Original Paper

HOXD3 Plays a Critical Role in Breast Cancer Stemness and Drug Resistance

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Key Words

Hoxd3 • Breast cancer • Stemness • Drug resistance • Integrin β 3 • Wnt/ β -catenin signaling

Abstract

Background/Aims: Homeobox D3 (HOXD3) is a member of the homeobox family of genes that is known primarily for its transcriptional regulation of morphogenesis in all multicellular organisms. In this study, we sought to explore the role that HOXD3 plays in the stem-like capacity, or stemness, and drug resistance of breast cancer cells. **Methods:** Expression of HOXD3 in clinical breast samples were examined by RT-PCR and immunohistochemistry. HOXD3 expression in breast cancer cell lines were analyzed by RT-PCR and western blot. Ability of drug resistance in breast cancer cells were elevated by MTT cell viability and colony formation assays. We examined stemness using cell fluorescent staining, RT-PCR and western blot for stem cell marker expression. Finally, activity of wnt signaling was analyzed by FOPflash luciferase assays. RT-PCR and western blot were performed for downstream genes of wnt signaling. **Results:** We demonstrated that HOXD3 is overexpressed in breast cancer tissue as compared to normal breast tissue. HOXD3 overexpression enhances breast cancer cell drug resistance. Furthermore, HOXD3 upregulation in the same cell lines increased sphere formation as well as the expression levels of stem cell biomarkers, suggesting that HOXD3 does indeed increase breast cancer cell stemness. Because we had previously shown that HOXD3 expression is closely associated with integrin β 3 expression in breast cancer patients, we hypothesized that HOXD3 may regulate breast cancer cell stemness and drug resistance through integrin β 3. Cell viability assays showed that integrin β 3 knockdown increased cell viability and that HOXD3 could not restore cancer cell stemness or drug resistance. Given integrin β 3's relationship with Wnt/ β -catenin signaling, we determine whether HOXD3 regulates integrin β 3 activity through Wnt/ β -catenin signaling. We found that, even though HOXD3 increased the expression of Wnt/ β -catenin downstream genes, it did not restore Wnt/ β -catenin signaling activity, which was inhibited in integrin β 3 knockdown breast cancer cells. **Conclusion:** We demonstrate that HOXD3 plays a critical role in breast cancer stemness and drug resistance via integrin β 3-mediated Wnt/ β -catenin signaling. Our findings open the possibility for improving the current standard of care for breast cancer patients by designing targeted molecular therapies that overcome the barriers of cancer cell stemness and drug resistance.

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Introduction

After skin cancer, breast cancer is the second most prevalent cancer in women worldwide, and the chance that a woman will die from breast cancer is 1 in 37 (about 2.7%), making it the second leading cause of cancer death in women [1]. In the developed world, the majority of cases (around 61%) are diagnosed when the disease is localized to the breast, around a third are diagnosed when the disease is regionally advanced, and the remainder are diagnosed during metastasis [2]. Even though the incidence and mortality rates of breast cancer have been improving in the past few decades, largely due to advances in diagnosis and adjuvant treatment strategies such as systemic chemotherapies and endocrine therapies, the disease's drug resistance and stem-like capacity, or stemness, continue to prevent successful treatment [3, 4]. In fact, for patients with recurrent breast cancer, the development of drug resistance is virtually inevitable, making the need for understanding the molecular bases underlying drug resistance particularly crucial [4].

The current standard of care for breast cancer patients is primary surgery with neoadjuvant systemic therapy (AST) based on factors such as the spread of disease, recurrence, and gene expression signatures [3, 5]. Generally, patients with early stage breast cancer receive primary surgery localized to the tumor site and regional lymph nodes, with or without breast irradiation [6]. Following localized treatment, patients may receive AST based on the aforementioned tumor characteristics [7]. Despite the use of AST and therapies targeted to the individual's gene expression profile, there remains a substantial residual risk of recurrence for patients diagnosed even in the earliest stages of breast cancer [8]. The use of platinum salts such as cisplatin (CDDP), in combination with systemic chemotherapies, are currently being investigated for use with metastatic tumors that have faulty DNA repair pathways, particularly in estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and Human Epidermal Growth Factor 2 (HER2)-negative breast cancer (also known as "triple negative" breast cancer) [9, 10]. Apart from CDDP, doxorubicin (DOX), an anthracycline antibiotic, is one of the most effective single agent treatments for metastatic breast cancer, as well as in combination with taxanes such as docetaxel [11-13]. Given the serious ramifications of developing resistance to these two drugs, which remain fundamental to current breast cancer therapies, in this study we sought to identify the gene responsible for breast cancer cell resistance to CDDP and DOX.

