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Heme Oxygenase-1 Inhibits Tumor Metastasis Mediated by Notch1 Pathway in Murine Mammary Carcinoma

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Heme oxygenase-1 (HO-1) plays an important role in the progression of several malignancies including breast cancer. However, its role in breast cancer metastasis is still ambiguous. In this study, we observed the effect of HO-1 on mouse mammary carcinoma metastasis using the in vivo tumor metastasis model. Our results revealed that overexpression of HO-1 strongly inhibits the lung metastasis of 4T1 cells. In in vitro analysis, associated indices for epithelial–mesenchymal transition (EMT), migration, and proliferation of 4T1 cells were evaluated. The results show that HO-1 inhibits EMT, migration, and proliferation of 4T1 cells. In addition, the Notch1/Slug pathway is found to mediate an antimetastasis role of HO-1 in mouse mammary carcinoma. In conclusion, since HO-1/Notch1/Slug axis plays an important role in breast cancer metastasis, induction of HO-1 could be used as a potential therapeutic strategy for breast cancer treatment.

Key words: Murine mammary carcinoma; Heme oxygenase-1 (HO-1); Tumor metastasis; Epithelial–mesenchymal transition (EMT); Notch1 signaling

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide, with an estimate of 1.7 million cases and 521,900 deaths in 2012[1]. In the recent years, encouraging progress has been made with regard to the diagnosis and treatment of breast cancer[2–4]. However, metastasis and recurrence still remain as the critical clinical events in breast cancer. Even in node-negative breast cancer patients, 25% of the patients develop metastasis. The 5-year survival rate is dramatically lowered among patients with distant metastasis[5]. Therefore, extensive efforts are required to explore novel therapeutic targets to control the metastasis and improve the prognosis among breast cancer patients.

Breast cancer is a malignant tumor with a strong tendency to metastasize, and epithelial–mesenchymal transition (EMT) is considered to play a vital role in cancer metastasis[6]. During tumorigenesis processes, changes in EMT regulatory pathways lead to a loss of cellular adhesions, changes in the polarization of the cell and cytoskeleton, detachment, migration, intravasation, survival in the vascular system, extravasation, and finally, metastasis[6]. The EMT is classically characterized by the dedifferentiation from an epithelial to mesenchymal phenotype, which is marked by the decrease in E-cadherin expression and the increase in vimentin expression[7]. Moreover, EMT-inducing transcriptional factors, such as Snail, Slug, Twist, and FOX2, are affected during the EMT process[8]. Slug and Snail are two known important transcription factors that initiate EMT through the downregulation of E-cadherin expression in breast cancer, and their expression has been shown to be regulated by Notch signaling[9]. Interestingly, Slug expression presents a much stronger correlation with loss of E-cadherin than Snail expression in breast cancer cell lines[10]. Shao et al. recently demonstrated that Notch1 signaling regulates the EMT and invasion of breast cancer cells in a Slug-dependent manner[11]. The Notch signaling pathway is dysregulated in many human malignancies. Moreover, overexpression of Notch1 is related to the poor survival of human breast cancer patients[12].

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Heme oxygenase (HO) is the rate-limiting enzyme in heme metabolism by catalyzing degradation of the heme group into carbon monoxide (CO), free iron, and biliverdin. At least three mammalian HO isoforms have been identified. HO-1 expression is highly increased by stressful conditions[13]. It participates in maintaining cellular homeostasis and plays an important protective role in the tissues by reducing the oxidative injury, attenuating the inflammatory response, inhibiting cell apoptosis, and regulating cell proliferation. Accumulating evidences demonstrate that HO-1 overexpression is implicated in the pathogenesis and progression of several types of malignancies, including breast cancer[14–16]. However, its role in tumor progression remains controversial.

In this study, we showed that overexpression of HO-1 could significantly decrease the number of lung metastatic nodules in a breast cancer metastasis model. We also demonstrated that overexpression of HO-1 may lead to suppression of EMT process, tumor growth, and migration using the in vitro model. Moreover, we explored the mechanisms by which HO-1 mediates EMT and cell migration in tumor cells. HO-1 suppressed Notch signaling pathway activation and its downstream target Slug transcription through downregulation of Notch1 mRNA and protein expression, which at least partially inhibits EMT and cell migration. These observations suggest that induction of HO-1 might be a promising therapeutic strategy for preventing breast cancer progression.

MATERIALS AND METHODS

Cell Culture, Treatment, and Drug Preparation

The mouse mammary carcinoma cell line 4T1 was purchased from Shanghai Sciencelight Biology Sci&Tech Co., Ltd. (Shanghai, P.R. China). 4T1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2. The γ-secretase inhibitor (DAPT; N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycinet-butylester, $C_{23}H_{26}F_2N_2O_4$) was purchased from MedChem Express (HY-13027; Shanghai, P.R. China). DAPT was dissolved in DMSO to a stock concentration of 10 mmol/L and was diluted to final concentrations of 20 μmol/L with conventional culture medium just prior to use. The control group was treated with conventional medium containing the mean volume of DMSO carrier only.

