The role of Src kinase in regulation of HDAC3 function

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Directed by Professor Ho-Geun Yoon

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ABSTRACT

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Histone deacetylase 3 (HDAC3) decreases acetylation level by removing acetyl group from histone. HDAC3 also binds to non-histone proteins and consequently regulates their cellular function. It has been well demonstrated that HDAC3 is phosphorylated at serine 424 increasing by casein kinase 2 (CK2), which subsequently increases its activity. Moreover, HDAC3 is shown to interact with Src kinase. Although the interaction between HDAC3 and Src is reported, it has been still remained uncertain whether Src phosphorylates HDAC3 and

affects the function of HDAC3.

Breast cancer is one of prevalent cancer to women in the world. Breast cancer is grouped into four subtypes - luminal A, luminal B, HER2 and triple negative breast cancer (TNBC) - depending on molecular genomic profiles. The treatment of breast cancer is performed appropriately in various ways such as mastectomy, radiation therapy, chemotherapy, hormone therapy and targeted therapy. Based on understanding of molecular pathway in cancer, there has been much attention toward targeted therapy that has high specificity to molecules responsible for cancer phenotypes. But, there are a lot of relapses of breast cancer in spite of treating by target therapy now that breast cancer avoids apoptosis using other pathways and shows heterogeneity.

In this study, it was found that HDAC3 directly interacted with Src by GST pull down assay and immunoprecipitation analysis. By domain mapping analysis, Src interacted with C-terminal domain (277-428 a.a.) of HDAC3 and phosphorylated tyrosine-325,328 and 331 of HDAC3. Importantly, overexpression of Src, but not inactive Src, increases the activity of HDAC3. In contrast, mutation of tyrosine residues at 325, 328 and 331 of HDAC3 abolished the overexpression effect of Src on HDAC3 activity. Moreover, a proliferation of breast cancer cell expressing phospho-deficient mutated HDAC3 is decreased when compared to control. Thus, it was suggested that phosphorylation of

HDAC3 by Src kinase regulates the proliferation of breast cancer cell.

Key words : histone deacetylase, HDAC3, Src, breast cancer, tyrosine phosphorylation

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I. INTRODUCTION

Histone deacetylase (HDAC) detaches acetyl group from histone, regulating gene expression at transcription level.[1] HDACs are classified into four classes, class Ⅰ,Ⅱ, Ⅲ and Ⅳ based on zinc or NAD^+ dependent mechanisms and their sequence similarity.[2] One of them, HDAC3 is categorized into class Ⅰ HDAC and exhibits distinct characteristics unlike other class Ⅰ HDACs.[1]

First, it recruits other proteins as component like SMRT(Silencing mediator of retinoic and thyroid receptors) / NCOR (nuclear receptor co-repressor) and MAPK1.[3,4] Second, it moves relatively freely from nucleus to cytoplasm.[1] Lastly, its catalytic domain is located comparatively closer to N-terminal region.[1]

HDAC3 can bind not only histone but transcription factors, oncogenes and tumor suppressors. It decreases acetylation level of target proteins so their expression and function are regulated in cellular level.[5] It is reported HDAC3 deacetylates RelA. Deacetylated RelA promotes complex formation with IκB. Then, RelA translocates from the nucleus to the cytoplasm.[6] HDAC3 competes with p300, regulating acetylation level of STAT3 in B-cell lymphoma.[7] In Ly3 cell, HDAC3 retains STAT3 protein in the nucleus and increases phosphorylation level of STAT3.[7]

HDAC is modified through phosphorylation, acetylation, ubiqutination and sumoylation called post translational modification (PTM).[8] PTM affects stability, location, activity and interaction of target proteins.[8] According to the previous studies, HDAC3 S424 is phosphorylated by casein Kinase (CK2) and its activity is increased in mitosis of cell cycle.[9] In contrast, N-terminal of HDAC3 is dephosphorylated by serine/threonine phosphatase (PP4) and its activity is decreased.[10] These findings suggested the phosphorylation status of HDAC3 regulates activity.

HDAC3 is also phosphorylated by other kinases.[11-14] One of them, Src kinase, an enzyme phosphorylates tyrosine residues in substrate, is located in cytoplasm mainly being inactivated state.[15] Src is activated by a number of

growth factor receptors such as EGFR, VEGFR and IGFR. It also switches on PI3K/AKT, RAS/RAF/MAPK, STAT and FAK signal pathway and modulates cellular processes. They are involved in regulation of cell survival,[16,17] proliferation,[18] angiogenesis[19-21] and migration.[22,23] Src is usually inactive by phosphorylation of Y530, but it can be activated by phosphorylation of Y419 at SH1 domain induced by conformational change through growth factors.[24]

Until now, impacts of Src kinase on tumorigenesis have been studied. It has been reported the activity of tyrosine kinase is increased by Src in malignant breast cancer.[25] The interaction between Src and EGFR promotes the tumorigenesis in MDA-MB-468 and MDA-MB-231 cell.[26] A tumor suppressor, RUNX3 is blocked to move into nucleus. Therefore, RUNX3 cannot function as tumor suppressor after phosphorylation by Src.[27]

There are reports on interaction between HDAC3 and Src kinase in cancer, but it still remains to be further investigated. The phosphorylation of HDAC3 by Src was discovered for the first time at membrane of keratinocyte by IP assay.[11] When MDA-MB-231 was treated with hepatocyte grow factor (HGF), phosphorylated HDAC3 by Src is localized at membrane and phosphorylation of HDAC3 decreased the expression of CXCR3, promoting metastasis of cancer cell.[12]

Breast cancer is the most common cancer in women throughout the world.[28] It is also fifth leading cause of cancer death.[28] In 2017, it is expected that about 257,000 new diagnoses of breast cancer in women.[28] In Korea, breast cancer is the second most prevalent cancer in women and the incidence of

breast cancer has steadily increased after 1999.[29]

Breast cancer is categorized into four basic groups (luminal A, luminal B, HER2 and triple-negative) based on genomic profiles. First, luminal A and luminal B type breast cancers express estrogen receptors (ER) and/or progesterone receptors (PR). Approximately, from 60% to 70% of breast cancers are involved in luminal A and luminal B type and they may grow dependent on estrogen and/or progesterone. The elevated activity of PI3K/AKT/mTOR signaling is observed in human ER positive breast cancer.[30] The mutation or amplification of gene related PI3K/AKT/mTOR pathway drives ER positive breast cancer more proliferative.[31-34] Unlike in other breast cancer subgroups, more than half of all disease recurrences in HR positive breast cancer occur 6 years or more after diagnosis, particularly following 5 years of adjuvant anti-estrogen therapy.[35] HER2 (human epidermal growth factor receptor 2) positive group constitutes from 15% to 20% of breast cancer.[36] It is reported that HER2 receptors are more amplified on cell surface in HER2 positive breast cancer and numerous HER2 receptors promote malignant tumor phenotypes.[37] Triple negative breast cancer (TNBC) accounts for from 10% to 20% of breast cancer.[38] It lacks expression of hormone receptor (ER and PR) and HER2.[39] Mutant p53 and p63 complex blocks anti-metastatic abilities by weakening the activity of Rab7 which is a molecular motor responsible for the transport of EGFR cargo to the lysosome for degradation in TNBC.[40-45] TNBC patients have a higher rate of distant recurrence and a poorer prognosis than women with other breast cancer subtypes.[46,47]

