCC (Tumor) is illustrated by scatterplot. The median expression level of each group is indicated by horizontal lines. Gene expression levels are shown on the ordinate (log_2 intensity). The differential HDAC6 expression for these two categories was determined by the unpaired *t* test (*$P < 0.0001$; two-tailed). (D) Western blot analysis of HDAC6 expression in six randomly selected human HCC tissues paired with histologically normal liver tissues (N) and dysplastic nodule (DN). All membranes were probed for GAPDH to confirm equal protein loading. (E) Endogenous HDAC6 expression in human liver cancer cell lines by northern and western blot analysis. β-Actin and GAPDH were used to control for RNA and protein loadings, respectively. Each experiment was repeated at least twice.

established oncogenic cell lines.[15] However, when we analyzed *HDAC6* gene expression from the microarray dataset that previously studied different histopathological grades of HCC,[16] we noted that *HDAC6* gene expression was significantly down-regulated in overt HCC as compared with premalignant lesion, dysplastic nodules (Supporting Fig. 1A). To confirm this, we employed a new subset of HCCs that include primary HCCs, defined by Edmondson grade 1 (TG1, n = 8), grade 2 (TG2, n = 9), grade 3 (TG3, n = 9), and dysplastic nodule (DN, n = 11) and chronic hepatic disease, liver fibrosis (LF, n = 12), and surrounding noncancerous liver tissues (N, n = 11), and subjected to whole genome expression microarrays. As shown in Fig. 1A, *HDAC6* gene expression was significantly down-regulated in overt HCC (TG3) as compared with normal or chronic liver disease (LF) or premalignant lesion (DN). This result was supported by immunohistochemical analyses of HCC tissue microarray (Supporting Table 1; Supporting Fig. 1B). Of the 32 normal hepatocyte samples tested, 30 (93.7%) showed moderate or strong immunopositivity for HDAC6 expression, whereas 15 (46.9%) of 32 HCCs tested were weak or negative-stained. Therefore, it seems that HDAC6 is down-regulated during hepatocarcinogenesis. To generalize our finding, we recapitulated *HDAC6* gene expression from the large cohorts of HCC patients that are available from the National Center for

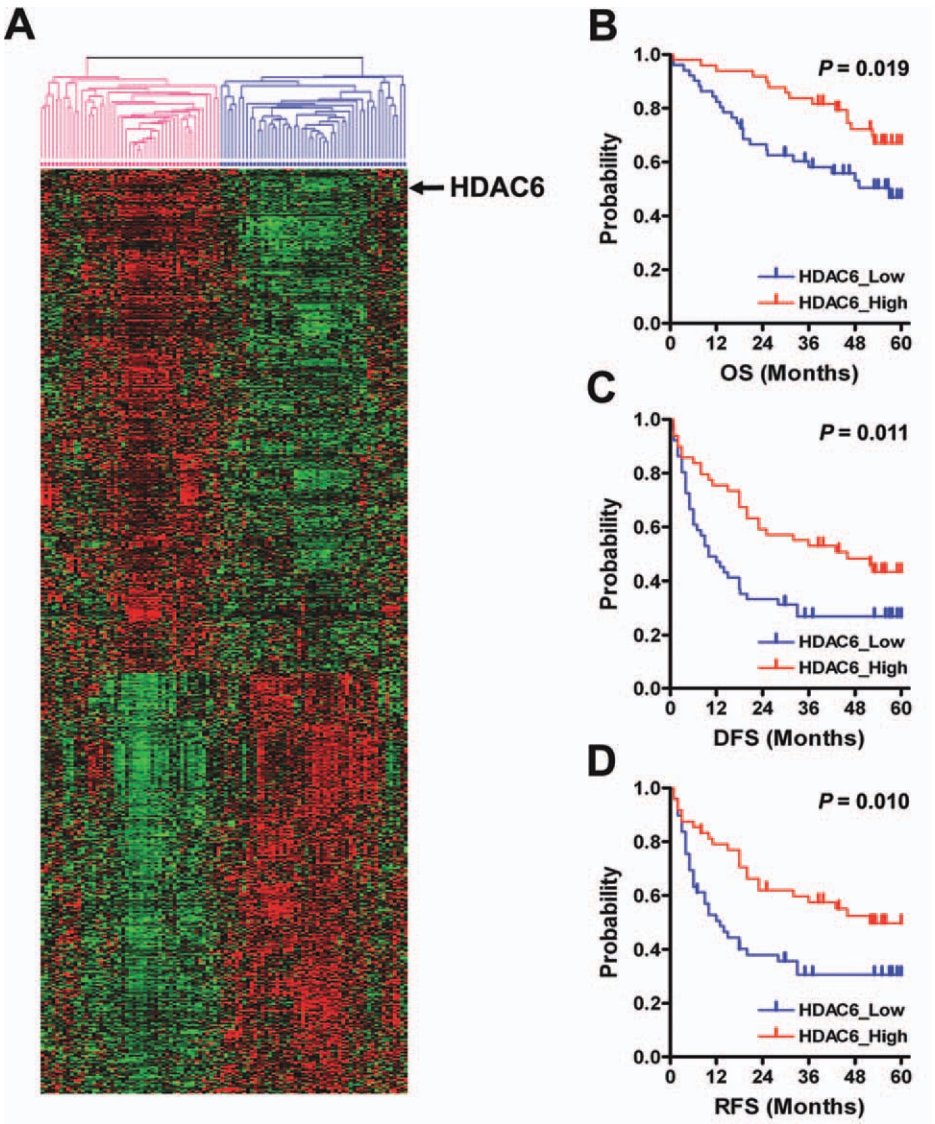

Fig. 2. Correlation of gene expression with HDAC6 expression and Kaplan-Meier survival curves of patients with HCC. (A) The microarray data were obtained from the GEO database (GSE16757). In all, 1,909 gene features that highly correlated with HDAC6 expression were selected for cluster analysis ($P < 0.001$, $r > 0.4$ or $r < -0.4$). Patients were divided into the following two groups: HDAC6 High cluster and HDAC6 Low cluster. (B) Kaplan-Meier survival curves of overall survival (OS), (C) diseases-free survival (DFS), and (D) recurrence-free survival (RFS). *P*-values were obtained with the log-rank test.

Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession numbers GSE14520 and GSE25097) and shown as scatterplots. Consistently, *HDAC6* gene expression was significantly down-regulated in two different HCC cohorts (Fig. 1B,C). Decreased expression of HDAC6 protein was confirmed by western immunoblotting of six randomly selected human HCC tissues paired with N and DN. As expected, HDAC6 was markedly down-regulated in all selected HCCs as compared with normal liver or dysplatic nodule tissues (Fig. 1D; Supporting Fig. 1C). Furthermore, endogenous expression of HDAC6 was investigated by northern and western blot analysis in nine different liver cancer cell lines (HepG2, Hep3B, PLC/PRF/5, SNU182, SNU354, SNU368, SNU387, SNU423, and SNU449), which were originally established from HCC or hepatoblastoma. The human liver cancer cell lines exhibited relatively low HDAC6 expression, with a few exceptions (Fig. 1E). These results strongly suggest that HDAC6 is suppressed in HCC.

