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Histone deacetylases 1, 6 and 8 are critical for invasion in breast cancer

SOON YOUNG PARK*, JI AE JUN*, KANG JIN JEONG, HOI JEONG HEO, JANG SIHN SOHN, HOI YOUNG LEE, CHANG GYO PARK and JAEKU KANG

Myunggok Medical Research Institute, College of Medicine, Konyang University, Daejeon 302-718, Republic of Korea

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Abstract. Histone deacetylases (HDACs) are associated with the development and progression of cancer, but it is not known which of the HDAC isoforms play important roles in breast cancer metastasis. This study identified the specific HDAC isoforms that are necessary for invasion and/or migration in human breast cancer cell lines. MDA-MB-231 cells were significantly more invasive and expressed higher levels of matrix metalloproteinase-9 (MMP-9) compared to MCF-7 cells. We compared the expression of HDAC isoforms between MCF-7 and MDA-MB-231 cells and found greater expression of HDAC4, 6 and 8 in MDA-MB-231 cells by RT-PCR and Western blot analyses. In addition, apicidin, a histone deacetylase inhibitor, was shown to attenuate the invasion, migration and MMP-9 expression in MDA-MB-231 cells. Using specific siRNAs directed against HDAC1, 4, 6 and 8, we show that inhibition of HDAC1, 6 and 8, but not HDAC4, are responsible for invasion and MMP-9 expression in MDA-MB-231 cells. We analyzed the invasiveness of MCF-7 cells overexpressing HDAC1, 4, 6 or 8 and found that overexpression of HDAC1, 6 or 8 increased invasion and MMP-9 expression. By developing HDAC isoforms as potential biomarkers for breast cancer metastasis, the present study can be extended to developing therapies for breast cancer invasion.

Introduction

Histone deacetylases (HDACs) are emerging as critical regulators of cell growth, differentiation and apoptosis. HDAC activity in human tumors leads to conformational changes within the nucleosome, which results in the transcriptional repression of genes during migration and metastasis (1). Changes in HDAC expression levels have been associated with clinical prognosis in patients with invasive cancer, including breast cancer (2,3). HDAC1 (4-6), HDAC2 (7,8) and HDAC3 (5) have been found to be expressed at higher levels in cancer tissues. Proliferation of lung, colon and cervical cancer cell lines is reduced after HDAC8 knockdown (9), and HDAC6 mediates estrogen-induced increase in cell motility (10). However, the molecular mechanisms underlying the HDAC isoform functions during invasion remain unclear.

Cancer invasion and metastasis are the leading causes of mortality in patients with breast cancer. Tumor metastasis consists of cell adhesion, invasion and angiogenesis (11). Matrix metalloproteinases (MMPs) have been recognized as important mediators of extracellular matrix (ECM) degradation (12). MMPs and MMP inhibitors have been extensively investigated in human breast cancer clinical studies (13,14). It has been reported that MMP-2 has a role in the H-ras-induced invasive phenotype of MCF10A human breast epithelial cells (15), analogous to the role of MMP-9 expression in the metastatic phenotype of transformed rat embryo cells (16).

This study was aimed at determining the role of HDAC-dependent invasion and migration in breast cancer and examining the HDAC-dependent regulation of MMP-9 expression in breast cancer cell migration and invasion.

Materials and methods

Cell culture. Human breast cancer MDA-MB-231 and MCF-7 cells were obtained from ATCC (Manassas, VA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and incubated in a humidified atmosphere containing 5% CO_2 at 37˚C.

Reagents. Apicidin was obtained from Sigma (St. Louis, MO). Antibodies against HDAC1 and 8 were purchased from Millipore (Billerica, MA). Antibodies against HDAC2, 3 and 4 were obtained from Abcam (Cambridge, MA). Antibodies against HDAC6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Reverse transcription-PCR. Total cellular RNA (1 µg) isolated from cultured cell lines was used for reverse transcription. The

Correspondence to: Dr Chang Gyo Park or Dr Jaeku Kang, Department of Pharmacology, College of Medicine, Konyang University, Daejeon 302-718, Republic of Korea
E-mail: aruso@konyang.ac.kr
E-mail: jaeku@konyang.ac.kr