Depending on subtype and grade, various methods are applied to treat breast cancer. Surgery, radiotherapy, chemotherapy, hormone therapy, and targeted therapy are used to manage the symptom. Surgery is considered as main therapy for breast cancer in combination with one or additional therapies. Radiotherapy uses high energy beam like X-ray and its aim is to minify tumor size before surgery. In comparison with surgery, radiotherapy has the advantage of being non-invasive and potentially organ preserving,[48] however, it was reported that adverse effects such as dermatitis, fatigue, swelling and heaviness occurred.[49-51] Chemotherapy is remedy for metastatic or recurrent breast cancer and uses cancer-killing drugs such as paclitaxel, 5-fluorouracil and Paraplatin. When paclitaxel was administered to breast cancer patients weekly, disease-free survival had been substantially meliorated.[52] However, diverse genetic heterogeneity of cancer cell correlated tumor progression, worse clinical outcome and this resulted in drug resistance.[53,54] To overcome hurdles, from two to four kinds of drugs have been combined to treat breast cancer.[55-58] Hormone therapy inhibits cancer cells from being proliferation which is based upon stimulation of estrogen and progesterone. Therefore, hormone therapy is only effective treatment for hormone positive breast cancer patients. Tamoxifen,[59] megestrol[60,61] and fulvestrant[62,63] have been widely used as anti-cancer drug in hormone therapy. It was also made the discovery about endocrine resistance arose by activating endocrine-independent growth and survival pathway resulted from genomic or epigenetic variation.[64] As contribution of research on molecular pathway in cancer biology, novel targets for developing cancer drugs like HER2,[65]

PI3K,[66] FGFR,[66] cyclin D,[66,67] CDK4,[66,67] HSP90,[68] BRCA1/2[69] and HDAC[70-75] have been identified. They are responsible for growth factor receptor, PI3K/mTOR pathway, cell cycle regulation and epigenetic pathway.[76] Trastuzumab, a fully humanized monoclonal antibody, targets HER2 extracellular domain and Trastuzumab is effective to block PI3K, MAPK signaling. The synthetic lethality in BRCAness termed as BRCA1 or BRCA2 mutation carriers is induced by PARP inhibitor, Olaparib.[77]

The DNA methylation status in CpG island of ESR1 promoter coding estrogen receptor gene induced the chromatin inactivation.[78,79] It was demonstrated that interplay between DNA methylation and chromatin inactivation is mediated by histone modification such as ESR1 silencing through interaction between DNA methyltransferase 1 (DNMT1) and HDACs in ER negative breast cancer.[80] The aberrant expression of HDACs contributes to maintain phenotypes of breast cancer. For this reason, HDACs are considered as one of attractive targets for breast cancer drug. HDAC inhibitors are divided into four groups - hydroxamates, benzamides, cyclic peptides and aliphatic acids - based on their chemical structure. They are also classified based on their specificity. It is reported that Panobinostat suppresses aromatase promoter and inhibits proliferation of H295R/MCF7 co-culture model.[73] Entinostat (SNDX-275), one of class Ⅰ HDAC inhibitor, diminishes cell growth by inducing apoptosis.[74] Tumor initiating cell (TIC) of triple negative breast cancer cell (TNBC) is attenuated in colony formation and metastatic outgrowth in treatment of a HDAC inhibitor,[75] however, it has been reported that breast cancer do not respond well targeted treatment by

activating mechanism of therapeutic resistance. There are other limitations such as a burden of medical expenses and a risk of overtreatment.

Previous studies demonstrated Src phosphorylates HDAC3 but it hasn't been proven the phosphorylation sites of HDAC3 by Src.[11,12] Furthermore, the effect of Src-mediated phosphorylation on HDAC3 function has to be explained.

In this study, tyrosine residues of HDAC3 phosphorylated by Src were identified. It was found that phosphorylation status of HDAC3 affect its activity. Furthermore, phosphorylation status of HDAC3 by Src was involved in proliferation of breast cancer cell.

II. MATERIALS AND METHODS

1. Cell culture and reagents

Human breast cancer cells (SKBR3, BT20, MDA-MB-231) and the human embryonic kidney cell (HEK293FT) cell were obtained from the American Type Culture collection (ATCC, Manassas, VA, USA). All cells were cultured in DMEM (Corning, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 1% penicillin/streptomycin (Hyclone, Logan, UT, USA) at 37℃ under 5% CO_2. Transient transfection was performed using TransIT 2020 (Mirus, Madison, WI, USA).

2. Plasmid and cloning

Wild-type, full-length HDAC3 and its mutant constructs were generated by PCR and cloned into the plasmid vectors pCDNA 3.1 myc his A (Invitrogen, Carlsbad, CA, USA) or pCDNA3 2X flag plasmid vector. Wild-type, full-length c-Src and its mutant constructs were generated by PCR and cloned into the plasmid vectors pCDNA3 HA plasmid vector. All plasmid constructs were verified by DNA sequencing.

3. GST fusion proteins purification and *in vitro* translation

GST or GST fusion protein was purified from *E.coli* BL21(DE3) (Real Biotech Corporation, Banqiao, Taiwan) transformed with GST or GST fusion proteins expression plasmid. The *E.coli* were induced with 0.1 mM

isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St.Louis, MO, USA) for 72 hours at 16℃. The cells were lysed by sonication in MBP buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, 1 mM DETA, 1 mM DTT. Lysates were vortexed and cleared by centrifugation at 13,000 rpm for 30 minutes at 4℃. Supernatants were purified using Glutathione-agarose 4 bead (Peptron, Daejeon, Korea). The purified proteins were resolved with SDS-PAGE to quantitate and assess purity. The same amount of aliquot of the protein-agarose bead complex was used in GST-fusion protein pull down assay.

All *in vitro* translations were performed by TNT T7 quick coupled transcription/translation kit according to the manufacturer's protocol. (Promega, San Luis Obispo, CA, USA). Plasmid DNA template, TNT T7 quick master mixture and [^{35}S]-methionine (1175.0 Ci/mol, PerkinElmer Life Sciences Inc., Waltham, MA, USA) were mixed and incubated at 30℃ for 90 minutes.

4. GST pull down assay

The purified GST fusion proteins (5 μg) were incubated with Glutathion-agarose 4 bead (Peptron) for 16 hours at 4℃ and washed three times with 1 ml MBP buffer. After that, GST fusion proteins incubated with 10 μl of *in vitro* translated products for 16 hours at 4℃. After incubation, mixtures were centrifuged at 2,000 rpm for 2 minutes at 4℃. The supernatants are removed and the pellets were washed five times with cold MBP buffer.

The bound proteins were eluted by heating at 100℃ for 3 minutes and separated by a SDS-PAGE. The SDS-PAGE gel was dried and exposed to X-ray film using image-intensifying screen (Kodak, Rochester, NY, USA).

