Hepatocarcinogenesis is a multistep process that involves alterations of various signaling cascades, which cause hepatocellular carcinoma to exhibit heterogeneous molecular signatures (1). Because of genetic and/or epigenetic alterations, several signaling cascades related to cell survival and proliferation are activated, and treatments targeting these activations have shown promise in preclinical and early clinical studies (2). With respect to proliferation cascades, epidermal growth factor receptor (EGFR) and Ras signaling are activated in more than 50% of hepatocellular carcinomas, whereas mTOR pathway is disrupted in 40% to 50% of liver cancers due to upstream signaling, inactivation of the tumor suppressor PTEN, or mutations of phosphoinositide-3-kinase (PI3K; ref. 3). Similarly, insulin-like growth factor receptor 1 (IGFRI) signaling is active in 20% of early hepatocellular carcinomas, and the deregulations of hepatocyte growth factor (HGF), vascular endothelial growth factor, Wingless, and c-MET pathway are common events (4).

EGF and autocrine signaling activator EGFR are associated with several malignancies, including hepatocellular carcinoma, and EGFR is among the best described therapeutic targets in cancer. Furthermore, preclinical evidence indicates that EGFR-related signaling is involved in hepatocarcinogenesis. EGFR is known to protect hepatocellular carcinoma cells from oxidative stress–induced apoptosis and to upregulate tumor angiogenesis by stimulating the Ras/Raf/ERK (extracellular signal-regulated kinase) and PI3K/v-akt murine thymoma viral oncogene homolog 1 (AKT)/mTOR signaling pathways (5). Thus, EGFR system and its chronic stimulation participate in...
the neoplastic conversion of liver, and EGF could be a mitogen required for hepatocarcinoma cell growth. However, the mechanisms responsible for malignant transformation induced by signals downstream of EGF are largely unknown.

Histone deacetylase 2 (HDAC2) is a member of the class I HDAC family, and is frequently dysregulated in cancers, including hepatocellular carcinoma (6). Our previous studies suggested that overexpression of HDAC2 plays a pivotal role in the development of hepatocellular carcinoma due to its regulation of cell-cycle components at the transcription levels (7). However, the underlying mechanisms linking HDAC2 and hepatocellular malignant progression and transformation have not been determined. Furthermore, the regulatory and signaling circuit controlling HDAC2 expression has not been defined. Accordingly, this study was undertaken to determine how growth factor signaling regulates oncogenic HDAC2 and to identify the mechanism responsible for the regulatory feedback of HDAC2 in hepatocarcinogenesis.

Materials and Methods

Tissue samples

Seven fresh surgical hepatocellular carcinoma specimens, including tumor tissues and peripheral tumor-free liver tissues, were obtained from the Liver Cancer Specimen Bank of the National Research Resource Program of the Korean Science and Engineering Foundation. This study was approved by Institutional Review Board (IRB) of Soonui Campus, College of Medicine, The Catholic University of Korea (Seoul, Korea; IRB approval number CUMC11U010).

Cell culture, treatments, and transfection

The human hepatocellular carcinoma cell line HepG2 (wt p53) was obtained from the American Type Culture Collection (ATCC). The Hep3B (p53 null), SNU-182, SNU-449, PLC/PRF/5, and HuH7 (mt p53) cell lines were purchased from the Korean Cell Line Bank (KCLB). All cell lines were maintained in RPMI supplemented with 10% FBS and antibiotics. Cells were authenticated by short tandem repeat profiling by ATCC and KCLB, and were used within 6 months after receipt or resuscitation. All cultures were maintained at 37°C in a 5% CO2–humidified atmosphere. For EGF stimulation, cells were starved in serum-free medium overnight and then stimulated with EGF (50 or 100 ng/mL) for the indicated times. To inhibit growth factor signaling, LY294002 (a PI3K inhibitor, 30 μmol/L; Cayman Chemicals), triciribine (an AKT inhibitor, 30 μmol/L; Cayman Chemicals), or rapamycin (an mTOR inhibitor, 25 μmol/L; Cell Signaling Technology) were treated for the indicated time points. Cells were transiently transfected with the siRNAs against AKT, HDAC2, FRAP1 (mTOR), NF-kBp50, or NF-kBp65 (RelA) or a negative control (Bioneer) using Lipofectamine2000 transfection reagent (Invitrogen), according to the manufacturer’s instructions.