*Contributed equally

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cDNA was then amplified with primer sets for HDAC1, 2, 3, 4, 6, 8, MMP-9, 2 and GAPDH. Primer sequences are as follows: HDAC1 forward, 5'-GGAAATCTATCGCCCTCACA-3' and reverse, 5'-TTGCCACAGAACCACCAGTA-3'; HDAC2 forward, 5'-ATAAAGCCACTGCCGAAGAA-3' and reverse, 5'-TCCTCCAGCCCAATTAACAG-3'; HDAC3 forward, 5'-ACGTGGGCAACTTCCACTAC-3' and reverse, 5'- GACTCTTGGTGAAGCCTTGC-3'; HDAC4 forward, 5'-AGCGTGAGCAAGATCCTCAT-3' and reverse, 5'-GCCAAGTACTCAGCGTCTCC-3'; HDAC6 forward, 5'-TATCTGCCCCAGTACCTTCG-3' and reverse, 5'-GGACATCCCAATCCACAATC-3'; HDAC8 forward, 5'-GGTGACGTGTCTGATGTTGG-3' and reverse, 5'-GACACTTGCCAATTCCCACT-3'; GAPDH forward, 5'-CATCTTCCAGGAGCGAGA-3' and reverse, 5'-CTGCTTCACCACCTTCTTGAT-3'; MMP-9 forward, 5'-CATCGTCATCCAGTTTGG-3' and reverse, 5'-GATGGATTGGCCTTGGAA-3' and MMP-2 forward, 5'-GGCCTCTCCTGACATTGACCTT-3' and reverse, 5'-GGC CTCGTATACCGCATCAATC-3'.

Western blotting. Cells were plated in 6-well plates and treated with apicidin for 24 h. After treatment, cells were harvested and washed twice with PBS at 4˚C. Total proteins were prepared by cell lysis in 200 μl of ice-cold RIPA buffer, and lysates were resolved by 10 or 12% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with blocking buffer containing 5% non-fat dry milk for 1 h at room temperature and incubated overnight at 4˚C with the antibodies indicated. After washing for 1 h with TBS containing 0.1% Tween-20, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-goat antibodies for 1 h. After washing three times with TBS containing 0.1% Tween-20, the immunoreactive bands were visualized using ECL.

Small interfering RNA (siRNA) and plasmid transfection. Specific siRNAs for HDAC1, 6, 8 (sc-29343, sc-35544, sc-35548, Santa Cruz Biotechnology) and HDAC4 (J-003497-07-0005, Dharmacon, Lafayette, CO) or a scrambled siRNA (Invitrogen, Carlsbad, CA) were obtained. HDAC1 and HDAC4 constructs were kindly provided by Dr S.L. Schreiber (Howard Hughes Medical Institute, Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA) and Dr E. Seto (Moffitt Cancer Center, University of South Florida, Tampa, FL), respectively. HDAC6 and HDAC8 constructs were purchased from Addgene (Cambridge, MA). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Invasion assay. Invasion assays were performed in triplicate, using a 48-well microchemotaxis chamber with 8-μm-pore membranes (Neuroprobe) pre-coated with 10 μg/ml Matrigel (BD Bioscience). The bottom chamber was filled with 1% FBS/RPMI (MDA-MB-231) or 20% FBS/RPMI (MCF-7), and then cells that had been treated with apicidin for 24 h were placed in the upper chamber and incubated at 37˚C for 24 h. The membranes were fixed and stained with Diff-Quik reagents (Dade Behring, Inc., Deerfield, IL).

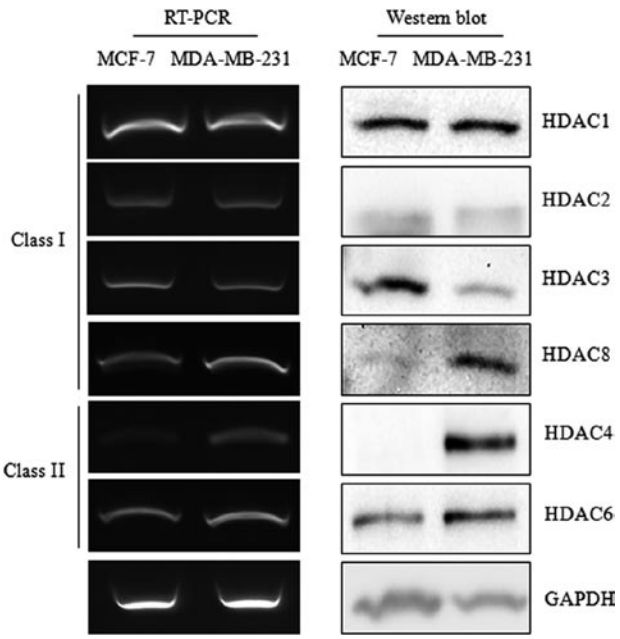

Figure 1. Expression of HDACs in breast cancer cells. Total RNA and cell lysates were prepared from MCF-7 and MDA-MB-231 cells. The levels of mRNA and protein were determined by RT-PCR (left) and Western blotting (right), respectively.