Cell Transfection

4T1 cells were seeded at a density of 2×10^5 cells in a six-well plate and grown to 60–70% confluency in growth media. We transfected 4T1 cells with pcDNA3.1(+) containing mouse HO-1 using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The stable cell lines were selected with 500 μg/ml G418 (Sigma-Aldrich, Shanghai, P.R. China). The pHAGE-mHO-1 and pHAGE were transiently transfected into 4T1 cells using the Lipofectamine 2000 transfection reagent.

RNA Interference

The vectors containing shRNA targeting mNotch1 were purchased from VectorBuilder (Guangzhou, P.R. China). They included mNotch1 shRNA#1 (ID: VB180621-1113puz), mNotch1 shRNA#2 (ID: VB180621-1114zmv), and Scramble shRNA (ID: VB151023-10034). Cells (5×10^4 per well) were seeded in six-well plates 24 h prior to transfection and transfected with 2.5 μg of DNA vector containing shRNA using Lipofectamine 3000 transfection reagent (Invitrogen), according to the manufacturer's protocol.

In Vivo Tumor Model

BALB/c female mice (aged 4 weeks) were purchased from Shanghai Laboratory Animal Center (Shanghai, P.R. China). All animal experiments were approved by the Institutional Animal Care and Use Committee of Harbin Medical University. The animal experiments were carried out in accordance with EU Directive 2010/63/EU. Mice housed under identical conditions were allowed free access to a standard diet and tap water with a 12-h light:12-h dark cycle. Four-week-old female mice were injected with 1×10^5 stably transfected pcDNA3.1(+)-mHO-1 cells or pcDNA3.1(+) in the left mammary fat pad[17]. Tumor size was measured using a caliper every 2 days. Both the maximum (L) and minimum (W) lengths of the tumors were measured, and the tumor size was calculated as $(L\times W^2)/2$. After 24 days, the mice were sacrificed and photographed. Four-week-old female mice were injected with 1×10^6 stably transfected pcDNA3.1(+)-mHO-1 or pcDNA3.1(+) cells via the tail vein. After 17 days, the mice were sacrificed and photographed. The lungs were harvested for paraffin embedding, sectioning, and hematoxylin and eosin (H&E) staining. Five animals were included in each group.

Histopathology

The H&E and immunohistochemical (IHC) staining were performed according to the classical procedures. The concentration of primary antibody was 1:400 for E-cadherin and 1:100 for vimentin.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated with an RNA extraction kit (AxyGen, Union City, CA, USA), according to the manufacturer's protocol, and the concentration of total RNA was measured with a Nanodrop 2000c. RNA (1 μg) was converted to cDNA by reverse transcriptase (Promega, Madison, WI, USA). With specific primers, the

quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using an ABI 7500 PCR System (Applied Biosystems, Mannheim, Germany). The annealing temperature for HO-1, E-cadherin, vimentin, Notch1, and Slug was 60°C. The annealing temperature for Hes-1 and Hey-1 were 61°C and 57°C individually. Actin was used as the endogenous control for the detection of mRNA expression level. The oligonucleotide primer sequences are shown in Table 1.

Western Blot Analysis

Total cell or tissue extracts were extracted using cell lysis buffer followed by immunoblotting with anti-E-cadherin (1:1,000; Cell Signaling Technology, Danvers, MA, USA), anti-vimentin (1:1,000; Cell Signaling Technology), anti-Slug (1:1,000; Cell Signaling Technology), anti-Notch1 (1:1,000; Cell Signaling Technology), anti-HO-1 (1:500; the ani-HO-1 antibody was provide by Professor Hong Zhou), and anti-β-actin (1:4,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunofluorescence

A total of 2×10^5 cells per well were grown on glass coverslips in a six-well plate overnight. The next day, when the cells were 50%–70% confluent, they were washed twice with PBS, fixed in 4% paraformaldehyde solution, and permeabilized in 0.03% Triton X-100 (Sigma-Aldrich) in PBS for 20 min. The cells were then washed three times with PBS and blocked with 5% BSA in PBS for 1 h at room temperature. The cells on the coverslips were incubated in a humidified box with the respective primary antibodies (E-cadherin, CST; vimentin, CST; F-actin, CST) at a 1:100 dilution overnight at 4°C. After which, the cells were washed three times in PBS and incubated for 1 h with secondary antibodies at a 1:50 dilution (Abcam, Cambridge, MA, USA) at room temperature in the dark. Finally, the cells were washed three times in PBS and incubated with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Roche, Indianapolis, IN, USA) for 5 min at room temperature in the dark. Then the slides were washed extensively with PBS and observed with a confocal microscope (Nikon, Tokyo, Japan) with identical exposure times at 400× magnification.