5. Western blot analysis

Cells were lysed in lysis buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.2% triton X-100, 0.3% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor cocktail (Gendepot, Barker, TX, USA) and incubated for 40 minutes on ice. During incubation, lysates was briefly vortexed every 10 minutes and sonicated. Lysates were cleared by centrifugation at 13,000 rpm for 30 minutes at 4℃. The supernatants were collected and transferred to fresh tubes. Concentration of proteins was quantified by 660 nm protein assay reagent (Thermo Scientific, Rockford, IL, USA). Equal amount of protein extracts was used to SDS-PAGE and then transferred to Nitrocellulose transfer membranes (Whatman, Dassel, Germany). The membranes were blocked in PBS containing 0.1% (v/v) Tween 20 (Amresco, solon, OH, USA) and 5% (w/v) nonfat Difco™ skim milk (BD Biosciences, Sparks, MD, USA) or TBS containing 0.1% (v/v) Tween 20 (Amresco) and 3% BSA (Affymetrix, Santaclara OH, USA) and probed with primary antibodies. The following antibodies were used: HDAC3 (Rabbit), HA (Rabbit), C-src (Mouse) (Santa Cruz Biotechnology Inc., Dallas, CA, USA), pan phospho Tyrosine conjugated HRP, Myc (Rabbit), phospho Y416 Src (Rabbit) (Cell signaling Technology, Danver, MA, USA), Flag (Mouse) β-actin (Mouse) (Sigma-Aldrich). The signals were developed by

substrate (Thermo Scientific) according to the manufacture's protocol.

6. Immunoprecipitation

Cells were lysed in lysis buffer as described above. After centrifugation, 500 μg of the clarified cell lysate was pre-cleared with G plus/protein A-agarose (Santa Cruz Biotechnology Inc.) and control IgG (Thermo scientific) by incubating for 1 hour. The supernatant was collected and 1 μg of antibody was added. After overnight incubation at 4℃, 20 μl of 50% slurry of G plus/protein A-agarose was added and the mixture was incubated for 1 hour. The agarose bead was centrifuged, washed four times with ice-cold lysis buffer, and suspended in electrophoresis sample buffer, and boiled for 3 minutes. After that, the samples were vortexed at RT for 15 minutes and immunoprecipitated protein was further analyzed by Western blotting.

7. Site-directed mutagenesis

The various mutants were created by PCR. 2X Q5 master mixture (NEB, Ipswich, MA, USA), DNA templates, 10 pM primer were mixed for PCR reaction. PCR cycling conditions used in site directed mutagenesis were 17 cycles of amplification of following reaction: denaturation at 98℃ for 10 seconds, annealing 55℃ or 58℃ for 30 seconds, and extension at 72℃ for 5 minutes. Amplified mixtures were treated with *Dpn* Ⅰ (Agilent Technologies, Santaclara, CA, USA) at 37℃ for 1 hour and PCR products were used to transform competent *E.coli* (Real Biotech Corporation). All the constructs were confirmed by DNA sequencing.

8. Establishment of overexpression stable cell line

HDAC3 WT and mutants with flag-tagged were cloned into plasmid vector pCDH-CMV-MCS-EF1-Puro (System Bioscience, Palo Alto, CA, USA). To generate lentiviral particles, cloned plasmids and lentiviral packaging plasmids (pSPAX2 and pMD2.G) are co-transfected into HEK293FT using TransIT 2020 (Mirus, Madison, WI, USA). After 48 hours incubation, supernatants were collected and filtered using 0.2 μm pore (Sartorius stedim biotech, Goettingen, Germany). Polybrene (8 μg/ml) is added to collected supernantant. Then, BT20 cell line was infected with lentivirus particles. After incubation with virus supernatant for 2 days, cells were selected with 1 μg/ml of puromycin (Sigma-Aldrich).

9. HDAC3 activity assay

7 mg of protein lysates were immunoprecipitated with 70μl of Flag M2 bead (Sigma-aldrich) and prepared for HDAC3 assay sample. The activity of HDAC3 was measured using HDAC3 assay kit (Biovision, Milpitas, CA, USA). 10 uM AFC was prepared to standardize fluorescent signal. 25 μl of Beads were added to 96 well black plate with duplicate. 2 μl of HDAC3 inhibitor (Trichostatin A) is added to the other well as background control. The samples were incubated at 37℃ for 10 minutes and agitated twice in every 5 minutes. After incubation, HDAC3 substrate was added and the samples were incubated at 37℃ for 30 minutes. The plate was shaken weakly to mix well every 5 minute. Developer was added to each well except standard and the samples were incubated at 37℃ for 5 minutes. The RFU was

measured at 380 nm and 500 nm for excitation and emission wavelength each using microplate reader (Flexstation 3, Molecular Devices, Union, CA, USA).

HDAC3 activity is measured as following calculation:

B / (30 x V) x dilution factor = U/ml

B is the AFC amount from the standard curve. 30 is the sample/substrate incubation time and V is the sample volume added into the reaction well.

10. Cell proliferation assay

1 x 10^4 - 2 x 10^4 cells were seeded in a 6 well plate (Corning incoporated, Corning, NY, USA). Cells were incubated 37℃ under 5% CO_2 for 3 days and detached every 24 hour. Cells were counted using hematocytometer.

11. RNA isolation and cDNA synthesis

Total RNA was extracted using Trizol reagent following the manufacturer's protocol (Takara Bio Inc., Otsu, Shiga, Japan). The 800 μl of Trizol was added to cells in cell culture dishes and cells were collected to tubes. The 200 μl of chloroform was added to samples and the samples were vortexed. The samples were incubated for 5 minutes at RT and centrifuged at 13,000 rpm for 15 minutes at 4℃. The 400 μl of supernatant was collected and transferred to fresh tubes. The 400 μl of isopropanol was added to samples and samples were thoroughly inverted. The samples were incubated for 20 minutes on ice and centrifuged at 13,000 rpm for 10 minutes at 4℃. The supernatant was removed and the 1 ml of 70% ethanol was added to samples. The samples were centrifuged at 13,000 rpm for 5 minutes at 4℃. The supernatant was

removed and pellet was dried at RT. The 50 μl of DEPC was added to dried pellet. The concentration of RNA was measured by Nanodrop1000 (Thermo scientific). After RNA isolation, the 3 μg of total RNA was mixed with 10 pM oligo dT and 2.5 mM dNTP (Takara). The samples were heated for 5 minutes at 65℃. Then, 5X buffer, MMLV-Reverse transcriptase (Takara) and distilled water were added to samples. The samples were incubated to anneal at 40℃ for 1 hour and to transcript at 65℃ for 10 minutes in PCR cycler (BioRed, Hercules, CA, USA). The concentration of cDNA was normalized by GAPDH. Primers used in PCR were as followed.

GAPDH : F-5'-GATGGCATGGACTGTGGTCA-3'

GAPDH : R-5'-GCAATGCCTCCTGCACCACC-3'

HDAC3 : F-5'-CCTGGCATTGACCCATAGCC-3'

HDAC3 : R-5'-CTCTTGGTGAAGCCTTGCATA-3'

Src : F-5'-TGGCAAGATCACCAGACGG-3'

Src : R-5'-GGCACCTTTCGTGGTCTCAC-3'

12. Statistical analysis

Statistical significance was examined using Student's t-tests. The two-sample t-test was used for two-group comparisons. Values were reported as mean ± standard deviations (SD). P values < 0.05 were considered significant.

III. RESULTS

1. Src Kinase binds to and phosphorylates HDAC3

According to previous studies, Src were interacted with HDAC3 at plasma membrane and is able to phosphorylate HDAC3. First, interaction between Src kinase and HDAC3 was investigated by performing GST pull down assay. It was found that Src kinase directly bound to HDAC3 (Fig. 1A). It was also checked that reciprocal interaction between Src kinase and HDAC3 by co-immunoprecipitation assay (Fig. 1B). Interestingly, there was a higher molecular weight band than we had expected when HDAC3 and Src is co-expressed. In general, it is well known that a higher molecular weight band is often seen when the target protein is posttranslationally modified such as phosphorylation. So, it was supposed that Src kinase may phosphorylate HDAC3. To verify whether a higher molecular weight band is phosphorylated HDAC3, western blotting was conducted using primary total phospho-tyrosine antibody. As a result, bands were detected horizontally on the blot, which signified that a higher molecular weight band was phosphorylated HDAC3 (Fig. 1C). SrcK298M, kinase dead mutant, was expressed whether Src activity is required for phosphorylation of HDAC3. As a result, kinase dead Src bound to HDAC3 but it did not phosphorylate HDAC3 (Fig. 1D). Taken together, Src directly binds to and phosphorylates HDAC3.

