Microtubule-Associated Histone Deacetylase 6 Supports the Calcium Store Sensor STIM1 in Mediating Malignant Cell Behaviors

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Abstract

Stromal-interaction molecule 1 (STIM1) is an endoplasmic reticulum Ca\(^{2+}\) storage sensor that promotes cell growth, migration, and angiogenesis in breast and cervical cancers. Here, we report that the microtubule-associated histone deacetylase 6 (HDAC6) differentially regulates activation of STIM1-mediated store-operated Ca\(^{2+}\) entry (SOCE) between cervical cancer cells and normal cervical epithelial cells. Confocal microscopy of living cells indicated that microtubule integrity was necessary for STIM1 trafficking to the plasma membrane and interaction with Orai1, an essential pore subunit of SOCE. Cancer cells overexpressed both STIM1 and Orai1 compared with normal cervical epithelial cells. HDAC6 upregulation in cancer cells was accompanied by hypoacetylated β-tubulin. Tubastatin-A, a specific HDAC6 inhibitor, inhibited STIM1 translocation to plasma membrane and blocked SOCE activation in cancer cells but not normal epithelial cells. Genetic or pharmacologic inhibition of HDAC6 blocked STIM1 membrane trafficking and downstream Ca\(^{2+}\) influx, as evidenced by total internal reflection fluorescent images and intracellular Ca\(^{2+}\) determination. In contrast, HDAC6 inhibition did not affect interactions between STIM1 and the microtubule plus-end-binding protein EB1. Analysis of surgical specimens confirmed that most cervical cancer tissues overexpressed STIM1 and Orai1, accompanied by hypoacetylated β-tubulin. Together, our results identify HDAC6 as a candidate target to disrupt STIM1-mediated SOCE as a general strategy to block malignant cell behavior. Cancer Res; 73(14); 4500–9. © 2013 AACR.

Introduction

Modulation of cytosolic Ca\(^{2+}\) levels provides versatile and dynamic signaling that mediates fundamental cellular functions, such as proliferation, migration, gene regulation, and apoptosis (1). Increase in cytosolic Ca\(^{2+}\) concentrations occurs as a result of Ca\(^{2+}\) entry from the extracellular space and Ca\(^{2+}\) release from intracellular stores, mainly from the endoplasmic reticulum. Store-operated Ca\(^{2+}\) entry (SOCE) is a major Ca\(^{2+}\) entry pathway in nonexcitable cells (2, 3). The SOCE activation includes several steps: (i) stimulation of G proteins or protein tyrosine kinases activates phospholipase C, which hydrolyzes phosphatidylinositol bisphosphate to release the second messenger inositol-1, 4, 5-trisphosphate (IP3). (ii) Binding of IP3 to IP3 receptor in the endoplasmic reticulum membrane causes rapid and transient Ca\(^{2+}\) release from endoplasmic reticulum lumen. (iii) The decrease of endoplasmic reticulum luminal Ca\(^{2+}\) activates store-operated Ca\(^{2+}\) (SOC) channels in the plasma membrane, leading to a sustained influx of extracellular Ca\(^{2+}\) across the plasma membrane (2, 4). Two genes, STIM1 (stromal-interaction molecule 1) and Orai1, are responsible for SOCE activation (2). STIM1 functions as an endoplasmic reticulum Ca\(^{2+}\) sensor that detects store depletion. Once endoplasmic reticulum Ca\(^{2+}\) is depleted, STIM1 aggregates into multiple punctate that translocates to the close proximity of plasma membranes. Orai1, an essential pore-forming component of SOC channel, translocates to the same STIM1-containing structures during endoplasmic reticulum Ca\(^{2+}\) depletion and opens to mediate Ca\(^{2+}\) entry.

