Inhibition of Notch1 reverses EMT and chemoresistance to cisplatin via direct downregulation of MCAM in triple-negative breast cancer cells

De Zeng1,2, Yuan-Ke Liang3,4, Ying-Sheng Xiao2,5, Xiao-Long Wei6, Hao-Yu Lin3, Yang Wu3, Jing-Wen Bai7, Min Chen2 and Guo-Jun Zhang6,7,8

1Department of Medical Oncology, Shantou University Medical College Cancer Hospital, Shantou, China
2Changjiang Scholar’s Laboratory, Shantou University Medical College (SUMC), Shantou, China
3Department of Thyroid and Breast Surgery, the First Affiliated Hospital of Shantou University Medical College, Shantou, China
4Department of Medical Oncology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
5Department of Thyroid Surgery, Shantou Central Hospital, Shantou, China
6Department of Pathology, Cancer Hospital of SUMC, Shantou, China
7The Cancer Center and the Department of Breast and Thyroid Surgery, Xiang’ an Hospital of Xiamen University, School of Medicine, Xiamen University, Xiamen, Fujian, China
8Fujian Anti-Cancer Center, Fujian, China

Resistance to chemotherapy continues to be a critical issue in the clinical therapy of triple-negative breast cancer (TNBC). Epithelial–mesenchymal transition (EMT) is thought to contribute to chemoresistance in several cancer types, including breast cancer. Identification of the key signaling pathway that regulates the EMT program and contributes to chemoresistance in TNBC will provide a novel strategy to overcome chemoresistance in this subtype of cancer. Herein, we demonstrate that Notch1 positively associates with melanoma cell adhesion molecule (MCAM), a unique EMT activator, in TNBC tissue samples both at mRNA and protein levels. High expression of Notch1 and MCAM both predicts a poor survival in basal-like/TNBC patients, particularly in those treated with chemotherapy. The expression of Notch1 and MCAM in MDA-MB-231 cells gradually increases in a time-dependent manner when exposing to low dose cisplatin. Moreover, the expressions of Notch1 and MCAM in cisplatin-resistant MDA-MB-231 cells are significantly higher than wild-type counterparts. Notch1 promotes EMT and chemoresistance, as well as invasion and proliferation of TNBC cells via direct activating MCAM promoter. Inhibition of Notch1 significantly downregulates MCAM expression, resulting in the reversion of EMT and chemoresistance to cisplatin in TNBC cells. Our study reveals the regulatory mechanism of the Notch1 pathway and MCAM in TNBC and suggesting that targeting the Notch1/MCAM axis, in conjunction with conventional chemotherapies, might be a potential avenue to enhance the therapeutic efficacy for patients with TNBC.

Introduction
Breast cancer is presenting with increasing incidence while with decreasing mortality in the past few decades, primarily attributed to early detection and emerging effective treatment.1 However, intrinsic or acquired drug resistance remains a significant problem and is one of the important reasons responsible for the treatment failure of breast cancer, particularly in patients with triple-negative breast cancer (TNBC), which is defined as absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2).2

A plethora of studies has progressively suggested that the acquisition of epithelial–mesenchymal transition (EMT) phenotype are interconnected and constitutively contributed to drug resistance in breast cancer.3 EMT is a process in which the epithelial cells gradually lose apical-basal polarity and decrease their tight junctions with the basement membrane. Meanwhile, the cytoskeleton is reconstructed, and eventually,
the cells transform into a phenotype featured with an expression of mesenchymal markers. This process is usually accompanied by an increasing number of cancer stem cells with active self-renewal capacity, and the tumor cells often become less sensitive to chemotherapy.

Recent studies have shown that there were shared regulatory mechanisms, including Notch, PI3K/Akt/GSK-3β/Snail and MAPK/JNK signaling pathways, between EMT and chemoresistance in a number of solid tumors. For example, by X. Gungor C and colleagues reported that replication stress-induced expression of Notch1 signaling midkine derived EMT and chemoresistance. In the present study, we sought to investigate the impact and regulatory effects of Notch1 signaling on MCAM, as well as the influence of EMT and chemoresistance in TNBC cells, with the ultimate goal of identifying a novel strategy to overcome chemoresistance in TNBC cells. The findings suggest that Notch1 regulates MCAM in EMT and contributes to cisplatin resistance in TNBC. Targeting the Notch1/MCAM axis might be a potential avenue to enhance therapeutic efficacy in patients with TNBC. The findings suggest that Notch1 regulates MCAM in EMT and contributes to cisplatin resistance in TNBC. Targeting the Notch1/MCAM axis might be a potential avenue to enhance therapeutic efficacy in patients with TNBC.

What’s new?

Epithelial-mesenchymal transition (EMT) likely contributes to chemoresistance in triple-negative breast cancers (TNBC), but the underlying mechanisms remain unclear. Here, the expression of Notch1 positively associated with melanoma cell adhesion molecule (MCAM), a unique EMT activator, in TNBC tissue samples. High expression of Notch1 and MCAM predicted poor survival, particularly in patients treated with chemotherapy. Notch1 and MCAM levels were significantly higher in cisplatin-resistant than wild-type TNBC cells. The findings suggest that Notch1 regulates MCAM in EMT and contributes to cisplatin resistance in TNBC. Targeting the Notch1/MCAM axis might be a potential avenue to enhance therapeutic efficacy in patients with TNBC.