Gene expression analysis

Expression profiling study related to HDAC2 was performed during our previous studies (7–9) using the Illumina HumanHT-12 V4 Expression BeadChip (Illumina Inc.). Gene expression data deposited in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database (accession number GSE31338 and GSE32070) were analyzed during the present study.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer’s instructions (Pierce Agarose ChIP Kit; Pierce). Approximately 1 × 10^7 cells were used for each immunoprecipitation. DNA was amplified by PCR using primers against two NF-kBp50 binding sites in the promoter of HDAC2 (binding positions: chr6 114292071–114292080 and chr6 114291629–114291638) and SYBR Green reaction mix (Bio-Rad Laboratories). Products were quantified using an IQ5 Real-Time PCR detection system (Bio-Rad Laboratories). Reactions were performed in triplicate, three independent experiments, and the means were normalized versus 10% chromatin input. Results are presented mean ± SEM.

Luciferase activity assay

Cells were grown to 50% confluence in 12-well plates, and then were transfected with siRNAs of NF-kBp50 and FRAP1 (mTOR). After 48 hours, serum-starved cells were transfected with 200 ng of pGL3-basic vector, which harbored HDAC2 promoter construct, and were stimulated with EGF for 24 hours. Firefly luciferase activity was measured with a luciferase assay kit (Promega).

Statistical analysis

All experiments were conducted at least three times, and all samples were analyzed in triplicate. Results are presented as mean ± SD. The statistical significance was calculated by unpaired Student t tests (GraphPad Prism 4.0).

Accession numbers

The microarray data reported herein have been deposited at the NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE14520, GSE16757, GSE25097, GSE31338, and GSE32070.

For additional details, refer to the Supplementary Materials and Methods.

Results

Aberrant HDAC2 expression is associated with the poor prognosis of patients with hepatocellular carcinoma and the upregulation of EGFR system–related signaling molecules

We recently reported that aberrant expression (highly expressed) of HDAC2 in hepatocellular carcinomas and its mitogenic functions are associated with the transcriptional regulations of cell-cycle regulators (7). To generalize our findings, we examined HDAC2 gene expression data in a large cohort of patients with hepatocellular carcinoma, available from the NCBI GEO database (accession numbers GSE14520, GSE16757, and GSE25097). Consistently, HDAC2 gene expression was significantly upregulated in three different hepatocellular carcinoma cohorts (Supplementary Fig. S1A). Because the mRNA and protein levels of HDAC2 were upregulated in overt hepatocellular carcinoma, we next assessed the prognostic implications of HDAC2 expression in a large cohort of
patients with hepatocellular carcinoma described in our previous study (10). First, 3,039 genes with expression patterns highly correlated with HDAC2 expression were selected for cluster analysis (P < 0.05, r > 0.4 or r < −0.4; Fig. 1A). Patients were then divided into HDAC2_high (n = 46) and HDAC2_low (n = 54) clusters. The Kaplan–Meier survival curves indicated that the 5-year overall survival (OS) and disease-free survival (DFS) rates of patients with hepatocellular carcinoma with high HDAC2 expression were significantly lower than those of patients with low HDAC2 expression (OS, P < 0.05; DFS, P < 0.05; Fig. 1B). Furthermore, the recurrence-free survival (RFS) rate of patients with hepatocellular carcinoma with high HDAC2 expression was also lower than that of patients with hepatocellular carcinoma with low HDAC2 expression. These results show that HDAC2 expression is strongly associated with prognosis in hepatocellular carcinoma and suggest that HDAC2 expression might be a valuable predictor of recurrence and survival.