Wound healing assay. Cells were grown to confluence in 6-well plates and serum starved overnight. Cells were scraped with a P200 pipette tip and treated with apicidin for 24 h. Migration of cells into the wound was examined by phase contrast microscopy. Photographic images were obtained immediately after scraping and after 24 h in the same locations.

Statistical analysis. Results are expressed as the mean ± standard deviation (SD), and an analysis was performed by a one-way Student's t-test.

Results

Apicidin inhibits invasion and migration in breast cancer cells. We first compared the expression of HDAC isoforms in two cell lines, MDA-MB-231 (a highly metastatic breast cancer cell line) and MCF-7 (a less invasive breast cancer cell line). HDAC1 and HDAC2 expression patterns were similar in both cell lines. However, the expression of HDAC4, 6 and 8 was higher in MDA-MB-231 cells compared to MCF-7 cells (Fig. 1).

We next examined whether apicidin, an inhibitor of histone deacetylases, inhibited invasion and migration of MDA-MB-231 cells. As shown in Fig. 2A and B, MDA-MB-231 cells exhibited greater invasion and migration than MCF-7 cells. Apicidin treatment of MDA-MB-231 cells inhibited invasion and migration in a dose-dependent manner.

Apicidin has been reported to inhibit MMP-2 activity more than MMP-9 in human breast epithelial cells (17). To test the effect of apicidin on MMP activity, we treated cells with increasing amounts of apicidin and observed mRNA expression using RT-PCR. Apicidin inhibited MMP-9 expression in MDA-MB-231 cells (Fig. 2C) but did not inhibit MMP-2 activity (data not shown).

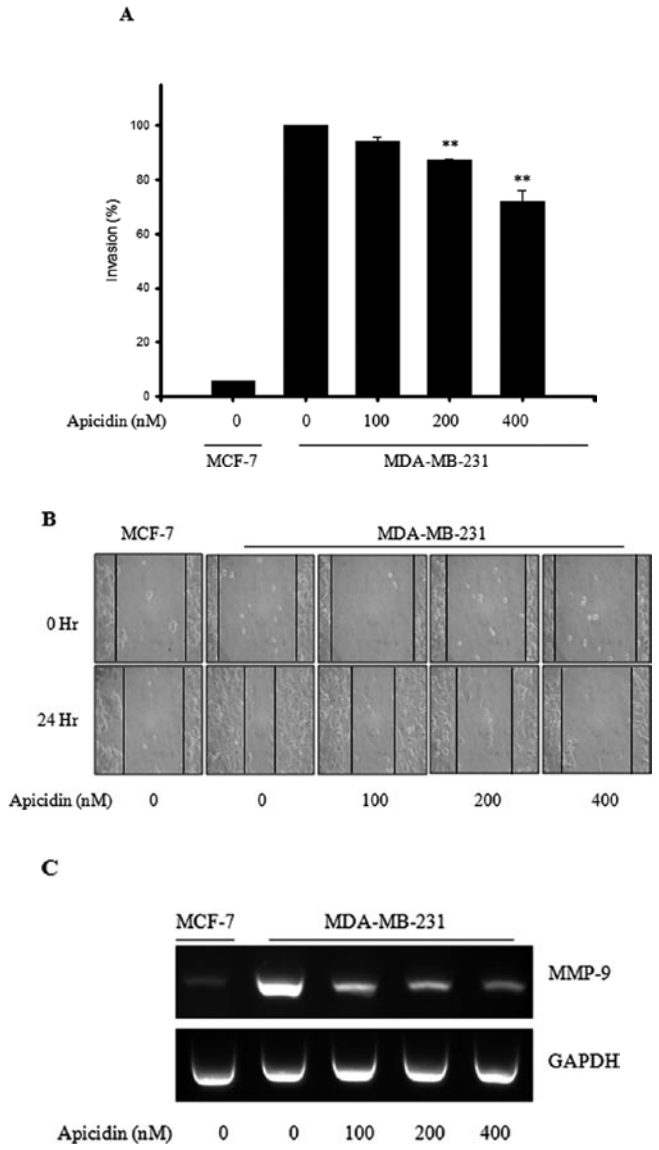

Figure 2. Apicidin inhibits invasion and migration in breast cancer cells. (A and B) Cells were treated with the indicated concentrations of apicidin for 24 h prior to an invasion assay (A) or a wound healing assay (B). (C) Cells were serum starved for 24 h and then treated with the indicated concentrations of apicidin for 24 h. Expression of mRNA was then determined by RT-PCR. Results show the mean ± standard deviation (SD) of three independent experiments performed in triplicate. $^{**}p<0.01$ vs. control.