Materials and Methods

Cell culture

The five human breast cancer cell lines, including T-47D (RRID: CVCL_0031), MCF-7 (RRID: CVCL_0033), MDA-MB-231 (RRID: CVCL_0062), SK-BR-3 (RRID: CVCL_0033) and BT-549 (RRID: CVCL_1092) were purchased from the American Type Culture Collection (ATCC). All the cells were cultured in DMEM medium containing 10% FBS. For construction of the cisplatin-resistant MDA-MB-231-DDPR cell line, 5 × 10⁶ of the parental wild-type MDA-MB-231 cells were cultured in the 10 cm dish and treated with cisplatin (Sigma-Aldrich, St. Louis, MO) and gradually increased the cisplatin concentration from 0.1 μg/l to 1 μg/l for 6 months (the cisplatin concentration was increased 0.1 μg/l every 2 weeks). Finally, the MDA-MB-231-DDPR cells were continuously cultured in 1 μg/l cisplatin and 10% FBS DMEM medium. The γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) was purchased from Sigma-Aldrich (Merck Millipore, Germany). All human cell lines have been authenticated using STR profiling within the last 3 years and all experiments were performed with mycoplasma-free cells.

In silico analysis (BcGenExMiner v4.1, TCGA and Kaplan–Meier-plotter)

The bcGenExMiner v4.1 database was used to analyze the relationship among the expression of Notch family members (Notch1-4), ERα (Estrogen receptor-α), E-Cadherin and Vimentin. The correlation of the mRNA levels between Notch1 and MCAM in breast cancer was analyzed by the cbioportal database (TCGA, nature 2012) (http://www.cbioportal.org/index.do) and Pearson correlation analysis was used to obtain their correlation coefficients. Prognostic values of Notch1 and MCAM were evaluated through survival analysis of 5,143 breast cancer patients derived from the Kaplan–Meier-plotter database (http://kmplot.com/analy-sis/index.php?p=service&cancer=breast).
Tumor tissue samples were obtained from 52 TNBC patients received surgery in the Cancer Hospital of Shantou University Medical College. Immunohistochemistry (IHC) assay was performed and analyzed as described previously. Briefly, tumor tissue sections were deparaffinized, rehydrated and antigen-retrieved. After blocking of the endogenous peroxidase with 3% hydrogen peroxide for 5 min, tumor sections were incubated with the primary antibody for 16 hr at 4°C, followed by incubating with HRP-conjugated secondary antibodies for 1 hr. Staining was visualized by using 3, 3’-diaminobenzidine (DAB), followed by counterstaining with hematoxylin. The primary antibodies of MCAM (dilution: 1:200), Notch1 (dilution: 1:200) were used in this assay (Supporting Information Table S4). The study and related procedures were approved by the Ethical Review Board of the hospital as indicated.

Proteins were extracted from culture cells (about 1 × 10⁶–5 × 10⁶ cells) by using radioimmunoprecipitation assay (RIPA) buffer. The Bio-Rad BCA protein assay was performed to quantify total protein. Cell protein aliquots were loaded on the SDS-PAGE gel, and then transferred to a PVDF membrane with subsequent blockade with 5% nonfat milk in TBST buffer. After 16 hr incubation with primary antibodies (Supporting Information Table S4) in 4°C, the blots were finally incubated with HRP-conjugated secondary antibody (Dilution: 1:5,000) for 1 hr and visualized using ECL Substrates (Applygen).

Total RNA was extracted from breast cancer cells using Trizol (Thermo Fisher) based on the manufacturer instructions. qRT-PCR assay was performed using primers as described previously. Reverse transcription was performed using the PrimeScript™ RT reagent kit (Takara Bio Inc., Dalian, China) on a CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA). Amplification was performed with the following cycling conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The sequences of forward and reverse primers used in the qRT-PCR assay were showed in Supporting Information Table S5.

About 1 × 10⁵ cells were seeded with 60% confluence in Millicell EZ 8-well glass slides (Merck Millipore, Germany) and fixed with 4% paraformaldehyde. After that, cells were treated with 0.5% Triton X-100, and then blocked with 10% BSA for 20 min. After incubated with primary antibodies (Notch1: 1:200; MCAM: 1:200) for 16 hr at 4°C, cells were then incubated with secondary antibodies (Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 594 donkey anti-rabbit IgG) at room temperature for 1 hr. Slides were finally mounted in Vectashield with DAPI (Life Technology, New York, NY). Images were visualized and captured with an immunofluorescence microscope (Carl Zeiss, Jena, Germany).

Then, 2 × 10⁶ MDA-MB-231 or MDA-MB-231-DDPR cells were seeded in 96-well plates and exposed to different treatments as described in the Figure legend. The Cell counting kit (CCK-8; Beyotime Institute of Biotechnology, Jiangsu, China) was added to the medium and incubated for 2 hr by following the manufacturer’s instructions before measuring the absorbance value (450 nm).

