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A yes-associated protein 1- Notch1 receptor positive feedback loop promotes breast cancer lung metastasis by attenuating the bone morphogenetic protein 4-SMAD family member 1/5 signaling

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Abstract

The Notch1 (Notch1 receptor) and yes-associated protein 1 (YAP1) signaling can regulate breast cancer metastasis. This study aimed at investigating whether and how these two signal pathways crosstalk to promote breast cancer lung metastasis. Here, we show that YAP1 expression was positively correlated with Notch1 in breast cancer according to bioinformatics and experimental validation. Mechanistically, YAP1 with TEA domain transcription factors (TEADs) enhanced Jagged1(JAG1)-Notch1 signaling. Meanwhile, Notch1 promoted YAP1 stability in breast cancer cells by inhibiting the β-TrCP-mediated degradation, thereby, forming a YAP1- JAG1/Notch1 positive feedback loop in breast cancer. Furthermore, YAP1 enhanced the mammosphere formation and stemness of MDA-MB-231 cells by attenuating the inhibition of the BMP4-SMAD1/5 signaling. *In vivo*, the YAP1- JAG1/Notch1 positive feedback loop promoted the lung colonization of MDA-MB-231 cells. Our data for the first time indicate that the YAP1-Notch1 positive feedback loop promotes lung metastasis of breast cancer by modulating self-renewal and inhibiting the BMP4-SMAD1/5 signaling.

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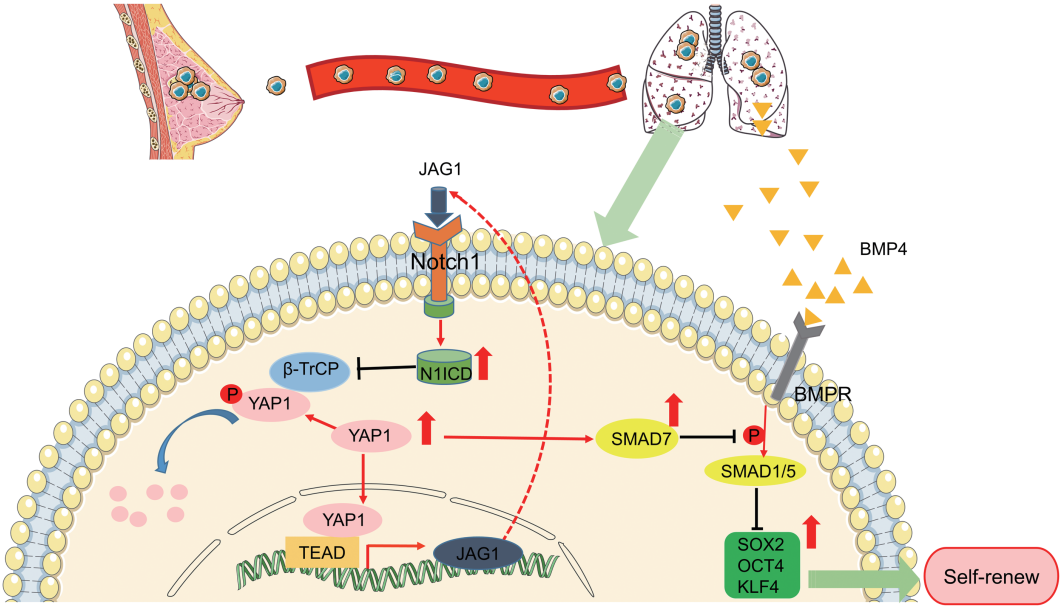
Graphical Abstract



Abbreviations: β-TrCP, beta-transducin repeat-containing protein; BMP4, Bone morphogenetic protein 4; CTGF, Connective tissue growth factor; Co-IP, Co-immunoprecipitation; JAG1, Jagged1 canonical Notch ligand 1; KLF4, Kruppel-like factor-4; mRNA, Messenger RNA; MMP9, Matrix metallopeptidase 9; Notch1, Notch1 receptor; N1ICD, Notch 1 receptor intracellular domain; OCT4, Octamer-binding transcription factor-4; RT-qPCR, Real-Time Quantitative Reverse Transcription polymerase chain reaction; SMAD1/5, SMAD family member 1/5; SMAD7, SMAD family member 7; SOX2, SRY-box transcription factor 2; TEADs, TEA domain family; YAP1, Yes-associated protein 1.

Introduction

Breast cancer remains the main malignant tumor threatening women's health (1). More than 90% of breast cancer-related deaths are related to metastasis, particularly into the lung and bone, lung metastasis is still difficult to treat (2). It is estimated that about 65% of breast cancer patients die of lung metastasis (1–4). Hence, the discovery of the molecular mechanisms underlying the lung metastasis of breast cancer will of significance in revealing new therapeutic targets and management of breast cancer patients.

Yes-associated protein 1 (YAP1) is the key transcriptional co-activators of the Hippo pathway. YAP1 can translocate into the nucleus, where they co-activate many transcription factors to initiate the expression of the downstream gene, such as *CTGF*. These transcription factors include the TEAD family members (TEAD1-4), SMAD family, RUNX2 family, and P73 (5–7). And motif analysis shows that TEADs are the main platform for YAP/TAZ to bind to chromatin (5). Conversely, YAP1 can be phosphorylated by activated LATS1/2 and undergo ubiquitin-dependent proteasomal degradation mediated by β-TrCP in the cytoplasm. In breast cancer, YAP1 has been proven to play a key role in breast cancer cell growth, invasion, and lung metastasis (8).

The dysregulation of Notch signaling contributes to the recurrence and metastasis of multiple malignant tumors, including breast cancer (9). Engagement of the Notch receptor (Notch1-4) by its ligands, Delta-like-1, Delta-like-3, Delta-like-4, Jagged1 (JAG1), and Jagged2, can trigger a sequence of proteolysis events. Ligand binding leads to cleavage of Notch ectodomain by ADAM (a disintegrin and metalloprotease), which activates γ-secretase complex to release the Notch receptor intracellular domain (NICD) (10). NICD translocates to the nucleus and binds transcription factors to induce the expression of related genes, including Hairy and enhancer of split (Hes), Hes-related with YRPW motif (Hey) (11). Previous studies have shown that aberrant activation of the Notch signaling can promote tumorigenesis (12) and high Notch1 and JAG1 expression is associated with poor prognosis of breast cancer (11,13).

It is well known that the crosstalk among different signal pathways can cooperatively regulate the development and progression of malignant tumors. The YAP1 signaling can interact with the TGF-β/Smad, Wnt/β-catenin, and G protein-coupled receptor (GPCR) signal pathways by directly or synergistically inducing the target gene expression (14–17). Similarly, the Notch signaling can crosstalk with the Wnt/β-catenin, TGF-β/Smad, NF-kB, and Jak/STAT signaling (18–20). Previous studies have demonstrated that the YAP1 could crosstalk with the Notch signaling to control cell fate and organ development as well as malignancy (14,18,21). Notch1 has been reported to induce YAP1 expression in neural stem cells (22), hepatocellular carcinoma (14), and glioma (23), while YAP1 enhances the Notch1 signaling to promote the differentiation and proliferation of hepatic progenitor cells. Conversely, the Notch1 signaling downregulates the transcription of YAP1 in the bile duct regeneration after hepatectomy (21). Furthermore, the YAP1 and NICD, together with their activated transcription factors, cooperatively regulate the expression of common target genes (21). However, such interactive regulation between the YAP1 and Notch signaling is probably dependent on cell type and its surrounding environment (21). In addition, the YAP1 also crosstalk with other signal pathways, such as the TGFβ/BMP/SMAD signaling, the activation of BMP-SMAD signaling inhibits the lung and bone metastasis of mouse breast cancer (24–26). However, there is little information on the crosstalk among these signal pathways that regulate the lung metastasis of human breast cancer.

In this study, we tested the hypothesis that the Notch1 receptor intracellular domain (N1ICD) can interact with YAP1 and stabilize the YAP1 protein. Although YAP1 promotes breast cancer cell self-renewal and lung metastasis by inhibiting BMP4-SMAD1/5 signaling. In breast cancer cells, we observed that YAP1 bound to TEAD family co-activators to activate JAG1 transcription, leading to Notch1 signaling and N1ICD released, N1ICD directly interacted with YAP1 and enhanced the stability of YAP1 protein by disrupting β-TrCP-mediated degradation of proteasome. Moreover, Overexpression of YAP1 enhanced inhibitory SMAD7, which inhibited BMP4-SMAD1/5 signaling. Therefore, Notch1 enhances the role of YAP1 in promoting self-renewal and lung metastasis of breast cancer cells by protecting YAP1 protein stability.