There is much evidence that supports the theory that chemoresistance arises from cancer stem cells (CSCs) [14-16]. Drug resistance can be inherent, acquired, or a combination of both [17]. Inherent drug resistance may arise from CSCs' natural tendency for quiescence, heightened DNA repair ability, and expression of ATP-binding cassette (ABC) transporters, which cause drug efflux [18, 19]. When CSCs survive an initial course of chemotherapy, this subpopulation initiates tumor recurrence, thus repopulating both CSCs and differentiated tumor cells [20]. Acquired resistance is thought to occur when the subpopulation of CSCs that survives a course of treatment accumulate mutations that confer a drug resistance phenotype [21, 22]. Breast cancer stem cells have specifically been shown to acquire therapeutic resistance through a variety of signaling pathways, including those dependent on MEK/ERK [23], TGF- β [24], VEGF [25] and Wnt/ β -catenin [26]. There are a variety of genes, including homeobox genes, that play crucial roles in tumorigenesis and that do so by interacting with signaling pathways [27, 28]. In the present study, we have identified the homeobox gene HOXD3 as a promoter of breast cancer cell stemness and drug resistance by acting through integrin β 3-mediated Wnt/ β -catenin signaling.

Materials and Methods

Breast cancer samples

A total of 87 breast cancer patients with histologically confirmed tumors at Harbin Medical University Cancer Hospital were enrolled in this study. Breast cancer tissue specimens were obtained from patients

undergoing primary mastectomies at the institution. The tissues were examined diagnostically by pathologists. The samples were collected immediately, snap-frozen in liquid nitrogen, and stored at -80°C for analysis. All protocols were reviewed and approved by the Ethical Committee of Harbin Medical University. Written consent was obtained from all participating patients.

Immunohistochemical staining

One representative section of the tissue was cut at 4 mm and placed on poly-L-lysine coated slides. The slides were deparaffinized, dehydrated, immersed in sodium citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0), pretreated in a microwave oven for 10 min, and rinsed with phosphate-buffered saline (PBS) for 10 minutes. After blocking with 3% hydrogen peroxide for 10 min at room temperature, the slides were incubated at 4°C overnight with primary anti-HOXD3 antibody (Santa Cruz Biotechnology, Santa Cruz, USA). The slides were then stained with the 2-step plus Poly-HRP anti-Rabbit IgG Detection System (ZSGB-Bio, Beijing, China). After visualization of the reaction with the DAB chromogen, the slides were counterstained with haematoxylin and covered with a glycerin gel. For negative controls, the primary antibody was substituted with PBS.

Cell culture, transfection, and treatment

Normal mammary epithelial cell line MCF-10A was maintained in DMEM medium (Gibco, Grand Island, NY, USA) containing 5% horse serum (Life Technologies, Carlsbad, CA, USA), 20 ng/mL human epidermal growth factor (hEGF; R&D Systems, Minneapolis, MN, USA), 0.5 mg/mL hydrocortisone (Sigma, St. Louis, MO, USA), 10 µg/mL insulin, 100 U/mL penicillin, and 100 U/mL streptomycin. Breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-435) were cultured in DMEM medium with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mmol/L L-glutamine. All cells were maintained at 37°C in a humidified 5% CO₂ atmosphere cell incubator. For stemness analysis, breast cancer cells (4×10⁴ cells/well) were seeded onto 6-well ultralow attachment plates (Corning, Corning, NY, USA) in serum-free DMEM medium supplying B27 supplement (Invitrogen, Carlsbad, CA, USA), 20ng/mL human fibroblast growth factor-basic (hFGF, R&D Systems), 20ng/mL human epidermal growth factor (hEGF, R&D Systems), and heparin (Sigma). The tumor spheres were counted after 7 days. All cells were from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China, and authenticated by STR genotyping. For drug resistance breast cancer cell establishment, the cells were continuously cultured and maintained in growing medium containing either 1.25 µM cisplatin (CDDP) or 25 mM doxorubicin (DOX, Sigma).

