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Clinical Cancer Research

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Cancer Therapy: Preclinical

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HDAC Inhibitors Augmented Cell Migration and Metastasis through Induction of PKCs Leading to Identification of Low Toxicity Modalities for Combination Cancer Therapy

Kuen-Tyng Lin[1], Yi-Wei Wang[1], Chiung-Tong Chen[3], Chun-Ming Ho[1,2,4], Wen-Hui Su[5], and Yuh-Shan Jou[1,2]

Abstract

Purpose: Histone deacetylase inhibitors (HDACi) are actively explored as new-generation epigenetic drugs but have low efficacy in cancer monotherapy. To reveal new mechanism for combination therapy, we show that HDACi induce cell death but simultaneously activate tumor-progressive genes to ruin therapeutic efficacy. Combined treatments to target tumorigenesis and HDACi-activated metastasis with low toxic modalities could develop new strategies for long-term cancer therapy.

Experimental Design: Because metastasis is the major cause of cancer mortality, we measured cell migration activity and profiled metastasis-related gene expressions in HDACi-treated cancer cells. We developed low toxic combination modalities targeting tumorigenesis and HDACi-activated metastasis for preclinical therapies in mice.

Results: We showed that cell migration activity was dramatically and dose dependently enhanced by various classes of HDACi treatments in 13 of 30 examined human breast, gastric, liver, and lung cancer cell lines. Tumor metastasis was also enhanced in HDACi-treated mice. HDACi treatments activated multiple PKCs and downstream substrates along with upregulated proapoptotic p21. For targeting tumorigenesis and metastasis with immediate clinical impact, we showed that new modalities of HDACi combined drugs with PKC inhibitory agent, curcumin or tamoxifen, not only suppressed HDACi-activated tumor progressive proteins and cell migration *in vitro* but also inhibited tumor growth and metastasis *in vivo*.

Conclusion: Treatments of different structural classes of HDACi simultaneously induced cell death and promoted cell migration and metastasis in multiple cancer cell types. Suppression of HDACi-induced PKCs leads to development of low toxic and long-term therapeutic strategies to potentially treat cancer as a chronic disease. *Clin Cancer Res; 18(17); 4691–701. ©2012 AACR.*

Introduction

Cancer is the leading cause of human mortality that resulted in 7.4 million (13% of all) deaths worldwide in 2004 and is projected to cause 12 million deaths in 2030 (1). During tumor progression, sequential accumulation of somatic alterations including genetic lesions and epigenetic aberrations has been attributed as the etiologic factors of tumorigenesis and metastasis (2). As a result, anticancer drugs are developed to target these altered cancer-progressive genes and pathways to kill or suppress cancer cells for therapy (3). Among epigenetic modulating agents, histone deacetylase inhibitors (HDACi) are attractive anticancer drugs due to frequent upregulation of HDACs in cancers and low toxicity to patients. HDACi commonly target multiple HDACs to cause accumulation of acetylated forms of histones and non-histone proteins, resulting in chromatin remodeling and altered transcription factor complexes. HDACi-induced expression of proapoptotic p21 and other program cell death proteins lead to an empirical conclusion that HDACi preferentially kill cancer cell through different mechanisms including cancer cell apoptosis, cell-cycle arrest, and antiangiogenesis (4, 5).

On the basis of diverse chemical structures identified from either chemical synthesis or natural products, HDACi are generally subdivided into 4 groups with a wide range of therapeutic potency (6). Because HDACi are well tolerated by patients with manageable side effects, numerous HDACi are under evaluation in preclinical or clinical trials of cancers (7, 8). In those HDACi, suberoylanilide hydroxamic acid (SAHA; Vorinostat) has been firstly approved by U.S.

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Translational Relevance

HDAC inhibitors (HDACi) are known to kill cancer cells through activation of tumor-suppressive genes and apoptotic pathways. However, low therapeutic efficacy and even progressive outcomes are frequently reported in cancer therapies. We analyzed a common but ignorant mechanism that treatment of HDACi could induce cell death but simultaneously activate tumor-progressive genes. We found that HDACi enhanced cell migration through induction of multiple PKCs and downstream pathways in 13 out of 30 (43%) tested cancer cell lines of liver, lung stomach, and breast to disrupt therapeutic effect in cell and mouse models. To improve the HDACi therapy against tumorigenesis and deadly metastasis, we suppressed the HDACi-enhanced cell migration and metastasis by combined treatments with PKC inhibitors; either dietary supplement curcumin or U.S. Food and Drug Administration–approved tamoxifen leaded to identification of new mechanism-based low toxic modalities for potential long-term treatment of solid tumors.

Food and Drug Administration (FDA) for treatment against a rare cancer cutaneous T-cell lymphoma. However, low therapeutic efficacy and progressive outcomes are frequently reported in patients with solid tumor who received HDACi treatments in clinical trials (9, 10). Recent emerging HDACi combination therapies with agents such as conventional cytotoxic drugs or inhibitors of molecular targets have been tested in preclinical models and clinical trials but with limited success and/or unaffordable costs (11–13).

The insufficiency or even progressive disease of patients with cancer who received HDACi treatment could be due to the global and simultaneous activation of proapoptotic and tumor-progressive genes in cancer cells. The HDACi-activated tumor-progressive genes might eventually diminish the therapeutic efficacy and make unfavorable outcomes. Because more than 90% cancer-related mortality is due to dissemination of primary cancer cells from the original tumor followed by metastasis and invasion to distant tissues (14), we therefore proposed to simultaneously target tumorigenesis and metastasis to prolong life of patients with patients.

After RNA-Seq transcriptome analysis with next-generation sequencing (NGS) technology, we profiled and validated several metastasis-related gene families in HDACi-treated cells with reverse transcriptase PCR (RT-PCR) technologies. We revealed that HDACi treatments induced not only the treatment control proapoptotic p21 (15) but also multiple PKCs and downstream-regulated matrix metalloproteinases (MMP). The serine/threonine PKC family consists of at least 10 isoforms, which are the first identified intracellular receptors to tumor-promoting agent phorbol esters and are aberrantly expressed in tumor progression and metastasis (16, 17). On the basis of structural and biochemical properties, PKCs were subclassified into 3 subfamilies: calcium-sensitive classic PKCs (cPKCs: PKC-α, PKC-βI, PKC-βII, and PKC-γ), calcium-independent novel PKCs (nPKCs: PKC-δ, PKC-ε, PKC-η, and PKC-θ), and atypical PKCs (aPKCs: PKC-ζ and PKC-ι). The activation of PKCs depends on ligand-stimulated tyrosine kinase receptors or G-protein–coupled receptors to activate phospholipase C (PLC), to generate lipid second messenger diacylglycerol (DAG) for PKC interaction, and to translocate the PKC–DAG complex to the membrane to trigger cellular signaling. Although different PKC isoforms expressed in different tumorigenic stages and in different tumors, simultaneous activation of multiple PKCs plays central and pivotal roles in various tumors to modulate subsequent downstream signaling pathways including PI3K/Akt, MEK/ERK, and IKK/NFκB for participation in cancer cell proliferation, cell migration, angiogenesis, and metastasis (18, 19).