Cell Migration Assay

To detect the ability of cells to migrate in vitro, we used the Transwell chamber assay. Briefly, 4T1 cells (2×10^5) were placed in the upper compartment of a 24-well Transwell unit with 8-μm polycarbonate nucleopore filters (Corning Costar, Cambridge, MA, USA). Medium containing 10% fetal bovine serum was added to the lower compartment, and the cells were incubated for 24 h. The cells were then fixed and counted as previously described[18].

Wound Healing Assay

Wound healing assay was also applied to evaluate the cell migratory ability. Cells were seeded in 3.5-cm plates and grown to a density of 70%–80%. Then a 200-μl pipette tip was used to create an artificial wound of scratched cells. The migrating distance was measured after 24 h.

Cell Viability Assay

Cell viability was determined using a 3-(4,5-dimethylthiazole-2-yl)-2.5- diphenyltetrazolium bromide (MTT) assay as described previously[18].

Colony Formation Assay

To investigate the colony formation ability, 4T1 cells were transfected with pcDNA3.1(+) or pcDNA3.1(+)-mHO-1 and subsequently seeded in 6-cm plates (1,000 cells/dish) and incubated for 2 weeks to allow for colony formation. The colonies were then fixed in methanol, stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA), and counted.

Statistical Analysis

SPSS V20.0 was used for the statistical analysis. All values are expressed as the mean ± SEM, and all experiments were repeated at least three times. Student *t*-tests were used to determine the statistical significance of the

Table 1. The Oligonucleotide Primer Sequences Used in This Study

Gene	Sense Primer	Antisense Primer
HO-1	5′-GAAGAACTTTCAGAAGGGTCAG-3′	5′-TCGTCGGAGACGCTTTACATAG-3′
E-cadherin	5′-AGCTTGCGGAAGTCAGTTCA-3′	5′-TGTAGCTCTCGGCGTCAAAG-3′
Vimentin	5′-AGCAGTATGAAAGCGTGGCT-3′	5′-CTCCAGGGACTCGTTAGTGC-3′
Notch-1	5′-GGAGCGTATGCACCACGATA-3′	5′-ACTTCTTCCTCCGTGCCTTG-3′
Slug	5′-CATCCTTGGGGCGTGTAAGT-3′	5′-ATGGCATGGGGGTCTGAAAG-3′
β-actin	5′-TACCTCATGAAGATCCTCACC-3′	5′-TTTCGTGGATGCCACAGGAC-3′
Hes-1	5′-CAACACGACACCGGATAAAC-3′	5′-TTCAGCTGGCTCAGACTTTC-3′
Hey-1	5′-GTTAACTCCTCCTTGCCCGC-3′	5′-CTCGATGATGCCTCTCCGTC-3′

differences between groups. Differences with a value of $p < 0.05$ were considered significant.

RESULTS

Overexpression of HO-1 Inhibits the Lung Metastasis of 4T1 Cells

To investigate the role of HO-1 in the metastasis of breast cancer in vivo, we constructed stable HO-1-transfected cells termed as mHO-1 (containing the mouse HO-1 expression vector) and mock (containing the empty vector). We confirmed the overexpression of HO-1 protein in the reconstituted 4T1 cell lines by Western blot analysis shown in Figure 1A. Clones 3 and 8 (mHO-1#3 and mHO-1#8) were selected for the subsequent lung metastasis model of murine mammary carcinoma in BALB/c mice. The mice were sacrificed after 17 days of inoculation. The lungs were separated to determine the metastasis potential of different HO-1 level cell clones. As shown in Figure 1B and D, significant reduction of lung metastasis nodules was observed in the mHO-1#3 and mHO-1#8 clone groups compared with the mock group. This result was confirmed by H&E stains of lung tissues in Figure 1C. These results suggest that HO-1 overexpression suppresses the lung metastasis of 4T1 cells in vivo.

HO-1 Suppresses the EMT Process of 4T1 Cells

The EMT process plays a pivotal role in the essential and initial step during the progression of tumor metastasis. To explore the role of HO-1 in the EMT process, we examined the expression of E-cadherin (epithelial marker) and vimentin (mesenchymal marker) in the lung metastasis model above by IHC staining. The expression of E-cadherin was significantly high, while the expression of vimentin was downregulated in mHO-1#8 group mice (Fig. 1E and F). Moreover, immunofluorescent staining and Western blot, together with qRT-PCR, were used to examine the expression of E-cadherin and vimentin, respectively, in the 4T1 cells to confirm the result shown in IHC. Consistent with the IHC finding, 4T1 cells that transiently transfected with pHAGE-mHO-1 (mHO-1) for 24 h showed a significant increase in E-cadherin expression together with the reduction of vimentin in the cell membrane compared