HOXD3 cDNA was amplified from MDA-MB-231 mRNA by polymerase chain reaction (PCR) and cloned into the pcDNA3.1 vector to generate HOXD3 overexpression plasmids. Integrin β3 siRNA and control siRNA were purchased from OriGene (Beijing, China). Wnt/β-catenin signaling reporter assays were carried out in 24-well plates with 100 ng of TOP Flash, FOP Flash, and Renilla plasmid transfection. The dual-luciferase reporter assay system (Promega, Madison, WI, USA) was used to detect luciferase activity. Lipofectamine 2000 (Invitrogen) was used for plasmids and siRNA transfection according to the manufacturer's instructions. Breast cancer cells were treated with different doses of CDDP or doxorubicin after transfection.

Real-Time PCR (RT-PCR)

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. RT-PCR assays were carried out in an ABI Prism 7900HT thermal cycler (Applied Biosystems, Foster City, CA, USA). RT-PCR amplification was performed in 20 µL reaction mixture containing 2 µL cDNA sample, 10 µL Quanti-Tect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), and specific primer sets. PCR began with a 15-min hot start at 95°C followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. Dissociation curve analysis (95°C for 15 s, 60°C for 15 s, and 95°C for 15 s) was performed at the end of the 40 cycles to verify the PCR product identity. Data were analyzed using Sequence Detector Systems version 2.0 software (Applied Biosystems). Quantification was performed using the standard curve method according to the method described in our previous reports [29]. Finally, relative gene expression levels were normalized to an internal reference gene (β-actin). The primers used are as follows: *HOXD3* sense: 5'-CCATAAATCAGCCGCAAGGAT-3', antisense: 5'-GATGGGTCTCAGACTTACCTTTGG-3'; *CD133* sense: 5'-CAGAGTACAACGCCAACC-3', antisense: 5'-AAATCACGATGAGGGTCAGC-3'; *Nanog* sense: 5'-ACAACTGGCCGAAGAATAGC-3',

antisense: 5'-AGTGTTCAGGAGTGGTTGC-3'; *Oct4* sense: 5'- ATAGACCGGTAATGGCGGGACACCTGGC-3',
antisense: 5'- CATAATGGCCGTCGACCAGTTTGAATGCATGGGAGA-3'; *SOX2*
sense: 5'-CGGTACCCGGGGATCCCCGCATGTACAACATGATGG-3', antisense:
5'-CATAATGGCCGTCGACCACATGTGTGAGAGGGGCA-3'; *Integrin β3* sense: 5'-GACTTTGGCAAGATCACGGG-3',
antisense: 5'-GCACATCTCCCCCTTGTAGC-3'; *β-catenin* sense: 5'-GCTGATTTGATGGAGTTGGA-3',
antisense: 5'-TCAGTACTTGTCTTGTAGTAA-3'; *myc* sense: 5'-TTGCAGCTGCTTAGACGCTG-3',
antisense: 5'-CCACATACAGTCCTGGATGA-3'; *cyclin D1* sense: 5'-GGATGCTGGAGGTCTGCCAG-3',
antisense: 5'-GAGAGGAAGCGTGTGAGGCG-3'; *β-actin* sense: 5'-TTGCCGACAGGATGCAGAA-3', antisense:
5'-GCCGATCCACACGGAGTACT-3'.

Western blot

Total proteins of cultured cells were extracted using RIPA buffer at 4°C for 30 min. The cell lysates were centrifuged at 4°C for 10 min at 12,000 g to separate soluble proteins. Proteins were resolved by 10% SDS/PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% (w/v) non-fat powdered milk in TBS containing 0.1% Tween for 1 h, washed with TBS/Tween, incubated overnight at 4°C with antibodies against HOXD3, CD133, Nanog, Oct4, SOX2, Integrin β3, β-catenin, myc, cyclin D1, or β-actin, and then incubated for 2 h with appropriate secondary antibodies. The primary antibodies were all from Santa Cruz Biotechnology. Signals were visualized by chemiluminescence (Beyotime, Beijing, China).