Because the messenger RNA (mRNA) and protein levels of HDAC6 showed down-regulation in overt HCCs, we next assessed the prognostic association of HDAC6 expression in a large cohort of 100 Korean HCC patients.[17] First, 1,909 genes with expression patterns that highly correlated with HDAC6 expression were selected for cluster analysis ($P < 0.001$, $r > 0.4$ or $r < -0.4$), and shown as heatmaps (Fig. 2A). Patients were then divided into the following two groups: HDAC6 High cluster and HDAC6 Low cluster. The Kaplan-Meier survival curves of patients with HCC indicated that the 5-year overall survival (OS) rate of HCC patients with low HDAC6 expression (50.9%) was significantly lower than that of HCC patients with high HDAC6 expression (69.4%, $P < 0.05$; Fig. 2B). The disease-free survival (DFS) rate of HCC patients

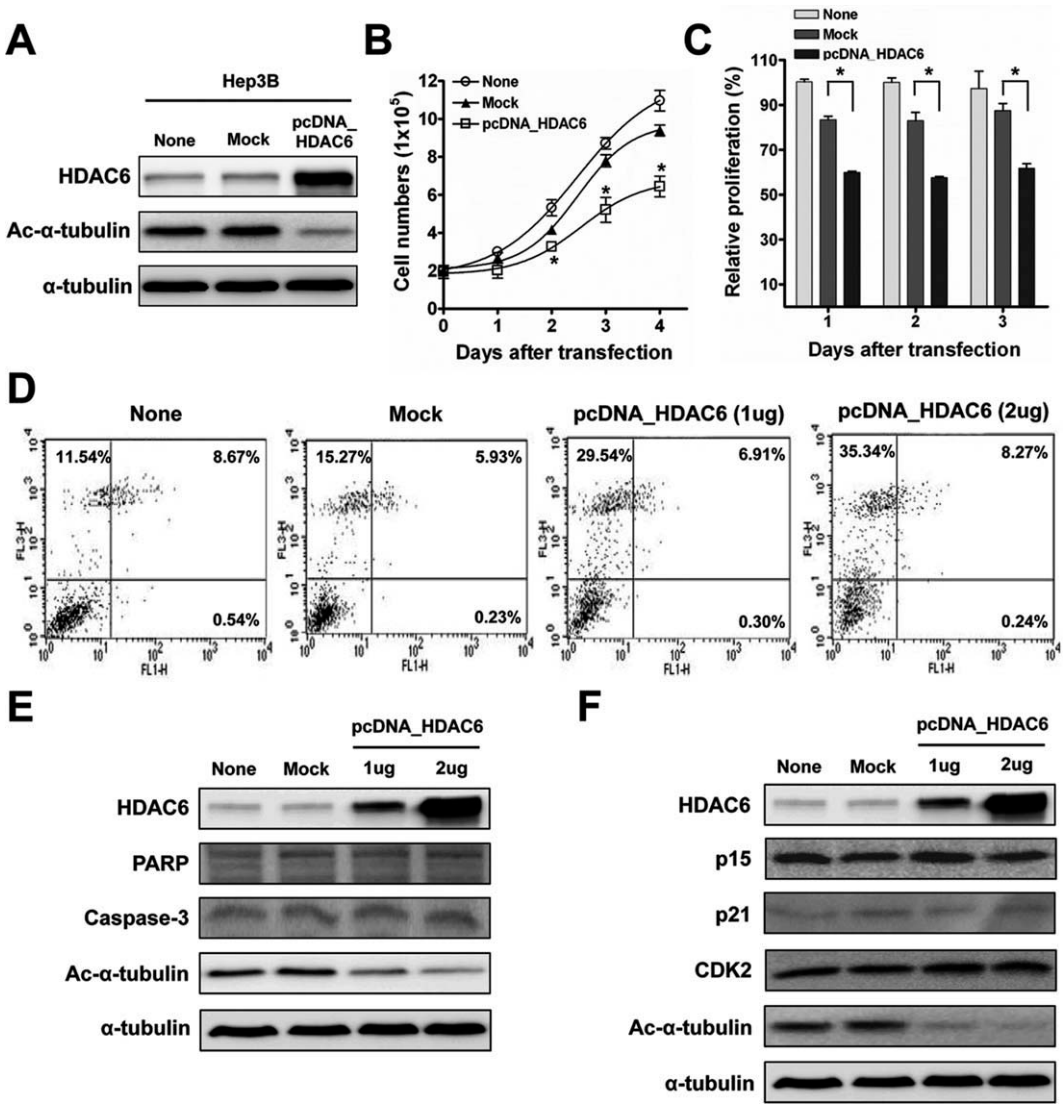

Fig. 3. Ectopic expression of HDAC6 induced caspase-independent cell death in Hep3B cells. (A) Cells were transfected with control (None), empty vector (Mock), or 1 μg of HDAC6 expression vector (pcDNA_HDAC6) for 48 hours. Expression levels of HDAC6 and acetylated α-tubulin (Ac-α-tubulin) were determined by western blot analysis. α-Tubulin was used as a loading control. (B) Effect of ectopic expression of HDAC6 on cell growth of Hep3B cells. Cells were transfected with control (None), empty vector (Mock), or 1 μg of pcDNA_HDAC6; the cell numbers were assessed by nuclear staining with trypan blue on the indicated days after transfection. Data are presented as the means $\pm$ standard deviations (SDs) of three experiments (unpaired Student *t* test, $^*P < 0.05$ versus Mock). (C) Cell proliferation rates were assessed by measuring MTT absorbance at A_{570} at the indicated times after transfection. Data are presented as the SDs of mean values in three experiments (unpaired Student *t* test, $^*P < 0.05$ versus Mock). (D) Flow cytometric analysis of Hep3B cells transfected with control (None), empty vector (Mock), or 1 or 2 μg of pcDNA_HDAC6. FITC-labeled Annexin V-positive cells (upper right and lower right) were considered apoptotic cells. Two independent experiments were performed. (E) Western blot analysis for apoptosis proteins in Hep3B cells transfected with control (None), empty vector (Mock), or 1 or 2 μg of pcDNA_HDAC6. α-Tubulin served as a loading control. (F) Western blot analysis was performed to determine the expressions of cell cycle proteins in Hep3B cells transfected with control (None), empty vector (Mock), or 1 or 2 μg of pcDNA_HDAC6. α-Tubulin was used as a loading control. The blot is typical of at least two individual experiments.

with low HDAC6 expression (27.5%) was also significantly lower than that of HCC patients with high HDAC6 expression (44.9%, $P < 0.05$; Fig. 2C). In addition, the recurrence-free survival (RFS) rate of HCC patients with low HDAC6 expression (35.3%) was significantly lower than that of HCC patients with high HDAC6 expression (53.1%, $P < 0.05$; Fig. 2D). These results demonstrated that HDAC6 expression is strongly associated with prognosis in HCC patients.