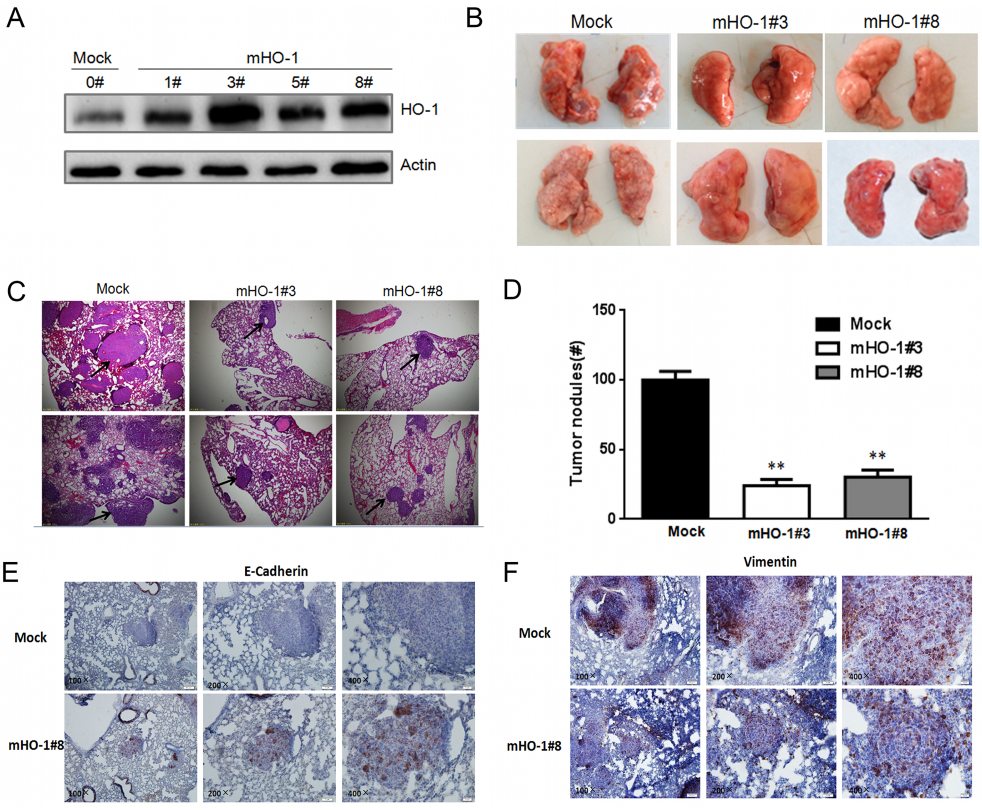

Figure 1. Overexpression of HO-1 inhibits lung metastasis of 4T1 cells. The BALB/c mice were injected with Mock, mHO-1#3 clone and mHO-1#8 4T1 cells via the tail vein. (A) The expression levels of HO-1 were determined by Western blot. (B) Lungs were removed from mice, representative images of lung were shown, and (C) the number of metastatic nodules on the lungs was counted. (D) Hematoxylin and eosin (H&E) (400×) staining of metastatic nodules in the lungs of mice bearing 4T1 mock or 4T1-mHO-1. (E, F) The expression levels of E-cadherin and vimentin in tumor tissue were assayed by immunohistochemical (IHC) staining. $^{**}p < 0.01$ versus the mock group.

with the control group (Fig. 2A–D). We analyzed the morphological alteration of 4T1 cells by F-actin immunofluorescent staining after transiently transfecting with pHAGE-mHO-1 or pHAGE for 24 h. Interestingly, mHO-1 4T1 cells were characterized by obvious stone pavement epithelial appearance, while the control 4T1 cells underwent a transformation into an elongated fibroblast-like cell morphology (Fig. 2E). Collectively, these results indicate that HO-1 can inhibit the EMT progress in 4T1 cells.

Overexpression of HO-1 Inhibits the Migration and Retards the Growth of 4T1 Cells

To confirm the role of HO-1 in migratory ability, we performed the Transwell and wound healing assays. As shown in Figure 3A and C, the cell migratory ability was significantly reduced in mHO-1#3 and mHO-1#8 4T1 cells compared to the mock group. Wound healing assays revealed a delayed wound closure ability of HO-1 overexpressing 4T1 cells (Fig. 3B and D). Taken together, these data demonstrate that HO-1 suppresses the migratory ability of 4T1 cells.

Next, we analyzed the effect of HO-1 on tumor growth in female BALB/c mice. Mice were injected with different tumor cell lines in the mammary fat pad. The volume of tumors was monitored every 2 days for growth curve. Mice were sacrificed after 24 days, and the tumors were dissected for photograph and weight (Fig. 3H and J). As shown in Figure 3I, the tumor volume of the mHO-1#3 clone seemed to be much smaller than that in the mock group. Additionally, colony formation assay (Fig. 3E and F) and MTT experiment (Fig. 3G) were performed to investigate the effect of HO-1 on cell growth in 4T1 cells.