On the basis of our observations that HDACi might activate tumor-progressive genes to interfere cancer therapies in multiple cancer cell types and the availability of low toxic inhibitors of PKCs (PKCi) under clinical trials for cancer therapies (16, 17), further suppression of HDACi-induced tumor-progressive genes with low toxic PKCi could allow us to develop new rational HDACi combination modalities for long-term treatment of cancers.

Materials and Methods

Cell viability assay

The culture conditions and authentication of cell lines were according to the previous report (20). Human liver (SNU-398, Huh6, HepG2, Hep3B, Huh7, PLC5, HCC36, TONG, HA59T, Sk-hep-1, HA22T, and Malhavu), lung (H358, H1437, H661, H226Br, H1299, CL1-3, CL1-0, H23, H928, and A549), gastric (NUGC, SC-M1, AZ521, AGS, and HR), and breast (MDA-231, Hs578T, and MCF-7) cancer cell lines were seeded into 6-cm dishes (1×10^5 cells per dish) and cultured for 24 hours. Fresh media contained various treatments (HDACi for 48 hours; MMPi or PKCi for 24 hours) were added into dishes in triplicate. After 48 hours treatments, cell viability was analyzed by alamar blue assay (BioSource International). In combination treatments, cells were treated with HDACi for 24 hours and then replaced with fresh media containing HDACi with MMPi or PKCi for another 24 hours.

Transwell migration assay

After HDACi treatment in 10-cm dishes for 24 hours, adherent cells were collected and resuspended into the HDACi-contained serum-free media. The number of viable cells was calculated by trypan blue dye exclusion method (Sigma-Aldrich). Cells (3×10^4 to 1×10^5) were seeded into migration assay (21). In combination treatments, various HDACi were combined with MMPi (doxcycline, Sigma-Aldrich) or PKCi [bisindolylmaleimide I, Santa Cruz; curcumin and tamoxifen, Sigma-Aldrich] in serum-free and 10% FBS-contained media. Each treatment was done in triplicate. After 24 hours of migration assay, cells on the

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insert membrane were stained with Giemsa's solution (Merck) and nonmigrated cells were removed with cotton swabs. Total migrated cells were counted in 100× microscopic fields (inverted microscope, Nikon).

Combined therapies with *in vivo* assays of tumorigenesis and metastasis in nude mice

For the combined therapy by measuring s.c. tumor xenograft formation, the detail protocol was described in Supplementary Materials and Methods. For the combined therapy of detecting tumor metastasis, we established an orthotopic spontaneous metastasis model by intrasplenic injection of cancer cells for liver metastasis (22). Hep3B-RFP cells (2×10^6) were injected into spleen of mouse. After 24 hours surgical recovery, mice were randomized into 4 groups in a blinded manner: vehicle control ($n = 10$), trichostatin A (TSA)-treated ($n = 11$), TSA/tamoxifen ($n = 12$)-treated, and TSA/curcumin ($n = 11$)-treated mice. The delivery dosages of TSA, tamoxifen, and curcumin were the same as that in s.c. tumor xenograft formation assays. After treatment for 2 months, all mice were euthanized. The red fluorescent cells on spleen and liver organs were revealed by fluorescence stereomicroscope and the tumor area was measured by Image-Pro Plus 5.0 software (Media Cybernetics).

Statistical analysis

The *in vitro* results were conducted in triplicate and shown as mean ± 95% confidence interval (CI) with statistical tests done by Student *t* test. The results from mouse models were shown as mean ± 95% CI and statistical tests were done by Students *t* test or Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

HDACi treatments enhanced cancer cell migration *in vitro* and metastasis *in vivo*

To test the mechanism that human cancer cells treated with HDACi not only caused cell death but also activated ignored tumor-progressive genes, a total of 30 human cancer cell lines including 12 liver, 10 lung, 5 gastric, and 3 breast cancer cell lines were treated with various doses of TSA and examined their cell migration activity. To our surprise, TSA treatments significantly enhance cell migration activity by up to several hundred folds with dose-dependent manners in 13 (Hep3B, PLC5, Huh7, and SNU-398 for liver cancer; H928, H226Br, H661, H23, CL1-0, and CL1-3 for lung cancer; NUGC and SC-M1 for gastric cancer; MCF-7 for breast cancer) of 30 (43%) human cancer cell lines (Fig. 1 and Supplementary Fig. S1). To investigate whether HDACi from different structural classes also promote cell migration, we examined cell migration activity using 6 HDACi including valporic acid, sodium butyrate, apicidin, MS-275, SAHA, and TSA on 6 aforementioned (Hep3B, Huh7, and SNU-398 for liver cancer; H23, CL1-0, and H928 for lung cancer) cell lines. Our results clearly showed that treatments of HDACi enhanced cancer cell migration and hastened their motility in dose-dependent manners for hundreds to a thousand-fold increase (Supplementary Fig. S2). Noticeably, the enhancement of cell migration is initiated at low and noncytotoxic dosages of HDACi (e.g., 1 μmol/L MS-275 or 0.5 mmol/L valporic acid treatment in Huh7 cells; 0.5 μmol/L SAHA treatment in H23 cells and others marked with arrowheads in Supplementary Fig. S2). Our results suggested that HDACi-enhanced cell migration is more likely resulted from the epigenetic activation of tumor-progressive genes in HDACi-treated cells rather than the selection of survived cells by HDACi toxicity.

Furthermore, to investigate whether treatments of HDACi also contributed to metastasis in mice model *in vivo*, we injected RFP-labeled Hep3B (Hep3B-RFP), H928-RFP, and GFP-labeled H23 (H23-GFP) cells into tail vein of mice for systemic metastasis. We found that tumor metastasis was observed in a total of 5 metastatic events in HDACi-treated mice with statistical significance ($P = 0.026$, Table 1 and Supplementary Fig. S3). Together, we concluded that different classes of HDACi including clinically approved SAHA can significantly enhance cell migration in subsets of liver, lung, breast, and gastric cancer cells *in vitro* or metastasis *in vivo*.