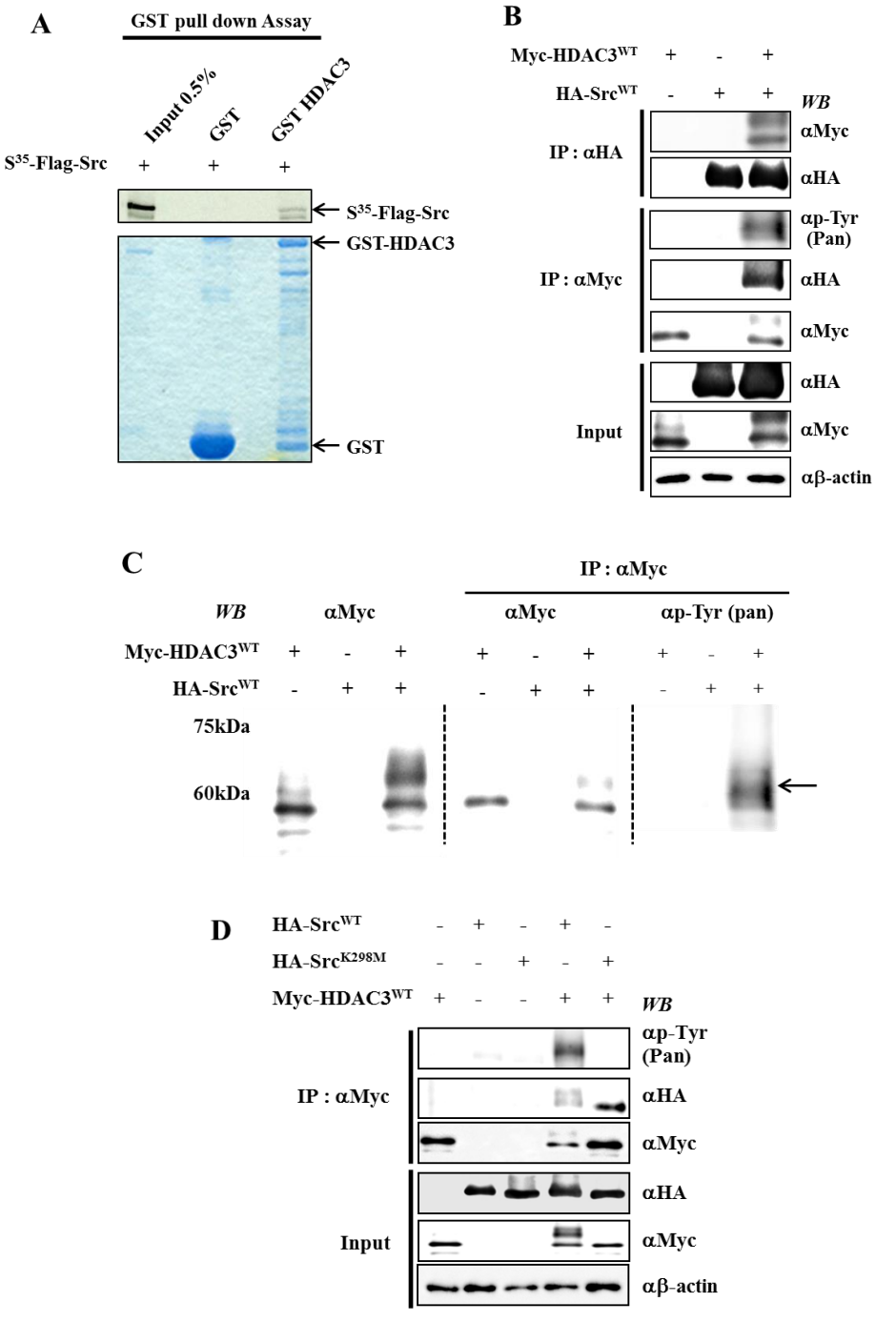


Figure 1. Src binds to and phosphorylates HDAC3. (A) Src directly binds to HDAC3. GST HDAC3 fusion protein was extracted in *E.coli* and Src was synthesized by *in vitro* translation. (B) Validation of HDAC3 and Src interaction. Src phosphorylates HDAC3. HEK293FT cells were transiently transfected with the indicated sets of plasmids. Whole cell lysates were immunoprecipitated with anti-myc antibody, anti-HA antibody and subsequently immunoblotted with indicated antibodies. (C) Slow moving band was identified as phosphorylated HDAC3. The vertical dot line indicates a repositioned gel lane from the same blot. The arrow indicates a higher molecular band. (D) Kinase dead SrcK298M binds to HDAC3 but fails to phosphorylate HDAC3. HEK293FT cells were transiently transfected with the indicated sets of plasmids. Whole cell lysates were immunoprecipitated with anti-myc antibody and subsequently immunoblotted with indicated antibodies.

2. The c-terminal region of HDAC3 (277-428 a.a.) is phosphorylated by Src kinase

Until now, the studies on domain of HDAC3 have been performed and the function of domain in HDAC3 is obvious, however the function of c-terminal region of HDAC3 has to be more elucidated. Lately, it is reported that c-terminal region of HDAC3 interacts with SMRT through Ins(1,4,5,6)P4 binding site.[81] Serine 424 was discovered as phosphorylation site in HDAC3 by CK2, which regulates the activity of HDAC3.[10] From previous studies, it was postulated that c-terminal region of HDAC3 may contribute as an essential hub for phosphorylation site by other kinases. So, it was examined which domain of HDAC3 is phosphorylated by Src kinase. Full length of HDAC3 was divided into three domains upon its function and the number of tyrosine residue (Fig. 2A). By domain mapping analysis, a higher molecular weight band was detected near 25kDa when HDAC3 (277-428 a.a.) and Src kinase were co-expressed (Fig. 2B). So, it was concluded that phosphorylation site by Src kinase is located in C-terminal region of HDAC3.