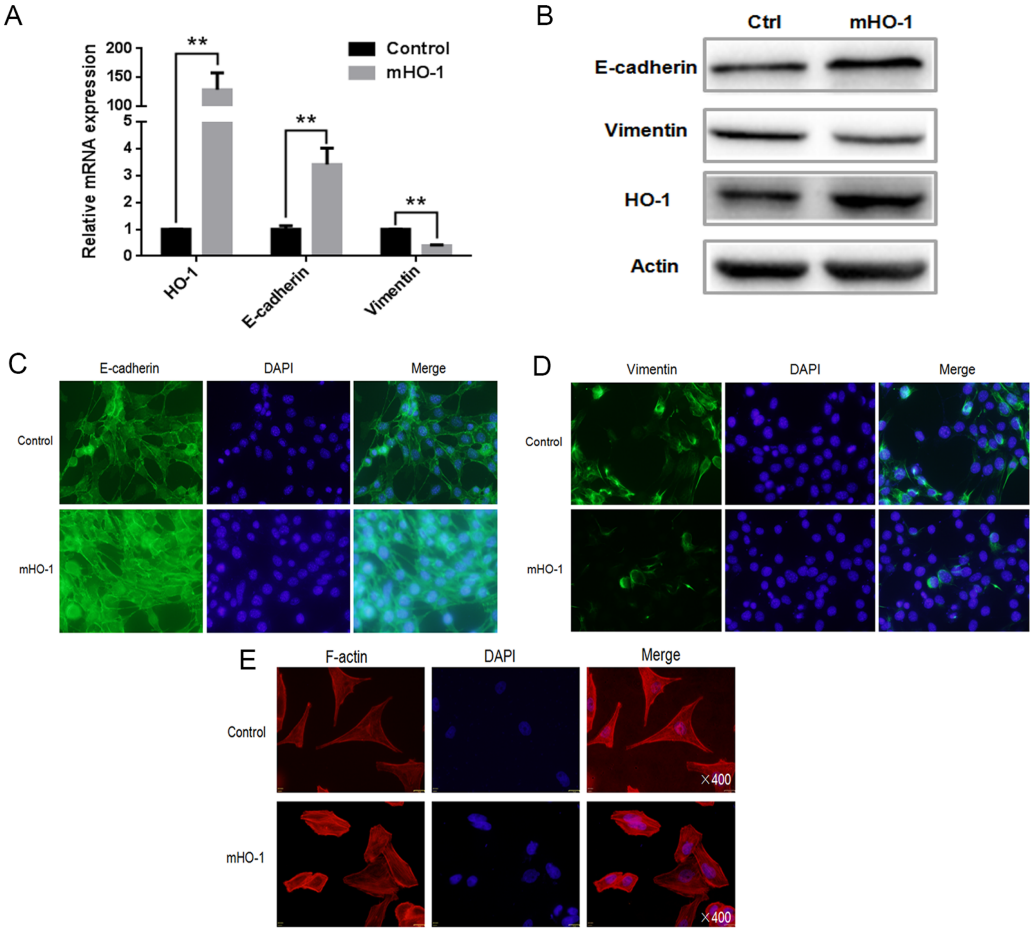

Figure 2. Characteristics of epithelial–mesenchymal transition (EMT) after the expression of HO-1 is upregulated in 4T1 cells. (A) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of HO-1 and the EMT-related markers E-cadherin and vimentin after 4T1 cells were transfected with pHAGE or pHAGE-mHO-1. Actin was used as a normalization control. (B) Total protein was extracted for Western blot analysis of HO-1 and the EMT-related markers E-cadherin and vimentin in 4T1 cells. Actin was used as a loading control. (C, D) Immunofluorescence analysis of E-cadherin and vimentin in 4T1 cells (magnification: 400×). DAPI staining was used to detect nuclei and is merged with E-cadherin and vimentin in their respective panels. (E) Morphological changes of 4T1 cells were observed by immunofluorescence analysis of F-actin (magnification: 400×). 4,6-Diamidino-2-phenylindole (DAPI) staining was used to detect nuclei, and the figures were merged with F-actin in their respective panels. $^{**}p<0.01$ versus the control group.