HDACi upregulated expressions of genes of *MMP* and *PKC* families

To target tumorigenesis and metastasis in the combined treatment, we conducted RNA-Seq transcriptome analysis by NGS technology between SAHA-treated and untreated H23 cells. We found that multiple PKCs and downstream MMPs were highly induced in SAHA-treated H23 cells (Fig. 2A). The consistency of HDACi-enhanced cell migration and induced *PKC* and *MMP* gene expressions were confirmed in TSA-treated Hep3B cells with concomitant upregulated p21 protein and decrease of cell viability (Fig. 2B and C and Supplementary Fig. S4A and S4B). Our results showed that multiple members of MMPs (1, 2, 9, 10, 12, and 13) and PKCs (βI, βII, γ, and θ) were simultaneously induced in TSA-treated Hep3B cell at both RNA and protein levels in a dose-dependent manner (Fig. 2B and C and Supplementary Fig. S4B). Interestingly, the PKC-δ, previously reported to confer proapoptotic and antiproliferative effects in several cancer cell types (23), is downregulated in TSA-treated Hep3B cells (Supplementary Fig. S4B). By using chromatin immunoprecipitation (ChIP) assays, we confirmed the acetylation status of histones H3 and H4 from promoter regions of *MMP* and *PKC* genes in association with TSA-regulated gene expression. TSA-upregulated *MMP* and *PKC* genes were associated with hyperacetylation of histone H3 and H4 (Fig. 2D); TSA-downregulated *MMP* and *PKC* genes were associated with hypoacetylation of histone H3 and H4 (Supplementary Fig. S4C). In contrast, the expression of PKCs and MMPs were downregulated with simultaneous detection of upregulated p21 protein in TSA-repressed cell migration of Malhavu and HCC36 cells (Supplementary Fig. S1A and S4D). Our results further elucidated the critical roles of

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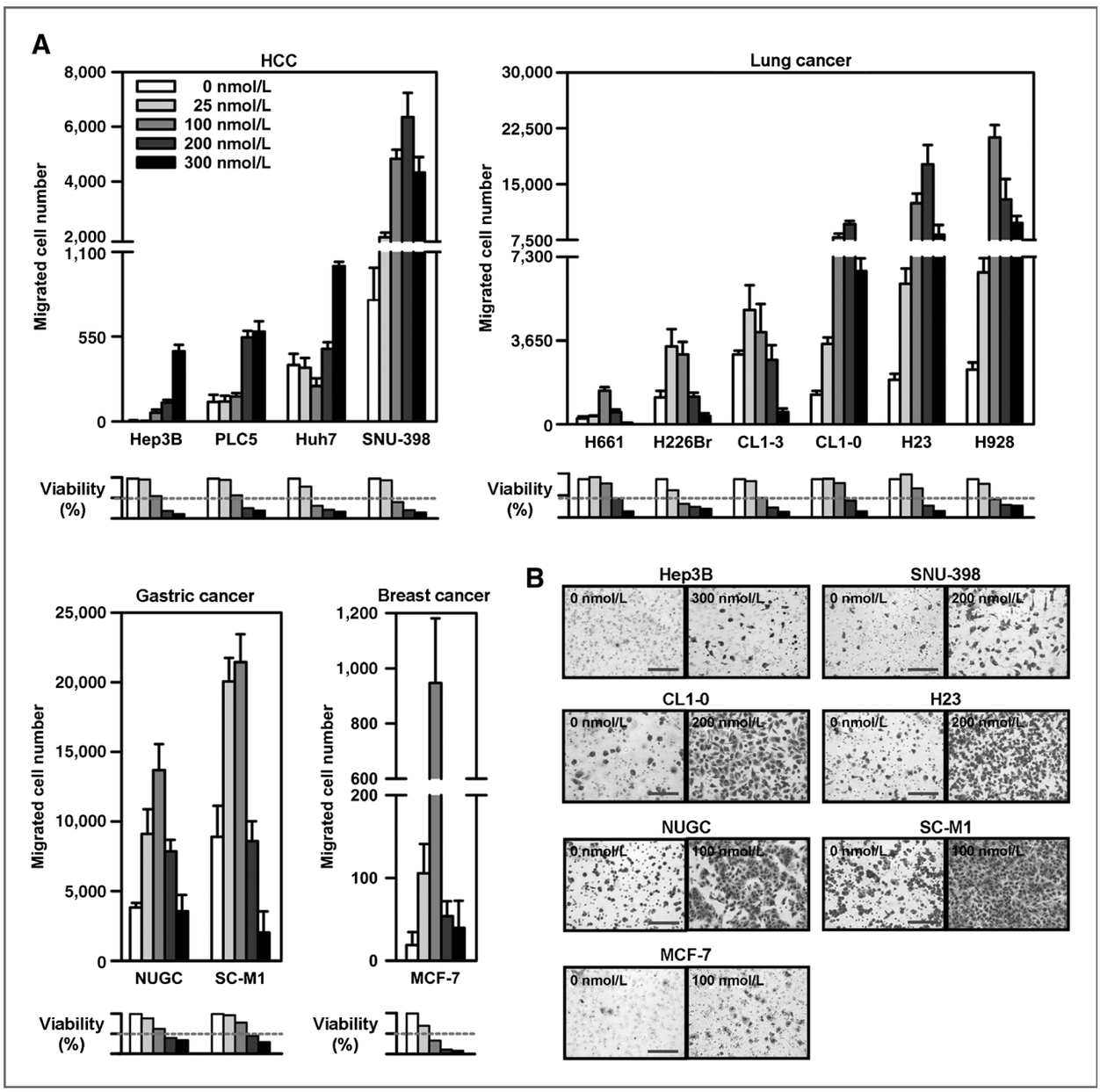

Figure 1. HDACi treatments enhanced cell migration activity of liver, lung, gastric, and breast cancer cells. A, TSA-enhanced cell migration in Transwell migration assay in dose-dependent manners ($n = 3$, mean ± 95% CI) with indication of 50% cell viability in red dash lines ($n = 3$, mean ± 95% CI). B, the representative images of migrated cells with and without TSA treatments (scale bar, 200 μm).

these PKCs and MMPs in regulating TSA-modulated cell migration function.