STIM1 is required for the development and function of regulatory T cells (5, 6) and STIM1 deficiency causes several autoimmune diseases and myopathy in human subjects and mouse models (7). The emerging importance of STIM1 in tumor biology has been highlighted in breast and cervical cancer (8–10). Inhibiting STIM1-mediated Ca\(^{2+}\) influx impaired focal adhesion turnover of breast cancer cells, which could be rescued by the small GTPases Ras and Rac (8). The reduction of Orai1 or STIM1 by RNA interference in highly
metastatic human breast cancer cells or treatment with a pharmacologic SOCE inhibitor decreased tumor metastasis in animal models. Our previous study showed that EGF induced the aggregation and trafficking of STIM1 toward the proximity of the plasma membrane, where STIM1 specifically interacted with Orai1 to mediate SOCE (9). The SOCE was necessary for the activation of Ca\(^{2+}\)-dependent protease calpain and tyrosine kinase Pyk2 in migratory cervical cancer cells (9). The activation of calpain and Pyk2 regulated multiple signaling events crucial for focal adhesion turnover and cervical cancer cell migration. More importantly, our findings from analyzing surgical specimens suggested that the pathologic significance of tumor STIM1 overexpression could be proposed to benefit the locomotion of cancer cells (9). Another study based on the microarray data analyses of patients with breast cancer showed that transcriptionally defined basal-like tumors, which are featured with a poor prognosis and a lack of effective therapies, are characterized by high STIM1 and low STIM2 mRNA expressions (10). To inhibit STIM1-dependent Ca\(^{2+}\) signaling by specifically targeting STIM1 activation and translocation in cancer cells is thus a potential target for cancer therapy.

The precise localization and trafficking of intracellular proteins mostly depend on the integrity of cytoskeleton (11). The morphology of endoplasmic reticulum is maintained through a tight interaction with microtubules (12). The microtubule property and function are critically regulated by the posttranslational modifications occurring on tubulin subunits, such as acetylation, detyrosination, polyglutamylation, and polyglycylation (13). Reversible acetylation of Lys40 on \(\alpha\)-tubulin is important for regulating microtubule stability and function and seems to have a role in cell motility. Histone deacetylase 6 (HDAC6), a unique cytoplasmic member of HDAC family, has been identified as a microtubule-associated protein and could function as the prominent \(\alpha\)-tubulin deacetylase (14–16). Here, we test the hypothesis that the modulation of microtubule dynamics by HDAC6-mediated \(\alpha\)-tubulin deacetylation plays an important role in controlling STIM1-dependent Ca\(^{2+}\) signaling. The results showed that microtubule-associated HDAC6 is a potential target to interfere with Ca\(^{2+}\) store sensor STIM1-mediated cancer malignant behaviors.

Materials and Methods

Surgical specimens

We enrolled 6 cases with pair-frozen tissues of cervical carcinoma and adjacent noncancer epithelia for immunoblotting analyses and another 21 cases of paraffin blocks containing tissues of cervical carcinoma and adjacent noncancer epithelia for immunofluorescent staining. All these cases are patients with early-stage (International Federation of Gynecology and Obstetrics staging Ib) cervical cancer who underwent radical hysterectomy and pelvic lymphadenectomy at National Cheng Kung University Hospital (NCKUH), Taiwan. Normal cervical epithelial cells were derived from the portio surface and transformation zone of the cervix uteri obtained from 35- to 45-year-old women with no history of cervical dysplasia and who underwent hysterectomy for benign uterine diseases at NCKUH. For explant culture of cervical carcinoma cells, tissue fragments of about 10 mm\(^3\) were obtained from the colposcopy-guided cervical biopsy to minimize the contamination of normal cervical epithelium at the Department of Obstetrics and Gynecology, NCKUH. The collection of surgical specimens was approved by the institutional review board of National Cheng Kung University Hospital.

Cell culture, transfection, and RNA interference

 Cultures of normal cervical epithelial cells, primary cancer cells, cervical cancer cell lines (SiHa, HT-3, CaSkI, and HeLa) and stable pools of cervical cancer cells overexpressing EGF-STIM1 were prepared as previously described (17, 18). Cell lines were authenticated by the short-tandem repeats analysis using the Promega StemElite ID System (GeneLabs Life Science Corp.). Primary cancer cells from 2 subjects were tested positive for type 16 and 58 human papillomavirus (HPV) genome, respectively, using the EASYCHIP HPV genotyping array (King Car Yuan Shan Institute, Taiwan). The EASYCHIP HPV blot simultaneously detects 39 types of HPV on the basis of reverse blot hybridization. The sequences of RNAi targeting human HDAC6 and EB1 are listed in Supplementary Materials and Methods.