Ectopic Overexpression of HDAC6 Causes Growth Retardation of Hep3B Cells. To better understand the molecular consequences of ectopic overexpression of HDAC6 in hepatocarcinogenesis, full-length human HDAC6 cDNA (pcDNA_HDAC6) was constructed and transiently transfected into Hep3B cells and cell viability and MTT cell proliferation assays were performed. The functional activity of HDAC6 was confirmed by detecting the hypoacetylation of α-tubulin

(Fig. 3A), and ectopic overexpression of HDAC6 resulted in reduction of growth rate and a significantly reduced proliferation rate of the Hep3B cells (Fig. 3B,C). This antimitotic effect of HDAC6 could be partially explained by the disruption of cell growth regulation. Thus, we next examined the effect of HDAC6 on the cell cycle distribution and on the apoptosis of Hep3B cells. Flow cytometry of Annexin V-stained cells showed no significant induction of apoptosis versus control (non- or empty vector-transfected) cells (Fig. 3D). In addition, HDAC6 overexpression did not affect the expressions of proapoptotic molecules, such as apoptosis-inducing factor (AIF), Bax, or Apaf-1 (data not shown), nor did it cause caspase-3 or poly (ADP-ribose) polymerase (PARP) cleavage of Hep3B cells (Fig. 3E). Moreover, when propidium iodide-stained HDAC6 transfected cells were performed using flow cytometry, no significant changes in cell cycle transition were observed versus control cells (Supporting Fig. 2). Likewise, the ectopic overexpression of HDAC6 did not affect the expressions of cell cycle proteins such as $p15^{INK4B}$, $p21^{WAF1/Cip1}$, or cyclin-dependent kinase 2 (CDK2) (Fig. 3F). These results suggest that HDAC6 overexpression induces a mitotic defect possibly mediated by caspase-independent cell death.

Restoration of HDAC6 Expression Activates Autophagic Cell Death in Hepatocarcinogenesis. It has been well established that autophagy is an evolutionarily conserved protein degradation process, which plays essential roles in cell survival or cell death, depending on the cellular context. The fact that HDAC6, a ubiquitin-binding deacetylase, is a central component of basal autophagy that targets protein aggregates and damages mitochondria[10] led us to investigate whether the ectopic expression of HDAC6 elicits autophagic cell death of HCC cells. Notably, it was found that ectopic expression of HDAC6 in Hep3B cells significantly increased the conversion of LC3B-I into LC3B-II (Fig. 4A,B), whereas treatment of 3-methyladenine (3-MA; a specific inhibitor of autophagy) effectively blocked LC3B-II conversion induced by HDAC6 in Hep3B cells (Fig. 4C). Consistently, reduced cell viability caused by ectopic HDAC6 expression was effectively blocked by 3-MA treatment (Fig. 4D). In addition, immunofluorescence staining for LC3B revealed that HDAC6 overexpression induced ring-shaped spots evenly distributed throughout cytoplasm, indicating an association between LC3 and autophagosomal membranes, and this association was completely blocked by 3-MA (Fig. 4E). Moreover, when cells were treated with HDAC6-sepcific inhibitors (Tubastatin A [Tub A] and Tubacin), ectopic overexpression of HDAC6 did not elicit hypoacetylation of α-tubulin, nor did it cause LC3B-II conversion in Hep3B cells (Fig. 4F). These results suggest that the restoration of HDAC6 expression activates autophagic cell death and the functional deacetylase activity of HDAC6 is required for the autophagy activation in hepatocarcinogenesis.

Next, to generalize our findings, we examined antimitotic effects of HDAC6 on different liver cancer cell lines. We selected the HepG2, SNU368, and SNU449 cells as they were found to express little or no HDAC6 by northern and western blot analysis (Fig. 1E), and transfected with pcDNA_HDAC6. As expected, ectopic expression of HDAC6 caused growth retardation and elicited increased LC3B-II conversion in these liver cancer cells as compared with control (non- or empty vector-transfected) cells (Fig. 5A-F). In contrast, for PLC/PRF/5 and SNU423 cells that exhibit relatively high expression of HDAC6 among liver cancer cell lines (Fig. 1E), the knockdown of HDAC6 significantly enhanced growth rates of these cell lines (Supporting Fig. 3). Similarly, when the same experimental approach was applied to newly established HDAC6-overexpressing Hep3B cell lines (Hep3B_HDAC6 Clone #1 and Clone #2), resilencing of HDAC6 also caused an increased growth rate compared to control cells (scramble sequence of siRNA transfectants).

Lastly, to investigate whether tumor suppressor activity of HDAC6 is HCC-specific, we analyzed *HDAC6* gene expressions of colon, gastric, and breast cancer patients from the NCBI GEO database. We selected two sets of microarray data for each colon, gastric, or breast cancer, and compared HDAC6 expression in cancer patients with that of nontumor tissues. There were no significant differences of HDAC6 expression between the normal and tumor group in both colon and gastric cancer datasets (Supporting Fig. 5A-D), whereas the HDAC6 expression in breast cancer was variable depending on cohort study (Supporting Fig. 5E,F). However, when ectopic overexpression of HDAC6 was performed in each of three different colon, gastric, or breast cancer cell lines, all cell lines exhibited no changes in growth rate and LC3B-II conversion (Supporting Figs. 6-8). These results clearly indicated that HDAC6 functions as a tumor suppressor by activating autophagic cell death, and tumor suppressor activity is specific to HCC.

Sustained Expression of HDAC6 Reduced the* In Vivo *Tumorigenic Potential of Hep3B Cells. To investigate whether the stable overexpression of HDAC6 suppresses liver tumorigenesis, we established