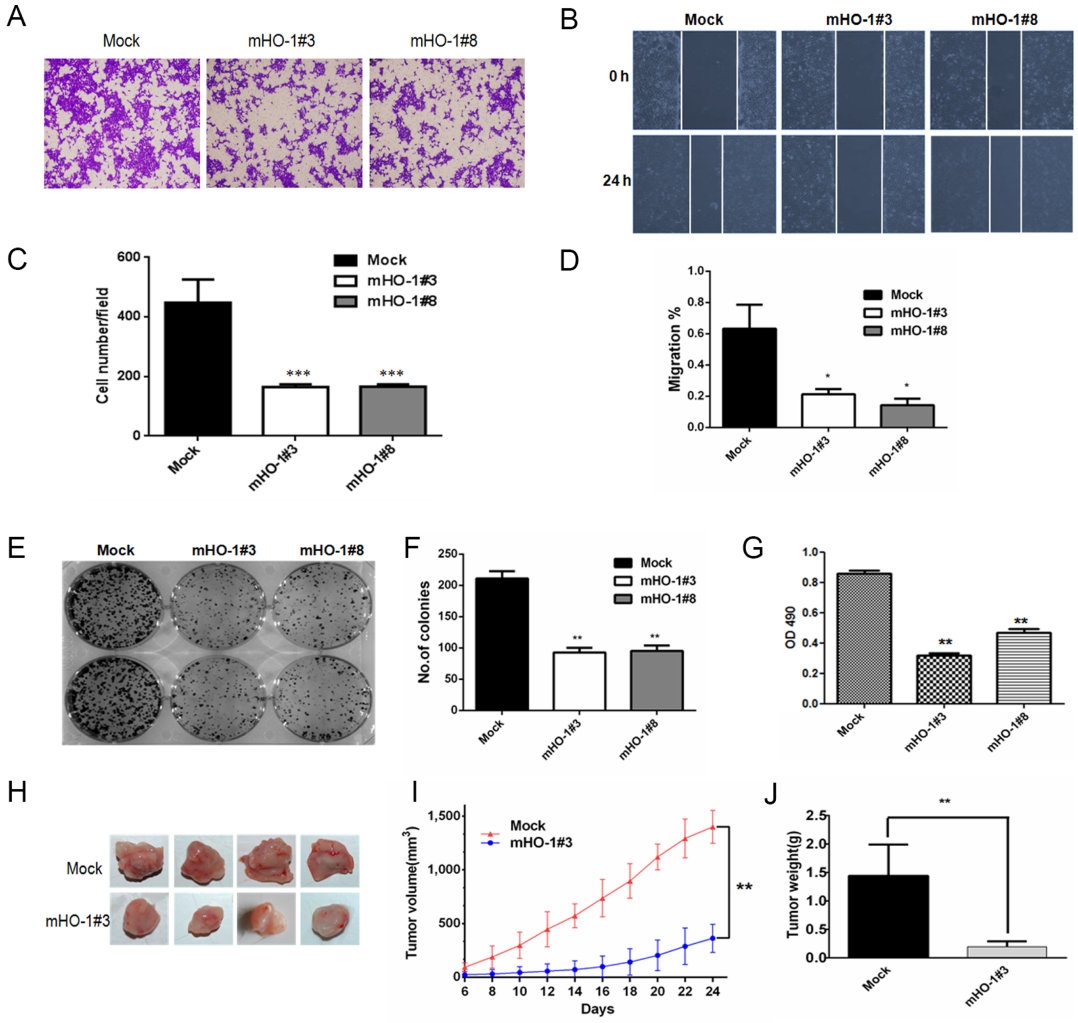

Figure 3. HO-1 inhibits the migration and retards the growth of 4T1 cells. The cells were seeded into a migration chamber and incubated for 24 h. The number of migrated cells was quantified by counting the numbers of cells from five random fields at magnification: 100×. Representative photos (A) and quantitative analysis (C) are shown. Wound healing assays were performed. Photos of the cells were taken at 0 and 24 h (B). The relative migration length was from three randomly selected locations (D). A clone formation assay was performed in Mock, mHO-1#3, or mHO-1#8 cells. Representative photos (E) and quantitative analysis of colony numbers (F) are shown. A cell viability assay was performed by MTT in mock, mHO-1#3, or mHO-1#8 cells (G). The mice were injected SC with mock or mHO-1#3 cells in the left mammary fat pad. Tumor size was measured using a caliper every 2 days. Tumors were harvested after 24 days. The representative photos of xenograft tumors (H) and the growth curve of tumor formation (I) are shown. Statistical analysis of tumor weight is shown (J). *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus the mock group.

We found that overexpression of HO-1 inhibited the growth of 4T1 cells. These findings suggest that overexpression of HO-1 inhibits tumor proliferation.

HO-1 Downregulates the Notch1 Expression and Inhibits the Notch1 Signal Pathway

Our results above showed that HO-1 could suppress the EMT process, and the migratory and proliferative ability of 4T1 cells. To understand the molecular basis of how downstream pathways of HO-1 is regulated in the progression of breast cancer cells, we first determined the expression of a series of breast cancer-related factors by qRT-PCR (data not shown). Serendipitously, Notch1 mRNA was obviously reduced (Fig. 4A). Consistent with the transcription level regulation, HO-1 also inhibited the expression of Notch1 protein (Fig. 4B). We then examined whether HO-1 influenced the Notch1 pathway activation, particularly Hes-1, Hey-1, and Slug, which are classical downstream molecules of Notch1signaling pathway by qRT-PCR. Interestingly, mRNA levels of Hes-1 and Hey-1 after HO-1 overexpression were slightly decreased without statistical significance. However, the expression of Slug mRNA and protein significantly decreased in the pHAGE-mHO-1 group compared with that of the control