Antibodies, chemicals, and immunoblotting

The detailed information of chemicals and antibodies are listed in Supplementary Materials and Methods. Immunoblots were detected with affinity-purified antibodies against various molecules and horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch). Bands in the immunoblots were quantified using Vision WorksLS software (UVP).

Immunofluorescence and confocal images

Immunofluorescent stainings were done using affinity-purified antibodies against various molecules and AlexaFluor-conjugated secondary antibodies (Invitrogen). The fluorophores were excited by laser at 405, 488, or 543 nm and detected by a scanning confocal microscope (FV-1000, Olympus). The intensity and distribution of immunofluorescent staining of STIM1, Orai1, and acetyl-\(\alpha\)-tubulin were graded as high or low in each surgical specimen. High grade indicates that immunofluorescent staining intensity and distribution are more than 30% of tumor area, whereas low grade indicates that the staining intensity and distribution are less than 30% of tumor area. A pixel-by-pixel analysis by the colocalization algorithm of FV-1000 software was used to assess the colocalization of different molecules in confocal images. Cell margin was visualized by differential interference contrast (DIC) images and by immunostaining with the endogenous plasma membrane marker Na-K-2Cl cotransporter 1 (NKCC1; ref. 19). The "juxta-plasma membrane region" was defined as 2 \(\mu\)m distances to the plasma membrane and STIM1 intensity within this region was defined as STIM1 membrane trafficking accordingly.

Single cell [Ca\(^{2+}\)]\text{measurement}

 Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was measured at 37°C with the Fura-2 fluorescence ratio method on a single-cell fluorimeter as previously described (9, 20). The Fura-2 was excited alternatively between 340 nm (\(I_{340}\)) and 380 nm (\(I_{380}\))
using the Polychrome IV monochromator (Till Photonics) and
images were detected by the Olympus IX71 inverted microsco-
scope equipped with a xenon illumination system and an
IMAGO CCD camera (Till Photonics). The fluorescence inten-
sity of excitation at 510 nm was monitored to calculate \([Ca^{2+}]_i\),
by TILLvisiON 4.0 program (Till Photonics).

Statistics
All values were reported as mean ± SEM. Student paired
t test or unpaired t test was used for statistical analyses.
Differences between values were considered significant when
\( P < 0.05 \).

Results
Microtubule is necessary for STIM1 membrane
trafficking
Serial Z-section of confocal images from living cells sug-
gested that STIM1 scattered at the middle or bottom plane
before EGF stimulation (Supplementary Fig. S1A). Upon EGF
stimulation, the general increase in STIM1 fluorescent signals
was observed in the serial Z-sections. The formation and
redistribution of STIM1 punctate toward plasma membrane
was more apparent at the bottom planes. Disruption of actin
filaments by cytochalasin D showed no effect on STIM1 puncta
formation and trafficking (Supplementary Fig. S1B). In con-
trast, STIM1 trafficking was significantly inhibited following
the collapse of microtubule network induced by colcemid
(Supplementary Fig. S1C). Consequently, the association
between STIM1 and Orai1 at the proximity of plasma mem-
brane was significantly attenuated (Supplementary Fig. S1D–
S1F), suggesting that the microtubule integrity is necessary for
STIM1 trafficking and the subsequent interaction with the SOC
channel Orai1.

HDAC6 inhibition induces the differential \( \alpha \)-tubulin
acetylation between normal and cancer cells
We studied whether the modulation of microtubule dynam-
ics by HDAC6 plays an important role in STIM1-dependent
cancer malignant behaviors. HDAC6 was localized exclusively
in the cytoplasm of cervical cancer cells, where it was mainly
associated with microtubules instead of actin cytoskeleton
(Supplementary Fig. S2A). To differentiate the posttranslation-
al modifications of microtubules between normal and cancer
cells, we analyzed the expression patterns of STIM1, Orai1,
HDAC6, and acetyl-\( \alpha \)-tubulin in cervical epithelial cells with
different malignant potential (Fig. 1A). The expression
levels of STIM1, Orai1, HDAC6, and acetyl-\( \alpha \)-tubulin were
similar among the primary cultures from different normal
subjects. Cervical cancer HT3 and SiHa cell lines clearly over-
expressed STIM1 and Orai1, compared with normal subjects.
Interestingly, HDAC6 was upregulated in cervical cancer cells, which was accompanied by hypo-acetylated α-tubulin. The coincident overexpression of HDAC6 and STIM1 in cervical cancer cells implies a possible role of α-tubulin acetylation in regulating STIM1 function. More importantly, tubastatin-A, a selective HDAC6 inhibitor (21), showed a differential effect on α-tubulin acetylation between cervical cancer cells and normal cervical epithelial cells (Fig. 1B–D). Incubation with a low concentration of tubastatin-A (0.5 μmol/L) for a short time period (< 5 hours) induced a significant increase in acetyl-α-tubulin of cervical cancer SiHa cells, whereas that of normal cervical epithelial cells was not affected (Fig. 1B and D). Moreover, incubation with a higher concentration of tubastatin-A (5 μmol/L) for 5 to 10 hours remarkably caused the acetylation of α-tubulin in cervical cancer SiHa cells, but only modestly induced that of normal cervical epithelial cells.