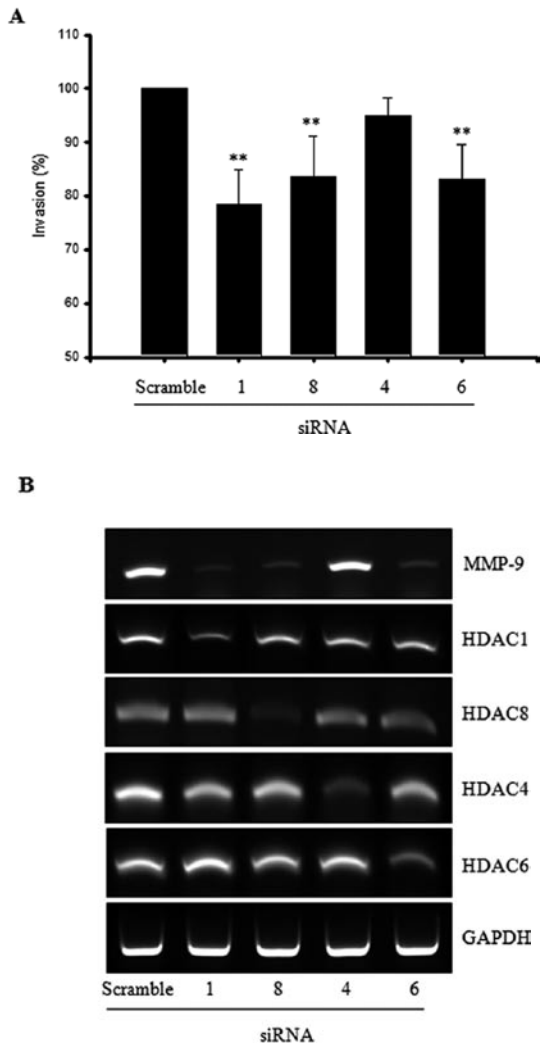

Figure 3. Specific HDAC siRNA decreases invasion and MMP-9 expression. MDA-MB-231 cells were transfected with either scrambled or specific HDAC siRNA for 48 h, followed by an invasion assay (A) and RT-PCR (B). Results show the mean ± standard deviation (SD) of three independent experiments performed in triplicate. $^{**}p<0.01$ vs. control.

Specific HDACs involved in invasion and MMP-9 expression. The comparison of HDAC expression between MDA-MB-231 and MCF-7 cells indicated that high expression of HDAC4, 6 and 8 was significantly associated with invasion. In addition, HDAC1 has been previously demonstrated to be associated with invasion (18). Based on these criteria, HDAC1, 4, 6 and 8 were selected for further study. We used specific HDAC siRNA to determine which HDACs were involved in invasion. Treatment of MDA-MB-231 cells with HDAC1, 6 and 8 siRNAs inhibited invasion (Fig. 3A). Each specific siRNA caused a marked decrease in specific mRNA levels without affecting the mRNA levels of the other HDACs. The knockdown of HDAC1, 6 and 8 caused decrease of MMP-9 mRNA expression (Fig. 3B). However, HDAC4 knockdown did not affect invasion or MMP-9 expression.

To confirm the effects of HDACs on cell invasion, we established MCF-7 cells that overexpress HDAC1, 4, 6 or 8 DNA constructs by transient transfection. After 48 h, we performed invasion assays and found that the overexpression of HDAC1, 6 and 8 in MCF-7 cells resulted in increased invasion (Fig. 4A). More importantly, overexpression of these HDAC isoforms in MCF-7 cells also increased MMP-9 mRNA expression (Fig. 4B).