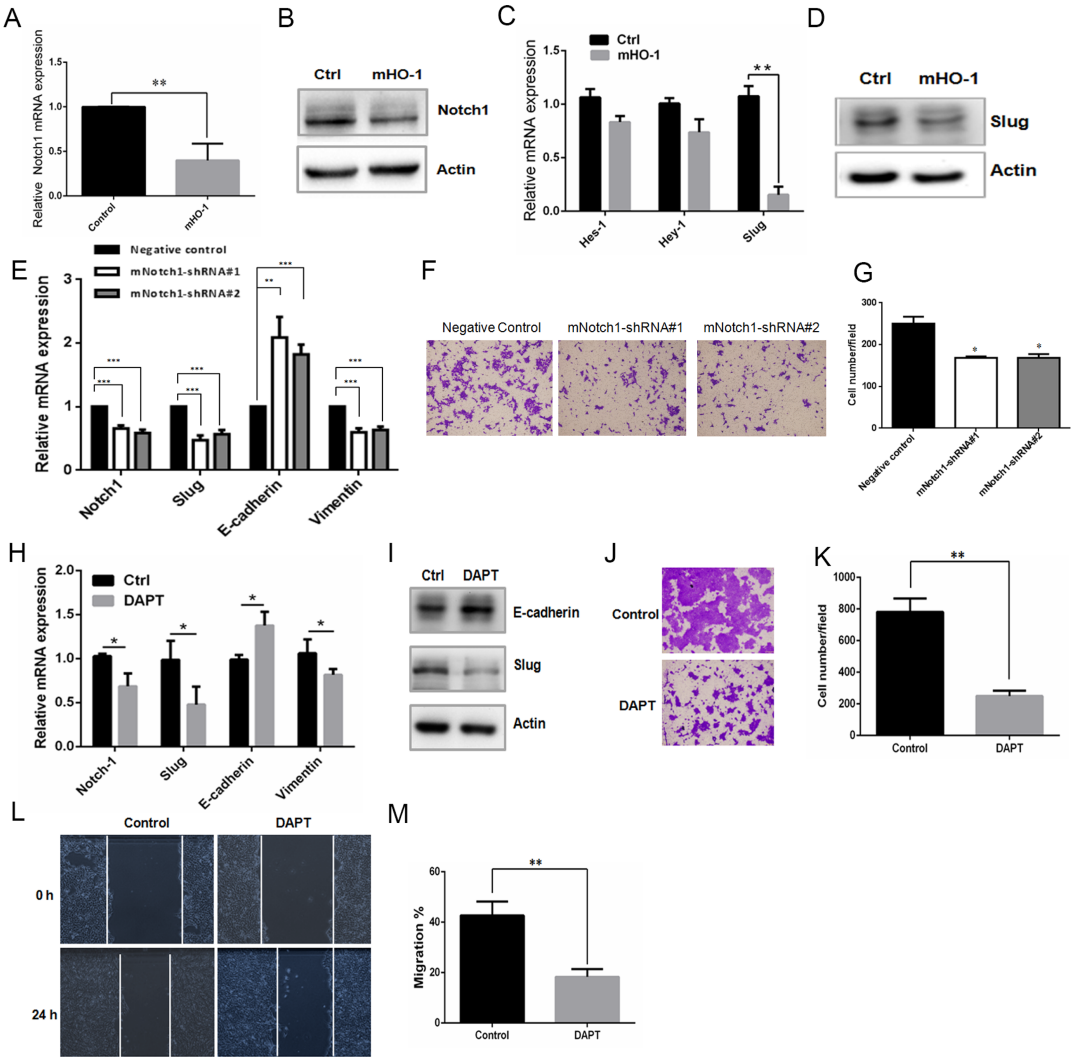

Figure 4. Notch-1 pathway mediates the inhibitory effect of HO-1 in EMT and cell migration. qRT-PCR assays (A) and Western blot analysis (B) were carried out to evaluate the expression of Notch1 when 4T1 cells were transfected with pHAGE or pHAGE-mHO-1 for 24 h. (C) qRT-PCR analysis of the classical downstream targets of Notch1 pathway including Hes-1, Hey -1, and Slug after 4T1 cells were transfected with pHAGE or pHAGE-mHO-1 for 24 h. (D) The protein expression of Slug was analyzed using Western blotting after HO-1 overexpression. (E) The expression of Notch1, Slug, E-cadherin, and vimentin after Notch1 was silenced using mNotch1 shRNA#1 or mNotch1-shRNA#2 were evaluated. (F, G) Transwell assay was performed to observe the cell migratory ability after Notch1 silencing. (H) qRT-PCR assays were performed to detect the expression of Notch1, Slug, E-cadherin, and vimentin after being treated with DAPT for 24 h. (I) Western blotting analysis of E-cadherin and Slug after being treated with DAPT for 24 h. Transwell assays (J, K) and wound healing assays (L, M) were performed after treatment with DAPT for 24 h. $*p<0.05$, $**p<0.01$, $***p<0.001$ versus the control group.

group (Fig. 4C and D). These data suggest that overexpression of HO-1 inhibits the activation of Notch1 signaling pathway. For the first time, we revealed that HO-1 could regulate the transcription level of Notch1.