To better understand the molecular role of HDAC2 in hepatocarcinogenesis, we next performed gene set enrichment analysis (GSEA) using an HDAC2-associated gene set (referred to as "the HDAC2 signature": 3,039 genes; Supplementary Table S1) as input. GSEA identified intracellular signaling pathways enriched in the HDAC2 signature of hepatocellular carcinoma, such as cell-cycle progression, proliferation, survival, migration, and metastasis (Supplementary Table S2). Of these pathways, a highly significant enrichment was detected toward known liver cancer–specific signatures (Supplementary Fig. S1B). We also noted that a part of the mitogen-activated protein kinase (MAPK) signaling gene set (MORF_MAP2K2) was enriched in the HDAC2 signature (Supplementary Fig. S1C). In addition, we performed GSEA using three different cancer cell lines (liver, lung, and gastric cancer) to confirm HDAC2 signatures in these cell lines (7, 9, 10). Indeed, GSEA showed that EGFR system–related gene elements were commonly enriched in HDAC2 signatures observed in these cell lines (Fig. 1C). These results indicate that HDAC2 functions as a tumor-promoting gene in association with EGFR-related signaling molecules in hepatocarcinogenesis.

**Activated mTORC1 is essential for growth factor–induced HDAC2 and its activation is sustained by HDAC2 in liver cancer**

The facts that EGFR and Ras signaling are activated in more than 50% of hepatocellular carcinomas and that our GSEA identified an EGFR-signaling gene subset in the HDAC2-associated molecular signature led us to hypothesize that EGF- or growth factor–related signaling molecules might be responsible for oncogenic HDAC2. Thus, we investigated whether the EGF treatment induces HDAC2 expression. Two hepatocellular carcinoma–derived and two immortalized normal hepatic cell lines were treated with EGF in serum-free medium for
different times and then immunoblotting was conducted on whole-cell lysates to examine EGF-signaling molecules. Treatment of the hepatocellular carcinoma cell lines (Hep3B and Huh7) with EGF (100 or 50 ng/mL, respectively) induced HDAC2 protein expression, but not in normal cell lines (MIHA and L-02; Fig. 2A and B). ERK seemed to be commonly activated in response to EGF stimulation in both hepatocellular carcinoma and hepatic cells. We also found that EGF treatment of hepatocellular carcinoma cells increased and sustained the phosphorylation levels of AKT and mTOR over the period we tested (24 hours). Although the significant phosphorylation of AKT and mTOR was also observed in L-02 or MIHA cells in the presence of EGF stimulation, those activities were immediately declined to levels similar to basal status within 24 hours. Furthermore, the phosphorylations of AKT and mTOR were sustained 24 hours after EGF treatment in association with the elevated level of HDAC2 expression in hepatocellular carcinoma cells. These results indicate that the activation of EGF signaling is sustained in hepatocellular carcinoma cells and that the activation of the activities of AKT and/or mTOR is necessary for the induction of HDAC2 in hepatocellular carcinoma cells. Therefore, we next investigated whether the activation of AKT and mTOR is required for EGF-stimulated HDAC2 expression in hepatocellular carcinoma cells. Treatment with LY294002 (a potent and specific inhibitor of PI3K/AKT signaling) dose dependently suppressed both EGF-stimulated AKT and mTOR phosphorylations and HDAC2 expression (Fig. 2C). Interestingly, treatment with triciribine (a specific inhibitor of AKT activation) neither abolished mTOR phosphorylation nor blocked the EGF-induced HDAC2 expression in Hep3B cells (Fig. 2D). In contrast, treatment with rapamycin, an mTOR inhibitor, attenuated EGF-induced HDAC2 expression in Hep3B cells (Fig. 2E). These results were then confirmed in SNU-449 cells (Fig. 2F). These findings demonstrate that EGF-stimulated HDAC2 expression in hepatocellular carcinoma cells is directly regulated by PI3K/mTOR signaling and indicate that EGF-induced activation of AKT and mTOR as well as their prolonged activities might explain the elevated levels of HDAC2 in hepatocarcinogenesis.