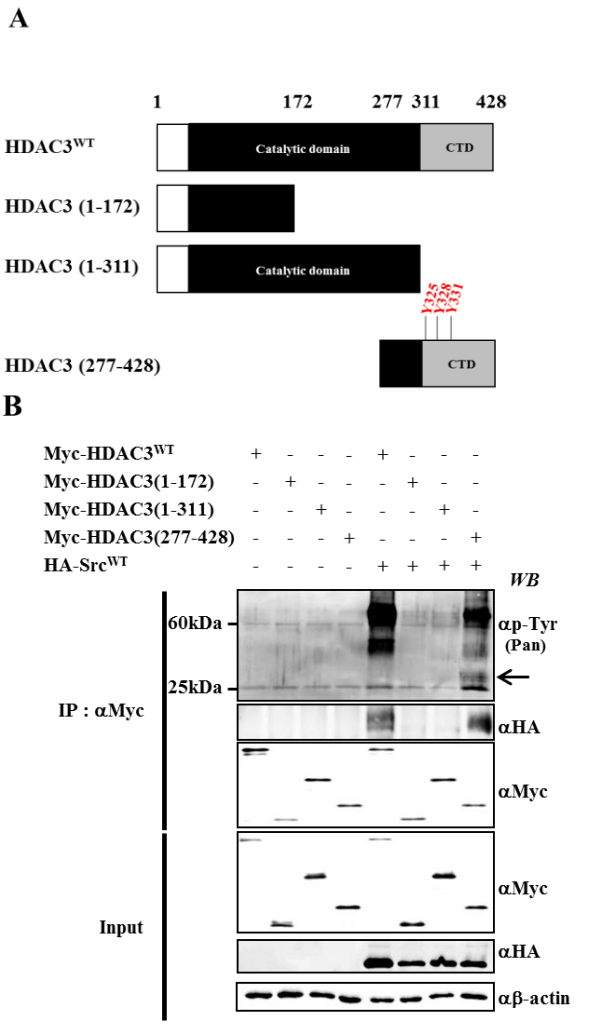


Figure 2. C-terminal region of HDAC3 is phosphorylated by Src. (A) The image shows design for domain mapping of HDAC3. HDAC3 full length was divided into three regions. (B) HDAC3 (277-428 a.a.) was key domain which is phosphorylated by Src. HEK293FT cells were transiently transfected with the indicated sets of plasmids. Whole cell lysates were immunoprecipitated with anti-myc antibody and subsequently immunoblotted with indicated antibodies. Arrow indicates a higher molecular weight band.

3. Three tyrosine sites (Y325, Y328, Y331) of HDAC3 are identified as phosphorylation sites by Src kinase

Based on a result of figure 2A, phosphorylation sites of HDAC3 by Src may be between 277 and 428 amino acids. To identify manifest phosphorylation site of HDAC3 by Src kinase, publicly available program, Netphos 3.1a, was exploited. This program can display the predicted phosphorylation sites by kinases. Interestingly, it was found that potential phosphorylation score of only two tyrosine sites (Y325 and Y331) in HDAC3 were over threshold (Fig 3A, table 1). So, it was examined whether these two sites are phosphorylation sites by Src kinase. To do this, these sites were mutated into phospho-deficient form (from tyrosine to alanine). Contrary to our expectation, HDAC3 Y325A, Y331A even 2YA (phospho-deficient mutant form of Y325 and Y331) were phosphorylated by Src kinase (Fig. 3B). It suggested that Y328 may be another candidate for phosphorylation site because this site also shows comparatively high phosphorylation potential score (Fig. 3A). Remarkably, the phosphorylation of HDAC3 by Src kinase was attenuated by mutation of three tyrosine sites (Y325, Y328 and Y331) (Fig. 3C). Therefore, tyrosine 325, 328 and 331 are essential sites for HDAC3 phosphorylation by Src.

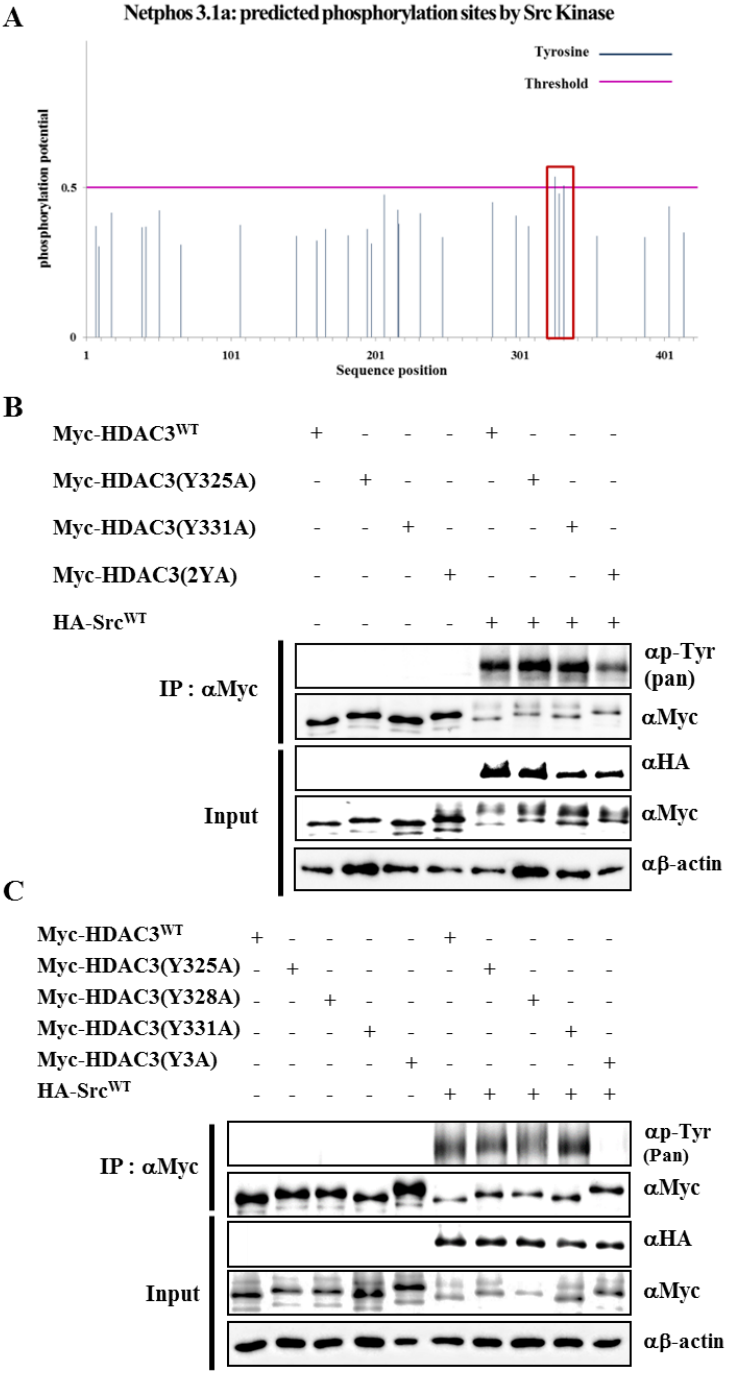


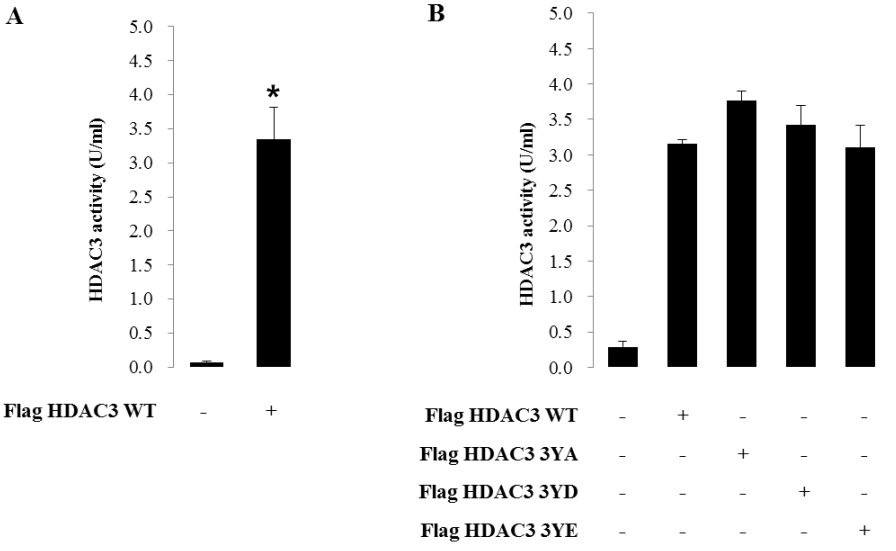
Figure 3. Three tyrosine sites (Y325, Y328, Y331) of HDAC3 are phosphorylated by Src. (A) The predicted phosphorylation sites by Src are located at HDAC3 C-terminal region. Src-mediated phosphorylation sites of HDAC3 were analyzed using NetPhos 3.1a program. The value of threshold is 0.5 and the box indicates tyrosine phosphorylation sites which can be phosphorylated by Src. (B) The tyrosine residue 325 and 331 are insufficient for blocking phosphorylation. HEK293FT cells were transiently transfected with the indicated sets of plasmids. Whole cell lysates were immunoprecipitated with anti-myc antibody and subsequently immunoblotted with indicated antibodies. (C) Phospho-deficient three tyrosine sites (Y325A, Y328A, Y331A – 3YA) impair phosphorylation of HDAC3. HEK293FT cells were transiently transfected with the indicated sets of plasmids. Whole cell lysates were immunoprecipitated with anti-myc antibody and subsequently immunoblotted with indicated antibodies.