To validate the role of PKCs and downstream MMPs participated in HDACi-induced cell migration is a common mechanism in subset of cancer cell lines; 4 different cell lines (Huh7 and SNU-398 of liver and H23 and CL1-0 of lung cancers) were treated with various HDACi doses with induction of highest migration activity to profile the HDACi-induced expression of PKCs and MMPs. Our results indicated that the enhancement of cell migration could be attributed to the upregulation of multiple but different members of classic and novel PKCs (cPKCs: PKC-α, PKC-βII and PKC-γ and nPKCs: PKC-ε, PKC-η, and PKC-θ) and their downstream MMPs (Supplementary Fig. S4E). Consistently, some of these PKCs and MMPs proteins were activated at nontoxic TSA dose (25 nmol/L) with 98% of cell viability in Hep3B cells (Fig. 2B and C and Supplementary Fig. S2A and S2B marked by arrowheads). In summary, different classes of HDACi epigenetically activated p21 and multiple members of metastasis-related cPKCs, nPKCs, and downstream MMPs to dramatically enhance cancer cell migration.

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Table 1. HDACi treatments and cancer metastasis in mice

Mice	No metastasis	Metastasis
None treated	42	0
HDACi treated	36	5[a]

[a]$P = 0.026$.

Combination treatments suppressed HDACi-enhanced cell migration and synergized killing of cancer cells

To exploit potential inhibitors targeting on HDACi-activated metastasis-related genes for combination therapies, we tested specific inhibitors either siRNAs, small chemicals, or antibodies against upregulated genes individually. Although inhibitory effects were detected to various levels, none of these single gene inhibitors combined with HDACi reached high efficiency to suppress augmented cell migration activity (data not shown). Because multiple members of PKCs and downstream MMPs were induced by HDACi (Fig. 2 and Supplementary Fig. S4E), we selected broad-spectrum inhibitors to target multiple members of MMPs or PKCs with MMP inhibitor (MMPi): (doxycycline, Dox) or PKC inhibitor (PKCi): (bisindolylmaleimide I, Bis), respectively, for improvement of inhibitory efficiency (24, 25). Our results indicated that broad spectrum but specific inhibitors Dox and Bis significantly suppressed TSA- and valporic acid-enhanced migration activity of Hep3B and Huh7 cells, respectively, compared with monotreatment ($P < 0.01$; Supplementary Fig. S5A).

Because PKCs are known to modulate expression of MMPs in metastasis-related signaling pathway (26) and since PKCi Bis was a more effective inhibitor than MMPi Dox to suppress HDACi-enhanced migration activity in our data ($P < 0.01$; Supplementary Fig. S5A), we therefore selected 2 drugs, curcumin (a dietary supplement) and tamoxifen (an FDA-approved drug), with PKCs inhibitory activity and low toxicity for potential cost-effective and long-term combination therapies (17). As predicted, when compared with monotreatment, our results showed that HDACi combined treatments with curcumin or tamoxifen can efficiently suppress cancer cell viability and HDACi-enhanced migration activity of Hep3B and Huh7 cells ($P < 0.01$; Supplementary Fig. S5A). Similarly, when applied, the combination treatments to other HDACi-treated liver, lung, gastric, and breast cancer cells, the HDACi and PKCi combined treatments showed more effective suppression of cancer cell viability and HDACi-enhanced migration activity than that of MMPi ($P < 0.01$; Fig. 3 and Supplementary Fig. S5B–S5D). We therefore concluded and focused on HDACi combined treatments with low toxic PKCi (curcumin and tamoxifen) as new modalities for

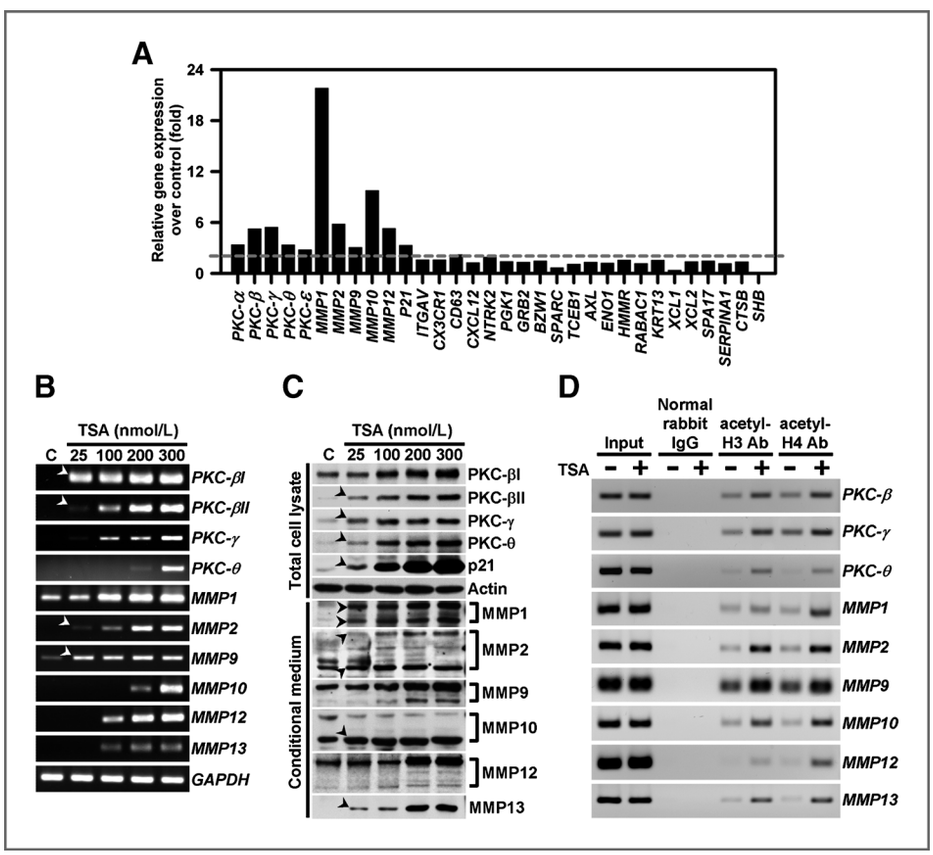

Figure 2. HDACi treatments increased RNA and protein expressions of MMPs and PKCs through hyperacetylation of chromatin histones at their promoter regions. A, RNA-seq transcriptome analysis of metastasis-related genes in H23 cells with and without treatment of SAHA. Dash line is the 2-fold increase expression compared with untreated control. The HDACi-induced PKCs and MMPs were validated by TSA-treated Hep3B cells (B) at RNA level by RT-PCR with *GAPDH* as internal control; (C) at protein level by Western bolt analysis and (D) at histone acetylation level of MMPs and PKCs promoters with 300 nmol/L TSA treatment by chromatin immunoprecipitation assay with input and normal rabbit IgG as loading and negative controls, respectively. Arrowheads indicated the upregulated genes induced by noncytotoxic TSA dose.