The pCMV-GFP-MCAM plasmids and corresponding empty vector pCMV-GFP were purchased from Sino Biological Inc. (Beijing, China). We are grateful to Prof. Yaochen Li sharing the N1ICD expression plasmid (LV201-N1ICD). MCAM promoter was amplified using primers from Supporting Information Table S5 and inserted in pGL3-SV40 Enhancer reporter vector using the restriction enzymes Xhol and SmaI. The Fast Mutagenesis System (TransGene Biotech, Beijing, China) was used to construct the mutant MCAM promoter-reporter (TGGGAA mutated to TGTCAA using primers from Supporting Information Table S5) according to the manufacturer’s protocols. The small interference RNA (siRNA) and short hairpin RNA (shRNA) were generated by GenePharma Company (Suzhou, China). Cells were transfected with the plasmids, siRNA and shRNA using Lipofectamine 3,000 reagent (Life Technology) according to the manufacturer’s instructions. The oligonucleotides sequences are shown in Supporting Information Table S5.

The chromatin immunoprecipitation (ChIP) experiment was performed with a ChIP assay kit (Beyotime, Shanghai, China) by following the manufacturer’s instructions, as described previously. Briefly, cells were cultured in 10 cm dish and crosslinked with 1% formaldehyde at room temperature for 10 min. Then, 10 ml ice-cold 1 mM PMSF PBS were added to cells and washed twice. Cells were centrifuged and resuspended with 1 mM PMSF SDS Lysis Buffer for 10 min and then sonicated to an average length of sheered genomic DNA of approximately 200–1,000 bp. Then, 1 μg Notch1 antibody was used in the ChIP assay and IgG was used as control. The lysate was incubated with 60 μg Protein A + G Agarose/Salmon Sperm DNA for 1 hr and washed with wash buffer. ChIP-purified DNA was quantified by using MCAM primers (Supporting Information Table S5).

The MDA-MB-231 cells and a series of oligonucleotides that contained the core sequence (TGGGAA) of CSL-binding elements were used and performed with an electrophoretic mobility shift assay (EMSA) Kit (Viagene, Tampa, FL) according to manufacturer’s protocols. In competition experiments, excessive amounts
of unlabeled competitors were added and incubated for 20 min, followed by the addition of labeled probes. In supershift assays, 5 μg of anti-Notch1 monoclonal antibody (Cell Signaling Technology) was added and incubated at 4°C for 1 hr. Sequences of the probes and mutated competitors utilized in the EMSAs are shown in Supporting Information Table S5.

Luciferase reporter assays
Then, 5 × 10^4 cells were seeded in 24-well plate and cotransfected with MCAM promoter luciferase reporter and LV201-N1ICD plasmid using Lipofectamine 3000. The control vector pRL-SV40 was also co-transfected in each sample to normalize transfection efficiency. Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used by following the manufacturer’s instructions.

Wound healing assay
Cells were seeded in a 6-well plate and allowed to grow to a 90% confluent monolayer. After serum-free starved for 12 hr, cells were wounded through scratching the monolayer with a 100 μl pipet tip in the middle of each well. After washed with PBS three times, Serum-free DMEM medium was added to the wells and then incubated at 37°C in 5% CO₂. All the data in the wound-healing assay are presented as a percentage of Day 0 wound width.

Transwell assay
Transwell assay was conducted to examine the cell migratory and invasive capacity, as described previously.²¹ Briefly, cells were seeded in upper transwell chambers (8 um pore size; BD, San Jose, CA) with 0.1% FBS medium. Medium with 10% FBS was added to the lower chamber. After cultured for 48 hr, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of cells from five fields in each well was counted by two investigators. Each assay was performed in triplicate.

Clonogenic survival assay
The sensitivity of MDA-MB-231 cells to cisplatin treatment was determined using the clonogenic assay in six-well plates. Two hundred cells were seeded in the plates and incubated overnight for cell adhesion. After that, cells were exposed to 0, 0.1 or 1 μM cisplatin. The medium were refreshed very 3 day. Two weeks later, cells were fixed and stained with crystal violet. Colonies consisting of minimum 50 cells were counted. Each assay was performed in triplicate.

Tumor xenograft models
The mouse tumorigenesis protocol was reviewed and approved by the Animal Care and Use Committee of Shantou University Medical College. Then, 2 × 10^6 MDA-MB-231-Fluc cells, MDA-MB-231-shNotch1-Fluc cells or MDA-MB-231–shNotch1+ pCMV-MCAM-Fluc cells were unilaterally injected into the mammary fat pad of 6-week-old Nu/Nu female mice (purchased from Vital River, Beijing, China). Tumor growth was monitored by measuring the width and length of tumors twice a week. The corresponding images were captured by using an IVIS Kinetic imaging system (PerkinElmer, Waltham, MA). Mice were euthanized after 42 days following tumor cells implantation.