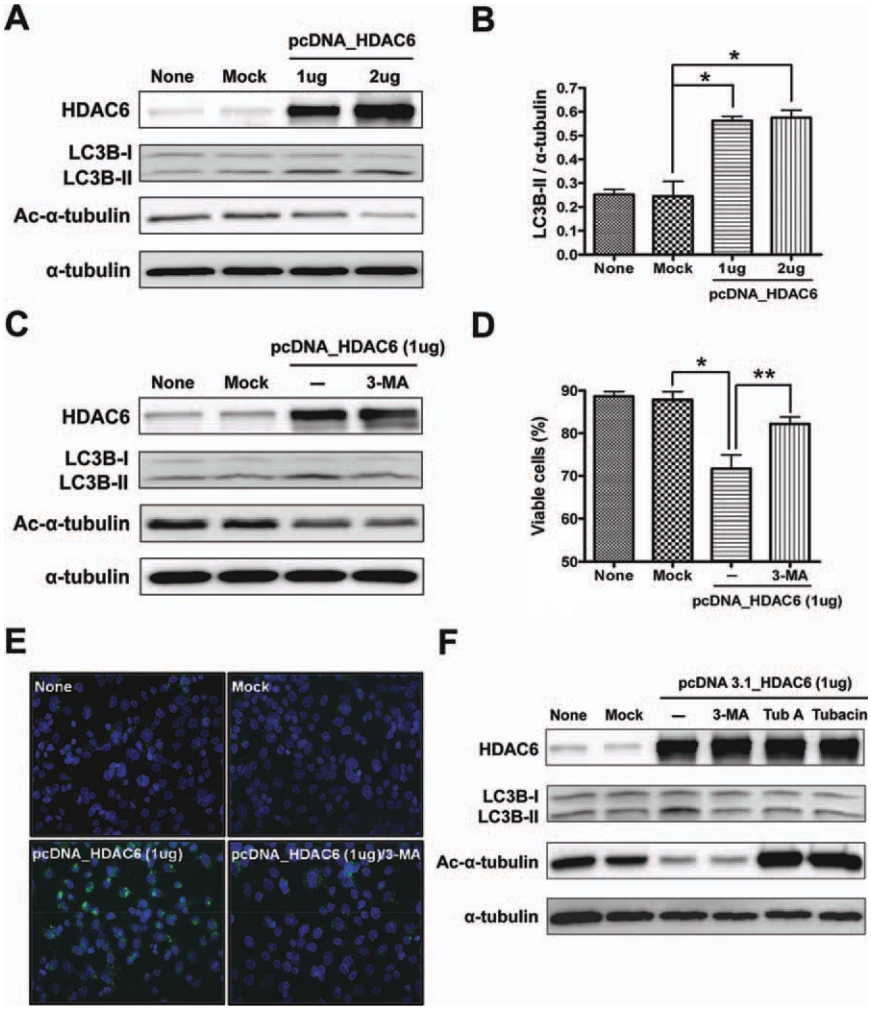

Fig. 4. The ectopic expression of HDAC6 induced autophagic cell death in Hep3B cells. (A) Cells were transfected with control (None), empty vector (Mock), or 1 or 2 μg of pcDNA_HDAC6. Expression levels of HDAC6, LC3B-II, and acetylated α-tubulin (Ac-α-tubulin) were determined by western blot analysis. The α-tubulin is shown as a loading control. (B) LC3B-II to α-tubulin ratios determined by densitometric analysis. Averaged LC3-II band intensities were normalized versus averaged α-tubulin band intensities for three replicate experiments (unpaired Student *t* test, $*P < 0.05$ versus Mock). (C) Hep3B cells were transfected with control (None), empty vector (Mock), or 1 μg of pcDNA_HDAC6. At 24 hours following transfection, cells were treated with or without 5 mM of 3-MA for another 24 hours. The levels of LC3B-II and acetylated α-tubulin (Ac-α-tubulin) were determined by western blot analysis. The membranes were probed for α-tubulin to confirm equal protein loading. This experiment was performed in duplicate and similar results were obtained. (D) Cells were transfected and treated as described in (C). Viable cells were trypsinized and counted by Trypan blue staining at 48 hours of transfection. The results shown are the means of three independent experiments and error bars represent SDs of mean values (unpaired Student *t* test, $*P < 0.05$ versus Mock, $**P < 0.05$ versus pcDNA_HDAC6). (E) Hep3B cells were transfected and treated as described in (C), then fixed, permeabilized, immunostained with a LC3B antibody, and visualized under a fluorescence microscope. In the resulting images the green fluorescence indicated LC3-FITC staining of autophagosomes and blue fluorescence indicated Hoechst-stained nuclei. Patterns of LC3-FITC staining in the cytosol changed from diffuse to predominantly punctate/vesicular. The results shown are representative of experiments performed in duplicate. (F) Functional deacetylase activity of HDAC6 is required for autophagy activation. Cells were transfected with control (None), empty vector (Mock), or 1 μg of pcDNA_HDAC6. At 24 hours following transfection, cells were treated with 3-MA (5 mM), Tubastatin A (Tub A, 10 μM), and Tubacin (10 μM) for another 24 hours. The levels of LC3B-II and acetylated-α-tubulin (Ac-α-tubulin) were determined by western blot analysis. α-Tubulin was used as a protein loading control. Two independent experiments with the same results were performed.

two cell lines stably overexpressing HDAC6 (Hep3B_HDAC6 Clone #1 and Clone #2). The functional HDAC6 expression was confirmed by detecting the hypoacetylated α-tubulin in these cell lines (Fig. 6A). These cells also exhibited lower growth rates than mock-transfected cells (Hep3B_Mock; Fig. 6B). The immunofluorescence analysis revealed the apparent accumulation of LC3B in Hep3B_HDAC6 cells, whereas almost no accumulation of LC3B was observed in Hep3B_Mock cells (Fig. 6C). In addition, when cells were examined ultrastructures by transmission electron microscopy, ≈40%-45% of

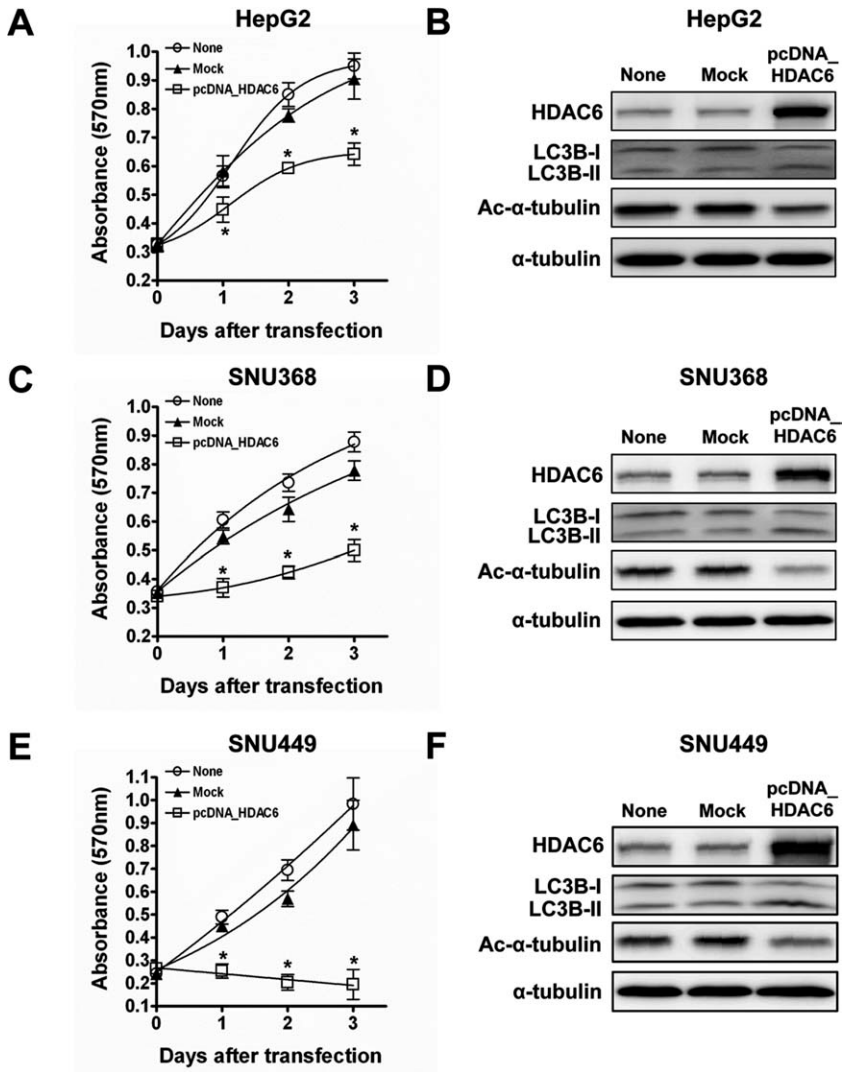

Fig. 5. HDAC6 induced autophagic cell death in liver cancer cell lines. (A) HepG2, (C) SNU368, and (E) SNU449 cells were transfected with control (None), empty vector (Mock), or 1 μg of pcDNA_HDAC6. The proliferation rates of liver cancer cell lines were determined by measuring MTT absorbance at A_{570} at the indicated times after transfection. Error bars show mean $\pm$ SD of three experiments (unpaired Student *t* test, $*P < 0.05$ versus Mock). At 48 hours following transfection, the LC3B-II conversion levels by HDAC6 overexpression in (B) HepG2, (D) SNU368, and (F) SNU449 cells were determined by western blot analysis. α-Tubulin was used as a loading control. Experiments were performed in duplicate.