To further confirm the regulatory effect of mTOR on HDAC2 expression, endogenous mTOR gene (FRAP1) knockdown was attempted by RNA interference in hepatocellular carcinoma cells. As was expected, mTOR knockdown blocked EGF-induced HDAC2 expression in both SNU-449 and Hep3B cells (Fig. 3A). Accumulating evidence shows that the mTOR pathway is dysregulated in 40% to 50% of liver cancers (3, 11).
agreement with that, we observed by immunohistochemistry (IHC) that both HDAC2 and p-mTOR were highly expressed in hepatocellular carcinoma cells compared with adjacent non-cancerous hepatic cells, whereas mTOR expression did not show a significant difference (Supplementary Fig. S2). Consistently, Western blotting of hepatocellular carcinoma subset tissues revealed that p-mTOR and HDAC2 expressions were higher in hepatocellular carcinomas than noncancerous tissues and that their expression levels were positively correlated (Fig. 3B). Next, to generalize the nature of mTOR-mediated HDAC2 expression in response to growth factors, hepatocellular carcinoma cells were treated with IGF-I (50 ng/mL), IGF-II (50 ng/mL), or HGF (50 ng/mL) and signaling molecules were observed. As a result, the effects of three growth factors on the HDAC2-expression level were different in two hepatocellular carcinoma cell lines that we tested. However, growth factor–stimulated HDAC2 induction was observed only when mTOR activation occurred in hepatocellular carcinoma cells (Fig. 3C). These results demonstrate that mTOR activation is essential for the growth factor–induced HDAC2 in hepatocellular carcinoma cells.

mTOR is present in at least two unique protein complexes, including mTOR complex 1 (mTORC1) and mTORC2 (12). These complexes are differentially regulated by distinct upstream signals and their activations and functional engagements result in the control of different downstream pathways. Therefore, to identify the mTOR complex responsible for growth factor–induced HDAC2 in hepatocellular carcinoma cells, we used a selective knockdown approach. The knockdown of the regulatory-associated protein mTOR (RAPTOR; a unique component of TORC1) markedly prevented the EGF- and FBS-induced phosphorylation of mTOR and HDAC2 expression, whereas the knockdown of rapamycin-insensitive companion of mTOR (RICTOR; a unique component of TORC2) did not affect mTOR activation or HDAC2 expression in hepatocellular carcinoma cells (Fig. 3D). Interestingly, we found that knockdown of RAPTOR increased the phospho-AKT level significantly. This result suggests that a mutual regulating mechanism exists between mTOR and AKT in hepatocellular carcinoma cells. In addition, immunoprecipitation analysis demonstrated that EGF stimulation activated mTOR by forming the mTOR/RAF/PI3K complex (mTORC1) in both Hep3B and Huh7 cells (Fig. 3E). Furthermore, immunoprecipitation analysis of HDAC2-depleted (Hep3B_shHDAC2) cells revealed that HDAC2 inactivation caused RAPTOR/mTOR dissociation, indicating that HDAC2 plays a pivotal role in mTORC1 stabilization in response to EGF stimulation in hepatocellular carcinoma cells (Fig. 3F).

Activated mTOR directly regulates HDAC2 expression by modulating NF-κBp50 transcriptional activity in hepatocellular carcinoma cells