Statistical analysis
Data are expressed as the mean ± SEM, unless otherwise stated, and were statistically analyzed using a two-sided Student’s t-test. p < 0.05 was considered statistically significant. Kaplan–Meier survival curve, HR with 95% confidence intervals and log-rank p value were calculated and plotted in R using Bio-conductor packages.

Data availability
For correlation analysis, a dataset of 825 cases for breast cancer from The Human Cancer Genome Atlas (TCGA, Nature 2012) were downloaded and analyzed from cbioportal (http://www.cbioportal.org/study/summary?id=brca_tcgapub). The data will be made available upon reasonable request.

Results
MCAM is associated with the expressions of Notch1 and key mesenchymal markers in TNBC
Previous studies have shown that both Notch signaling and MCAM were critical driver genes in EMT activation,²²,²³ as well as contributed to chemoresistance in multiple tumor types.²⁴ Furthermore, it has also been reported that MCAM was significantly involved in Notch signaling,²⁵ however, which specific Notch family member has the most intimate connection and potential regulatory effects with MCAM in breast cancer is not yet determined. Hence, we initially conducted an extensive analysis of public databases with plenty of breast cancer cases to probe the association between Notch family members and MCAM, as well as key EMT markers. We found that, among the four Notch family members, only Notch1 expression was significantly correlated to MCAM in breast cancer (Fig. 1a).

Subsequently, the mRNA and protein levels of Notch1 are similar in all breast cancer cell lines while MCAM was highly expressed in ER-negative breast cancer cell lines (MDA-MB-231, SK-BR-3 and BT-549), but relatively low expressed or absent in ER-positive breast cancer cell lines (MCF-7 and T-47D; Figs. 1b and 1c). Immunofluorescence assay in MDA-MB-231 cells demonstrated that Notch1 primarily located in the nucleus, and MCAM mainly located in the cellular membrane (Fig. 1d). The result was in line with the finding in immunohistochemistry assay in TNBC tissue samples, which showed that MCAM predominantly located in the cellular membrane and particularly aggregated in the margin of tumor nest (Fig. 1e), implying that MCAM might act as a critical factor that facilitate cancer cells invasion and dissemination.
Analysis of a dataset with 528 breast cancer cases derived from TCGA, which demonstrated the mRNA level of Notch1 was positively associated with that of MCAM ($r = 0.4450$, $p < 0.0001$; Fig. 1e). The positive rate of Notch1 was statistically higher in ER-negative tumors than those in ER-positive tumors ($p < 0.00$). The positive rate of Notch1 was statistically higher in basal-like
Figure 2. The prognostic values of Notch1 and MCAM expressions breast cancer patients. (a–c) The Kaplan–Meier survival curves of high and low expressions of Notch1 in basal-like subtype breast cancer patients (a: n = 718, HR = 1.56, p = 0.00054), and those treated with chemotherapy (b: n = 230, HR = 1.82, p = 0.02) and without chemotherapy treatment basal-like subtype breast cancer patients (c: n = 184, HR = 1.17, p = 0.53). (d–f) The Kaplan–Meier survival curves of high and low expressions of MCAM in basal-like subtype breast cancer patients (d: n = 718, HR = 1.29, p = 0.048), and those treated with chemotherapy (e: n = 230, HR = 1.66, p = 0.045) and without chemotherapy treatment basal-like subtype breast cancer patients (f: n = 184, HR = 1.22, p = 0.41). [Color figure can be viewed at wileyonlinelibrary.com]
Figure 3. Notch1 induces EMT by upregulating MCAM. (a) Notch1, MCAM, E-cadherin and Vimentin protein levels were analyzed by western blot in MDA-MB-231 and BT-549 when knockdown Notch1, with or without re-overexpressing MCAM. (b,c) Representative images (b) and quantitative (c) wound recovery data of 0 hr and after 24 hr in MDA-MB-231 cells transfected with shNotch1 and with or without overexpressing MCAM. (d,e) Representative images (d) and quantitative (e) wound recovery data of 0 hr and after 24 hr in BT-549 cells transfected with shNotch1 and with or without overexpressing MCAM. (f,g) Representative images (f) and quantitative (g) migration and invasion assays in MDA-MB-231 cells transfected with shNotch1 and with or without overexpressing MCAM. (h,i) Representative images (h) and quantitative (i) migration and invasion assays in BT-549 cells transfected with shNotch1 and with or without overexpressing MCAM. Mean ± SEM, ns = no significance; *p < 0.05; **p < 0.01; ***p < 0.001 by Student's t-test. [Color figure can be viewed at wileyonlinelibrary.com]
Figure 4. Notch1 promotes cisplatin-chemoresistance in MDA-MB-231 cells by regulating MCAM. (a) The protein levels of Notch1 and MCAM were measured by Western blot in MDA-MB-231 cells treated with cisplatin. Please see Supporting Information Figure 1a for quantification of Notch1 and MCAM protein levels. (b) Cell viability analysis of cisplatin-resistant MDA-MB-231 cells (MDA-MB-231-DDPR) and parental MDA-MB-231 cells after treatment with cisplatin. (c) Protein level of Notch1, MCAM, Vimentin, pAKT, P-glycoprotein (P-gp) and multidrug resistance protein 1 (MDR1) detected by Western blot analysis in MDA-MB-231 and MDA-MB-231-DDPR cells. (d,e) Representative micrographs and quantitative migration and invasion transwell assays. (f) IC50 analysis of cisplatin-resistant MDA-MB-231-DDPR cells transfected with shNotch1, shMCAM or pCMV-MCAM after treatment with cisplatin. (g) IC50 analysis of MDA-MB-231 cells transfected with shNotch1, shMCAM or pCMV-MCAM after treatment with cisplatin. (h) Representative images and quantitative of colony formation in MDA-MB-231 cells treated with or without cisplatin are shown. Mean ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by Student's t-test. [Color figure can be viewed at wileyonlinelibrary.com]
subtype than nonbasal-like subtype of breast cancer ($p < 0.00$; Supporting Information Table S1). A similar tendency was found in the mRNA profile of MCAM, which showed that the positive rate of MCAM was notably higher in ER-negative tumors than those in ER-positive tumors ($p = 0.003$). The positive rate of MCAM was significantly higher in basal-like subtype than nonbasal-like subtype of breast cancer ($p < 0.00$; Supporting Information Table S2).