Active SOCE with hypoacetylated α-tubulin in cervical cancer

To study the clinical relevance, the expression pattern of STIM1, Orai1, HDAC6, and acetyl-α-tubulin were examined in the surgical specimens of cervical cancer (Fig. 2A–E). Compared with that of noncancerous tissues, the expression level of STIM1 in tumor tissues was increased in all cases analyzed by immunoblotting (Fig. 2A), which is consistent with our previous study (9). STIM1 overexpression in tumor tissues was accompanied by HDAC6 upregulation in most cases (67%; Fig. 2A). We also enrolled another 21 cases with paraffin blocks for immunofluorescent staining (Fig. 2B–E). Cervical cancer tissues clearly expressed STIM1 and Orai1, whereas these proteins were rarely detected in the adjacent noncancerous cervical epithelia. On the other hand, acetyl-α-tubulin was more obvious in noncancerous cervical epithelia, when compared with cervical cancer tissues (Fig. 2C). We graded the tumor tissues by the distribution and intensity of immunofluorescent staining (Fig. 2D and E). The majority of these enrolled cases showed low grade of acetyl-α-tubulin and high grade of STIM1 and Orai1. We have tested the selectivity of Orai1 antibody by using an Orai1 blocking peptide (Supplementary Fig. S2B–S2C). Evidence from images and immunoblotting suggests that the Orai1 antibody is more selective with immunofluorescent staining than immunoblotting.

A key question that remained unanswered is "if there is a difference in endoplasmic reticulum Ca2+ release and SOCE between normal cervical cells and cervical cancer cells." To answer this question and to avoid bias from the clonal effect of cell lines, we did the primary tissue cultures from normal subjects and patients with cervical cancer.

Figure 2. Active SOCE with hypo-acetyl-α-tubulin in cervical cancer. A, expression pattern of STIM1 and HDAC6 in early-stage cervical cancer. Cervical cancer with pair-frozen tissues of carcinoma and adjacent noncancer epithelia were analyzed by immunoblotting. N, noncancer epithelia; T, tumor tissues. Expression of STIM1 and HDAC6 was normalized against β-actin. B and C, immunofluorescent stainings of STIM1, Orai1, and acetyl-α-tubulin in immunofluorescent stainings. Case numbers are indicated in parentheses beside each point. F and G, representative [Ca2+]i measurement in normal cervical cells (from Subject 5) and primary cervical cancer cells (from Subject 2). Mean traces of [Ca2+]i measurement from at least 30 different cells in each subject. The SOCE amplitude indicates the rise of [Ca2+], in replenishment of [Ca2+], from 0 to 2 mmol/L. Each value represents mean ± SEM of at least 30 cells. Similar results were observed in primary tissue cultures from 6 cases of normal cervix and 2 cases of cervical carcinoma. Arrow, adding 2 μmol/L thapsigargin (TG); SKF96365, SOCE inhibitor; *, P < 0.01.
cancer cells were cultured in the same condition for 3 days before experiments. As shown in Fig. 2F–G, thapsigargin-induced \(\text{Ca}^{2+}\) release from endoplasmic reticulum store was not significantly different between normal cervical cells and primary cervical cancer cells. Both normal cervical cells and primary cervical cancer cells displayed SOCE that was sensitive to SOCE inhibitor SKF96365. However, the SOCE of primary cervical cancer cells was more active, which is consistent with the molecular evidences showing overexpressed STIM1 and Orai1 in cervical cancer tissues.