Materials and methods

Detailed descriptions of materials and methods, including HE staining, immunohistochemical staining, lentiviral infection, and siRNA transfection, were described in Supplementary Materials and Methods available at *Carcinogenesis* Online.

Breast cancer cells

Human breast cancer MDA-MB-231, MDA-MB-453, BT-549, T47D, and MCF-7 cells were obtained from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China) and identified by STR. MDA-MB-231, MDA-MB-453, BT-549, T47D, and MCF-7 cells were cultured in L-15 (#KGM41300N-500, KeyGEN BioTECH, China), RPMI1640 (#SH30809.01B, HyClone, USA)and DMEM (#SH30022.01B, HyClone, USA) supplemented with 10% fetal bovine serum (FBS) (#04-001-1A,Biological Industries, Israel), 100 Units/ml penicillin and 100 μg/ml streptomycin(#SV30010, HyClone) (complete medium) at 37°C 5% CO_2, respectively.

Some cells were pretreated in triplicate with 100 ng/ml BMP4 (#120-05ET, Peprotech, Rocky Hill, USA) in an FBS-free medium for 72 h. MDA-MB-231 cells were treated in triplicate with 10 μg/ml of anti-JAG1 (#AF1277-SP, R and D, USA) in complete medium for 48 h.

Patients

The experimental protocol was approved by the Human Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. Patients or family members of patients signed a written informed consent form. A total of 41 female breast cancer patients were recruited and their surgical specimens were obtained when they underwent surgical treatment at the Breast Cancer Surgery Department from 2016 to 2018 and follow-up until December 31, 2019. Individual breast cancer patients were diagnosed by histopathology and radiological images. The tumor-lymph node metastasis (TNM) stage and Molecular subtype were evaluated by pathologists, according to the American Joint Committee on Cancer (AJCC) (8th edition, 2021). Their clinical characteristics are summarized in Supplementary Table S1 available at *Carcinogenesis* Online.

Western blotting

The different groups of cells were harvested and lysed in RIPA cell lysis buffer (#FD008, Fdbio science, Hangzhou, China). After centrifuging, their proteins were determined using a BCA kit (#P0010S, Beyotime, Shanghai, China). The cell lysate samples (30–50 μg/lane) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred onto polyvinylidene difluoride (PVDF) (Millipore, Boston, MA, USA). Block the membrane with 5% nonfat dry milk in TBST for 2 h at room temperature, then incubate with the primary antibody overnight at 4°C and the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using the enhanced chemiluminescent reagents. The data were normalized to the internal control GAPDH and analyzed by densitometric scanning using ImageJ software.

The used primary antibodies included anti-Notch1 (#3068, 1:1000 dilution, Cell Signaling Technology, Boston, MA, USA), anti-YAP1 (#14074, 1:1000 dilution, Cell Signaling Technology), anti-JAG1 (#70109, 1:1000 dilution, Cell Signaling Technology), anti-TEAD1 antibody (#YT4596, 1:1000 dilution, ImmunoWay Biotechnology, Plano, USA), anti-TEAD2 antibody (#YT4597, 1:1000 dilution, ImmunoWay Biotechnology), anti-TEAD3 antibody (#YT4598, 1:1000 dilution, ImmunoWay Biotechnology), anti-TEAD4 antibody (#12418-1-AP, 1:1000 dilution, Proteintech), anti-β-TRCP antibody (#13149-1-AP, 1:1000 dilution, Proteintech), anti-Flag (#20543-1-AP, 1:2000 dilution, Proteintech), anti-HA (#M20003, 1:3000 dilution, Abmart), anti-N-cadherin (#13116,1:1000 dilution, Cell Signaling Technology), anti-occludin (#27260-1-AP,1:1000 dilution, Proteintech), anti-MMP-9 (#10375-2-AP, 1:1000 dilution, Proteintech) and anti-GAPDH (Santa Cruz Biotechnology, USA), anti-phospho-SMAD1/5 (#9516,1:1000 dilution, Cell Signaling Technology), anti-total SMAD1/5 (#YT4325,1:1000 dilution, ImmunoWay Biotechnology), anti-SMAD7 (#ab216428,1:1000 dilution, Abcam).

RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from breast cancer cells using RNAfast 200 (#220011, Fastagen, Shanghai, China) and the RNA samples (1–3 μg each) were reversely transcribed into cDNA using PrimeScript RT reagent kit (#RR037A, TaKaRa, Dalian, China) with random primers (Tsingke Biotechnology, Nanjing, China). The relative levels of the targeted gene to the control GAPDH mRNA transcripts were quantified by RT-qPCR using the SYBR Green PCR Premix Ex TaqIIkit (#RR820A, TaKaRa), and specific primers (Supplementary Table S2 available at *Carcinogenesis* Online).

Co-immunoprecipitation

The potential direct interaction among the interested proteins was determined by Co-immunoprecipitation (Co-IP). Briefly, the different groups of cells were harvested and lysed. After centrifuged, individual groups of cell lysates (100 μg each) were used as the input samples or reacted with control IgG, anti-Notch1 (#3068, 1:150 dilution, Cell Signaling Technology), anti-YAP1 (#14074, 1:100 dilution, Cell Signaling Technology), or anti-HA (#M20003, 1:100 dilution, Abmart) at 4°C overnight for 1 hour. The reactions were mixed with 20 μL Protein A/G PLUS-Agarose (#sc-2003, Santa Cruz Biotechnology) overnight at 4 °C and centrifuged. After being extensively washed, the immunobeads were re-suspended in 40 μL of sample cracking buffer and the bound proteins and the input samples were resolved by

SDS-PAGE, followed by identified by Western blot using anti-N1ICD, anti-YAP1, and anti-β-TrCP.

Mammosphere formation assay

The different groups of cells (0.5×10^4 cells/well) were cultured in triplicate in 6-well ultra-low binding plates (Corning) with DMEM-F12 medium (Heart, China), supplemented with 20 ng/ml EGF, 20 ng/ml basic FGF, 1× B27 for 14 days. Spheres over 100 mm diameter (MDA-MB-231) or 50 mm diameter (MCF-7) were counted and pictured using Nikon microscope.

Transwell migration and invasion assays.

MDA-MB-231 (2×10^5 cells/well) and MCF-7 (3×10^5 cells/well) cells in 200 μL serum-free medium were cultured in triplicate in the upper chamber of 24-well transwell plates (8 μm, Millipore, Billerica, USA). The lower chambers contain 600 μL 10% FBS complete medium. After being cultured for 24 h, noninvaded cells on the top side of the filter membrane were removed by scraping with a cotton swab. Next, 4% paraformaldehyde was applied to fix the membrane, and 0.1% crystal violet was used to stain the invaded cells. The invaded cells were counted and photographed at 200 × magnification for six randomly selected fields per membrane under the optical microscope. The invaded cells were counted in a blinded manner. Similar transwell invasion assays were performed, except for culturing cells for 36 h on the upper chamber membranes that had been coated with the Matrigel (BD Biosciences, San Diego, USA).

Animal study

The experimental protocol was approved by the Animal Care and Use Committee of Xi'an Jiaotong University. Female BALB/c nude mice (4-week-old) were purchased from Silaike Laboratory Animals (Shanghai, China) and housed in a specific pathogens-free facility. The mice were randomized and injected intravenously with 5×10^6 MDA-MB-231-NC, MDA-MB-231-N1ICD, or MDA-MB-231-YAP1 cells through their tail vein. On day 49 post-inoculation, the mice were injected intraperitoneally with D-Luciferin potassium salt (Abcam), and the luminescent signals in individual mice were imaged using the IVIS imaging system (IVIS Spectrum, Xenogen, USA) and the mice were euthanized. Their lung tissues were dissected for H and E staining.

Bioinformatics

The STRING database (functional protein association networks, https://www.string-db.org/) is a website that integrates all publicly available sources of protein-protein interaction information (27). We use this database to conduct a preliminary exploration of the relationship between YAP1, Notch1, and JAG1. Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/) is a website for gene expression analysis based on tumor and normal samples from TCGA and GTEx databases (28). We use this database to evaluated the correlation between YAP1 expression and Notch1 mRNA expression. JASPAR2018 website(http://jaspar.genereg.net/) (29) was used to predict that TEAD1-4s are possible transcription factors for JAG1 in Vertebrate, and we used the matrix alignment tool to perform hierarchical clustering on these transcription factors, then the clustering results are presented as a radial tree. Cistrome DB (http://cistrome.org/db) is a relatively comprehensive and open human and mouse ChIP-seq and open chromatin information resource database (30). We use this database to analyze the regulatory relationship between JAG1 and TEADs.