Cell fluorescence staining

Breast cancer cells were cultured in chamber slides and then fixed with 4% polyphosphate formaldehyde for 15 min and washed with PBS after HOXD3 transfection. Permeabilization was then performed with 1% Triton for 30 min, followed by blocking with 5% bovine serum albumin (BSA) for 30 min at room temperature. After washing, the cells were then incubated with anti-HOXD3 antibody (Santa Cruz Biotechnology) at 4 °C overnight and were subsequently incubated with Texas Red Conjugated Secondary Antibody for 30 min in the dark. DAPI was used for nuclear staining.

MTT assay

Breast cancer cells were grown on 96-well plates at an initial concentration of 5×10^3 cells/mL per well. 20 μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) were added to the growing medium of each well at days 0, 1, 2, 3, 4, 5 and 6. Cells were then incubated at 37 °C in the dark. After 4 h, the MTT solution was removed and replaced with 100 μL of dimethyl sulfoxide (DMSO). Absorbance values were determined at a wavelength of 590 nm in a microplate reader.

Colony formation

Breast cancer cells (500 per well) were plated into each well of a 6-well plate and incubated at 37 °C for 2 weeks. The cells were then fixed with 4% paraformaldehyde and stained with 1% crystal violet. The number of colonies was counted.

Statistics

All analyses were performed using the statistical software GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA). For experimental data, one-way analysis of variance (ANOVA) was performed for serial analysis, while two treatment groups were compared using the unpaired Student's *t*-test. All experiments were performed at least three times. Data were expressed as mean ± standard deviation (SD). *P* values of <0.05 were considered statistically significant in all of the analyses.

Results

The expression of HOXD3 is upregulated in breast cancer tissues and cell lines

To explore the function of HOXD3 in breast cancer tumorigenesis, immunohistochemical staining of HOXD3 was performed in human breast cancer tissues and paired adjacent normal tissues. We found that HOXD3 staining in breast cancer is stronger than in normal tissues (Fig. 1A). We then performed RT-PCR to analyze *HOXD3* mRNA expression in clini-

cal samples. Across a total of 87 human tissues, expression levels of *HOXD3* in breast cancer tissues are higher than normal tissues (Fig. 1B). We further examined *HOXD3* expression in three breast cancer cell lines (MDA-MB-231, MDA-MB-435 and MCF-7). As compared to one normal mammary epithelial cell line MCF-10A, both mRNA and protein levels in breast cancer cells are higher (Fig. 1C). Taken together, these findings suggest that *HOXD3* is upregulated in breast cancer tissues and cell lines and that the protein plays a role in breast cancer cell survival.

HOXD3 is required for multiple drug resistance in breast cancer cells

To analyze the role of *HOXD3* in breast cancer drug resistance, we first examined the IC₅₀s of MDA-MB-231, MDA-MB-435, and MCF-7 cells treated with CDDP or DOX. We found that MDA-MB-231 cells have the highest IC₅₀ values for both CDDP and DOX treatment (Fig. 2A). We then established CDDP or DOX resistant cells using the MDA-MB-231 and MDA-MB-435 cell lines. Each resistant cell line has a higher IC₅₀ compared to the original cell line (Fig. 2B). *HOXD3* expression was examined in drug resistant cells. Higher levels of *HOXD3* were observed in both CDDP and DOX resistant cells by RT-PCR and western blot (Figs 2C and 2D). Consistently, *HOXD3* fluorescent staining in CDDP and DOX resistant cells is stronger than in the

Fig. 1. The expression of *HOXD3* is upregulated in breast cancer tissues and cell lines. (A) Representative immunostaining for *HOXD3* expression in breast cancer and paired adjacent normal tissues. (B) Relative *HOXD3* mRNA expression in 87 breast cancer and paired adjacent normal tissues. $P < 0.001$. (C, D) Relative *HOXD3* mRNA (C) and protein (D) expression in one normal mammary epithelial cell line MCF-10A and three breast cancer cell lines MDA-MB-231, MDA-MB-435 and MCF-7. $*p < 0.05$.