Figure 5. Legend on next page.
Immunohistochemistry for 52 pathologically diagnosed TNBC tissue samples further confirmed that Notch1 expression was positively associated with MCAM in TNBC (r = 0.356, p = 0.01; Fig. 1f, Supporting Information Table S3). Therefore, we postulated that both Notch1 and MCAM might act as pivotal roles in TNBC and jointly participate in the regulation of mesenchymal attributes.

High expression of Notch1 and MCAM predicted a poor prognosis in TNBC patients, particularly in the subgroup receiving chemotherapy

We used Kaplan–Meier-plotter, an online database with 5,143 breast cancer patients, to elucidate the association of Notch1 and MCAM with survival outcomes in TNBC patients, with a particular focus on those who received chemotherapy. In patients with triple-negative/basal-like subtype breast cancer, elevated Notch1 expression was significantly associated with reduced recurrence-free survival (RFS, p = 0.00054, HR = 1.56; Fig. 2a). Of note, subgroup analysis indicated that higher expression of Notch1 was significantly associated with a shorter RFS in those patients who received chemotherapy (p = 0.02, HR = 1.82; Fig. 2b). However, no significance was found in the group without chemotherapy (p = 0.53, HR = 1.17; Fig. 2c). In addition, high level of MCAM was also associated with poor RFS in triple-negative/basal-like subtype breast cancer patients (p = 0.048, HR = 1.29; Fig. 2d) as well as those patients received chemotherapy (p = 0.045, HR = 1.66; Fig. 2e). However, no statistically significant difference was found in those without receiving chemotherapy (p = 0.41, HR = 1.22; Fig. 2f). Thus, these data suggested that high expression of Notch1 and MCAM might contribute, at least in part, to the chemoresistance of TNBC.

Notch1 induces EMT by upregulating MCAM

It has been reported that Notch1 was involved in the modulation of EMT and promoted aggressive ability in breast cancer.22,26 We found that knockdown of Notch1 in MDA-MB-231 and BT-549 cells reduced MCAM and mesenchymal marker Vimentin expressions but increased the epithelial marker E-cadherin expression, while re-expressing MCAM in Notch1-knockdown MDA-MB-231 cells abrogated these expression changes (Fig. 3a). Next, we examined the influence of the Notch1/MCAM axis on the migration and invasion of TNBC cells. Knockdown of Notch1 showed decreased migratory ability in wound healing assay, while re-overexpressing MCAM expression could restore the aggressive property (Figs. 3b–3e). Transwell assay showed that the migratory and invasive ability shift reduced by knockdown of Notch1 was almost prevented by restoration of MCAM expression (Figs. 3f–3i). Collectively, these data suggest that Notch1 induces EMT through regulating MCAM in TNBC cells.

Notch1 and MCAM expressions are higher in cisplatin-resistant MDA-MB-231 cells

To explore the role of Notch1 and MCAM in chemoresistance of breast cancer cells, we treated parental wild-type MDA-MB-231 cells with 0.5 μM/l cisplatin from 0 hr to 12 hr. We found that both Notch1 and MCAM expressions were increased time-dependently when exposing to cisplatin (Fig. 4a and Supporting Information Fig. S1a). Next, we established a cisplatin-resistant MDA-MB-231 cell line (MDA-MB-231-DDPR) by continuously exposed to increasing concentrations of cisplatin for 6 months until a stable resistant phenotype was obtained. The IC_{50} value of cisplatin to MDA-MB-231-DDPR cells (77 ± 0.7148 μM/l) was about 6.02 times higher than that of parental cells (12.85 ± 0.599 μM/l; Fig. 4b). Then, we examined Notch1 and MCAM expressions in MDA-MB-231-DDPR cells. As shown that both protein and mRNA of Notch1 were higher in the MDA-MB-231-DDPR cells as compared to the parental MDA-MB-231 cells (Fig. 4c; Supporting Information Fig. S1b). In addition, the mesenchymal marker Vimentin and major drug resistance-related proteins including pAKT, P-glycoprotein (P-gp) and multidrug resistance protein1 (MRP1) were also activated (Fig. 4c). Furthermore, the transwell assays showed that the migratory and invasive capabilities were enhanced in MDA-MB-231-DDPR cells as compared to parental MDA-MB-231 cells (Figs. 4d and 4e). These results suggest that Notch1 and MCAM may play essential functions in acquiring cisplatin resistance and inducing EMT programs in the MDA-MB-231 cell line.