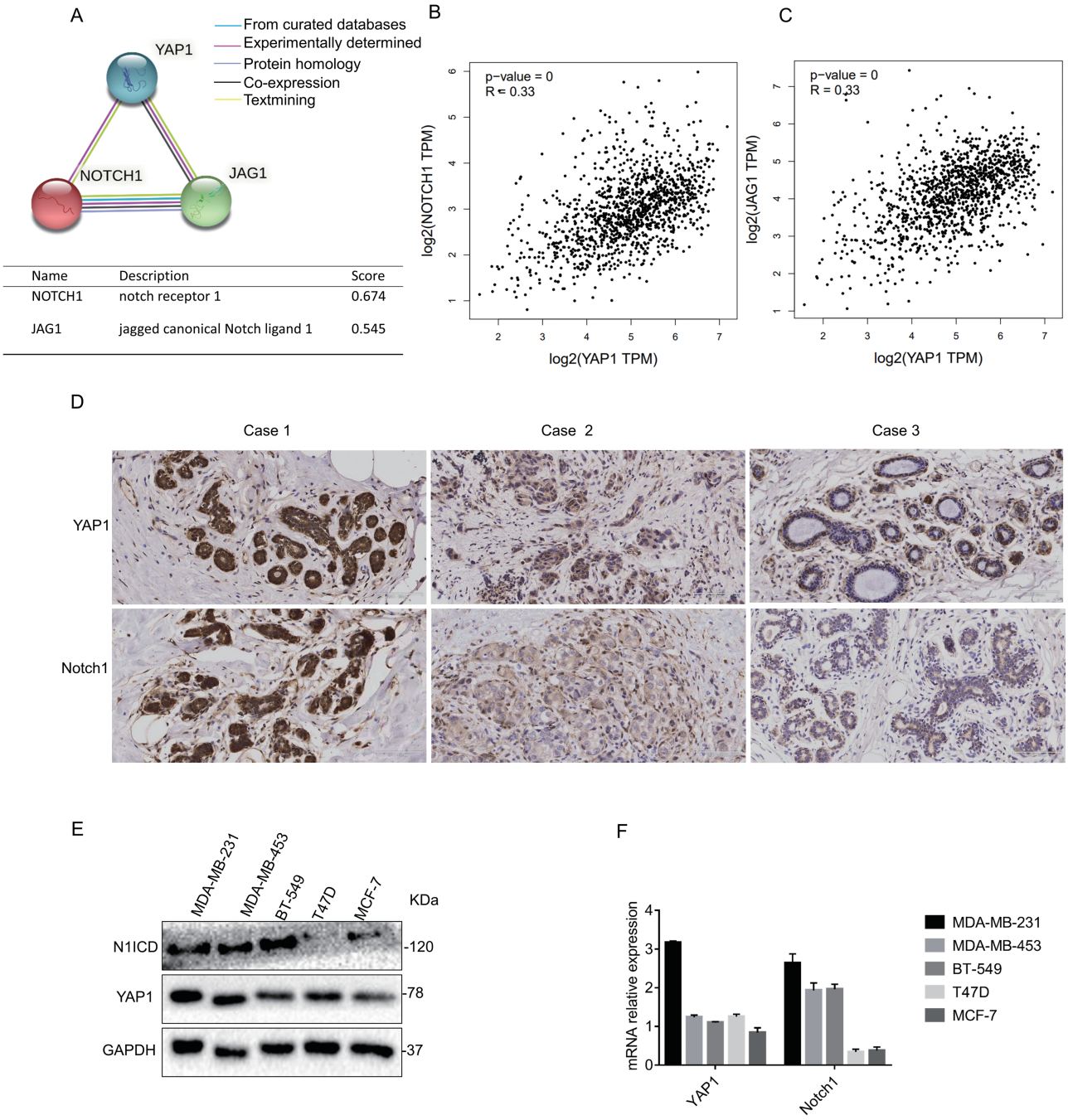
Statistics

Data are obtained from at least three separate experiments and expressed as mean ± standard deviation (SD). The difference among multiple groups was analyzed by one-way ANOVA analysis and between two groups was analyzed by Student's *t*-test. The relationship between YAP1/Notch1 and clinicopathologic characteristics were analyzed by a two-tailed Chi-square or Fisher's test. And the correlation between YAP1 and Notch1 expression were analyzed with Spearman's Rank test. All statistical analyses were performed using SPSS 21.0 (SPSS, Chicago, USA) and GraphPad 7.0 (GraphPad Software). A *P*-value of < 0.05 was considered statistically significant.

Results

Positive correlation between Notch1 and YAP1 expression in breast cancer

To understand the potential relationship between the YAP1 and JAG1/Notch1 signaling, we searched publically available databases to explore the potential interaction of JAG1/Notch1 with YAP1 proteins in breast cancer. In the STRING: function protein association networks (https://www.string-db.org) (27), we found that Notch1 was predicted to interact with YAP1 and YAP1 were co-expressed with JAG1, a ligand of Notch1 (Figure 1A). Furthermore, using GEPIA (http://gepia.cancer-pku.cn/) (28), we found that the relative levels of YAP1 mRNA transcripts in breast cancer tissues were positively correlated with Notch1 ($r = 0.33$ $P < 0.001$, Figure 1B) and JAG1 ($r = 0.33$ $P < 0.001$, Figure 1C). To investigate the relationship between YAP1 and Notch1 protein levels in mammary tumors, we performed immunohistochemistry to analyze the levels of Notch1 and YAP1 expression in 41 human breast cancer specimens (Supplementary Table S1 available at *Carcinogenesis* Online). It was seen that the positive signals of YAP1 and Notch1 can be seen in the cytoplasm and cell membrane (Figure 1D). As shown in Supplementary Table S1 available at *Carcinogenesis* Online, YAP1 expression was correlated with tumor size ($P = 0.05$), lymph node metastasis ($P = 0.015$), and distant metastasis ($P = 0.02$) of breast cancer. Consistently, statistical analysis showed that the expression of Notch1 was also correlated with tumor size ($P = 0.035$), lymph node metastasis ($P < 0.001$), and distant metastasis ($P < 0.001$) in breast cancer. Then, we further analyzed the expression relationship between YAP1 and Notch1 in 41 human breast cancer specimens, and statistical analysis showed that there was a positive correlation between YAP1 and Notch1 staining ($R = 0.547$, $P < 0.001$, Supplementary Table S4 available at *Carcinogenesis* Online). Together, these data suggest that the JAG1/Notch1 and YAP1 signaling may crosstalk in breast cancer. The expression levels of both YAP1 and Notch1 were relatively low in MCF-7 and high in MDA-MB-231 cells (Figure 1E and F),



Name	Description	Score
NOTCH1	notch receptor 1	0.674
JAG1	jagged canonical Notch ligand 1	0.545

Figure 1. Notch1 expression is positively correlated with yes-associated protein 1 (**YAP1) expression in breast cancer.** (**A**) STRING: function protein association networks (https://www.string-db.org/) predicted the relationship among Notch1, YAP1 and JAG1 (Upper panel). Correlation score of YAP1 with Notch1 and JAG1 in STRING (Lower panel). (**B**) and (**C**) Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/) analysis of breast cancer specimen in the TCGA database displayed the positive correlation between JAG1/Notch1 and YAP1 mRNA expression. (**D**) Immunohistochemistry analysis of the association among YAP1, and Notch1 expression in human breast invasive ductal carcinoma tissues (100 × magnification and scar bar:200 μm); (**E**) and (**F**) and Western blot and RT-qPCR analyses of the relative levels of N1ICD and YAP1 to the control GAPDH in the indicated human breast cancer cell lines. Data are representative images or expressed as the mean ± SD from each cell line from three separate experiments.

so we selected MCF-7 and MDA-MB-231 for subsequent experiments.

YAP1 enhances the JAG1/Notch1 signaling by co-activating the TEADs in breast cancer cells.