Discussion

In this study, we examined whether HDAC isoform status plays a role in the differential responses of MCF-7 and MDA-MB-231 cells. Many reports have correlated HDAC expression levels with clinical prognosis in patients with invasive cancers, including breast cancer (2,3,6,10,19). This study is the first to investigate the expression of various HDAC isoforms. It has been proposed that HDAC expression leads to increased MMP expression and cell invasion. Apicidin prevents an H-ras-induced invasive phenotype (17). Our data suggest that treating

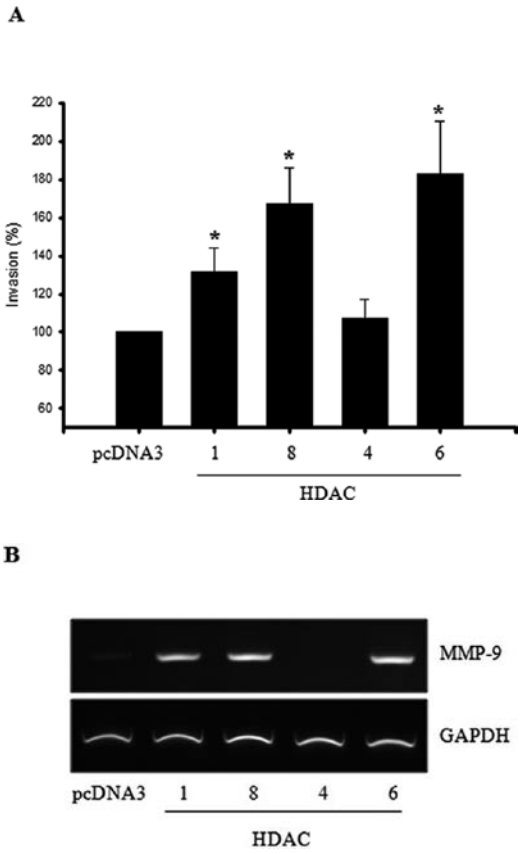

Figure 4. HDAC1, 6 and 8 are involved in invasion and MMP-9 expression. MCF-7 cells were transfected with pcDNA3 (vector) or HDAC1, 4, 6 or 8 for 48 h, followed by an invasion assay (A) and RT-PCR (B). Results show the mean ± standard deviation (SD) of three independent experiments performed in triplicate. $^{*}p<0.05$ vs. control.

MDA-MB-231 cells with apicidin inhibits invasion, migration and MMP-9 expression in a dose-dependent manner.

HER-2/neu inhibits the metastasis suppressor RECK via an Sp1- and HDAC1-dependent mechanism, to promote cell invasion (20). In addition, HDAC1 has been found to be involved in the migration and invasion of cancer cells (18). In this study, HDAC1 expression was similar in both MDA-MB-231 and MCF-7 cell lines but was also found to be important for invasion. Cell lines representative of ER-positive (MCF-7) and ER-negative (MDA-MB-231) human breast cancer cells can both differentiate to invasion. An ER-negative and invasive human breast cancer cell line, MDA-MB-231, has constitutively higher mitogen-activated protein kinase (ERK1&2/MAPK) when compared to the ER-positive and non-invasive MCF-7 (21). Thus, the importance of HDAC1 in breast cancer cells will be the topic of future investigations.

We found that MDA-MB-231 cells express higher levels of HDAC4, 6 and 8 than MCF-7 cells. Specific knockdown of HDAC isoforms in MDA-MB-231 cells (silencing of HDAC1, 6 and 8) abolished invasion and MMP-9 expression. Reconstitution of HDAC1, 4, 6 and 8 in MCF-7 cells, as well as overexpression of HDAC1, 6 and 8 but not 4, increased invasion and MMP-9 expression. HDAC4 is a key mediator of p53-dependent cancer cell growth arrest in response to DNA damage (22-24); therefore, we propose that HDAC4 acts as an anti-proliferative and a pro-survival factor for cancer cells.

Our data suggest that inhibitor and siRNA of HDACs abolished breast cancer invasion by regulating the expression of factors involved in the degradation of the ECM. MMP-2, 9, 11, 13 and 14 are known to enhance the invasiveness of MDA-MB-231 cells (25,26). HDACs are known to regulate the expression of MMPs (27,28). Therefore, the identification of cancer-related HDAC isoforms has therapeutic interest in oncology for more specific HDAC inhibitors with fewer side effects for patients.

In conclusion, we have examined the functional role of HDACs in invasion according to their expression in MCF-7 and MDA-MB-231 cells. Our findings implicate HDAC1, 6 and 8 in the progression of breast cancer cells.

Acknowledgements

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