Table 1. Tyrosine residues at c-terminal region of HDAC3 (277-428 a.a.) and their potential phosphorylation score by Src kinase

Tyrosine site	Amino acid sequence	Score	Possibility
282	ECVEYVKSF	0.452	
298	GGGGYTVRN	0.406	
309	RCWTYETSL	0.372	
325	EELPYSEYF	0.537	Yes
328	PYSEYFEYF	0.481	
331	EYFEYFAPD	0.507	Yes
354	NSRQYLDQI	0.338	
387	DLLTYDRTD	0.335	
404	PEENYSRPE	0.438	
414	PNEFYDGDH	0.351	

4. Src kinase increases the activity of HDAC3

There is a positive correlation between phosphorylation level of HDAC3 and its activity.[10] It was hypothesized that the phosphorylation of HDAC3 by Src can also regulate the activity of HDAC3 like CK2 manner. First, the activity of wild type HDAC3 is checked (Fig. 4A). It was expected that phospho-defective HDAC3 (HDAC3 3YA) shows lower activity than wild type HDAC3. Against our prediction, the activity of phospho-defective HDAC3 is higher than the wild type HDAC3 (Fig. 4B). It was speculated that unknown other PTMs may countervail phospho-defective effects and maintain activity as a level of wild type HDAC3. To confirm the role of Src kinase in regulating activity of HDAC3, wild type HDAC3 and wild type Src kinase were co-expressed and the activity was analyzed. When wild type HDAC3 and wild type Src kinase are co-expressed, the activity of HDAC3 is increased (Fig. 4C). However, wild type Src fails to increase phospho-defective HDAC3 (3YA), indicating that Src activates HDAC3 by phosphorylating Y325, Y328 and Y331 of HDAC3. To assure the effect of Src kinase in regulation of HDAC3 function, the HDAC3 activity is examined in condition which kinase dead mutant Src (SrcK298M) and wild type HDAC3 are co-expressed. As expect, wild type Src, but not kinase dead mutant Src, increases HDAC3 activity (Fig. 4D). Taken together, the activity of HDAC3 is increased by Src.



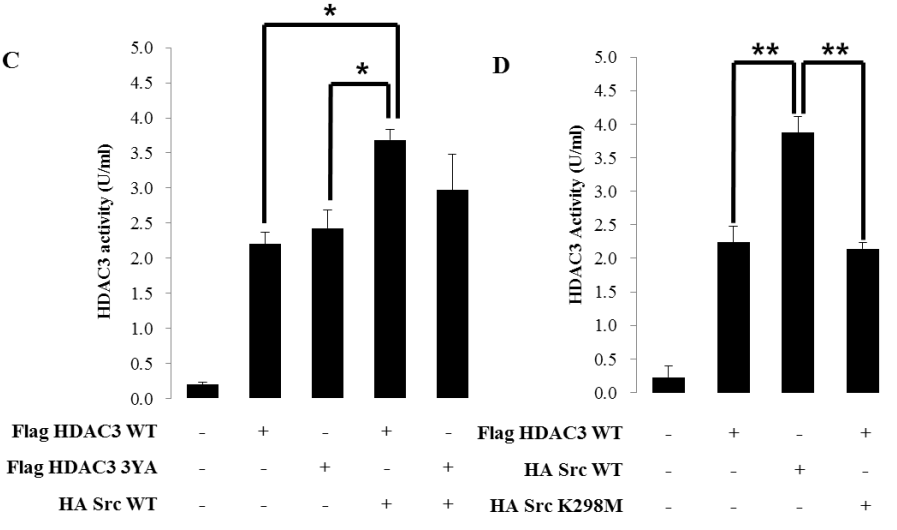


Figure 4. Src increases the activity of HDAC3. (A) Validation of HDAC3 activity using HDAC3 assay kit. HEK293FT cells were transiently transfected with the indicated sets of plasmids. Whole cell lysates were immunoprecipitated with flag M2 beads. They were prepared for sample of HDAC3 assay. Error bars, SD (n=3) *P<0.05. (B) The activity of wild type HDAC3 does not show notable difference with phospho-deficient HDAC3 (3YA). HEK293FT cells were transiently transfected with the indicated sets of plasmids. Whole cell lysates were immunoprecipitated with flag M2 beads. They were prepared for sample of HDAC3 assay. (C) Phospho-deficient mutant HDAC3 (3YA) blocks enhancement of HDAC3 activity mediated by Src kinase. HEK293FT cells were transiently transfected with the indicated sets of plasmids. Whole cell lysates were immunoprecipitated with flag M2 beads. They were prepared for sample of HDAC3 assay. Error bars, SD (n=3) *P<0.05. (D) Kinase dead Src cannot increase HDAC3 activity. HEK293FT cells were transiently transfected with the indicated sets of plasmids. Whole cell lysates were immunoprecipitated with flag M2 beads. They were prepared for sample of HDAC3 assay. Error bars, SD (n=3) **P<0.005.

5. Src-mediated phosphorylation of HDAC3 regulates cancer cell proliferation

Src is upregulated in various cancers like breast cancers and promotes proliferation of cancer cells. HDAC inhibitors are used as cancer drugs in breast cancer and the efficacy of HDAC inhibitors is proved.[73-75,82] Based on these reports, It was hypothesized that HDAC3 phosphorylation sites by Src may affect cancer cell proliferation. First, endogenous gene expression level of HDAC3 and Src was checked in breast cancer cells (SKBR3, BT20 and MDA-MB-231). It was confirmed that SKBR3, one of HER2 subtype breast cancer cell, maintained high phosphorylation level of Src. (Fig. 5A) It was also checked that protein and mRNA expression of Src was higher than the others. (Fig. 5A,B) On the other hand, protein and mRNA expression of HDAC3 was broadly similar among three breast cancer cell. (Fig. 5A,B) It was required that the stable overexpression of HDAC3 to magnify proliferation effect by phosphorylated HDAC3. Hence, SKBR3, BT20 and MDA-MB-231 were created to stable overexpression cell line. It was found that the cells expressing mutant HDAC3 (3YA) were inhibited to be proliferated in SKBR3 (Fig. 5C) but not in BT20 and MDA-MB-231 (Fig. 5 D,E), suggesting a functional effect of Src-mediated HDAC3 phosphorylation on breast cancer cell proliferation. It was observed that the growth rate of breast cancer cells shows a distinct difference depending on the subtype of breast cancer cells.