**HDAC6 is involved in SOCE activation of cervical cancer cells**

We further studied whether HDAC6 is involved in the regulation of SOCE. As shown in Fig. 3A, tubastatin-A dose-dependently induced \(\alpha\)-tubulin acetylation in cervical cancer SiHa and CaSki cell lines as well as primary cervical cancer cells. Thapsigargin- or EGF-induced SOCE activation in cervical cancer cells was also inhibited by tubastatin-A in a dose-dependent manner (Fig. 3B–E). Importantly, normal cervical epithelial cells displayed less active SOCE, which was insensitive to tubastatin-A (Fig. 3F and G). We also studied the role of other HDAC members on SOCE activation. Neither entinostat (MS275, an inhibitor for HDAC1, 2, and 3) nor sodium butyrate (NaB, a broad spectrum inhibitor for HDACs excluding HDAC6 and 10) affected \(\alpha\)-tubulin acetylation and SOCE activation in cervical cancer cells (Supplementary Fig. S3A–S3C). These results suggest that HDAC6, rather than other HDACs, is critically involved in the regulation of SOCE activation of cervical cancer cells.

**Figure 3.** HDAC6 is important for SOCE activation in cervical cancer cells. A, tubastatin-A induces \(\alpha\)-tubulin acetylation in different types of cervical cancer cells. Cells were incubated with different concentrations of tubastatin-A for 10 hours. Acetyl-\(\alpha\)-tubulin was normalized against \(\alpha\)-tubulin levels. B and C, tubastatin-A inhibited thapsigargin-associated SOCE in cervical cancer SiHa cells with a concentration-dependent manner. Mean traces for \([\text{Ca}^{2+}]_i\) measurement from at least 80 different cells. Arrow, adding 2 \(\mu\)mol/L thapsigargin (TG). Each point in the dose–response curve (C) represents mean \pm SEM mean from at least 3 different experiments. D and E, tubastatin-A dose dependently inhibits EGF-associated SOCE in cervical cancer SiHa and CaSki cell lines and primary cervical cancer cells from tissue cultures. D, mean traces for \([\text{Ca}^{2+}]_i\) measurement from at least 60 different cells. Arrow, adding 100 ng/mL EGF. E, quantitative analyses of changes in \([\text{Ca}^{2+}]_i\) levels (\(\Delta[\text{Ca}^{2+}]_i\)). Each value represents mean \pm SEM of at least 60 cells. *, \(P < 0.01\). F and G, tubastatin-A showed no effect on SOCE activation in normal cervical cells. F, mean traces for \([\text{Ca}^{2+}]_i\) measurement from at least 30 different cells. G, quantitative analyses of \(\Delta[\text{Ca}^{2+}]_i\). Each value represents mean \pm SEM of at least 30 cells. *, \(P < 0.01\).
The mechanism by which HDAC6 regulates SOCE was studied. Thapsigargin or EGF stimulated the aggregation and trafficking of STIM1 proteins toward the proximity of plasma membrane in cervical cancer SiHa, CaSki, and HeLa cells (Fig. 4, Supplementary Figs. S4 and S5A and S5B), without altering the acetylation level of α-tubulin (Supplementary Fig. S2D). HDAC6 inhibitor tubastatin-A caused apparent STIM1 aggregations in cytosol, which was not correlated with STIM1 movement toward juxta-membrane region induced by thapsigargin or EGF (Fig. 4, Supplementary Figs. S4 and S5A and S5B). In contrast, neither MS275 nor NaB affected the puncta formation and membrane trafficking of STIM1 (Supplementary Fig. S3D). These results indicate that STIM1 movement toward plasma membrane is critically regulated by HDAC6. To dissect the role of microtubule dynamics in STIM1 trafficking in a more quantitative way, we monitored EGFP-STIM1 movements by the total internal reflection fluorescence (TIRF) microscopy (Supplementary Movies 1 and 2), which visualizes the fluorescence restricted to within approximately 100 nm from the plasma membrane of living cells (22). STIM1 was clearly located in the TIRF plane. EGF stimulated STIM1 trafficking toward the cell periphery, which was abolished by tubastatin-A (Supplementary Fig. S4A). The [Ca\textsuperscript{2+}] measurement by TIRF also showed that EGF-induced Ca\textsuperscript{2+} spikes was inhibited by tubastatin-A in cervical cancer SiHa cells (Supplementary Fig. S4B).