Recently, it was demonstrated that unsaturated fatty acids trigger steatosis by downregulating the PTEN expression in
hepatocytes via the activation of an mTOR/NF-κB complex (13). This led us to hypothesize that growth factor–stimulated HDAC2 expression could be regulated by mTOR/NF-κB signaling in hepatocellular carcinoma cells. Thus, we assessed the subcellular localizations of NF-κBp50 and NF-κBp65 in the presence of EGF stimulation. As was expected, EGF treatment enhanced the nuclear translocation of NF-κBp50 and NF-κBp65 in Hep3B and SNU-449 cells (Fig. 4A). It is well established that NF-κB moves rapidly to the nuclear compartment when stimulated by growth factors. Therefore, we then performed a short-term confocal imaging analysis for NF-κBp50, NF-κBp65, and HDAC2 in liver cancer cells in the presence of EGF stimulation. Both NF-κB subunits (NF-κBp50 and NF-κBp65) were mainly localized in cytoplasm in the absence of growth factor stimulation, whereas the treatment with EGF caused nuclear translocation of NF-κBp50 and NF-κBp65 in Hep3B cells within 1 hour of stimulation. This result reflected the observation that HDAC2 expression was gradually induced from the time point 0.5 hour of EGF stimulation in both Hep3B and Huh7 liver cancer cells (Fig. 2A). Next, to identify the functional subunit of NF-κB that leads to the induction of HDAC2 expression, specific siRNA for each subunit of NF-κB was introduced into Hep3B and SNU-449 cells in the presence of EGF stimulation. Notably, we found that only NF-κBp50 knockdown was able to block EGF-induced HDAC2 expression in these hepatocellular carcinoma cells (Fig. 4C). Furthermore, to clarify whether EGF stimulation induces HDAC2 expression via mTOR-dependent NF-κBp50 nuclear translocation, we examined phospho-mTOR and NF-κBp50 levels in the presence or absence of rapamycin in hepatocellular carcinoma cells. As expected, EGF stimulation enhanced phospho-mTOR and the nuclear localizations of NF-κBp50, whereas rapamycin prevented EGF-stimulated NF-κBp50 activation in both Hep3B and SNU-449 cells (Fig. 4D).

Next, we considered that NF-κBp50 might elicit the transcriptional activation of HDAC2. As illustrated in Fig. 5A, two NF-κBp50 binding sites (GGGAGCCCAT and GGGTGCCCGG) are located in the HDAC2 gene near its transcription start site (TSS). To determine whether NF-κBp50 interacts with these two HDAC2 loci and functions as a transcription factor for HDAC2, we performed ChIP and quantitative real-time PCR (qRT-PCR) in hepatocellular carcinoma cells. The ChIP–qRT-PCR analysis demonstrated that EGF stimulation significantly provoked NF-κBp50 binding to the two HDAC2 loci, 0.2 kb (+221) and 0.7 kb (+663) 3’ of the HDAC2 gene TSS, whereas LY294002 or rapamycin treatment blocked the NF-κBp50 binding to these loci in hepatocellular carcinoma cells (Fig. 5B). We then investigated whether mTOR activation directly regulates HDAC2 expression through the NF-κB transcriptional activity in liver cancer cells. To this end, we constructed a luciferase reporter plasmid that harbors the HDAC2–promoter region and transfected it into Hep3B cells in the presence or absence of EGF. The luciferase activity was significantly upregulated in the presence of EGF, while the treatment of rapamycin attenuated EGF-induced luciferase activity in Hep3B cells. We then performed qRT-PCR analysis to validate the regulatory role of mTOR in HDAC2 transcription. Similarly, the expression of HDAC2 mRNA was significantly induced by the treatment of EGF and this induction was blocked by rapamycin treatment (Fig. 5C). In addition, to investigate the
regulatory effect of mTOR activation or NF-κBp50 binding on HDAC2 promoter, the experiment was carried out in NF-κBp50- or mTOR-depleted Hep3B cells. As shown in Fig. 5D, the targeted disruption of NF-κBp50 (siNF-κBp50) or mTOR (siFRAP1) elicited the suppression of EGF-induced luciferase activity in Hep3B cells. A similar result was obtained from the same set of experiments for HDAC2 mRNA assessment by using qRT-PCR. These results suggest that activated mTOR directly regulates HDAC2 expression by modulating NF-κBp50 transcriptional activity in hepatocellular carcinoma cells.