Hep3B_HDAC6 cells exhibited autophagic vacuoles, some of which accumulated to form larger cytoplasmic vacuoles (Fig. 6D-b), and at higher magnifications most vacuoles were found to contain electron-dense material and degraded organelles (Fig. 6D-c,d). In contrast, fewer vacuoles were observed in cytoplasm of control cells (Hep3B_Mock) and these did not contain electron-dense material or degraded organelles (Fig. 6D-a). Thus, to determine whether HDAC6 overexpression has a tumor suppressive effect *in vivo*, we subcutaneously injected these cells (Hep3B_HDAC6 Clone #1 and Clone #2) into athymic nude mice. At 45 days postinoculation, tumors were detected in animals injected with mock-transfected cells (Hep3B_-Mock). In contrast, tumors were observed in animals injected with Hep3B_HDAC6 Clone #1 or Clone #2 cells from 55 days postinoculation (Fig. 6E). Overall, tumor growth was significantly lower in animals injected with Hep3B_HDAC6 Clone #1 or Clone #2 cells than that of Hep3B_Mock cells ($P \leq 0.05$; Fig. 6E), and average tumor volume at sacrifice was much smaller in the Hep3B_HDAC6 group than Hep3B_-Mock group ($P \leq 0.05$; Supporting Fig. 9). The overexpression of HDAC6 and reduced acetylation status of α-tubulin were then confirmed in tumor tissues of animals treated with Hep3B_HDAC6 Clone #1 or Clone #2 cells (Fig. 6F). Interestingly, it was found that tumor tissues treated with Hep3B_HDAC6, Clone #1 or Clone #2 cells exhibited increased Beclin 1 expression, and it has been well established that Beclin 1 participates during the early stages of autophagy.[18] This result implies that HDAC6 mediates autophagic cell death by way of Beclin 1-induction pathway.

HDAC6 Activated Autophagy by Way of c-Jun NH2-Terminal Kinase-Mediated Beclin 1 Expression. Recent studies have demonstrated that the induction of Beclin 1 occurs during autophagy in various