Previous studies have suggested that YAP1 can enhance the expression of JAG1 in non-tumor cells (14,31). To understand the role of YAP1 in regulating the JAG1/Notch1 signaling in breast cancer, we generated stably YAP1-silencing MDA-MB-231-shYAP1 and MCF-7-shYAP1 cells and YAP1 over-expressing MDA-MB-231-YAP1 and MCF-7-YAP1 cells as well as their corresponding control cells (Figure 2A–D). Functionally, YAP1 silencing significantly decreased the relative levels of Notch1 and JAG1 mRNA transcripts and

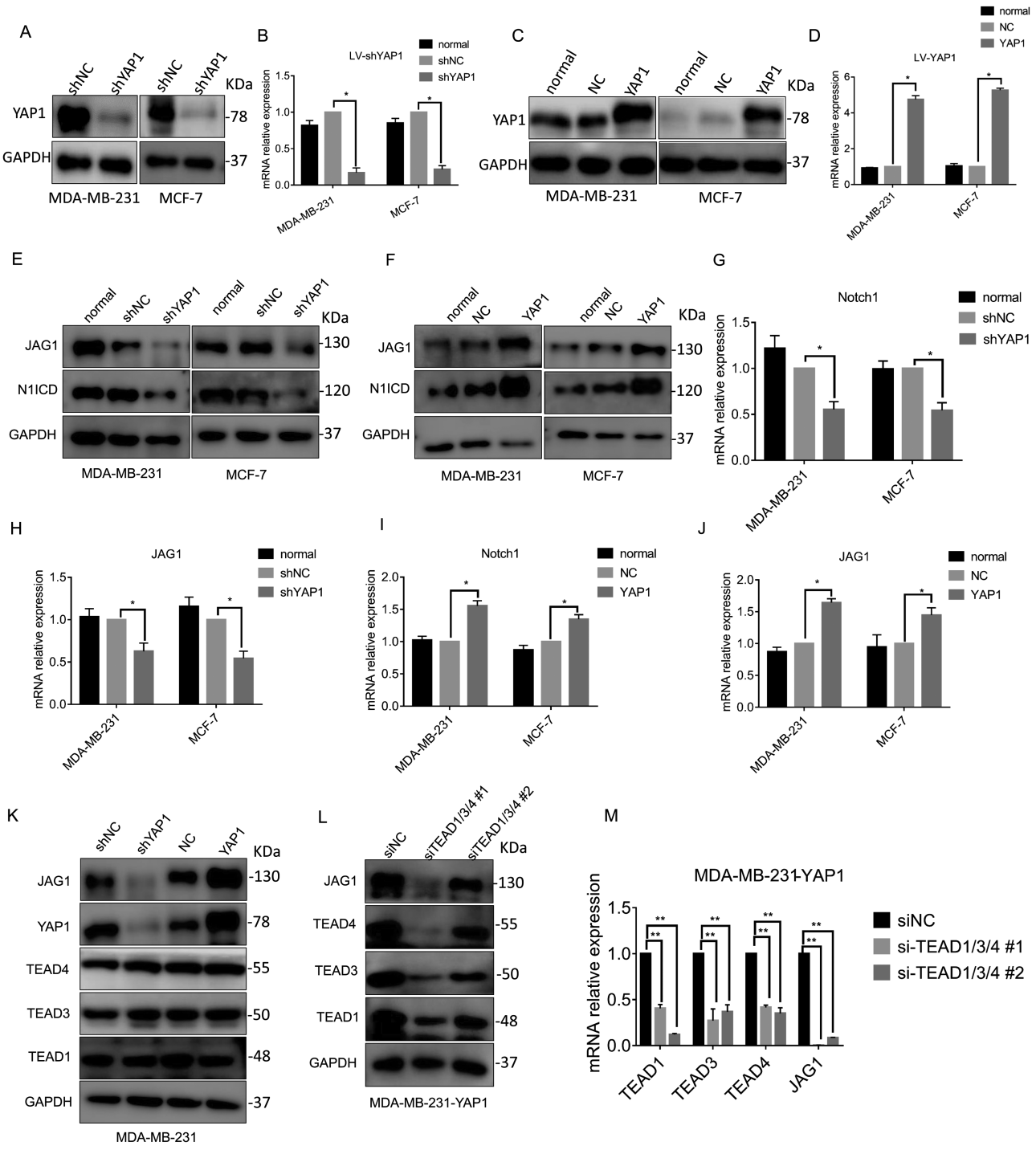


Figure 2. Yes-associated protein 1 (**YAP1) enhances the JAG1/Notch1 signaling by co-activating the TEADs in breast cancer cells.** (**A**–**D**) Western blot and RT-qPCR verification of altered YAP1 expression in the indicated breast cancer cells. (**E**) and (**F**) Western blot analysis of the relative levels of JAG1 and N1ICD to the control GAPDH expression in the indicated cells. (**G**–**J**) The expression levels of Notch1 and JAG1 mRNA transcripts were determined by RT-qPCR. (**K**) Western blot analysis of TEAD1/3/4, YAP1, and JAG1 expression in the indicated cells. (**L**) and (**M**) Simultaneous knockdown of TEAD1/3/4 effectively decreased JAG1 expression in MDA-MB-231 cells, determined by Western blot (**L**) and RT-qPCR (**M**). Data are representative images or expressed as the mean ± SD from each cell line from three separate experiments. *$P < 0.05$, ** $P < 0.01$. Nomal: Unmanipulated MCF-7 or MDA-MB-231; shNC: MCF-7 or MDA-MB-231 transfected with scramble shRNA; shYAP1: MCF-7 or MDA-MB-231 cells transfected with YAP1-shRNA. NC: MCF-7 or MDA-MB-231 transfected with scramble lentivirus; YAP1: MCF-7 or MDA-MB-231 cells transduced with over-expression lentivirus of YAP1.

protein expression while YAP1 over-expression had opposite effects in both MDA-MB-231 and MCF-7 cells ($P < 0.05$ for all, Figure 2E–J), consistent with the positive correlation between these molecule expressions in breast cancer tissues (Figure 1D, Supplementary Table S4 available at *Carcinogenesis* Online). Thus, we validated that YAP1 positively regulates the JAG1/Notch1 signaling in breast cancer cells.

YAP1 co-activates a panel of transcription factors, such as TEADs and SMADs, to regulate the expression of their downstream target genes (32). To explore the transcription factors responsible for YAP1-enhanced JAG1 expression, searching the JASPAR 2018 website (29) revealed that YAP1 predominantly co-activated the TEADs to mediate JAG1 transcription (Supplementary Figure S1A available at *Carcinogenesis* Online). In addition, YAP1 did not affect the expression of TEADs in MDA-MB-231 cells (Figure 2K). Given that the DNA binding sequences of TEAD1-4 are highly similar with a homology of 93.4% (33), we designed two specific siRNAs (TEAD1/3/4 #1 and TEAD1/3/4 #2) for simultaneously targeting TEAD1, TEAD3, and TEAD4 (31). Transfection with either TEAD1/3/4 siRNA effectively decreased the relative levels of TEAD1, TEAD3 and TEAD4 in MDA-MB-231-YAP1 cells (Figure 2L–M), these TEAD1/3/4 siRNAs strongly blocked the induction of JAG1 by YAP1 overexpression (Figure 2L–M). Next, we used the CistromeDB database to analyze the previously published ChIP-seq datasets. In MDA-MB-231 (Supplementary Figure S1B available at *Carcinogenesis* Online) and MCF-7 (Supplementary Figure S1C available at *Carcinogenesis* Online) cells, we found that H3K4me1, H3K4me3, H3K27ac, and TEAD4 were enriched around the JAG1 binding regions. In particular, H3K4me1 and H3K4me3 mark enhancers and promoters, respectively. H3K27ac is related to gene activation. Both in MDA-MB-231 and MCF-7 cells, TEAD4 ChIP-seq data showed that a small part of TEAD4 were located at the promoter region of JAG1, and most were bound to the enhancer region of JAG1 (Supplementary Figure S1B–C available at Carcinogenesis Online). Chip-seq studies have shown that YAP1/TEAD activates transcription by binding to introns or genome enhancers (31,34). Taken together, these data suggest that TEADs play an important role in YAP1-induced JAG1 expression, and YAP1 enhances the Notch1 signaling by co-activating the TEADs to induce JAG1 expression in breast cancer cells.

Notch1 stabilizes YAP1 by inhibiting the β-TrCP-mediated YAP1 degradation in breast cancer cells

To further explore the correlation between Notch1 and YAP1 signaling in breast cancer, we established stable Notch1 silencing MDA-MB-231-shNotch1 and MCF-7-shNotch1 cells and N1ICD over-expressing MDA-MB-231-N1ICD and MCF-7-N1ICD as well as their controls, respectively (Supplementary Figure S2A–D available at *Carcinogenesis* Online). Next, we tested how altered Notch 1 expression modulated YAP1 expression in breast cancer cells. Interestingly, while Notch1 silencing or N1ICD over-expression did not significantly alter the relative levels of YAP1 mRNA transcripts (Supplementary Figure S2E–F available at *Carcinogenesis* Online), Notch1 silencing dramatically decreased the relative levels of YAP1 proteins and N1ICD over-expression increased slightly the relative levels of YAP1 proteins in both MDA-MB-231 and MCF-7 cells (Figure 3A and 3C). Additionally, treatment with neutralizing anti-JAG1 antibody remarkably reduced the relative levels of N1ICD, Hes1, and YAP1 expression in MDA-MB-231 cells, regardless of YAP1 over-expression (Figure 3B). Furthermore, in the presence of cycloheximide (CHX, 35 μg/ml) to inhibit new protein synthesis, N1ICD over-expression significantly reduced YAP1 protein degradation in both MDA-MB-231 cells, relative to their corresponding controls ($P < 0.05$, Figure 3D). Similarly, compared to the corresponding control, knockdown of N1ICD accelerated the degradation of YAP1 protein in MDA-MB-231 cells (Supplementary Figure S2G available at *Carcinogenesis* Online). Hence, the Notch1 signaling enhanced the YAP1 signaling by stabilizing YAP1 proteins in breast cancer cells.