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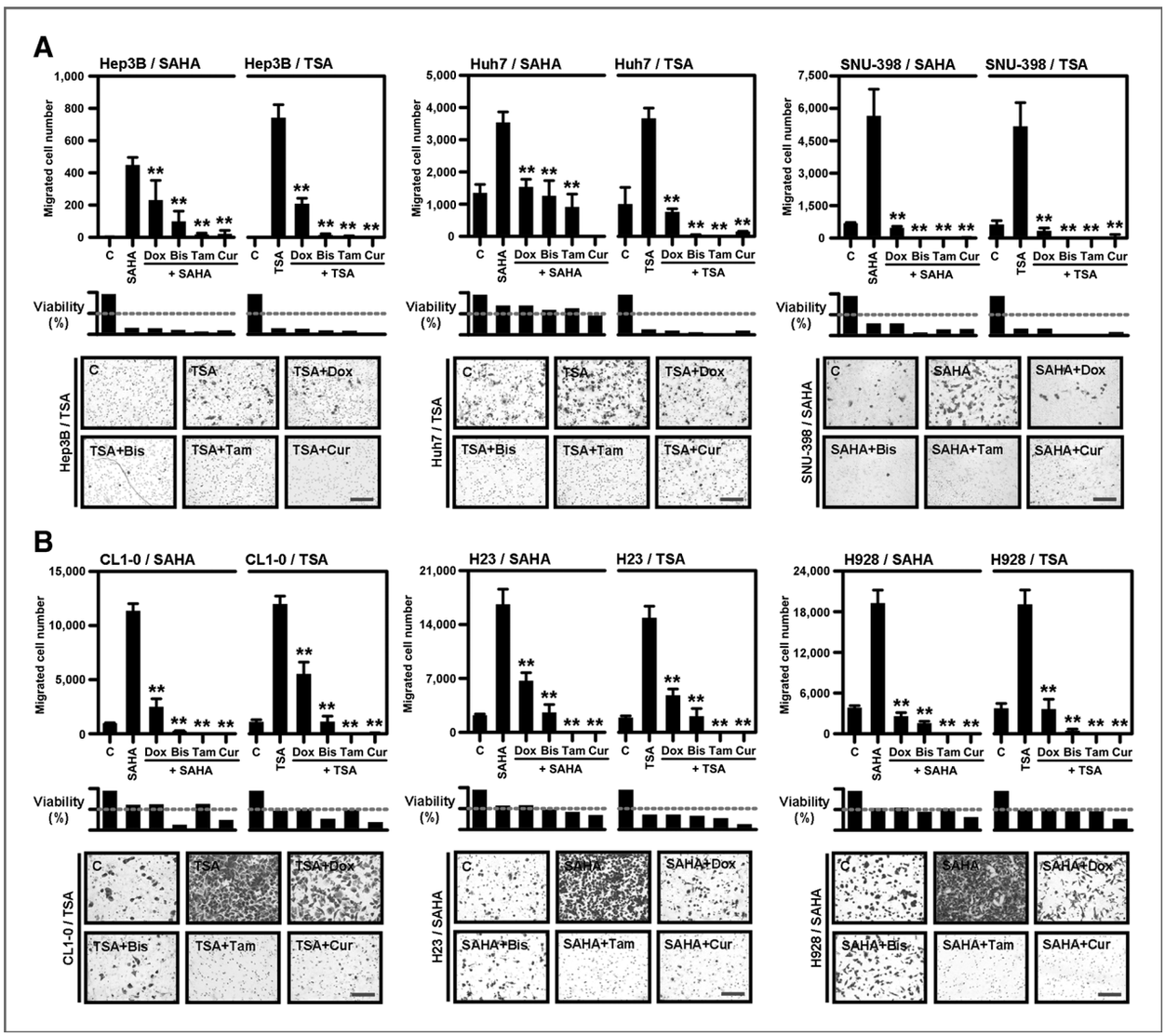

Figure 3. Combination treatments of HDACi with MMPi or PKCi suppressed HDACi-enhanced cell migration. Transwell migration assays were applied for HDACi (TSA or SAHA) combination treatments with MMPi (Dox) or PKCi (Bis, curcumin, and tamoxifen) in (A) liver and (B) lung cancer cells (**, $P < 0.01$; $n = 3$, mean ± 95% CI). Cell viability with indication of 50% cell viability in red dash lines and representative images of migrated cells (scale bar, 200 μm) were presented ($n = 3$, mean ± 95% CI). The doses for TSA and SAHA treatments for 24 hours were 300 nmol/L and 6 μmol/L for Hep3B; 300 nmol/L and 0.5 μmol/L for Huh7; 200 nmol/L and 6 μmol/L for SNU-398; 200 nmol/L and 4 μmol/L for H23; 200 nmol/L and 2 μmol/L for CL1-0; and 100 nmol/L and 2 μmol/L for H928, respectively. The doses for other inhibitors were Dox (25 μg/mL), Bis (15 μmol/L), tamoxifen (15 μmol/L), and curcumin (50 μmol/L). The "C" stands for control or untreated cells.

potential cost-effective and long-term combination cancer therapies.

HDACi-augmented cell migration, PKC activity, and phosphorylation of PKC substrates are suppressed by combination treatments with PKCi

To provide molecular evidence that PKCi suppresses HDACi-enhanced cell migration, we measured the PKC activity and the activated downstream PKC substrates for validation. Our results showed that combined treatments with PKCi suppressed HDACi-induced MMP and PKC protein expressions and total PKC activity in liver and lung cancer cells ($P < 0.05$; Fig. 4A and B). To ensure the HDACi-activated PKC phosphosubstrates, we used TSA-treated Hep3B and SAHA-treated H23 cell as examples for detection of various upregulated and phosphorylated PKC substrates. We showed that PKC phosphorylated proteins with tumor-progressive features were indeed upregulated by TSA or SAHA treatment but downregulated only by combined treatments with PKCi including Bis, curcumin, or tamoxifen (Fig. 4B and Supplementary Fig. S5E). For example, p-Bcl-2 (Ser 70), p-Bim (Ser 65), p-Connexin 43 (Ser 368), and p-p38 MAPK (Thr 180) were phosphorylated by their specific HDACi-activated PKCs to potentially facilitate their