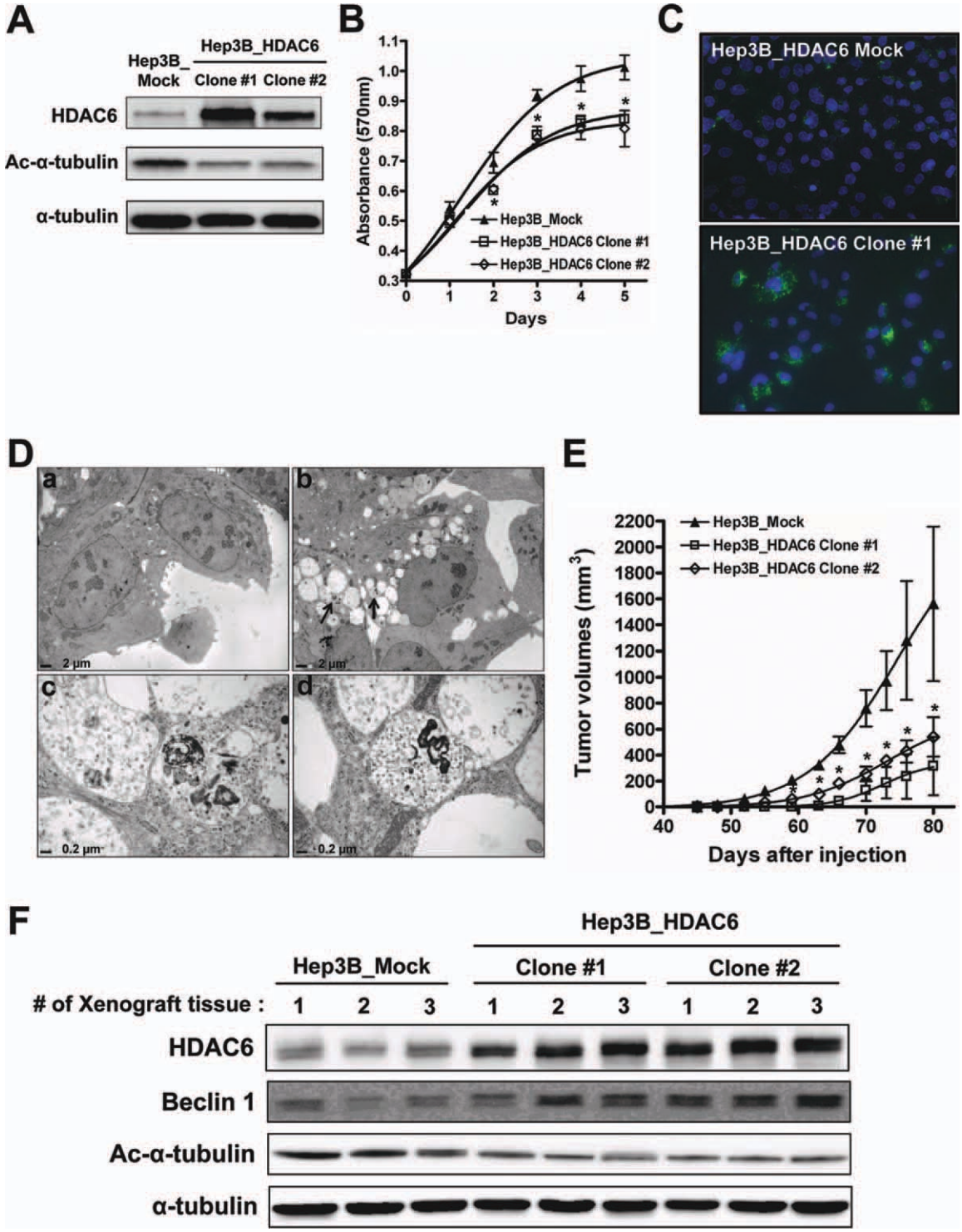

Fig. 6. The sustained overexpression of HDAC6 attenuated the tumorigenic potential of Hep3B cells *in vitro* and *in vivo*. (A) Stable HDAC6-overexpressing cell lines were established as described in Supporting Materials and Methods. Expression of sustained-functional HDAC6 was confirmed by detecting the hypoacetylation of α-tubulin in Hep3B_HDAC6 Clone #1 or Clone #2 cells. (B) Cell growth analysis of the established HDAC6-overexpressing cell lines (Hep3B_Mock, Hep3B_HDAC6 Clone #1 and Clone #2). Relative growth rates were determined using MTT assays at the indicated timepoints. Data are the SDs of mean values in three experiments (unpaired Student *t* test, $*P < 0.05$ versus Mock). (C) Immunofluorescence analysis of LC3B in Hep3B_HDAC6 Clone #1 cells. This experiment was repeated twice with similar results. A typical result is shown. (D) Transmission electron microscopy showed the ultrastructures of (a) control (empty vector transfectant) and (b) HDAC6-overexpressing cells (Hep3B_HDAC6 Clone #1) under normal growth conditions. Electron microscopy image analysis was conducted as described in Supporting Materials and Methods. Arrows indicate the presence of autophagic vacuoles, such as autophagosomes or autophagolysosomes. (c-d) The regions indicated by arrows are presented at higher magnification and show autophagosome and autophagolysosome structural details. Experiments were performed in triplicate. (E) Tumor cell growth rates of animals injected Hep3B_Mock cells, Hep3B_HDAC6 Clone #1 and Clone #2 cells. Tumors sizes were measured with calipers in three dimensions on the indicated days. Tumor volumes were calculated using the formula: tumor volume (mm^3) = L × W^2 / 2, where L is length and W is width. Values shown are mean ± SD (Hep3B_Mock, n = 5; Hep3B_HDAC6 Clone #1, n = 5; Hep3B_HDAC6 Clone #2, n = 5). $*P < 0.05$, compared with Hep3B_Mock. (F) HDAC6, Beclin 1, and acetylated α-tubulin (Ac-α-tubulin) expressions in xenograft tumors by western blot analysis. The results showed levels of HDAC6, the acetylation status of α-tubulin, and Beclin 1 expression in three randomly selected xenograft tumors. A representative result of duplicate experiments is shown.

cell types. However, it has not been determined how Beclin 1 expression is regulated.[19] To investigate whether HDAC6 induces Beclin 1 expression during autophagy in liver cancer cells, we examined the endogenous expressions of Beclin 1 and LC3B-II in Hep3B cells stably expressing HDAC6 (Hep3B_HDAC6 Clone #1 or Clone #2 cells). As shown in Fig. 7A, both Hep3B_HDAC6 Clone #1 and Clone #2 cells expressed markedly more Beclin 1 and LC3B-II than Hep3B_Mock cells. This effect of HDAC6 on autophagy was confirmed by treating Hep3B cells with ceramide, a potent inducer of autophagic cell death. The increased expression of Beclin 1 and LC3B-II conversion were repressed by HDAC6 knockdown in Hep3B_HDAC6 Clone #1 cells (Fig. 7B). Consistently, treatment of 3-MA suppressed Beclin 1 induction and LC3B-II conversion in Hep3B_HDAC6 Clone #1 and Clone #2 cells (Fig. 7C,D). Thus, to explore whether Beclin 1 plays a critical role in HDAC6-mediated autophagy, we performed knockdown of Beclin 1 in Hep3B_HDAC6 Clone #1 and Clone #2 cells. As expected, Beclin 1 knockdown markedly blocked LC3B-II conversion in both Hep3B_HDAC6 Clone #1 and Clone #2 cells (Fig. 7E,F). Overall, these results suggest that HDAC6 exerts its tumor suppressing effect by way of Beclin 1 and LC3-II processing-dependent autophagy in liver cancer cells.

Recent investigations have indicated that JNK is activated during autophagy and that this is required for autophagosome formation, although the underlying mechanism has not been determined.[20] It has also been reported that the activation of JNK-mediated Beclin 1 expression by anticancer agents induces autophagic cell death in cancer cells.[21] Because we observed that Beclin 1 expression is significantly up-regulated during HDAC6-induced autophagy, we next examined phosphorylated-JNK (p-JNK) levels to determine whether the JNK pathway is activated in HDAC6-overexpressing cells. As shown in Fig. 8A, the p-JNK level increased both Hep3B_HDAC6 Clone #1 and Clone #2 cells as compared with control cells (Hep3B_Mock). We also found that phosphorylation of the transcription factor c-Jun, the target substrate of JNK, was enhanced in these HDAC6-overexpressing cells. Thus, to determine whether JNK activation is involved and required for Beclin 1 induction during HDAC6-mediated autophagy, HDAC6 was resilenced in Hep3B_HDAC6 Clone #1 cells. As shown in Fig. 8B, the knockdown of HDAC6 reduced the phosphorylation of JNK and c-Jun without changing the basal level, and suppressed Beclin 1 induction and LC3B-II conversion. Lastly, we observed that the treatment of SP600125, a JNK-specific inhibitor, effectively blocked Beclin 1 induction and LC3B-II conversion of Hep3B_HDAC6 Clone #1 cells (Fig. 8C). Collectively, these results demonstrate that HDAC6 induces autophagic cell death by way of JNK-mediated Beclin 1 pathway in liver cancer cells.

Discussion

In this report we present evidence that HDAC6, a cytoplasmic deacetylase, functions as a tumor suppressor by mediating caspase-independent autophagic cell death by way of the JNK-activated Beclin 1-dependent pathway in human liver cancer cells. The expression of HDAC6 is suppressed or negative in overt HCC and significantly associated with poor prognosis of HCC patients. It was found that the ectopic expression of HDAC6 inhibited the tumor growth rate of cells *in vitro* and *in vivo*, and it was also demonstrated that HDAC6 activates the JNK/c-Jun signaling pathway, which activates Beclin 1/LC3B-II-dependent autophagy in liver cancer cells. These findings define a central role for HDAC6 in liver tumorigenesis and suggest that HDAC6 has potential therapeutic value for the treatment of liver cancer.

The acetylation of histones by lysine is one of the major epigenetic regulators of chromatin conformation and gene expression. The dynamic nature of histone acetylation is determined by the balance between the activities of histone acetyltransferase (HAT) and HDAC enzymes.[5] Several studies have shown that certain HDAC family members are aberrantly expressed in some tumors and that they have nonredundant functions in controlling the hallmarks of cancer cells.[7,22] Abnormal HDAC activity has been implicated in tumorigenesis and, therefore, considerable effort has been put into developing HDAC inhibitors that enable histone acetylation status to be modified and that induce the reexpressions of aberrantly silenced tumor suppressor genes. Although HDAC6 is a cytoplasmic deacetylase that associates with microtubules and deacetylates α-tubulin, HDAC6 has also been reported to be overexpressed in a number of human cancer cell lines and in mouse tumor models. Additionally, HDAC6 has been suggested as a therapeutic target due to its essential role in many signaling pathways that provide survival advantages to malignant cells and maintain their phenotypes.[15] For example, HDAC6 expression has been shown to be up-regulated in primary oral squamous cell carcinoma[13] and human breast cancer tissues,[23] and in primary acute myeloid