Next, we explored whether Notch1 affects the degradation process of YAP1. Notch1 signaling stabilizes TAZ proteins by interfering with β-TrCP-mediated degradation in liver cancer (18). Therefore, we examined whether N1ICD affects the β-TrCP-mediated degradation process of YAP1. The Co-IP revealed that N1ICD interacted with YAP1 and reduced its poly-ubiquitination (Supplementary Figure 3E–F available at *Carcinogenesis* Online). It is well known that the β-TrCP (an E3 ligase)-mediated proteasome degradation is crucial for the stability of YAP1 proteins (35). And the Co-IP revealed that anti-YAP1 pulled down β-TrCP and YAP1(Figure 3G), further verifying that β-TrCP was involved in the protein degradation process of YAP1. Furthermore, we found that in breast cancer cells over-expressing N1ICD, the level of β-TrCP pulled down by anti-YAP1 was reduced, compared with normal group (Figure 3H). Besides, we determined that gradually increased N1ICD expression decreased the levels of YAP1-interacted β-TrCP in MDA-MB-231 cells (Figure 3I). Collectively, these data indicate that the Notch1 signaling stabilizes the YAP1 proteins in breast cancer cells by inhibiting the binding of β-TrCP to YAP1 and consequent YAP1 degradation in breast cancer cells. These, together with the fact that YAP1 with TEADs enhance JAG1-Notch1 signaling, formed the YAP1-JAG1/Notch1 positive feedback loop in breast cancer cells.

YAP1 attenuates the inhibition of BMP4-SMAD1/5 signaling on breast cancer cell self-renewal

Among the 41 cases, 21 cases with bone metastases (51.2%), 15 cases with lung metastases (36.6%), 8 cases with liver metastases (19.5%), and 4 cases with brain metastases (9.8%), which basically in line with previous studies (2). In the breast cancer in situ specimens with lung metastasis, 80% (12/15) of the specimens showed positive staining of YAP1, while positive staining for Notch1 was seen in 73.3% (11/15) of those specimens. Correlation analysis shows that there was a stronger positive correlation between Notch1 and YAP1 protein in the breast cancer specimens with lung metastasis ($R = 0.787$, $P < 0.001$, Supplementary Table S5 available at *Carcinogenesis* Online). Breast cancer with lung metastasis caught our attention because of its refractory properties and poor prognosis.

Breast cancer cells, particularly for cancer stem cells (CSC), can migrate and colonize in the targeted organ to complete the metastatic process by overcoming the various local antimetastatic signals, as breast CSC have the ability of self-renewal and differentiation (36,37). Notably, the BMP4-related SMAD signaling in the lung can inhibit the self-renewal ability of migrated breast cancer cells (38). What's more, BMP4 plays an important role in the self-renewal and differentiation of human embryonic stem cells (39,40). Besides, it is known that YAP1/TAZ plays an important role in the Epithelial-mesenchymal transition (EMT) process induced by BMP4 (41,42). Therefore, we explored the relationship between YAP1 and BMP4 in breast cancer cells. Unexpectedly, BMP4 treatment failed to significantly alter the migration of MDA-MB-231 cells and their expression of N-cadherin, Occludin, and MMP9 (Supplementary Figure

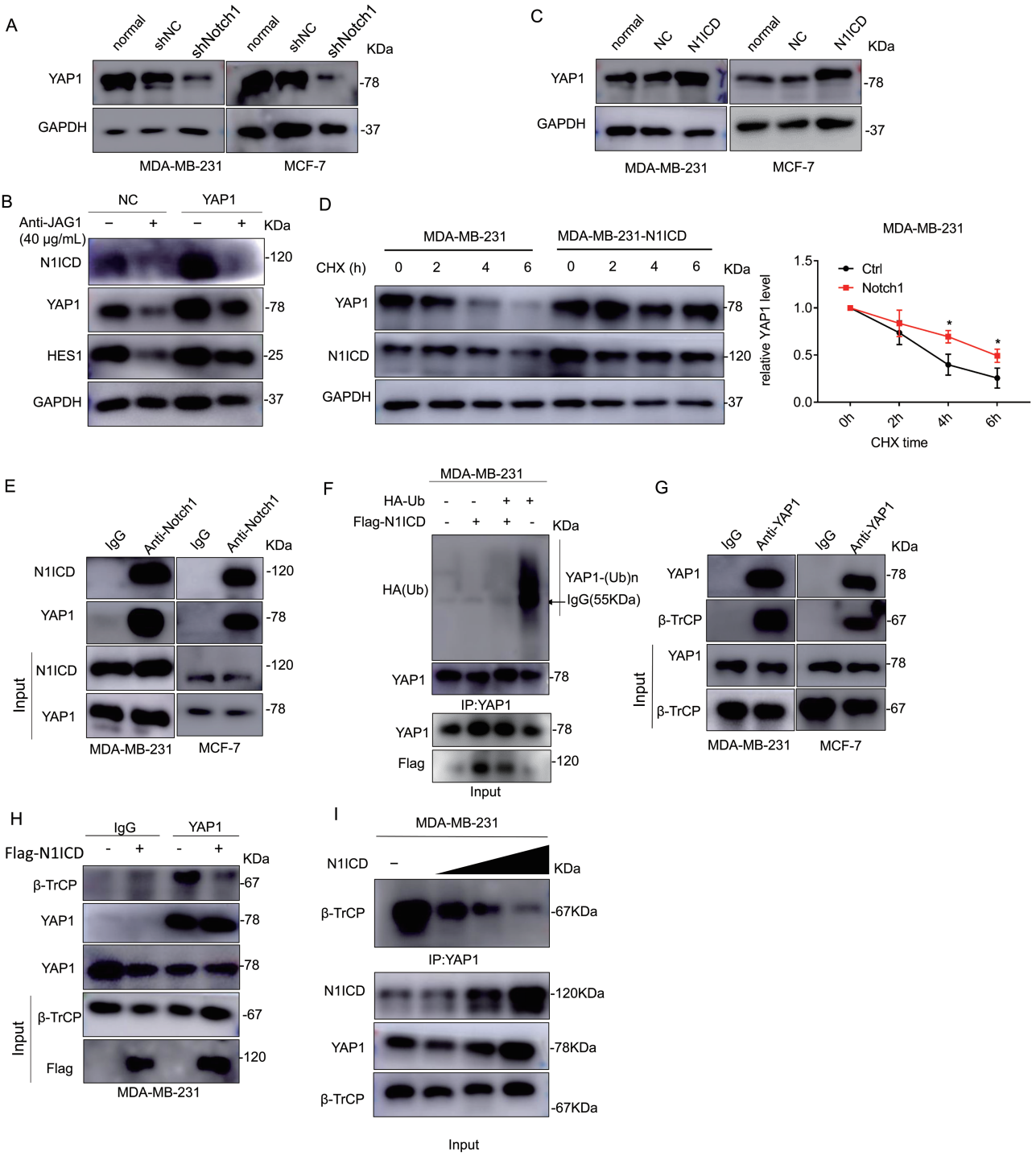


Figure 3. Notch1 stabilizes yes-associated protein 1 (**YAP1) proteins by inhibiting the β-TrCP-mediated YAP1 degradation in breast cancer cells.** (**A**,**C**) Western blot analysis of the relative levels of YAP1 proteins. (**B**)MDA-MB-231-NC and MDA-MB-231-YAP1 cells were treated with 10 μg/ml of JAG1-neutralizing antibody or anti-IgG for 48 h, and the relative levels of interested proteins were quantified by Western blot. (**D**) N1ICD over-expression significantly mitigated the degradation of YAP1 proteins in MDA-MB-231 cells. The cells were treated with, or without, 35 μg/ml CHX for the indicated time periods and the relative levels of YAP1 and N1ICD proteins were analyzed by Western blot. (**E**) Co-IP analysis of direct interaction between YAP1 and Notch1 proteins using the Notch1 antibodies in breast cancer cells. (**F**) N1ICD over-expression reduced the ubiquitination of YAP1 in MDA-MB-231 cells. The cells were induced for the over-expression of HA-Ubiquitin and Flag-tagged N1ICD, lysed and treated with 20 μM MG132 for 8 h. The YAP1 protein was immunoprecipitated, and analyzed by Western blot using anti-HA. (**G**) Co-IP analysis of direct interaction between YAP1 and β-TrCP proteins using the YAP1 antibodies in breast cancer cells. (**H**) Flag-tagged N1ICD was transfected into MDA-MB-231 cells and treated them with 20 μM MG132 for 8 h. Use anti-YAP1 antibody for IP to detect interaction with β-TrCP. **(I**) N1ICD increased YAP1 protein dose-dependently by reducing the binding of β-TrCP to YAP1 in MDA-MB-231 cells. The cells were transfected with 0.5, 1, and 2 μg plasmid for expression of Flag-tagged N1ICD, lysed, immunoprecipitated with anti-YAP1 antibody and analyzed by Western blot. Data are representative images or expressed as the mean ± SD from each cell line from three separate experiments. *$P < 0.05$, **$P < 0.01$ versus the control by 2-tailed Student's t test. Normal: Unmanipulated MDA-MB-231 or MCF-7 cells; shNC: MDA-MB-231 or MCF-7 cells transduced with scramble shRNA; shNotch1: MDA-MB-231 or MCF-7 cells transduced with Notch1-shRNA; N1ICD: MDA-MB-231 or MCF-7 cells transduced with over-expression lentivirus of N1ICD.