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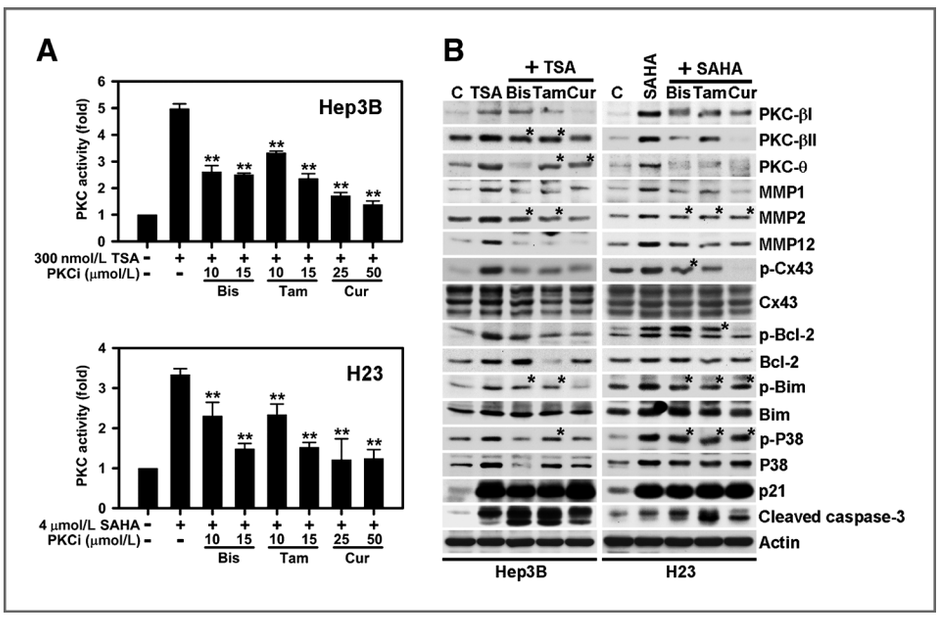

Figure 4. PKCi represses HDACi-activated PKC activity and expression of PKCs, MMPs, and PKC-specific phosphosubstrates. A, total PKC activity of Hep3B or H23 cells after combined treatments of HDACi (300 nmol/L TSA or 4 μmol/L SAHA) with PKCi. (**, $P < 0.01$; $n = 3$, mean ± 95% CI). B, expressions of HDACi-activated PKCs, MMPs, and PKC phosphosubstrates (300 nmol/L TSA for Hep3B; 4 μmol/L SAHA for H23) in combination treatments with PKCi by Western blot analysis (*, $P < 0.05$; $n = 3$, mean ± 95% CI). ** or * represented significant change relative to the HDACi monotreatment. p21, caspase-3, and actin served as controls for HDACi treatments, cell death, and internal, respectively. The concentrations of inhibitors were Bis (15 μmol/L), tamoxifen (15 μmol/L), and curcumin (50 μmol/L). The "C" stands for untreated control.

corresponding tumor-progressive effects (27–29). Furthermore, these HDACi-induced PKC phosphosubstrates could be suppressed again by combined treatments with low toxic PKCi, curcumin, and tamoxifen, with increased cleavage of caspase-3 and without affecting the sustained upregulated p21 protein. We concluded that HDACi combined treatments with PKCi can efficiently suppress HDACi-activated PKC activity, the phosphorylation of PKC substrates, and the mediated cell migration and tumor-progressive activities.

Combination therapies of HDACi with curcumin or tamoxifen suppress xenografted tumor growth

Next, to examine the therapeutic efficacy of low toxic modalities of HDACi combined with curcumin or tamoxifen in preclinical animal studies, we tested the new modalities with reported dosages in xenograft models by s.c. injection of Hep3B or H23 cells into nude mice (30, 31). To mimic tumor growth in disease condition, Hep3B or H23 cells were inoculated (Fig. 5A and B; step 1) and allowed for tumor growth to 40 to 80 mm^3 before injection of HDACi (TSA or SAHA; step 2). When therapeutic effect of HDACi-shrunk tumors of all mice reached distinguishable sizes compared with that of HDACi-untreated control mice ($P < 0.05$), combination therapies were launched by injection of HDACi with curcumin or tamoxifen (step 3). Our results indicated that monotherapy of TSA in Hep3B or SAHA in H23 xenografted mouse model suppressed tumor volume and weight as previous reports ($P < 0.01$; Supplementary Fig. S6A and S6B). In contrast, individual treatment of curcumin or tamoxifen showed nonsignificant suppressive trend as compared with that of vehicle control mice (Supplementary Fig. S6A and S6B). However, xenografted tumor volume and weight were further suppressed when tamoxifen or curcumin was added into HDACi combination therapies in Hep3B or H23 xenografted mouse model with no significant changes of mice body weight during therapeutic procedure ($P < 0.01$; Fig. 5A and B, Supplementary Fig. S6C).

To further validate therapeutic efficacies and mechanisms of combined inhibitors of HDACs and PKCs, we collected treated and untreated tumors from Hep3B xenografted model for Western blot analysis. Our results evidenced that upregulation of MMPs, PKCs, and PKC phosphosubstrates can be detected in all collected TSA-treated xenografted tissues (Supplementary Fig. S6D). TSA-combined treatments with curcumin or tamoxifen could downregulate aforementioned PKC downstream protein and substrates with tumor-progressive effects *in vivo* (Supplementary Fig. S6D). Noticeably, characterization of affected genes in treated xenografted tumor tissues further support our results that HDACi can simultaneously activate p21 and tumor-progressive proteins *in vitro* and *in vivo*. Hence, our results suggested that combined treatments of HDACi with PKCi can improve the monotherapeutic efficacy on xenografted tumor growth through resuppression of HDACi-activated multiple tumor-progressive proteins.