Notch1 promotes cisplatin resistance in MDA-MB-231 cells by regulating MCAM

To evaluate the effects of Notch1 and MCAM on chemoresistance of breast cancer cells, we treated cisplatin-resistant MDA-MB-231
cells (MDA-MB-231-DDPR) with varying doses of cisplatin and measured the IC50 values. Knockdown of Notch1 or MCAM enables MDA-MB-231-DDPR cells to become more sensitive to cisplatin (IC50 were \(33.85 \pm 0.5773 \mu M/l \) and \(35.73 \pm 1.732 \mu M/l \), respectively), as compared to the cisplatin-resistant (MDA-MB-231-DDPR) control group (IC50 = \(59.10 \pm 1.155 \mu M/l \)). It was

Figure 6. MCAM is a pivotal mediator in Notch1 induced tumor formation and proliferation in breast cancer. (a) \(2 \times 10^6\) MDA-MB-231-Luciferase cells were injected into the mammary fat pad tissue of immunodeficient NU/NU mice (control group \(n = 8\); Notch1 knockdown group \(n = 8\); Notch1 knockdown and MCAM re-overexpressing group \(n = 8\)). Representative bioluminescence images of mice tumor were shown. (b) Tumor size was monitored every 3 days. (c) Primary tumors dissected from each group of mice are shown. (d) Weight of primary tumors in the different groups. (e) Schematic signaling model of Notch1/MCAM axis promoting EMT, invasion and chemoresistance in TNBC cells. [Color figure can be viewed at wileyonlinelibrary.com]
more obvious that combinative inhibition of Notch1 and MCAM by using RNAi significantly enhanced cisplatin sensitivity (IC50 = 26.99 ± 1.842 μM/l). However, the effect of siNotch1 in chemo-sensitive can be partly nullified by overexpressing MCAM (IC50 = 47.58 ± 1.039 μM/l; Fig. 4f; Supporting Information Fig. S1c). In MDA-MB-231 cells, knockdown of Notch1 decreased IC50 value to cisplatin (IC50 = 7.600 ± 0.2887 μM/l) compared to the control group (IC50 = 16.00 ± 0.5774 μM/l), while re-expressed MCAM abrogated this effect (IC50 = 15.80 ± 0.4041 μM/l; Fig. 4g; Supporting Information Fig. S1d). Furthermore, we demonstrated that re-expressing MCAM could restore the inhibition of colony formation ability by depletion of Notch1, especially in the cells treated with cisplatin (Figs. 4h and 4i). These findings indicate that Notch1 acts as a pivotal mediator of cisplatin resistance via modulating MCAM in TNBC cells.

**Notch1 ICD directly binds to the MCAM promoter and activates its expression**

In MDA-MB-231, knockdown of Notch1, but not Notch2 and Notch4, decreases the protein level of MCAM, which were further verified by qRT-PCR in mRNA level (Figs. 5a–5c; Supporting Information Figs. S2a–S2c). The Notch target gene Hey1 is also downregulated when knockdown of Notch1 (Supporting Information Fig. S2d). Next, we used the gamma-secretase inhibitor (DAPT) to block Notch1 intracellular domain (ICD) in MDA-MB-231 cells. It was found that both Notch1 ICD and MCAM expressions were decreased dose-dependently by treating with gamma-secretase inhibitor (Fig. 5d). These findings suggest that Notch1 positively regulates MCAM in TNBC cells.

To explore the regulatory mechanism of MCAM by Notch1, we further evaluated whether Notch1 regulated MCAM in transcription level. As expected, four Notch family CSL binding sites were confirmed to be present in the promoter of MCAM. Among them, three continuous CSL binding sites were located in −3.5 kb of MCAM promoter, the other one was located at −477b (Supporting Information Fig. S2f). ChIP assay demonstrated that Notch1 directly bind to the promoter of MCAM, especially in the region of −3.5 kb upstream of MCAM exon 1 (Figs. 5e and 5f). We next constructed the wild type and mutant MCAM promoter-luciferase reporter. Overexpression of Notch1 ICD induces nearly 15 folds of luciferase activities in MCAM promoter-luciferase reporter, which contains three CSL binding sites, and more than threefold in the reporter that contains one CSL binding site compared to the control reporter. However, mutated CSL binding sites can abrogate the increasing luciferase activities by Notch1 (Figs. 5g and 5h), suggesting that Notch1 signaling is responsible for trans-activating MCAM expression in TNBC cells. Next, electrophoretic mobility shift assays (EMSAs) was used to explore the elements required for Notch1 binding within the promoter of MCAM. In competition assays, we found that the addition of excess unlabeled nucleotides eliminated the Notch1 shifting band. However, this effect was not observed in the presence of an excess of unlabeled mutated oligonucleotide. After adding labeled probe and anti-Notch1 antibody to MDA-MB-231 nuclear extracts, a super-shifted band was observed, suggesting that Notch1 was capable of binding to the typical core bound element within the promoter of MCAM. Our findings indicate that Notch1 is a component of a complex that binds the MCAM promoter (Figs. 5i and 5j).