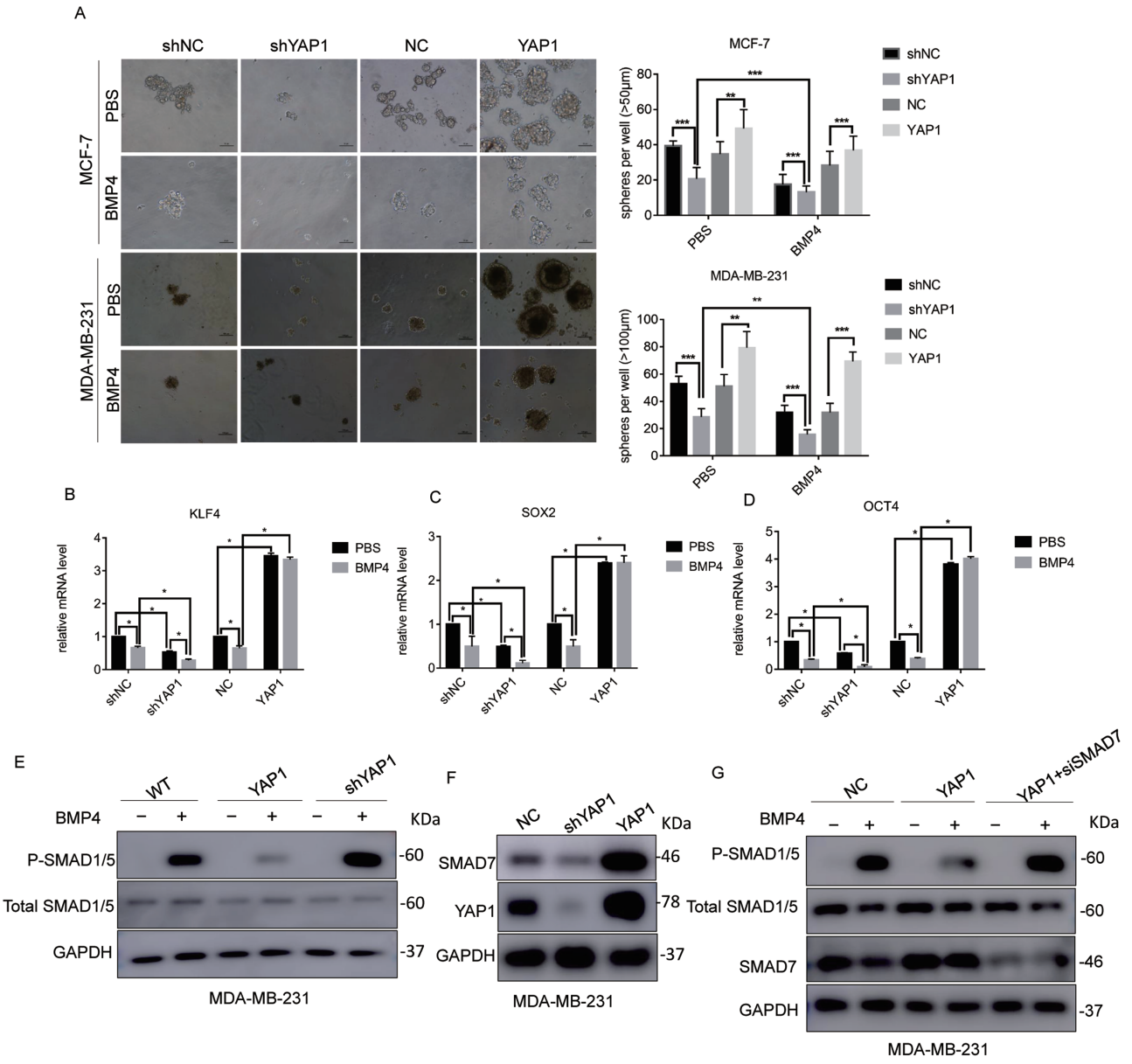


Figure 4. Yes-associated protein 1 (**YAP1) attenuates the inhibition of BMP4 on the self-renewal of breast cancer cells.** (**A**) Mammosphere formation analysis of the self-renewal of the indicated breast cancer cells in the presence or absence of BMP4. The numbers of mammosphers (MCF-7, 50 μm) or (MDA-MB-231, 100 μm) were counted in a blinded manner ($n = 6$ per group). (**B**–**D**) YAP1 over-expression abrogated the BMP4 decreased KLF4, SOX2, and OCT4 mRNA transcription in the indicated MDA-MB-231 cells, determined by RT-qPCR. (**E**) YAP1 over-expression attenuated total SMAD1/5 activation in MDA-MB-231 cells. The indicated MDA-MB-231 cells were treated with PBS or BMP4 (100 ng/ml), and their proteins were analyzed by Western blot. (**F**) Western blot analysis of SMAD7 expression in MDA-MB-231 cells. (**G**) YAP1 inhibits BMP4-SMAD1/5 signal through SMAD7. The indicated MDA-MB-231 cells were treated with PBS or BMP4 (100 ng/ml), and their proteins were analyzed by Western blot. Data are presentative images or expressed as the mean ± SD of each group from three separate experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. shNC: MDA-MB-231 transduced with scramble shRNA; shYAP1: MDA-MB-231cells transduced with YAP1-shRNA; NC: MDA-MB-231 transfected with scramble lentivirus; YAP1: MDA-MB-231 cells transduced with over-expression lentivirus of YAP1; YAP1 + siSMAD7: MDA-MB-231 cells transduced with over-expression lentivirus of YAP1 and SMAD7-siRNA.

S3A–B available at *Carcinogenesis* Online). Then we tested whether YAP1 modulated the BMP4-regulated self-renewal of breast cancer cells by mammosphere formation assays. As shown in Figure 4A, YAP1 silencing significantly decreased the numbers of mammospheres while YAP1 over-expression increased the numbers of formed mammospheres in MDA-MB-231 and MCF-7 cells. Treatment with BMP4 (100 ng/ml) significantly reduced the numbers of mammospheres in all groups of cells, especially in the YAP1-silenced breast cancer cells. In contrast, BMP4 treatment only achieved moderate inhibitory effects in the YAP1 over-expressing breast cancer cells, suggesting that the YAP1-related signaling mitigated the inhibition of BMP4 on the mammosphere formation of breast cancer cells.

The stemness transcription factors, such as kruppel-like factor-4 (KLF4), SRY-box transcription factor 2 (SOX2), and octamer-binding transcription factor-4 (OCT4) are crucial for mammosphere formation (37). Accordingly, we tested the effect of BMP4 on the expression of KLF4, SOX2, and OCT4

in YAP1 silencing and over-expressing MDA-MB-231 cells by RT-qPCR. First, YAP1 silencing and over-expression did change the relative levels of KLF4, SOX2, and OCT4 mRNA transcripts in MDA-MB-231 cells (Figure 4B–D). Second, treatment with BMP4 significantly decreased the relative levels of KLF4, SOX2, and OCT4 mRNA transcripts in the control and YAP1 silencing MDA-MB-231 cells ($P < 0.05$ for all), but not in the YAP1 over-expressing MDA-MB-231 cells. These data indicated that YAP1 attenuated the inhibition of the BMP4-related signaling on the self-renewal of breast CSC *in vitro*.