TSA combination therapies with curcumin or tamoxifen suppress liver metastasis

To further investigate the efficacy of combination therapy on tumor metastasis that is known to cause more than 90%

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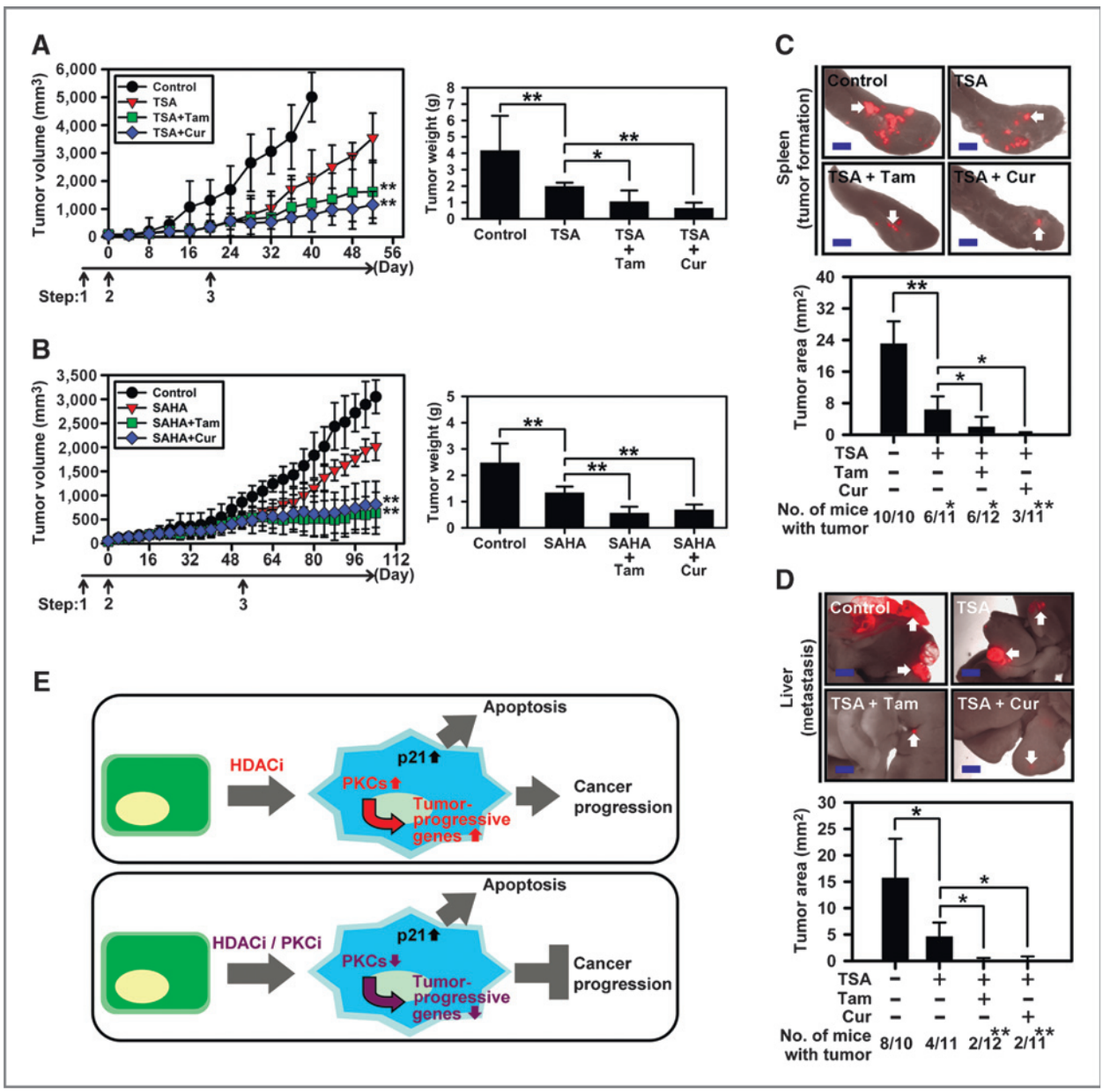

Figure 5. Combination therapies of HDACi and PKCi repress s.c. tumor growth and liver metastasis in mice. A, Hep3B-RFP and (B) H23-GFP s.c. tumor growth model treated with TSA (10 mg/kg/d) and SAHA (100 mg/kg/d), respectively, in combination therapy with curcumin (40 mg/kg/d) or tamoxifen (10 mg/kg/d). (*, $P < 0.05$ and **, $P < 0.01$; Student t test, means ± 95% CI). An orthotopic liver metastasis model after intrasplenic injection of Hep3B-RFP cells was conducted to determine the therapeutic efficacy of HDACi/PKCi combination treatments by measuring tumor areas in spleen for tumorigenesis (C) and in liver for metastasis (D). Tumor area was measured by Image-Pro Plus 5.0 software (mean ± 95% CI; Student t test; *, $P < 0.05$; **, $P < 0.01$; scale bar, 2 mm). The number of mice bearing tumor cells was calculated by Fisher's exact test (*, $P < 0.05$; **, $P < 0.01$). E, model of rational combination therapies of HDACi/PKCi. Top, HDACi activated tumor-progressive proteins including PKCs to increase cell migration. Bottom, HDACi/PKCi combination therapies suppress cancer progression through PKCi resuppression of HDACi-induced tumor-progressive PKCs and downstream MMPs.

mortality in patients with cancer, we applied the liver metastasis model by intrasplenic injection of Hep3B-RFP cells into athymic nude mice for therapy (Fig. 5C and Supplementary Fig. S7). Our results showed that TSA alone can suppress both intrasplenic tumor growth and liver metastasis compared with vehicle control mice by measuring tumor areas ($P < 0.05$; Fig. 5C and D). However, TSA combination therapies with tamoxifen or curcumin compared with TSA monotreatment or vehicle control mice can further suppress both tumorigenicity in spleen and metastasis in liver in both tumor size and number of loci with statistical significance ($P < 0.05$). Together, we concluded that combined treatments of HDACi with FDA-approved or dietary supplement PKCi not only suppress tumorigenesis

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and metastasis but also revealed potential new therapeutic modalities for long-term cancer therapy.

Discussion

With the increases of cancer mortality and therapeutic cost, development of preconceived rational combination modalities with low toxicity and cost is the urgent task to improve cancer management. Treatment with low toxic drugs to constrain tumor mass to an indolent tumor could reduce side effects and treat cancer as a chronic disease for better life quality (32). In this study, we provided *in vitro* and *in vivo* evidence that global treatment of HDACi to subset of liver, lung, gastric, and breast cancer cells could modulate expression not only proapoptotic genes but also ignored tumor-progressive genes to counter antitumor activity of HDACi (Fig. 5E, top). After establishment of HDACi-induced cell migration and metastasis assays and metastasis-related genes profiling, we found multiple members of PKCs and downstream pathways were induced by HDACi and suitable for developing new HDACi/PKCi combination modalities for cancer therapy (Fig. 5E, bottom). More importantly, 2 clinical and low toxic drugs with PKCi activity, curcumin, and tamoxifen were combined with HDACi for realization of inexpensive modalities for potential long-term treatment of cancers.