To generalize our hypothesis, we prepared diethylnitrosamine (DEN)–induced liver cancer mouse and rat models and examined mTOR/NF-κBp50 activation and HDAC2 expression in hepatocellular carcinoma tissues. Both DEN-induced mouse...
and rat hepatocellular carcinoma tissues exhibited highly activated mTOR/NF-κBp50 levels and HDAC2 expression compared with corresponding normal hepatic tissues (Fig. 5E and F). However, no significant difference in phospho-AKT levels was observed between hepatocellular carcinoma and normal hepatic tissues in these two models. These results demonstrate that the NF-κBp50 activation and mTOR are essential for the activation of oncogenic HDAC2 in hepatocarcinogenesis.

Elevated HDAC2 expression triggers the stabilization of mTOR and the activation of AKT in liver cancer

We found that HDAC2 contributed to the association between RATOR and mTOR to form mTORC1 in hepatocellular carcinoma (Fig. 3F). To clarify the role of HDAC2 in mTOR activation in vivo, we examined phospho-mTOR levels in xenograft tissues from mice injected with HDAC2-depleted (Hep3B_shHDAC2) or control (Hep3B_shScr) cells. As expected, phospho-mTOR levels were markedly lower in Hep3B_shHDAC2 xenograft tissues than in Hep3B_shScr xenograft tissues. In addition, we also noted that phospho-AKT levels were remarkably decreased in Hep3B_shHDAC2 xenograft tissues (Fig. 6A). Similarly, another HDAC2-depleted cell line (Huh7_shHDAC2) confirmed that sustained suppression of HDAC2 results in reduced AKT and mTOR phosphorylation (Fig. 6B). These results indicate that HDAC2 is required to sustain the active statuses of mTOR and AKT in hepatocellular carcinoma cells. This is further supported by the observation that rapamycin abrogated EGF-stimulated mTOR activation and HDAC2 expression. Furthermore, rapamycin simultaneously enhanced AKT phosphorylation in Hep3B cells. This negative regulatory function of mTOR toward AKT phosphorylation was more clearly seen in HDAC2-depleted Hep3B cells (Fig. 6C).

We then hypothesized that by positive feedback, HDAC2 regulates AKT activation in liver cancer. To explore this possibility, HDAC2 was knocked down in four different liver cancer cell lines, and immunoblotting was performed with anti–phospho-AKT antibody (Supplementary Fig. S3A). As expected, p-AKT levels were suppressed by HDAC2 knockdown in all tested hepatocellular carcinoma cell lines (Hep3B, PLC/PRF/5, SNU-182, and SNU-449). Conversely, the ectopic expression of HDAC2 increased phospho-AKT levels in same cell lines (Supplementary Fig. S3B). Notably, the ectopic expression of HDAC2 sustained the elevated phospho-AKT levels even in AKT knockdown cells (Fig. 6D). We then investigated the mRNA expression patterns of several genes encoding a specific kinase or phosphatase in the phosphoinositide signaling pathway (these genes were identified during our previous microarray analysis of human hepatocellular carcinoma tissues (10). We found that the expressions of kinase-coding genes (PIK3CB, PIK3R2, PIP5K2C, and PIP5K2C) were upregulated, whereas the expressions of phosphatase-encoding genes (PTEN, INPP5B, and INPP5F) were downregulated in overt hepatocellular carcinoma (Supplementary Fig. S4). To determine whether HDAC2 regulates phosphoinositide signaling molecules and thereby contributes to the activation of AKT, we compared their mRNA levels in Hep3B and in HDAC2-
depleted Hep3B cells. As expected, HDAC2 knockdown significantly induced the mRNA expressions of INPP5E and INPP5F, and simultaneously inhibited the expressions of PI3KCB and PIP5K2C (Fig. 6E). From another set of qRT-PCR after the ectopic overexpression of HDAC2, we also found the direction of steady-state level change was reversed without exception in all the genes we tested in the knockdown experiment (Fig 6F). These results demonstrate that HDAC2 plays a pivotal role in the positive feedback activation of AKT via regulation of phosphoinositide signaling components at the transcription level (Supplementary Fig. S5).