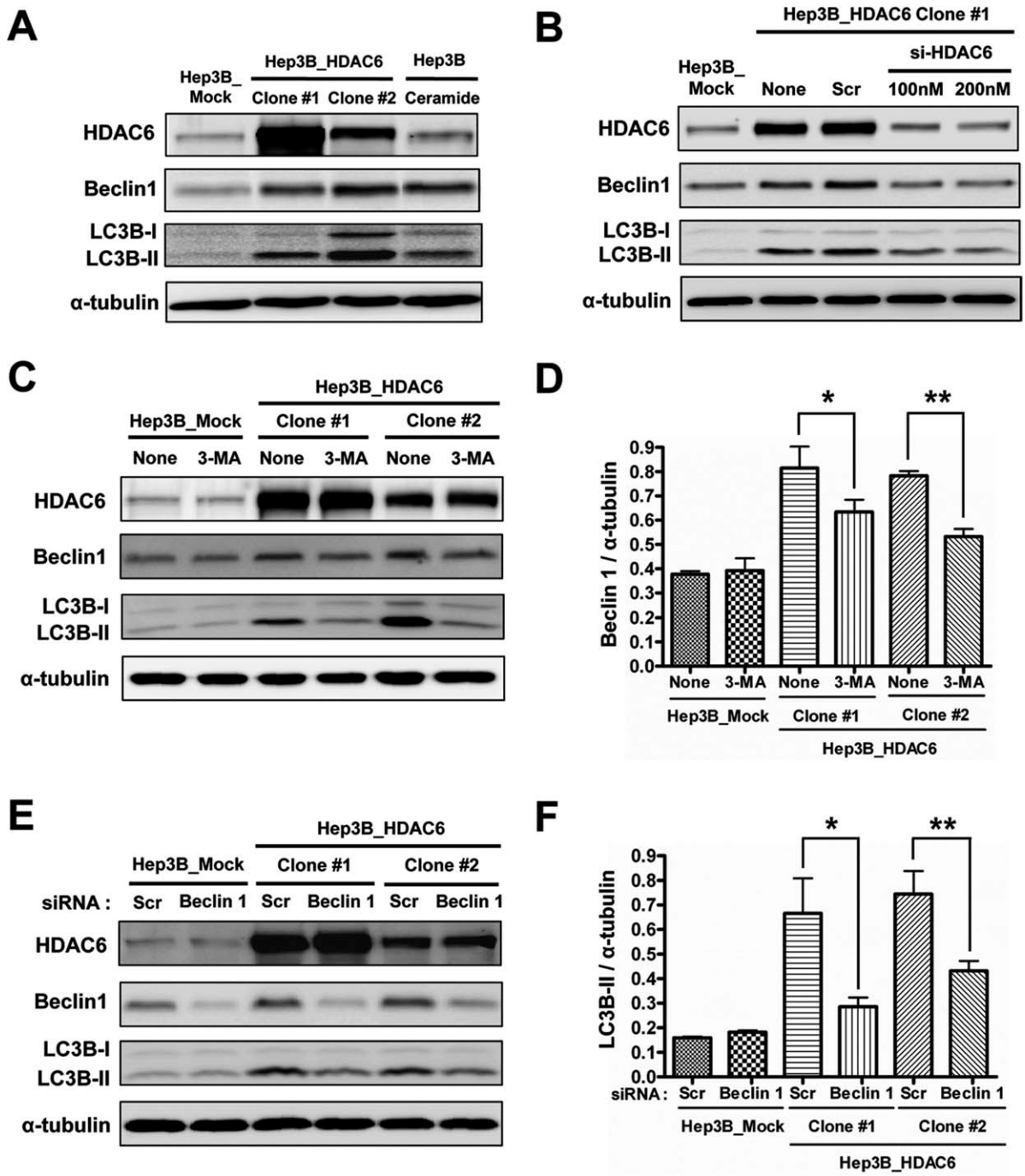

Fig. 7. HDAC6 overexpression activates autophagy by way of the Beclin 1-dependent LC3B-II pathway. (A) The induction of Beclin 1 expression was examined by western blot analysis in control (Hep3B_Mock) and HDAC6-overexpressing cell lines (Hep3B_HDAC6 Clone #1 and Clone #2). Hep3B cells treated with 20 μM of ceramide for 24 hours were used as a positive control for Beclin 1 induction. (B) HDAC6-overexpressing cells (Hep3B_HDAC6 Clone #1) were transfected with control (None), 100 nM of scrambled siRNA (Scr), or 100 nM or 200 nM of HDAC6-specific siRNA (si-HDAC6) for 48 hours. Beclin 1 expression and LC3B-II conversion levels were then evaluated by western blot analysis. (C) Hep3B cell lines stably overexpressing HDAC6 (Hep3B_HDAC6 Clone #1 and Clone #2) and empty vector (Hep3B_Mock) transfected cells were treated with 5 mM of 3-MA to inhibit autophagy. The levels of Beclin 1 expression and LC3B-II conversion were determined by western blot analysis. (D) The graph bar shows the ratios of Beclin 1 protein expression relative to α-tubulin (unpaired Student t test, $^{***}P < 0.05$ versus None in Hep3B_HDAC6 Clone #1 and Clone #2). (E) Control (Hep3B_Mock) and HDAC6-overexpressing cell lines (Hep3B_HDAC6 Clone #1 and Clone #2) were transfected with 100 nM of scrambled siRNA (Scr) or 100 nM of Beclin 1-specific siRNA. At 48 hours following transfection, the expression levels of Beclin 1 and LC3B-II were determined by western blot analysis. (F) The graph bar shows the ratios of LC3-II/α-tubulin (unpaired Student t test, $^{*,**}P < 0.05$ versus Scr in Hep3B_HDAC6 Clone #1 and Clone #2). α-Tubulin served as a loading control in all experiments. A typical result of three experiments is shown. The signal intensities of protein bands were scanned from images derived from three independent western blot experiments and quantified. In all experiments, averaged intensities of Beclin 1 and LC3-II bands were normalized versus averaged intensities of α-tubulin bands.