Finally, we explored the mechanisms by which YAP1 attenuated the BMP4-mediated inhibition of breast cancer cell self-renewal by analysis of the BMP4-related SMAD1/5 activation. Western blot analysis revealed that compared with that in the control cells, YAP1 over-expressing dramatically reduced the levels of BMP4-induced SMAD1/5 phosphorylation while YAP1 silencing enhanced SMAD1/5 phosphorylation (Figure 4E). In order to further explore the mechanism by which YAP1 impairs BMP4-SMAD1/5 signaling, we explored the interaction between YAP1 and BMP4 signaling pathway components using the STRING database. SMAD7, a negative regulator of BMP4 signaling, was closely related to YAP1 (Supplementary Figure S3C available at *Carcinogenesis* Online). Consistently, YAP1 and SMAD7 showed a positive correlation in expression level (Supplementary Figure S3D available at *Carcinogenesis* Online, $r = 0.8$, $P < 0.001$). Western blot analysis showed that YAP1 over-expressing promoted the expression of SMAD7, which was opposite in the YAP1 silence group (Figure 4F). We next investigated whether the SMAD7 is responsible for inhibiting BMP4 signaling, which occurs in response to YAP/TAZ over-expression. We found that knockdown of SMAD7 reversed SMAD1/5 phosphorylation induced by recombinant human BMP4 (Figure 4G and Supplementary Figure S3E–F available at *Carcinogenesis* Online). Collectively, such novel data indicated that the over-expression of YAP1 enhanced inhibitory SMAD7, which in turn inhibited the BMP4-SMAD1/5 signaling.

YAP1-Notch1 positive feedback loop promotes lung metastasis of breast cancer cells *in vivo*

Next, we tested how the YAP1-Notch1 signaling modulated the metastasis of MDA-MB-231 cells *in vivo*. We found that either N1ICD or YAP1 over-expression significantly enhanced the migration and invasion of MDA-MB-231 cells, while YAP1 + shNotch1 or N1ICD + shYAP1 significantly attenuated the migration and invasion of MDA-MB-231 cell compared with the N1ICD or YAP1 over-expression group *in vitro* ($P < 0.05$ for all, Figure 5A–B), extending previous observations (8,11). We further examined the effect of YAP1 and Notch1 on the lung metastasis of MDA-MB-231 tumors *in vivo*. We injected intravenously with the same number of MDA-MB-231-YAP1, MDA-MB-231-N1ICD, MDA-MB-231-YAP1 + shN1ICD, MDA-MB-231-N1ICD + shYAP1 or control MDA-MB-231-NC (NC + shNC) cells into 4-week-old BALB/c nude mice. Luminescent imaging (Figure 5C) and HE staining (Figure 5D–E) displayed that either N1ICD or YAP1 over-expression increased the numbers of lung metastatic nodules in mice, while YAP1 + shNotch1 or N1ICD + shYAP1 significantly decreased the numbers of lung metastatic nodules, compared with the N1ICD or YAP1 over-expression group mice. In addition, IHC staining further verified the expression of YAP1 and Notch1 in the lungs of each group. (Figure 5F). Thus, YAP1-Notch1 positive feedback loop promoted the lung colonization of MDA-MB-231 cells.

Discussion

In this study, our findings revealed that the YAP1-JAG1/Notch1 positive feedback loop promoted lung metastasis of breast cancer cells. Mechanically, firstly, N1ICD promotes the stability of YAP1 by inhibiting the β-TrCP-mediated proteasomal degradation in breast cancer cells. Secondly, YAP1 co-activates TEADs to target *JAG1* gene transcription. Thus, A YAP1-JAG1/ Notch1 positive feedback loop is formed. In addition, YAP1 enhanced inhibitory SMAD7, which in turn obstructed BMP4-SMAD1/5 signaling, to promote the self-renewal and lung metastasis of breast cancer cells. More importantly, our findings may shed new light on the molecular mechanisms underlying the pathogenesis of breast cancer lung metastasis and suggest the YAP1- JAG1/ Notch1 positive feedback loop may be therapeutic target for the prevention and intervention of breast cancer lung metastasis.

There are several studies focused on the YAP1-Notch positive feedback loop in cancers (14,18,21,23,43). However, there is less evidence of breast cancer, and the mechanism of this loop is still controversial. In this study, we verified a YAP1-Notch1 positive feedback loop in breast cancer cells and provided new evidence that N1ICD directly interacted with YAP1 to enhance the stability of YAP1 in breast cancer cells. These findings extended previous observations that NICD protects TAZ (WWTR1, WW domain containing transcription regulator 1) protein stability in hepatocellular carcinoma (23). Differently, previous studies suggest that Notch1 can directly induce YAP1 expression in smooth muscle (44), and both Notch1 and YAP1 can directly transcriptionally regulate each other in rhabdomyosarcoma (43). However, we found that N1ICD over-expression failed to increase YAP1 mRNA transcripts in breast cancer. The discrepancy may be from different types of cells with varying genetic backgrounds. Interestingly, in another study, Notch3 inhibits epithelial-mesenchymal transition by activating Hippo/YAP signaling in MCF-7 cells (45). However, there are different roles between Notch1 and Notch3 in breast cancer. After being treated with TGF-β, the expression of Notch1 increased and Notch3 decreased in MCF-7 cells (45). Therefore, it is still necessary to explore the relationship between Notch1 and YAP1 in breast cancer. What's more, our study supported the notion that enhancing the stability of key signal events by a molecule in another signal pathway is a common mechanism underlying the crosstalk between two signal pathways.

A genome-wide analysis indicated that *JAG1* is a direct YAP1-TEAD gene and TEAD1 with YAP1 occupies more than 80% of the promoters of *JAG1* in MCF-10A cells (31). However, the relationship between YAP1 and JAG1 protein in breast cancer cell is imprecise due to the lack of experimental validation. Our study demonstrated that YAP1 enhanced JAG1 expression, which was significantly attenuated by simultaneous knockdown of multiple TEADs in MDA-MB-231 cells. And chromatin immunoprecipitation (ChIP) sequencing identifies that YAP1 preferred to co-activate TEAD4 in MDA-MB-231 cells (Supplementary Figure S1B available at