**MCAM is a pivotal mediator in Notch1-induced tumor formation and proliferation in MDA-MB-231 cells**

We next performed tumor xenograft model to explore the role of Notch1 and MCAM in tumor formation and proliferation. MDA-MB-231 cells were injected into the mammary fat pad of NU/NU mice. It showed that Notch1-knockdown MDA-MB-231 cells developed fewer tumor formation (4/8) than the control group. In addition, the proliferation rate was slower and tumor weight was less in the Notch1-knockdown group. However, re-overexpressing MCAM in Notch1 depleted MDA-MB-231 cells restored the tumor formation and proliferation capability (Figs. 6a–6d, Supporting Information Fig. S2g). These results showed that Notch1 inhibition suppressed tumor formation and proliferation in MDA-MB-231 cells by downregulating MCAM.

**Discussion**

Systemic chemotherapy remains a mainstay in the treatment of women with TNBC, either at adjuvant or metastatic setting, due to the absence of a valid target (73). Generally, TNBCs are more susceptible to chemotherapy as compared to luminal-A, luminal-B or HER2-positive tumors.27 At present, taxanes and anthracyclines are the cornerstone of adjuvant chemotherapy for TNBC patients. Platinum has also been investigated in clinical trials and showed a beneficial effect on this subset of patients, particularly in BRCA-mutation carriers.28,29 However, the risk of relapse for patients with TNBC is markedly higher than those with hormone-positive subtypes, primarily attributed to the aggressive biological nature and in a large part to chemoresistance, which accounts for over 90% of treatment failure in patients presenting with advanced and metastatic diseases.30

Currently, the established molecular mechanisms of chemoresistance in TNBC include (i) ABC transporter,31 (ii) alterations in genes involved in apoptosis inducer such as p53,32 (iii) aberrant activation in NF-κB or PI3K/AKT signaling pathways,33,34 (iv) mutations in DNA repair enzymes such as DNA mismatch repair enzymes,35 (v) autophagy36 and more recently, (vi) EMT.3 EMT has been recognized to be an essential attribute in cancer biology, in which cancer cells obtain a more aggressive phenotype to adapt to the microenvironmnet with possibly low oxygen and poor nutrition supply and facilitate migration to a more habitable location within the host.37 Increasing evidence has indicated that activation of the EMT program contributes critically to the development of drug resistance in a variety of cancer types, thereby permitting clinical relapse.3

In the present study, in-depth exploratory analysis on an array of publicly accessible databases, including BcGenExMiner v4.1, TCGA and Kaplan–Meier-plotter, revealed a strong link between Notch1 and MCAM mRNA expression, which also demonstrated...
an intimate connection with major EMT markers in TNBC. This remarkable association was also verified in the correlation analysis of immunohistochemical examination for tissue samples from our institution (Fig. 1). Analysis of the prognostic value of the expression levels of Notch1 and MCAM in breast cancer patients indicated that RFS was significantly shorter in patients with high expression of Notch1 in basal-like breast cancer patients, especially those treated with chemotherapy. Similarly, high MCAM expression was significantly correlated with shorter RFS in patients with TNBC subtype breast cancer, particularly in the subgroup undergoing chemotherapy (Fig. 2). Therefore, we postulated that high expression of Notch1 and MCAM might contribute, at least in part, to the chemoresistance of TNBC.

Strikingly, *ex vivo* experiments revealed that, when exposing to low dose cisplatin (nonlethal), the expression of Notch1 and MCAM in TNBC cells gradually increased in a time-dependent manner. The expression of Notch1 and MCAM in cisplatin-resistant TNBC cells (MDA-MB-231-DDPR) were significantly higher than wild-type counterparts. In addition, the expression of mesenchymal marker Vimentin and classic chemoresistance-associated proteins, such as pAKT, P-gp and MRP1, were also found to significantly upregulate in MDA-MB-231-DDPR cells (Fig. 4). A similar phenomenon was observed in a recent study by researchers from MD Anderson Cancer Center, demonstrating that MCAM markedly upregulated in chemoresistant small cell lung cancer (SCLC) cell lines that exhibited a mesenchymal phenotype and in chemoresistant patient-derived xenografts (PDxs) compared to matched treatment-naive tumors.16 Hence, we postulated that Notch1 and MCAM might jointly participate in the modulation of EMT program, and play essential roles that enable TNBC cells to acquire anticancer drug resistance.