Fig. 2. *HOXD3* expression is increased in breast cancer cells of multiple drug resistance. (A) IC₅₀ of cisplatin (CDDP) and doxorubicin (DOX) in three breast cancer cell lines MDA-MB-231, MDA-MB-435 and MCF-7. (B) IC₅₀ of CDDP and DOX in CDDP or DOX resistant MDA-MB-231 and MDA-MB-435 cells, respectively. $*p < 0.05$. (C, D) Relative *HOXD3* mRNA (C) and protein (D) expression in CDDP or DOX resistant MDA-MB-231 and MDA-MB-435 cells analyzed by RT-PCR and western blot, respectively. $*p < 0.05$. (E) Cell fluorescence staining of *HOXD3* in CDDP or DOX resistant MDA-MB-231 and MDA-MB-435 cells.

Fig. 3. HOXD3 enhances multiple drug resistance in breast cancer cells. (A, B) HOXD3 mRNA (A) and protein (B) expression in MDA-MB-231 and MDA-MB-435 cells transfected vector or HOXD3 plasmids by RT-PCR and western blot. $**p < 0.01$. (C, D) Cell viability in HOXD3 overexpression MDA-MB-231 and MDA-MB-435 cells treated with different doses of CDDP (C) or DOX (D) indicated, respectively. (E, F) Colony formation in HOXD3 overexpression MDA-MB-231 and MDA-MB-435 cells treated with different doses of CDDP (E) or DOX (F) indicated, respectively. $*p < 0.05$.

Fig. 4. HOXD3 augments breast cancer cell stemness traits. (A) Tumor sphere formation assays in HOXD3 overexpression MDA-MB-231 and MDA-MB-435 cells. Sphere number was quantified. $*p < 0.05$. (B, C) Relative mRNA (B) and protein (C) expression of stem cell markers (CD133, Nanog, Oct4 and SOX2). $*p < 0.05$. (D) Cell fluorescence of CD133 staining in HOXD3 overexpression MDA-MB-231 and MDA-MB-435 cells. (E) MTT assays were performed to analyze cell proliferation in HOXD3 overexpression MDA-MB-231 and MDA-MB-435 cells.

original cells (Fig. 2E). We therefore hypothesized that HOXD3 might play a role in breast cancer drug resistance. MDA-MB-231 and MDA-MB-435 cells were transfected with HOXD3 overexpression plasmid (Figs 3A and 3B). We then evaluated the drug resistance ability of breast cancer cells by treating them with different doses of CDDP or DOX. MTT cell viability and colony formation assays demonstrated that overexpression of HOXD3 significantly increased cell viability and colony formation under CDDP or DOX treatment in both MDA-MB-231 and MDA-MB-435 cells (Figs 3C to 3F). Overall, our investigation into the impact of HOXD3 expression on drug resistance strongly suggested that HOXD3 is required for multiple drug resistance in breast cancer cells.

HOXD3 augments breast cancer cell stemness traits

Breast cancer stem cells exhibit stem cell properties, including resistance to multiple drugs. In order to assess whether HOXD3 affects breast cancer cell stemness, sphere formation assays were performed. Upregulation of HOXD3 in MDA-MB-231 and MDA-MB-435 cells led to

Fig. 5. Silencing of integrin $\beta 3$ abolished HOXD3-induced drug resistance and stemness. (A, B) HOXD3 and integrin $\beta 3$ mRNA (A) and protein (B) expression in integrin $\beta 3$ knockdown MDA-MB-231 and MDA-MB-435 cells with or without HOXD3 overexpression. $**p < 0.01$. (C, D) Cell viability in integrin $\beta 3$ knockdown MDA-MB-231 and MDA-MB-435 cells with or without HOXD3 overexpression treated with different doses of CDDP (C) or DOX (D) indicated, respectively. (E) Tumor sphere formation in integrin $\beta 3$ knockdown MDA-MB-231 and MDA-MB-435 cells with or without HOXD3 overexpression. $*p < 0.05$. (F) Stem cell marker expression in integrin $\beta 3$ knockdown MDA-MB-231 and MDA-MB-435 cells with or without HOXD3 overexpression. $*p < 0.05$.

a significant increase in sphere number (Fig. 4A). The stem cell biomarkers including CD133, Nanog, Oct4, and SOX2 were analyzed by RT-PCR and western blot. The expression levels of stem cell biomarkers in both MDA-MB-231 and MDA-MB-435 cells significantly increased after HOXD3 overexpression (Figs 4B and 4C). Furthermore, CD133 cell fluorescence assays in breast cancer cells consistently showed stronger staining in HOXD3 overexpression MDA-MB-231 and MDA-MB-435 cells (Fig. 4D). MTT assays also showed that upregulation of HOXD3 promotes breast cancer cell proliferation (Fig. 4E).