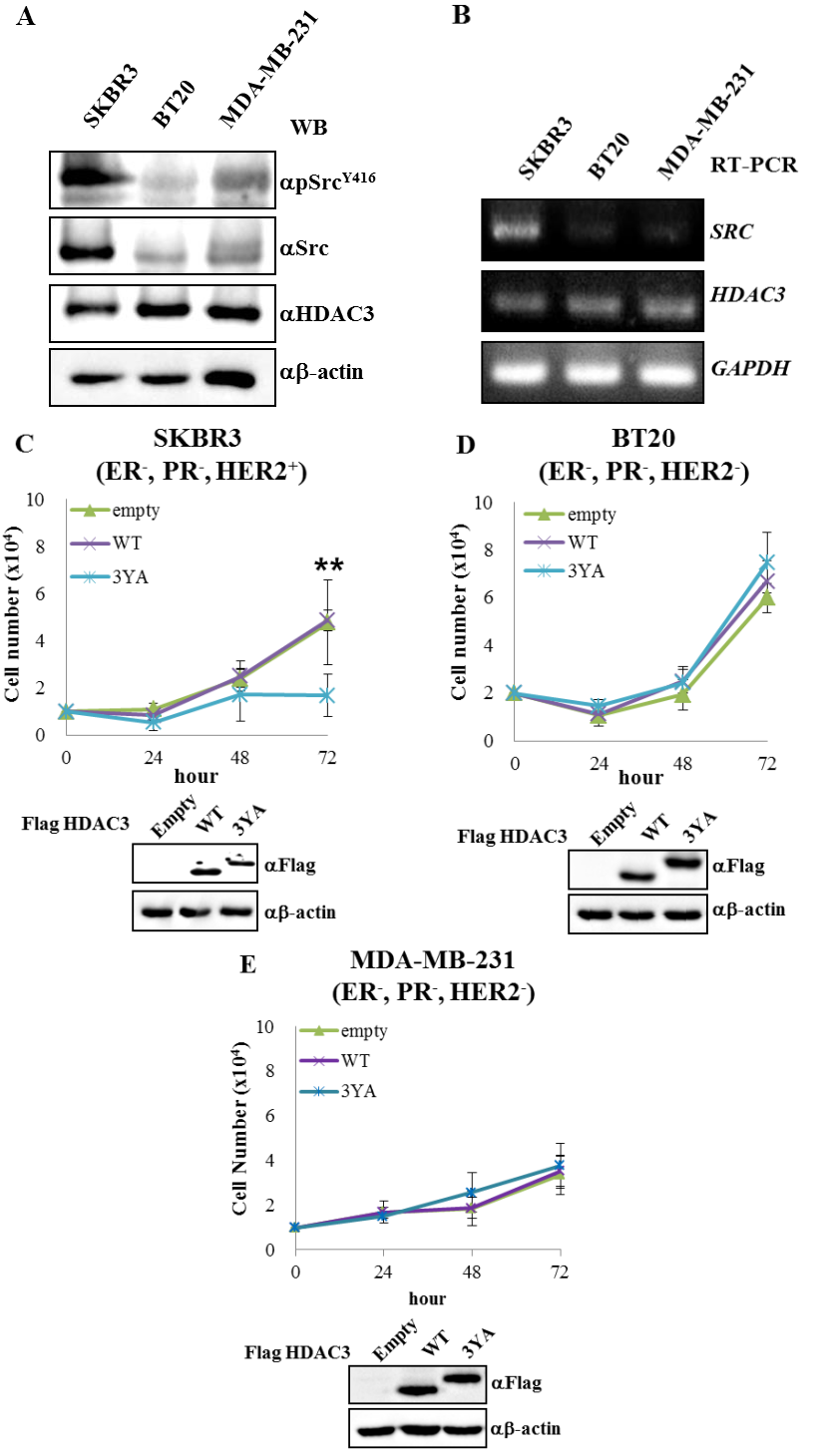


Figure 5. Src-mediated phosphorylation of HDAC3 increases the growth of breast cancer. (A) Endogenous activated Src in SKBR3 is higher than in BT20 and MDA-MB-231. Cells were harvested at 48 hours after cell seeding. Phosphorylated Src, total Src and HDAC3 protein level was analyzed by western blot. (B) The mRNA expression level of Src in SKBR3 is higher than in others. Cells were harvested at 48 hours after cell seeding. RNA was isolated from three breast cancer cell (SKBR3, BT20 and MDA-MB-231). After RNA isolation, cDNA was synthesized to analyze mRNA expression level by RT-PCR. (C, D, E) The growth rate of cell expressing phospho-deficient mutant of HDAC3 is lower than HDAC3 WT in SKBR3. SKBR3, BT20 and MDA-MB-231 stable cells were seeded at 6 well plate and incubated for 72 hours. After 48 hours, cells were supplemented with new media. Error bars, SD **P<0.005 (n=4).

IV. DISCUSSION

Histone deacetylase 3 (HDAC3) is an enzyme that catalyze the removal of acetyl groups from the lysine residues of both histone and non-histone proteins. Until now, it has been demonstrated that the activity of HDAC3 is increased phosphorylation by CK2 in a phosphorylation-dependent manner. In this study, it was found that other kinases, Src increases the activity of HDAC3 by phosphorylating tyrosine residues of HDAC3.

HDAC3 has been researched on various fields. HDAC3 was reported that it was major regulator in medullary thymic epithelial cells (mTEC) development. HDAC3 is considered as a switch in mTEC differentiation through NF-κB mediated pathway.[83] In the aspects of circadian rhythm, HDAC3 plays a role in regulating transcription of E-box which is related to circadian clock gene.[84] HDAC3 augmented the stability of BMAL1 and increased the complex of BMAL1 and CLOCK in a day, however, CRY1 which is accumulated by BMAL1-CLOCK complex competed BMAL1 and inhibited BMAL1-CLOCK complex in a night.[84] Therefore, HDAC3 controlled circadian rhythm in distinct two phases.[84] HDAC3 was highlighted as a regulator of white adipose tissue browning. In liver specific HDAC3 knockout mouse model, acetylation level of enhancer in *Pparg* and *Ucp1* gene is increased.[85] This promoted activation of *de novo* fatty acid synthesis and β-oxidation and white adipose tissues are turned into browning like adipose tissue.[85] On behalf of nerve system, HDAC3 performed a pivotal role on pathology of Huntington disease. Huntington disease is result from amplified CAG repeats in the huntingtin (*HTT*) gene,

causing to induce mutant huntingtin (*mHTT*) protein that consists of redundant glutamine residue.[86,87] HDAC3 stimulated expansions of CAG repeats in human cell.[88,89] The HDAC3 selective inhibitor alleviated Huntington disease by preventing aggregation of defective Huntington protein.[90] Regarding cardiovascular disease, HDAC3 results in enhancing recruitment of mineralocorticoid receptor (MR) and polymerase 2 on target gene promoter including glucocorticoid-induced leucine zipper (*GILZ*) and serum and glucocorticoid-regulated kinase 1 (*SGK-1*) which are component of modulating kidney function.[91] HDAC3 was also introduced as a regulator of MCP1 and target of allergic skin inflammation under triphasic cutaneous reaction.[92] Monocyte chemoattractant protein 1 (MCP1) was induced by HDAC3 and Lyn which is RTK in c-Src family.[92] It was mediated by recruitment of SP1 and c-Jun on MCP1 promoter. Finally, induction of MCP1 released histamine and showed angiogenesis which are marker of allergic skin inflammation.[92]