**HDAC2 enhances the metastatic potential of hepatocellular carcinoma cells**

The global molecular profiling of HDAC2-knockdown cells has shown that the HDAC2 signature includes gene elements involved in cell proliferation–related pathways such as cell cycle and apoptosis (7). To gain further insights into the biologic roles of HDAC2, we integrated a comprehensive collection of cancer-related gene expression signatures and analyzed the HDAC2 signature using the Molecular Concept Map (14). This analysis allowed us to navigate a network of associations involving HDAC2-related gene elements and to identify an enrichment network linking the HDAC2 signature with cellular signaling, migration, inflammation, and gene expression regulation programs (Fig. 7A). We also noted that the signature of HDAC2 is highly linked to cell migration or cell migration–related signatures. Thus, to elucidate the role of HDAC2 in the malignant behavior of liver cancer cells, we performed in vitro motility assays. A modified Boyden chamber assay revealed that HDAC2 knockdown significantly suppressed the EGF-stimulated migratory responses of Huh7 cells (Fig. 7B). Similarly, a scratch wound healing assay also showed that HDAC2 knockdown reduced EGF-stimulated wound-healing efficacy of various hepatocellular carcinoma cells (Fig. 7C; Supplementary Fig. S6). Notably, Snail expression, a hallmark of epithelial–mesenchymal transition (EMT), was dramatically increased in response of EGF stimulation, whereas E-cadherin...
expression was diminished in Huh7 cells (Fig. 7D). Further experiments demonstrated that HDAC2 regulated EGF- or serum-stimulated Snail expression in liver cancer cells (Fig. 7E). These results suggest that the metastatic potential of HDAC2 could be attributed to the induction of EMT in hepatocellular carcinoma cells. Furthermore, gene expression analysis of large cohort of hepatocellular carcinoma patient from the NCBI GEO database (accession number GSE20140) consistently showed that HDAC2 was significantly upregulated in patients with hepatocellular carcinoma with vascular invasion (Fig. 7F; left). In agreement with this result, another NCBI GEO dataset (accession number GSE26391) for two newly established hepatocellular carcinoma cell lines (HCC1.2 with epithelial properties and HCC1.1 with mesenchymal properties) showed that HDAC2 expression was significantly upregulated in the hepatocellular carcinoma cell line exhibiting a mesenchymal phenotype (Fig. 7F; right).

Discussion

Histone acetyltransferases and HDACs are two opposing classes of enzymes, which finely regulate histone acetylation and, thus, affect chromatin packaging and gene expression. Imbalanced acetylation has been associated with carcinogenesis and cancer. Recently, we demonstrated that HDAC2 overexpression is implicated in hepatocellular carcinoma cell proliferation, while the loss of HDAC2 negatively affects the proliferation of tumor cells leading to p21WAF1/CIP1 upregulation in liver cancer (7). Our data show that growth factor stimulation followed by mTOR activation causes HDAC2 expression in hepatocellular carcinoma cells and further in vitro and in vivo experiments provided evidence indicating that growth factor–stimulated mTORC1 and NF-κBp50 are key regulators for the activation of oncogenic HDAC2 in liver cancer. In addition, we observed that phospho-mTOR induced by EGF stimulation remained activated in liver cancer cell lines, whereas the induction of phospho-mTOR by EGF stimulation gradually diminished in normal hepatic cell lines (Fig. 2). This observation explains how growth factor–stimulated mTOR signaling provokes the elevated expression of HDAC2 in hepatocellular carcinoma cells and suggests the presence of a positive feedback mechanism to sustain active mTOR signaling in hepatocellular carcinoma cells. Indeed, endogenous HDAC2 in hepatocellular carcinoma cells was found to be required for the association between RAPTOR and mTOR, which leads to the activation of mTORC1 in response to EGF stimulation (Fig 3F).