Notch1 Pathway Blocking Inhibits EMT and Migration Similar to the Effect of HO-1 Overexpression in 4T1 Cells

It has been studied so far that the Notch1 pathway activation could induce the EMT process, and the evidences have shown that the Notch pathway facilitates the EMT process through regulating the expression of Slug in breast cancer[11]. To further corroborate our findings on the crosstalk between EMT and Notch signaling, Notch1 shRNA or DAPT was used to block the EMT process and cell migration. We first examined the effect of mNotch1shRNA#1and mNotch1shRNA #2 on Notch1 gene silencing. After 4T1 cells were transfected with different shRNAs for 48 h, the mRNA expression of Notch1, Slug, E-cadherin, and vimentin were detected by qRT-PCR. The Transwell was performed as well to confirm

the effect of Notch1 silencing on cell migration. Notably, Notch1 silencing could inhibit the mRNA expression of Slug and vimentin and increase the mRNA expression of E-cadherin (Fig. 4E). As we expected, blocking Notch pathway suppressed the migratory ability of 4T1 cells (Fig. 4F and G). DAPT, the inhibitor of Notch pathway activation, was also used to verify whether Notch signaling inhibition could suppress the EMT process. The protein expression of E-cadherin and Slug was detected in 4T1 cells. The expression of Slug was reduced. Nevertheless, E-cadherin was significantly elevated (Fig. 4H and I), and the cell migratory ability was also reduced after DAPT treatment (Fig. 4J–M). These results are close to the outcome of HO-1 overexpression, which support our hypothesis that HO-1 overexpression is involved in cell migration suppression through Notch1 inhibition at least in part.

DISCUSSION

Heme oxygenase-1 (HO-1) participates in cellular homeostasis maintenance and plays an important protective role in the tissues via reducing oxidative injury, attenuating the inflammatory response, inhibiting cell apoptosis, and regulating cell proliferation. A growing body of evidence indicates that HO-1 activation may play a role in carcinogenesis and can potently influence the growth and metastasis of tumors[19]. Our previous study finds that HO-1 overexpression inhibits the migratory ability of the liver cancer cells[18]. Several studies observe the role of HO-1 in breast cancer progression, but unfortunately these results are conflicting. Kim et al. found that 15-deoxy-D12, 14-prostaglandin J2 upregulate matrix metalloproteinase-1 (MMP-1) expression via induction of HO-1, which may contribute to increased metastasis and invasiveness of the human breast cancer cells[15]. Nevertheless, other researches indicate that HO-1 inhibits breast cancer invasion via suppressing the expression of MMP-9[16], and the results from both experiment were obtained in vitro. As we know, tumor metastasis is a complex process that involves the interaction between tumor cells and the microenvironment. Therefore, we believe that it is essential to detect the role of HO-1 in breast cancer metastasis in vivo. In our study, we first utilized the in vivo tumor metastasis model to observe the effect of HO-1 on mouse mammary carcinoma. The results demonstrate that overexpression of HO-1 strongly inhibits the lung metastasis of 4T1 cells.

Metastases represent the end products of a multistep cell biological process, including EMT, cell migration, invasion, intravasation, anoikis, extravasation, and growth in the distant organ site[20]. Our primary goal is to identify which steps HO-1 participates in. The phenotype of cell morphology changes after HO-1 overexpression provides us with the clue that HO-1 may take part in regulating the EMT process in 4T1 cells. EMT is currently in the limelight for investigating the onset of cancer cell migration, invasion, and metastatic dissemination. The expression and location of E-cadherin and vimentin by Western blot and immunofluorescence confirmed our hypothesis. HO-1 overexpression inhibits the EMT process in 4T1 cells. A few previous articles discussed the role of HO-1 in EMT process, such as the ability of HO-1 in attenuating the EMT in peritoneal fibrosis and renal fibrosis[21,22]. Recent study in prostate cancer demonstrates that initiation and progression of cancer xenografts in the presence of macrophages lacking HO-1 results in the loss of E-cadherin[23]. The data suggest that HO-1 is closely related to the EMT process both in tumor cells and in macrophage. Our results provide a new evidence that EMT could be adjusted by HO-1 in murine mammary carcinoma.

It has been shown that overexpression of Notch1 indicates a poor prognosis for breast cancer patients[16]. Furthermore, the Notch signaling pathway is related to several protumorigenic activities in breast cancer cell lines, which can also cause mammary hyperplasia and carcinogenesis in mice[24,25]. Recently, Notch signaling has been also implicated in the EMT process in human breast cancer cells by activating the transcription factor Slug, a potent repressor of E-cadherin gene expression[15]. In the process of exploring the mechanism of HO-1 antimetastasis effect, we screened some genes related to breast cancer progression. Fortunately, we found that Notch1 transcription was strongly suppressed by HO-1 overexpression in 4T1 cells. Interestingly, Nemeth et al. showed that HO-1 silencing enhances the Notch1 cleavage by high expression of P-Erk1/2 in stroma cells of lung cancer[26]. These data further suggest that HO-1 may be a negative regulator of Notch1 signaling both in tumor cells and stroma cells of tumors. In conclusion, our data show that HO-1 exerts the inhibitory effect on EMT process through the Notch1 pathway.

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