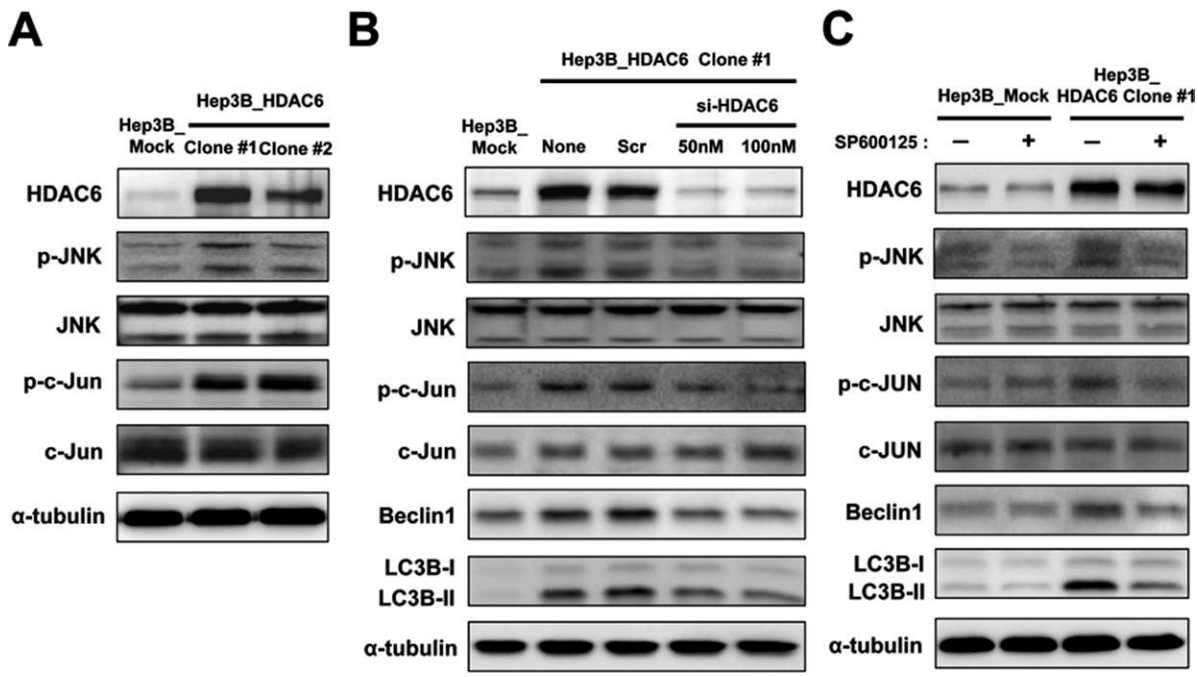

Fig. 8. HDAC6 activates JNK/c-Jun activation-mediated Beclin 1-dependent autophagy in liver cancer cells. (A) The expression levels of JNK, phospho-JNK (p-JNK), c-Jun, and phospho-c-Jun (p-c-Jun) were evaluated by western blot analysis in control (Hep3B_Mock) and HDAC6-overexpressing cell lines (Hep3B_HDAC6 Clone #1 and Clone #2). (B) HDAC6-overexpressing cells (Hep3B_ HDAC6 Clone #1) were transfected with control (None), 100 nM of scrambled siRNA (Scr), or 50 nM or 100 nM of HDAC6-specific siRNA (si-HDAC6) for 48 hours. Immunoblotting was used to determine the expression levels of HDAC6, JNK, phospho-JNK (p-JNK), c-Jun, phospho-c-Jun (p-c-Jun), Beclin 1, and LC3B-II protein after transfection with HDAC6-specific siRNA. (C) Control (Hep3B_Mock) and HDAC6-overexpressing cell clones (Hep3B_HDAC6 Clone #1) were treated for 24 hours with or without 10 μM of SP600125 to inhibit JNK signaling, and then the expression levels of HDAC6, JNK, phospho-JNK (p-JNK), c-Jun, phospho-c-Jun (p-c-Jun), Beclin 1, and LC3B-II protein were determined by immunoblotting. All membrane blots were reprobed for α-tubulin to ensure equal loading. A typical result of three experiments is shown.

leukemia blasts.[24] However, no detailed analysis of the biological roles of HDAC6 in human HCC has been conducted to date.

In a previous study, we performed transcriptomic changes in different histopathological grades of HCC.[16] Based on gene expression data of multistep histopathological grades of HCC, we noted that *HDAC6* gene expression is significantly down-regulated in HCC, and that it is also significantly down-regulated in a large cohort of HCC patients. This loss or negative expression of HDAC6 was confirmed in a subset of human HCC tissues and in various liver cancer cell lines (Fig. 1; Supporting Fig. 1). Notably, it was found that low expression of HDAC6 was significantly associated with a poor prognosis in HCC patients in 5-year OS, DFS, and RFS (Fig. 2). Molecular hepatocarcinogenesis has been reported to be different according to etiologies such as hepatitis B virus, hepatitis C virus, alcohol, etc.[2,3] However, our results showed no difference of HDAC6 expression according to such etiologies (Supporting Fig. 10). These results caused us to speculate that HDAC6 has an antitumor function during liver tumorigenesis. Subsequently, we demonstrated that the ectopic expression of HDAC6 suppressed liver cancer cell growth and proliferation without affecting cell cycle progression or cellular apoptosis (Fig. 3). Accordingly, our findings contradict previous reports regarding the oncogenic functions of HDAC6 in cancer development and progression, as it suggests that HDAC6 acts as a tumor suppressor in hepatocarcinogenesis.

HDAC6 is localized exclusively in the cytoplasm, where it associates with the microtubule and actin cytoskeletons. Unlike other HDAC family members, HDAC6 has intrinsic ubiquitin-binding activity and associates with both microtubules and the F-actin cytoskeleton.[9,25] Recently, it was suggested that HDAC6 controls the fusion of autophagosomes and lysosomes and, thus, regulates autophagy.[10] During the early stage of tumor development, autophagy suppresses tumor growth and during cancer therapy many cells undergo autophagic cell death.[26] In the present study, we demonstrated that ectopic expression of HDAC6 elicited LC3B-II conversion and reduced viable Hep3B cells counts, and that this was blocked by 3-MA, a specific inhibitor of autophagy (Fig. 4). In addition,

similar results were consistently obtained from different liver cancer cell lines (Fig. 5) and the *in vivo* mouse tumor xenograft experiment (Fig. 6E). Today, autophagy is considered a multifaceted process, and alterations in autophagic signaling pathways are frequently found in cancer and many other diseases. Although autophagy has been reported to paradoxically promote cell survival and death during tumor development and in cancer therapy,[27] our results demonstrate that HDAC6 functions as a tumor suppressor by activating autophagic cell death in liver cancer.

Earlier studies on the mechanism underlying the regulation of autophagy in cancer cells showed that autophagy is regulated by multiple diverse signaling pathways, such as the class II PI3K, the protein kinase mammalian target of rapamycin (mTOR), extracellular signal-regulated kinase (ERK), and the p38 pathways.[26] In this regard, we noted with interest a report that the activation of JNK can mediate Beclin 1 expression, which is known to play a key role in the autophagic cell death of cancer cells.[21] Concordantly, Beclin 1 expression was found to be induced in mouse xenograft tumor tissues injected with HDAC6-overexpressing Hep3B cells (Fig. 6F). It was also found that sustained expression of HDAC6 induced Beclin 1 expression, whereas its resilencing suppressed Beclin 1 induction and autophagy, as determined by reduced LC3B-II conversion in Hep3B_HDAC6 Clone #1 and Clone #2 cells (Fig. 7A,B). Thus, we postulated that HDAC6 could activate the JNK pathway and mediate Beclin 1-dependent autophagy in liver cancer cells. To clarify this hypothesis, we focused on the relationships between JNK pathway activation and Beclin 1 expression in HDAC6-induced autophagy, and demonstrated that during HDAC6-induced autophagy the JNK pathway is activated in liver cancer cell lines, and that this induced Beclin 1 expression. On the other hand, the JNK-specific inhibitor SP600125 and HDAC6 knockdown inhibited Beclin 1 induction and autophagy activation (Fig. 8). Moreover, we observed that c-Jun was also involved in the regulation of Beclin 1 in response to HDAC6-induced autophagy. These results suggest a novel mechanism for the regulation of Beclin 1 expression in HDAC6-induced autophagy in liver cancer. Although it is not clear whether HDAC6 directly activates JNK/c-Jun signaling, it is obvious that HDAC6 causes autophagic cell death by way of JNK-mediated Beclin 1 expression in liver cancer cells.

Taken together, the present study shows that HDAC6 expression is suppressed or lost in HCC, and that the ectopic expression of HDAC6 inhibits *in vitro* and *in vivo* tumor growth by promoting autophagic cell death and by activating a JNK-mediated Beclin 1 pathway. Future detailed analyses of the molecular mechanisms governing HDAC6 inactivation should illustrate how HDAC6 influences the balance of autophagic signals. Here, we propose for the first time that HDAC6 functions as a tumor suppressor by activating caspase-independent autophagic cell death during hepatocarcinogenesis, and thus, our findings might support the clinical potential of HDAC6 for the treatment of liver cancer.

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