Negative regulatory function of mTOR toward AKT activation is not a well-known mechanism in cancer cells. However, Guertin and Sabatini have demonstrated that mTORC1 activation leads to the suppression of PI3K-AKT signaling upstream in the PI3K pathway in many types of cells (15). In addition, Chen and colleagues have shown that the negative regulatory function of mTOR could be dependent on the cellular context or the dose of rapamycin. According to their result, a low dose of rapamycin induced AKT phosphorylation not in MCF-7 cells but in DU-145 cells. Moreover, in DU-145 cells, a high dose of rapamycin did not activate AKT (16). From our result, the abrogation of mTOR (more specifically TORC1) activates AKT signaling, but HDAC2 level was still downregulated by mTOR inhibition (Fig. 6C). However, negative regulatory function of mTOR was neither observed in rapamycin-treated Huh7 cells (Fig. 2F) nor in the DEN-induced mouse and rat hepatocellular carcinoma model (Fig. 5E and F). These data show that there could be cell-specific nature of negative regulation of AKT phosphorylation by mTOR.

A high chemoresistance is one of the main obstacles to curative treatment in hepatocellular carcinoma. For example, clinical trials on sorafenib (a tyrosine kinase inhibitor), which showed survival benefit in advanced hepatocellular carcinoma, also found that drug-induced EGFR activation diminished cell response to sorafenib (17). This finding indicates that molecular regulation by a specific signaling pathway is a key determinant of drug sensitivity in chemoresistant tumors. Furthermore, it has recently been suggested that HDAC2 is involved in the EGF-induced phosphorylations of ERK and AKT by regulating EGFR expression in colorectal cancer (18). It has been reported that selective depletion of HDAC2 enhances hormonal drug sensitivity via the downregulation of estrogen receptor (ER) and progesterone receptor (PR) in ER/PR–positive breast cancer cells (19). Although it is unclear whether HDAC2 directly regulates EGFR in hepatocellular carcinoma cells, our study revealed that targeted inhibition of HDAC2 attenuated AKT activation in hepatocellular carcinoma cells. We also demonstrate that elevated HDAC2 expression mediates the transcriptions of entities associated with phosphoinositide signaling molecules and thereby activates AKT phosphorylation in hepatocellular carcinoma cells. According to our results, rapamycin potentiated AKT phosphorylation of EGF-stimulated hepatocellular carcinoma cells (Fig. 5C). Siriolimus (also known as rapamycin) has potent antiproliferative activity against hepatocellular carcinoma cell lines in vitro, but in vivo evidence is currently lacking. This lack of evidence may be due to the weaker effect of Sirolimus, where inhibition of the mTORC1 activates AKT to a certain extent. Therefore, dual mTOR/HDAC2 inhibition, which theoretically could more potently suppress the mTOR pathway due to additional inhibition of AKT upstream of mTOR, should be considered for the treatment of hepatocellular carcinoma. In the present study, HDAC2 knockdown was found to act synergistically with sorafenib to suppress hepatocellular carcinoma cell proliferation (Supplementary Fig. S7).

Taken together, this study shows that growth factor–stimulated mTORC1 causes nuclear translocation of NF-κBp50 and transcriptional activation of oncogenic HDAC2. Elevated HDAC2 expression plays a critical role in sustaining active mitogenic signaling in hepatocellular carcinoma cells by stabilizing mTORC1 and by positive feedback maintaining the activation of AKT (Supplementary Fig. S8). Although further research is required to identify additional regulatory mechanisms involving oncogenic HDAC2 in liver cancer, our findings indicate that the mTORC1/NF-κBp50/HDAC2 axis plays a central role in malignant progression and transformation in liver cancer. In addition, we propose a regulatory feedback loop whereby mTORC1/NF-κBp50–mediated HDAC2 positively
regulates mTORC1 and AKT during hepatocarcinogenesis, which makes HDAC2 a promising therapeutic target.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: J.H. Noh, J.Y. Lee, S.W. Nam

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.H. Noh, K. Lee

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.H. Noh, H.J. Bae, J.W. Eun

Writing, review, and/or revision of the manuscript: J.H. Noh, H.J. Bae, S.W. Nam

**References**


HDAC2 Provides a Critical Support to Malignant Progression of Hepatocellular Carcinoma through Feedback Control of mTORC1 and AKT

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