The major molecular hallmark of EMT in breast cancer is the downregulation of E-cadherin and upregulation of cytoskeleton protein vimentin.38,39 Lost- and gain-of-function experiments in TNBC cells demonstrated that inhibition of Notch1 substantially decreased MCAM expression both at mRNA and protein levels, along with significant reduction of MCAM expression. Subsequent rescue experiments in two TNBC cell lines demonstrated that Notch1 was able to modulate the EMT program with corresponding changes in the expression of E-cadherin and Vimentin, as well as alter their migratory and invasive capabilities. More importantly, MCAM was found to act as a key mediator during these regulatory processes (Fig. 3). The results were in line with previous studies recognizing that Notch1 signaling pathway was a pivotal signal pathway constitutively involved in EMT regulation in breast cancer,23,26 and agree with the latest perspective that MCAM is a unique EMT inducer.13,26

The intriguing findings prompt us to further evaluate whether the Notch1/MCAM signaling pathway can also influence chemo sensitivity. *In vitro* drug resistance assays revealed that, as compared to the control group, knockdown of Notch1 in MDA-MB-231 cells significantly enhanced their sensitivity to cisplatin, along with significantly reduced IC_{50}. While enforced expression of MCAM, the sensitivity of MDA-MB-231 cells to cisplatin decrease again, with IC_{50} significantly increased. The clonal formation assay also found a similar tendency. These results verified that inhibition of the Notch1/MCAM signaling pathways not only reversed the EMT in TNBC cell, but also increased their sensitivity to cisplatin (Fig. 4). The viewpoint was supported by the findings in the study by Ren et al.,40 who suggested that targeted inhibition of ZEB1 in pancreatic cells can restore epithelial cell characteristics, and enhance the sensitivity of antitumor drugs. Moreover, recent clinical studies have shown that EMT-targeted therapeutic strategies can reverse cancer resistance to the chemotherapeutic drug, including carboplatin and gemcitabine.41,42

Previous studies in melanoma by Pinnix et al. have reported that Notch1 signaling could regulate MCAM expression, however, the detail regulatory mechanism remains not fully elucidated. In the present study, we found the core sequence of CSL binding element in Notch1 signaling in the MCAM promoter. ChiP and EMSA experiments mutually validated that Notch1-ICD can directly bind to the CSL binding site in MCAM promoter in vitro. Moreover, dual-fluorescent reporter assay confirmed that Notch1-ICD can drive the activity of MCAM promoter in MDA-MB-231 cells (Fig. 5). The above results suggested that Notch1 transcriptionally activated MCAM expression in TNBC cells.

The mediating effect of MCAM in Notch1 signaling was also found in *in vivo* experiments. Xenograft models demonstrated that, compared to the control group, knockdown of Notch1 significantly reduced tumor size than the control group. While enforced-expressed of MCAM significantly enhanced tumor formation ability and tumor size, which indicated that MCAM mediated the Notch1 signaling in promoting the proliferative ability of breast cancer cells, as well as facilitating xenograft tumor formation (Fig. 6).

One possible explanation for the Notch1/MCAM axis promoting EMT and contributing to cisplatin resistance of breast cancer cells, is that the EMT process could enable the cancer cell to acquire self-renewal capacity, with features resemble those of cancer stem cells,43 since Notch1 and MCAM has been recognized to be essential contributors of cancer stemness.44,45 Another reason is that the Notch1/MCAM axis is able to activate the PI3K/AKT pathway, as well as upregulation of several classic chemoresistance proteins, such as P-gp and MRP1, which has been demonstrated in cisplatin-resistant MDA-MB-231 cells in the present study. The previous study on the mechanism of chemoresistance in breast cancer suggested that canonical Notch1 pathway can promote chemoresistance by regulating PTEN/PI3K/AKT pathways.46 Several studies suggested that the constitutive and inducible PI3K/AKT activities involved in chemoresistance of breast cancer.47,48 A study by Singel and colleagues showed that KLF14 promoted AKT phosphorylation, thereby enhancing chemoresistance in TNBC.49 Kim et al. reported that chemotherapy induces Notch1-dependent MRP1 upregulation, suppression of which sensitizes breast cancer cells to chemotherapy.24 Finally, MCAM is a key activator of the PI3K/AKT pathway, which has been widely recognized to
contribute to drug resistance in multiple cancers,\textsuperscript{14,50} as shown in the representative signaling model in Figure 6e.

In summary, Notch1 was positively correlated with MCAM in TNBC both at mRNA and protein levels. High expression of Notch1 and MCAM both predicted a poor prognosis in basal-like/TNBC patients, particularly in those treated with chemotherapy. Notch1 promotes EMT and chemoresistance, as well as invasion and proliferation of TNBC cells via direct activating MCAM promoter. Inhibition of Notch1 significantly downregulated MCAM expression, resulting in the reversion of EMT and chemoresistance to cisplatin in TNBC cells. Our study might help to better understand the regulatory mechanism of EMT in contribution to chemoresistance in breast cancer and provides evidence that employing Notch inhibitor or MCAM monoclonal antibody, in conjunction with conventional chemotherapies, might be a potential avenue to enhance the therapeutic efficacy for patients with TNBC.

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Conflict of interest
No potential conflicts of interest were disclosed.

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