Silencing of integrin $\beta 3$ abolished HOXD3-induced drug resistance and stemness

In a previous study, we observed that the expression of HOXD3 was positively associated with integrin $\beta 3$ levels [29]. Breast cancer cells were regulated by integrin $\beta 3$ [30]. We thus hypothesized that HOXD3 may affect breast cancer cells through integrin $\beta 3$ regulation. To test this hypothesis, we knocked down integrin $\beta 3$ in both MDA-MB-231 and MDA-MB-435 cells with or without HOXD3 overexpression. Downregulation of integrin $\beta 3$ did not affect HOXD3 expression (Fig. 5A and 5B). The cells were then treated with different doses of CDDP or DOX and subjected to cell viability assays. Knockdown of integrin $\beta 3$ significantly reduced cell viability under CDDP or DOX treatment while HOXD3 overexpression did not restore cell viability (Figs 5C and 5D). Moreover, HOXD3 could not rescue tumor sphere formation and stem cell marker expression inhibited by integrin $\beta 3$ silencing (Fig. 5E and 5F).

HOXD3 activates integrin $\beta 3$ -mediated Wnt/ β -catenin signaling

Since integrin $\beta 3$ function is related to Wnt/ β -catenin signaling and Wnt/ β -catenin signaling plays an important role in breast cancer cell drug resistance and stemness, we hypothesized that HOXD3 would promote breast cancer cell stemness and chemoresistance through Wnt/ β -catenin signaling [31]. We examined downstream genes of Wnt/ β -catenin signaling including β -catenin, myc, and cyclin D1 by RT-PCR and western blot after HOXD3 overexpression. HOXD3 significantly increased the expression of these downstream genes

Fig. 6. HOXD3 activates integrin $\beta 3$ -mediated Wnt/ β -catenin signaling. (A, B) mRNA (A) and protein (B) expression of Wnt/ β -catenin signaling protein (β -catenin, myc and cyclin D1) in HOXD3 overexpression MDA-MB-231 and MDA-MB-435 cells. * $p < 0.05$. (C) TOP/FOP ratio in HOXD3 overexpression MDA-MB-231 and MDA-MB-435 cells. * $p < 0.05$. (D) TOP/FOP ratio in integrin $\beta 3$ knockdown MDA-MB-231 and MDA-MB-435 cells with or without HOXD3 overexpression. * $p < 0.05$.

(Fig. 6A). Furthermore, we examined Wnt/ β -catenin signaling activity using TOP/FOP flash assays. Overexpression of HOXD3 significantly stimulated Wnt/ β -catenin signaling (Fig. 6B). In integrin $\beta 3$ knockdown MDA-MB-231 and MDA-MB-435 cells, Wnt/ β -catenin signaling activity was inhibited (Fig. 6C). However, overexpression of HOXD3 did not further rescue Wnt/ β -catenin signaling activity (Fig. 6D). These findings suggest that both HOXD3 and integrin $\beta 3$ expression are necessary for Wnt/ β -catenin signaling and the breast cancer cell stemness and chemoresistance.

Discussion

In agreement with the role of homeobox gene mutation and epigenetic changes in a variety of cancers, including prostate cancer [32], lung cancer [33], and breast cancer [34], our findings support the hypothesis that HOXD3 overexpression contributes to breast cancer cell stemness and chemoresistance. Using human tumor and paired adjacent normal samples from 87 patients, as well as several breast cancer cell lines, we demonstrated with real-time PCR (RT-PCR), western blotting, and immunohistochemistry that HOXD3 is overexpressed in breast cancer cells as compared to normal cells. After determining that HOXD3 is required for multiple drug resistance and increases stemness traits in breast cancer cells, we used CDDP- and DOX-resistant breast cancer cell lines, tumor sphere formation assays, TOP/FOP flash assays, and western blot assays to demonstrate that HOXD3 promotes these phenotypes through integrin $\beta 3$ -mediated Wnt/ β -catenin signaling.