It has been estimated that HDACi treatments altered expression of approximately 10% genes from previous microarray studies with preferential activation of proapoptotic and tumor-suppressive genes such as p21 protein served as a biomarker of HDACi treatment in cancer cells (33). Nevertheless, contradictory observations of HDACi-induced cell migration were reported from researchers. Treatments of HDACi in some cancer cells including prostate, melanoma, and hepatocellular carcinoma (HCC) were shown to upregulate E-cadherin and some cell contact proteins to suppress cell migration (33–36). On the other hand, HDACi-enhanced cell migration was sporadically reported through activation of genes such as CCR7, CXCR4, integrins, glycodelin, and uPA by our group and others (21, 37–40). Interestingly, except 4 cancer cell lines of Huh6, H358, H1437, and HR with difficulty to judge the HDACi-induced cell migration effects, equal number of the remaining 26 cancer cell lines could be classified into 2 groups: HDACi-induced promotion and suppression of cell migration (Fig. 1 and Supplementary Fig. S1). Among 13 of 30 (43%) cell lines with HDACi-augmented cell migration activity, majority of them (Hep3B, PLC5, Huh7 and SNU-398 for liver; H928, H226Br, H661, H23, CL1-0, and CL1-3 for lung; NUGC and SC-M1 for gastric; and MCF-7 for breast cancer cells) can be classified into relatively low migration cell lines in Transwell migration assay compared with other high migration cell lines of the same tissue origins (HCC36, HA22T, and Malhavu for liver; A549, CL1-5, and H1299 for lung; AZ521 and HR for gastric; and Hs578T for breast cancer cells). Our observations suggested that the metastatic potential of cancer cells may be epigenetically activated to enhance cell migration during tumor progression in multiple cancer types.

Noticeably, in contrast to the drug resistance or side population cells selected after treatment of high doses of chemotherapeutic drugs, we consistently observed induction of p21 and metastasis-related proteins including gene families of *PKCs* and *MMPs* in low doses of HDACi treatments. Previous observations of individual PKC upregulation to counter HDACi-induced apoptotic effects were observed in leukemia/lymphoma cells (41, 42). However, our data showed that HDACi-enhanced cell migration was associated with multiple upregulated PKCs in solid tumors, which provided the rationale of abrogating HDACi-enhanced cancer cell migration through combination treatments with multiple PKC targeting drugs. Moreover, other tumor-progressive proteins including cytoskeleton-related proteins (Rac 1 and Rho C), transcription factors (SLUG and Snail), and multidrug-resistant proteins (ABCG2) were also activated by treatments of low doses of HDACi and resuppressed by combination treatments with PKCi (data not shown). Together, these observations further support us to target the reactivated metastasis-related genes, especially upstream PKCs, for developing new rational strategies for targeting both tumorigenesis and metastasis to improve cancer patient survival.

We noticed that there are potential contradictory results of HDACi-induced metastasis *in vivo* in 2 different metastasis models: the experimental metastasis model with tail vein injection of cancer cells showed HDACi-enhanced metastasis (Table 1 and Supplementary Fig. S3); and the orthotopic spontaneous metastasis model after intrasplenic injection of Hep3B-RFP cells showed HDACi-suppressed tumorigenesis and metastasis (Fig. 5C and D and Supplementary Fig. S7). The experimental metastasis model, with tail vein injection of cancer cells directly into circulation eliminated early steps of metastasis cascade, might directly reflect the capability of HDACi-induced metastasis but with lower efficiency than that of *in vitro* Transwell migration data (43). In contrast, the orthotopic intrasplenic injection of Hep3B-RFP cells resulted in a model that more resembled to human cancer with complicated spontaneous metastasis cascade. When TSA-treated Hep3B-RFP cells were transplanted into spleen, large number of tumor nodules formed in spleen and subsequently metastasized to the adjacent liver. We speculated that TSA treatment of Hep3B cells remained its capability to enhance cell metastasis but restricted by the steps of metastasis cascade and overshadowed by the tumor-suppressive effect of TSA monotherapy. Although future experiments are needed to clarify the discrepancy of HDACi-induced metastasis in between 2 *in vivo* mice metastasis models, our results of TSA-induced metastasis-related genes expression in Hep3B xenografted tumor tissues further support our speculation (Supplementary Fig. S6D).

Recent emerging theory of early metastasis model indicated that metastatic-prone cancer cells might leave the primary tumor site much earlier than canonical theory of late stages tumor metastasis model (44). The colonized and expanded cancer cells from early metastasis could confer additional changes and become heterogeneous compared

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with cancer cells in primary tumor site. Therefore, the early metastasis theory implicates that drug treatments including HDACi targeting to primary tumor cells might be insufficient for cancer therapies. However, according to our rational combination therapies, simultaneously targeting to tumorigenic and metastatic pathways with low toxic and low cost modalities might potentially overcome premetastatic niche in early metastasis and become an ideal approach for treating cancer as a chronic disease.

Although curcumin and tamoxifen are used mainly as PKCi in our work, we cannot exclude other innate antitumor effects from both multiple targeted agents (45, 46). For example, there are results showing that HDACi can enhance the antitumor effects of curcumin and tamoxifen in human cancer cells through HDACi epigenetic modulation and/or posttranslational modification (ex. acetylation) of the heat shock protein 90 (Hsp90) and estrogen receptor, respectively (47, 48). With the concept of HDACi to reverse the tamoxifen/aromatase inhibitor resistance in hormone receptor–positive breast cancer, a recent phase II clinical trial of estrogen receptor–positive patients with metastatic breast cancer who received at least one prior aromatase inhibitor treatment were selected for combination therapy of SAHA/tamoxifen (48). An objective response rate of 19% and the clinical benefit rate of 40% over 24 weeks' combination treatment with median response duration of 10.3 months among 43 patients implicated an encouraging therapeutic potential of HDACi/PKCi combined therapy in our study. Hence, the *in vivo* suppression of tumorigenesis and metastasis by preconceived HDACi rational combination therapies with curcumin or tamoxifen from our preclinical experiments should be warrant for further investigation in the clinic to translate our results for patients. Furthermore, the low toxic agents in our study either synthesized or derived from natural products have been individually used in clinic for decades (49, 50). Therefore, our results provide not only the established assays for rational combination therapies targeting to reactivated tumor-progressive genes but also the opportunity to develop low cost-efficacy combination modalities for long-term cancer therapies to prolong life of patients with cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K.-T. Lin, Y.-S. Jou
Development of methodology: K.-T. Lin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-T. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.-T. Lin, Y.-W. Wang, C.-M. Ho, W.-H. Su, Y.-S. Jou
Writing, review, and/or revision of the manuscript: K.-T. Lin, C.-T. Chen, Y.-S. Jou
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.-T. Lin
Study supervision: K.-T. Lin, Y.-S. Jou

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