HDAC6 regulates STIM1 membrane trafficking

The important role of HDAC6 on SOCE activation and STIM1 trafficking was further shown by the knockdown approaches (Fig. 5 and Supplementary Fig. S5C–S5E). HDAC6 knockdown by different duplexes of siRNA in cervical cancer SiHa and CaSki cells was accompanied by increasing α-tubulin acetylation (Fig. 5A and Supplementary Fig. S5C). Moreover, the SOCE activation was significantly inhibited by HDAC6 silencing (Fig. 5B and C and Supplementary Fig. S5D and S5E). In the presence of HDAC6-specific siRNAs, EGF or thapsigargin caused obvious STIM1 puncta without trafficking toward juxta-plasma membrane (Fig. 5D and E). Importantly, the inhibition of STIM1 membrane trafficking by HDAC6 knockdown or inhibitor decreased the proliferation and migration of cervical cancer cells (Supplementary Fig. S5F and S5G), suggesting that STIM1 membrane trafficking is a potential target to manipulate STIM1-mediated cancer malignant behaviors.

Microtubule plus-end tracking proteins are necessary for STIM1 trafficking

The interaction between STIM1 and the microtubule plus-end-binding protein EB1 at the growing plus end of microtubules plays an important role in the remodeling of endoplasmic reticulum morphology (23–25). We studied the role of microtubule plus-end-tracking complexes on STIM1 membrane trafficking and the subsequent SOCE activation in cervical cancer SiHa, CaSki, and HeLa cells. SOCE activation
was significantly inhibited by the EB1-specific siRNA (Fig. 6A–C). Consistently, EB1 silencing blocked STIM1 trafficking toward plasma membrane and resulted in the accumulation of STIM1 aggregation in cytosol (Fig. 6D and E). However, the association between STIM1 and EB1 was not affected by tubastatin-A (Fig. 7). These results suggest that the association between STIM1 and microtubule plus-end-tracking complex is necessary for STIM1 trafficking and the subsequent SOCE activation, but HDAC6 is not involved in the binding of STIM1 with EB1.

Discussion

This study highlights an important role of microtubule-associated HDAC6 that is required for SOCE activation by optimizing the localization of endoplasmic reticulum Ca\(^{2+}\) sensor STIM1 toward plasma membrane. This conclusion is supported by the following evidences. (a) The interaction between STIM1 and the SOC channel Orai1 at the proximity of plasma membrane was significantly attenuated by the disruption of microtubule network. (b) The siRNA-mediated knockdown of HDAC6 expression or the pharmacologic blockade of HDAC6 activity significantly decreased STIM1 membrane trafficking and the subsequent SOCE. We also found that Ca\(^{2+}\) depletion from endoplasmic reticulum store enhanced the interaction between STIM1 and the microtubule plus-end-binding protein EB1, which was required for STIM1 trafficking toward plasma membrane. α-Tubulin acetylation is a posttranslational modification of stabilized microtubules (13, 26). This is the first study showing the important function of HDAC6-mediated α-tubulin deacetylation in regulating STIM1-dependent SOCE, as evidenced by confocal images, TIRF images, and single-cell [Ca\(^{2+}\)]\(_i\) measurement. STIM1-dependent Ca\(^{2+}\) signaling plays an important role in controlling cancer cell growth, migration, and angiogenesis (9). STIM1 involves the activation of Ca\(^{2+}\)-regulated calpain and Pyk2, which regulates the focal-adhesion dynamics of migratory cervical cancer cells. Because of an increase of p21 protein levels and a decrease of Cdc25C protein levels, STIM1-silencing in cervical cancer cells significantly inhibits cell proliferation by arresting the cell cycle at the S and G2–M phases. STIM1 also regulates the release of vascular endothelial growth factor from cervical
cancer cells. Interference with STIM1 expression or blockade of SOCE activity inhibits tumor angiogenesis and growth in animal models, confirming the crucial role of STIM1-mediated Ca\(^{2+}\) influx in aggravating tumor development in vivo. To block STIM1-dependent Ca\(^{2+}\) signaling is thus an alternative for cancer adjuvant therapy. To target STIM1-mediated cancer malignant behaviors could be achieved by RNAi-mediated reduction of STIM1 and Orai1, the pharmacologic inhibition of SOCE activity, or the interference with STIM1 trafficking toward Orai1-containing juxta-plasma membrane area. Although RNAi holds a hope as a novel nucleic acid-based therapeutic against a wide variety of diseases, to date most RNA therapeutics are still in early clinical trials and only the modest and circumscribed successes have been shown in animals (27). Furthermore, the physiologic and pathophysiologic functions of STIM1-mediated SOCE have been implicated in several tissues (2, 28), including immune system and cardiovascular system. The systemic administration of SOC channel blockers to patients with cancer may induce unexpected adverse effects. Therefore, to specifically target STIM1 membrane trafficking of cancer cells is a feasible strategy to inhibit STIM1-mediated cancer malignant behaviors.