Homeobox genes are crucial to the normal physiological development of animals and plants, though they also play important roles in the initiation and maintenance of a number of pathologies, including cancers [34]. In normal physiological development, these genes encode a highly conserved family of transcription factors that regulate morphogenesis and cell differentiation in all multicellular organisms [35]. HOXD3 is one of four mammalian homeobox gene clusters that are expressed in a tissue-specific manner in normal adult tissues and that contribute to maintaining architectural integrity by regulating cell motility and cell-cell interactions [33]. A study by Miyazaki, et al. showed that, in human erythroleukemia and lung cancer cells, overexpressing HOXD3 increases the activity of TGF- β -dependent and -independent signaling pathways to promote cancer cell motility and invasiveness [36]. Since then, it has become clear that HOXD3 plays an important role in invasive breast cancer and can even serve as a prognostic indicator for breast cancer patients [29]. In the present study, we have not only confirmed that HOXD3 contributes to breast cancer cell stemness and drug resistance, but also we have established that it does so by activating $\beta 3$ -integrin-mediated Wnt/ β -catenin signaling.

Integrin $\beta 3$ is a member of the integrin family of cell surface receptors that regulates interactions across the cell membrane, largely between the extracellular matrix and cell cytoskeleton [37]. Members of the integrin family are crucial for cell adhesion, proliferation, and mobility, among other foundational physiological cell functions [38]. However, they are also implicated in a growing number of pathologies, including a variety of cancers [39, 40]. Integrin $\beta 3$ is one part of the heterodimeric integrin transmembrane receptor, composed of αv and $\beta 3$ subunits [41]. $\alpha v\beta 3$ -integrin became of interest largely in the context of vasculature, where it was found to be upregulated in cancers, including cervical, pancreatic, and breast, and has been shown to promote tumor cell migration, invasion, and growth factor release [42-45]. In our previous study, we demonstrated that, in breast cancer cells, integrin $\beta 3$ levels were positively correlated with HOXD3 expression, which increased cell metastasis and was associated with high histological grade and poorer survival in breast cancer patients [29]. The present study builds on our prior work, as well as the work of other scientists investigating the role of integrin $\beta 3$ in breast cancer, by revealing that, without integrin $\beta 3$, HOXD3 expression could not induce breast cancer cell stemness or chemoresistance.

We sought to determine HOXD3's mechanism of action by first determining what signaling pathway could give rise to the established phenotypes while being contingent upon integrin $\beta 3$ activity. Previous studies have shown that Wnt signaling and integrin $\beta 3$ act in a coordinated manner to increase tumorigenesis [46]. Specifically, it has been shown in a mouse model of mammary tumorigenesis that integrin $\beta 3$ is expressed prominently in transgenic mice with wnt-1 signaling pathway overexpression [30]. This finding, along with the subsequent identification of HOXD3 as a prognostic marker in humans with breast cancer, encouraged us to explore the Wnt/ β -catenin pathway as the mechanism by which HOXD3 and integrin $\beta 3$ promote breast cancer cell stemness and chemoresistance. In the present study, we have demonstrated that HOXD3 significantly promotes Wnt/ β -catenin signaling activity. Furthermore, in integrin $\beta 3$ knockdown cells, Wnt/ β -catenin signaling activity was abolished and could not be rescued by HOXD3 overexpression.

In the present study, we have not only furthered scientific understanding of the role of Wnt/ β -catenin signaling in breast cancer cell stemness and drug resistance, but also established for the first time a role for homeobox gene HOXD3 in promoting these phenotypes through integrin $\beta 3$ -mediated Wnt/ β -catenin signaling. These findings open the door for more precisely targeting the molecular causes of resistance to breast cancer chemotherapies, as well as for potentially ablating the CSC subpopulations that allow for tumor progression and recurrence. It will be important to determine to what degree the relationship between HOXD3, integrin $\beta 3$, and Wnt/ β -catenin established here applies to treatments other than CDDP and DOX, as well as whether this relationship is consistent across tumor grades and stages.

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Disclosure Statement

The authors declare that there is no Disclosure Statement in this study.

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