Not only physiological function of HDAC3, cellular function of HDAC3 has been researched. Altered expression of HDAC3 in various cancers affects a gene expression which is related to cell cycle,[93] apoptosis[94] and metastasis.[12,95] HDAC3 regulates the stability of cyclin A by decreasing acetylation level in S phase and this process inhibits G2/M transition by blocking proteasomal degradation.[93] HDAC3 prevents p53 from binding the promoter of PUMA. Thus, HDAC3 blocks p53 mediated apoptosis.[94] When the acetylation level of transcription factor forkhead box O3 (FoxO3) is lowered by HDAC3, transcriptional activity of FoxO3 is decreased.[95] Downstream of FoxO3, Dicer that suppresses metastasis is reduced and results in being more aggressive breast

cancer.[95] HDAC3-PPARγ direct association on the E-cadherin promoter demonstrated inverse correlation in activity of PPARγ in prostate cancer. [96] This repressive activity of PPARγ induced by HDAC3 downregulates E-cadherin, promoting invasiveness and being aggressive prostate cancer.[96]

Recently, the role of c-terminal region of HDAC3 has been gradually clarified. It was notable that inositol tetraphosphate (IP_4) bound to tyrosine residues at C-terminal region of HDAC3.[81] Surprisingly, the phosphorylation sites by Src kinase which was found in this study were included in IP_4 binding sites.[81] IP_4 also contributed to increase the activity of HDAC3.[81] Moreover, phosphatidylinositol metabolism and formation of IP_4 were associated with Src kinase.[97-99] Based on these reports, it was needed to study more precise molecular mechanism on regulation of HDAC3 activity at its C-terminal domain.

The connection with HDAC3 and Src may be considered in aspect of physiological effect. It was reported that the expression level of HDAC3 is periodically changed in circadian rhythm, regulating the hepatic lipid metabolism.[100] IP_4 level was changed dependent on cell cycle. It was confirmed association of HDAC3 and Src from this study, it was speculated that HDAC3 and Src may be associated in regulating circadian rhythm and cell cycle.

It was interested that HDAC3s were detected at different molecular weight dependent on mutation. It was suggested that other PTM may affect the activity of HDAC3 at c-terminal region mediating other proteins. It was also considered that conformational change of mutant HDAC3 may affect.

It was confirmed that the phosphorylation status of HDAC3 by Src is

essential for cancer cell growth. Recently, HDAC inhibitors have been highlighted as cancer drugs. A pan HDAC inhibitor, Panobinostat, and aromatase inhibitors inhibited proliferation of aromatase resistant breast cancer cell, regulating cell cycle and inducing apoptosis.[101] It is reported that combination of SNDX-275, a class I HDAC3 inhibitor, and Herceptin increases the efficacy in Herceptin resistant patients switching off Erbb3 and Akt signaling pathway.[82] According to previous study, the elevation of activated Src kinase was confirmed in Herceptin resistant breast cancer cell and patients.[102] Src kinase was considered as key modulator retaining Herceptin resistance. It was hypothesized that the HDAC3 phosphorylation by Src kinase can be involved in Herceptin resistance pathway and potentially molecular target for cancer therapies. Molecular mechanism of HDAC3 and Src in proliferation of cancer cell is needed to be further studied.

V. CONCLUSION

Src kinase phosphorylates HDAC3 and increases the activity of HDAC3. The results showed Src was one of kinase that bound to HDAC3. By domain mapping analysis, Src phosphorylated HDAC3 at C-terminal (277-428 a.a.) Interestingly, it was identified three tyrosine sites (Y325, Y328 and Y331) were critical for HDAC3 phosphorylation by Src. It was also found out these noble sites are involved in increase of HDAC3 activity by phosphorylation. Finally, the association between HDAC3 and Src expedited cancer cell proliferation. Taken our findings together, noble phosphorylation sites that were discovered may be promising target for cancer therapy under more concrete investigation.

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ABSTRACT (IN KOREAN)

타이로신 인산화 효소 c-Src 단백질에 의한 HDAC3 기능 조절 연구

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국 가 람

히스톤 탈아세틸화 효소 3(HDAC3)는 히스톤에 있는 아세틸기를 제거하여 아세틸화 수준을 감소시킨다. HDAC3는 히스톤 뿐만 아니라 다른 여러 단백질들과 결합하며 세포 기능을 조절한다. Casein kinase 2(CK2)에 의해 HDAC3 424번 세린 잔기의 인산화가 이뤄지고 HDAC3의 활성이 증가되는 것이 잘 알려져 있다. CK2뿐만 아니라 타이로신 인산화

단백질인 Src이 HDAC3와 결합하여 인산화 한다는 것이 보고되었다. 하지만 Src에 의한 HDAC3를 인산화 자리가 규명되지 않았으며 HDAC3의 활성을 조절에 대한 연구도 진행되지 않았다.

유방암은 전 세계적으로 여성들에게 발병률이 높은 암이다. 유방암은 단백질의 발현 형태에 따라 크게 luminal A, luminal B, HER2 및 triple negative breast cancer (TNBC)의 네 가지 아형으로 분류된다. 현재까지 시행되는 유방암 치료는 유방 절제술, 방사선 요법, 화학 요법, 호르몬 요법 및 표적 치료가 있으며 아형에 따라 다양한 방법으로 적절하게 수행되고 있다. 암에서의 분자 경로에 대한 이해를 바탕으로 암에서 발현이 높은 단백질에 대한 높은 특이성을 가진 표적 치료에 많은 관심을 가져 이를 토대로 치료방법이 발전 되었다. 하지만 암의 치료과정에서 암세포의 이질성에 따라 항암제에 저항성을 갖는 암세포가 생긴다. 저항성을 지닌 암세포는 성장에 관여하는 여러 신호 전달 경로를 활성화하고 세포 사멸을 피하는 기전으로 증식하여 표적 치료 요법으로 치료가 쉽지 않다. 또한 치료 후에도 재발이 많이 일어난다고 보고되었다.

본 연구에서는 Src과 HDAC3의 직접적인 결합을 GST pull down assay와 면역침강법을 통해 제시하였다. 또한 domain

mapping을 통해 Src이 HDAC3 카르복실기 말단부위(277-428번 아미노산) 와의 결합하여 3개의 타이로신 잔기(325번, 328번 331번 아미노산)를 인산화한다는 것을 밝혔다. 또한 Src과 HDAC3가 함께 고발현 될 때 HDAC3 활성이 증가되지만 HDAC3 325번, 328번, 331번의 타이로신 잔기가 알라닌으로 치환될 때 Src을 고발현 하여도 HDAC3의 활성이 증가하지 않는 것을 확인하였다. 또한 kinase 기능을 못하는 Src이 고발현 되어도 HDAC3의 활성이 증가되지 않는다는 것을 확인하였다. 이를 통해 Src이 HDAC3의 활성을 증가시키는 것을 확인하였다. 또한 유방암세포에서 인산화 일어날 수 없는 돌연변이 HDAC3가 stable expression 될 때 대조군에 비해서 증식 속도가 뚜렷하게 낮아졌다. 이를 통해 HDAC3와 Src의 발현이 유방암세포 증식에 영향을 주는 것을 확인할 수 있었다.

핵심되는 말 : 탈아세틸화효소, 유방암 , 타이로신 인산화, HDAC3, Src