Our clinical study has shown that STIM1 overexpression was noted in cervical cancer tissues and the tumor expression level of STIM1 was associated with the clinical outcome of early-stage cervical cancer (9). Here we studied the cell type-specific regulation of STIM1-dependent Ca\(^{2+}\) influx by using normal cervical epithelial cells and cervical cancer cells as the pair. Consistent with the previous findings in surgical specimens, STIM1 and Orai1 were differentially expressed between normal cervical epithelial cells and cervical cancer cell lines. Consequently, cervical cancer cells exhibited upregulated SOCE activity, compared with normal cervical epithelial cells and cervical cancer cell lines. Consistently, cervical cancer cells exhibited upregulated SOCE activity, compared with normal cervical epithelial cells. Moreover, the close association between SOCE activity, \(\alpha\)-tubulin acetylation, and HDAC6 abundances suggest the possibility that HDAC6-mediated \(\alpha\)-tubulin deacetylation contributes to the cancer cell–specific regulation of STIM1 activation. Interestingly, normal cervical epithelial cells and cervical cancer cells showed a different sensitivity to HDAC6 inhibitor. The differential inhibitory effects of tubastatin-A on SOCE activation can be explained by the fact that tubastatin-A induced the

Figure 6. Microtubule end-binding protein EB1 is necessary for SOCE activation and STIM1 membrane trafficking. A, representative immunoblots showing siRNA-mediated EB1 knockdown in cervical cancer SiHa and HeLa cells. B and C, EB1 silencing inhibits thapsigargin-induced SOCE activation. Mean traces of \([\text{Ca}^{2+}]_i\) measurement from at least 60 different cells. C, quantitative analyses of \(\Delta[\text{Ca}^{2+}]_i\). Each value represents mean \(\pm\) SEM from at least 60 cells from 3 different experiments. D and E, EB1 silencing inhibits EGF or thapsigargin-induced STIM1 membrane trafficking in cervical cancer SiHa cells. Nuclei were stained with Hoechst 33258 (blue). Lower, the enlargements of areas indicated by rectangles in whole-cell images. Arrowhead, STIM1 puncta at juxta-plasma membranes. Arrow, the aggregation of STIM1 in cytosol. Scale bar, 10 \(\mu\)m. E, quantitative analyses of STIM1 fluorescent intensity at juxta-plasma membranes. Each value represents mean \(\pm\) SEM from at least 50 different cells. *P < 0.01.
different α-tubulin acetylation between normal and cancer cells. Most importantly, cervical cancer tissues overexpressed STIM1 as well as HDAC6 that provides a niche to inhibit the interaction between STIM1 and Orai1 through microtubule-associated HDAC6 pathways.

In conclusion, the microtubule-associated HDAC6 is necessary for STIM1 trafficking toward juxta-membrane regions. Tubastatin-A blocks STIM1 trafficking and inhibits SOCE activation in cervical cancer cells but not in normal cervical epithelial cells. This makes the HDAC6-mediated STIM1 recruitment toward the plasma membrane as an attractive target to interfere with STIM1-mediated cancer malignant behaviors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
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References

6. Qu B, Al-Ansary D, Kummerow C, Kummerow C, Schwarz EC. ORAI1 and STIM1 de

HD6C in STIM1 Membrane Trafficking

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