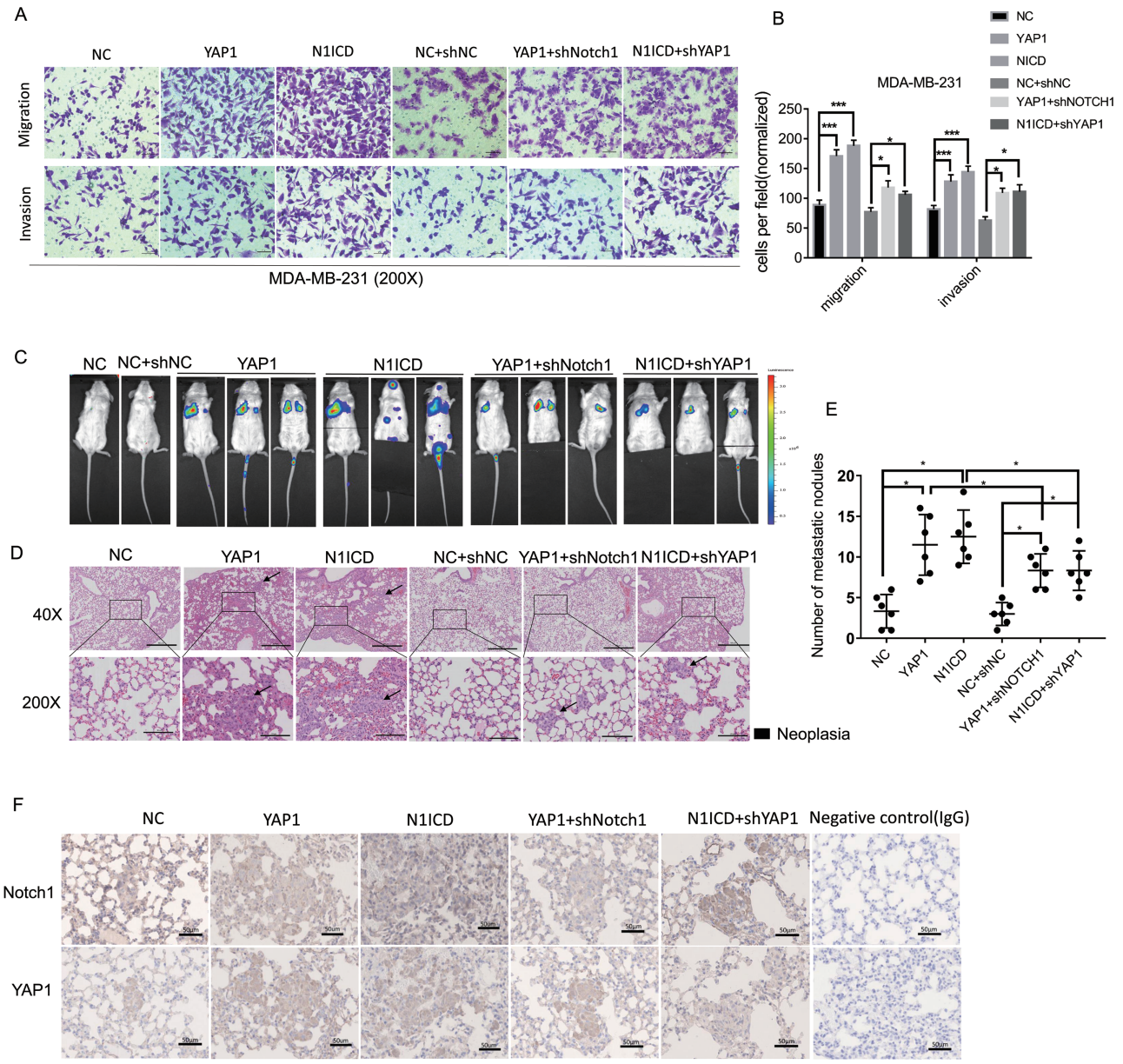


Figure 5. Blocking the yes-associated protein 1 (**YAP1)-Notch1 loop impairs breast cancer cell lung metastasis.** (**A**) and (**B**) Blocking the YAP1-Notch1 loop weakened the migration and invasion of MDA-MB-231 cells. Six representative images (200 × magnification and scar bar: 50 μm) were randomly selected for quantification in a blinded manner. (**C**) Bioluminescent imaging of the lung metastatic breast cancer. Female BALB/c nude mice were injected intravenously with MDA-MB-231-NC, MDA-MB-231-NC + shNC, MDA-MB-231-YAP1, MDA-MB-231-N1ICD, MDA-MB-231-YAP1 + shNotch1, and MDA-MB-231-N1ICD + shYAP1 cells and 49 days later, the lung metastatic breast cancers were characterized by IVIS imaging. (**D**) Lung tumor nodules (black arrows) were analyzed by **H** and **E** staining (Right column, 40 × magnification and scar bar: 500 μm, and 200× magnification and scar bar: 100 μm). (**E**) Quantitative analysis of the number of lung metastatic nodules. (**F**) Immunohistochemistry analysis of the lung metastatic nodules using anti-YAP1 and anti-Notch1 (400 × magnification and scar bar: 50 μm). Data are representative images or expressed as the mean ± SD each group from two separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NC: MDA-MB-231 cells transduced with NC; NC + shNC: MDA-MB-231 transduced with NC and scramble shRNA; YAP1: MDA-MB-231 cells transduced with over-expression lentivirus of YAP1; N1ICD: MDA-MB-231 cells transduced with over-expression lentivirus of N1ICD; YAP1 + shNotch1: MDA-MB-231cells transduced with over-expression lentivirus of YAP1 and Notch1-shRNA; N1ICD + shYAP1: MDA-MB-231 cells transduced with over-expression lentivirus of N1ICD and YAP1-shRNA.

Carcinogenesis Online) (7). Complementally, it has recently been shown that YAP/TEAD mainly binds to distal enhancers rather than promoters. The effect of YAP/TAZ/TEAD on enhancers goes beyond their promoter-related functions, which expands the prior knowledge of transcriptional regulation of these proteins (7,46). Furthermore, those results complemented the research that YAP1-TEAD regulates JAG1/Notch1 signaling and together with the fact that YAP1 promoted JAG1 expression, formed a positive feedback regulatory loop in breast cancer cells.

Breast cancer can metastasize to multiple organs, including the lung, liver, bone, and brain. During the metastatic process, metastatic breast cancer cells, such as breast CSC, have to migrate and colonize in the organ, which are regulated positively by many oncogenic factors (26,37,47). At the same time, the metastatic breast cancer cells also need to

overcome the anti-metastatic signals produced in the organ (26,48). In our study, we observed that the size and number of breast cancer lung metastatic nodules were reduced in the YAP1$^+$Notch1$^-$ and N1ICD$^+$YAP1$^-$ groups *in vivo*. Breaking the YAP1-Notch1 positive feedback loop weakens the effect of YAP1/Notch1 on the promotion of breast cancer lung metastasis, further confirming that the Notch1-YAP1 positive feedback loop promoted the lung metastasis of breast cancer.

It is well known that BMP4 plays an important role in osteogenesis, but also as a differentiation factor for pluripotent stem cells (39). In cancer, BMP4 promoted CSC differentiation and reduced oncogenic capability and metastasis in vitro in glioma, hepatocellular carcinoma, and colorectal cancer models (49,50). However, the role of BMP4 and its contribution to tumorigenesis is still controversial, because some studies have shown that BMP4 can play a dual role. On the one hand, it can promote migration and invasion of breast cancer cells (51,52), on the other hand, BMP4 inhibits breast cancer metastasis (38,53,54). We show here that BMP4 treatment significantly reduced the mammosphere formation and the stemness transcription factors expression in MDA-MB-231 cells, and over-expression of YAP1 could weaken the inhibitory effect of BMP4 on breast CSC. However, there are different views on the crosstalk between YAP1 and BMP4 signaling. Lai, Dulcie et al. proposed that TAZ, homologous analogs of YAP1, could activate BMP4 transcription to promote cell migration in mammary epithelial cells (42). What's more, it is worth noting that BMP4 signaling could crosstalk with Notch1 signaling (51,55,56). Choi et al. reported that BMP4 activates Notch1 signaling by upregulating JAG1 expression, promoting EMT and stem cell properties in MDA-MB-231 cells (51). Similarly, in mesenchymal-subtype colorectal cancer, BMP4 signaling cross-talks with Notch1 signaling, and this correlates with poor patient prognosis (55). In particular, BMP4 functions as a downstream target gene of Notch1 signaling in epidermal cells (56). However, our study proposed that BMP4 inhibited the microsphere-forming ability of MDA-MB-231 cells, and BMP4 did not exhibit significant effect on the expression of N-cadherin, occludin, or MMP-1, which play important roles in EMT. Although the reasons for these conflicting effects are unclear, the context-dependent and genetic constraints of BMP4 signaling might play an important role (55), and the experimental approaches employed in each report may have influenced BMP function.

Interestingly, in another whole-gene expression analysis that studied the transcriptional response of BMP4 signaling in breast cancer, none of the EMT-related genes showed a consistent response to BMP4 in most cell lines after six human breast cancer cell lines were exposed to BMP4 (24,53).

SMAD7, a classically induced inhibitory SMAD (iSMAD), antagonizes the type I BMP and TGF-β receptor complex (53). Furthermore, we provide new evidence that YAP1 attenuates BMP4-SMAD1/5 signaling by inducing the inhibitor SMAD7 expression. Consistently, a previous study reported that SMAD7 inhibited BMP4-SMAD1/5 signaling and blocked breast cancer metastasis (53). Besides, *CTGF*, a target gene downstream of YAP1, and CTGF protein can bind to BMP4 or TGF-β to prevent BMP4 from binding to its receptor to antagonize BMP4 signaling, but it can enhance TGF-β binds to its receptor and activates TGF-β signaling (57). Therefore, these observations further suggest that YAP1 inhibits the BMP4 signaling pathway.

Conclusion

In summary, these data revealed that the YAP1-Notch1 positive feedback loop promoted the lung metastasis of breast cancer by enhancing breast cancer stemness, what's more, YAP1 over-expression attenuates the inhibition of breast cancer stemness by BMP4-SMAD1/5 signaling through inducing SMAD7 expression in breast cancer cells. We are interested in further exploring the mechanisms of YAP1/SMAD7/BMP4 in regulating the stemness and metastasis of breast CSC. Our novel data emphasize the importance of interaction between signaling pathways in promoting breast cancer lung metastasis, and may provide new insights into the mechanisms underlying the context-dependence of the Notch1 and YAP1 signal pathways in regulating breast cancer lung metastasis. Conceivably, our novel findings may aid in the design of new therapeutics by targeting multiple signal pathways simultaneously for intervention of metastatic breast cancer.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

Author Contributions

S.S., X.Z., and S.G. performed the study proposal and design. L.Z., S.S., and J.L. were involved in all aspects of the study, including data analysis and interpretation, and manuscript writing. Y.Z., Q.N., M.L., and Y.Q. provided assistance in the analysis and interpretation of data and statistical analysis, and L.W. and L.Z. performed the animal experiments. All authors read and approved the final manuscript.

Ethics Committee Approval and Patient Consent

This study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University Consent for publication and was carried out in accordance with the approved experimental guidelines. All patients had informed consent to be included in this study. All institutional and national guidelines for the care and use of laboratory animals were followed.

Data Availability

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

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