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Original Research

NEDD9 is transcriptionally regulated by HDAC4 and promotes breast cancer metastasis and macrophage M2 polarization via the FAK/NF-κB signaling pathway

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ABSTRACT

Background: Breast cancer is a malignancy with a generally poor prognosis. With the advancement of molecular research, we have gained deeper insights into the cellular processes that drive breast cancer development. However, the precise mechanisms remain elusive.

Results: Based on the CPTAC database, we found that NEDD9 expression is up-regulated in breast cancer tissues and is associated with poor prognosis in breast cancer patients. Functional experiments showed that NEDD9 promotes tumor growth and metastasis both in vitro and in vivo. Overexpression of NEDD9 disrupts mammary epithelial acinus formation and triggers epithelial-mesenchymal transition in breast cancer cells, effects that are reversed upon NEDD9 gene silencing. Mechanistically, NEDD9 upregulates its expression by inhibiting HDAC4 activity, leading to enhanced H3K9 acetylation of the NEDD9 gene promoter and activation of the FAK/NF-κB signaling pathway. Furthermore, NEDD9 overexpression promotes IL-6 secretion, which further drives breast cancer progression. Notably, NEDD9 activation fosters the pro-tumoral M2 macrophage polarization in the tumor microenvironment. NEDD9 stimulates IL-6 secretion, polarizes monocytes towards an M2-like phenotype, and enhances BC cell invasiveness.

Conclusions: These findings suggest that NEDD9 upregulation plays a pivotal role in breast cancer metastasis and macrophage M2 polarization via the FAK/NF-κB signaling axis. Targeting NEDD9 may offer a promising therapeutic approach for breast cancer treatment.

Introduction

Breast cancer is still a serious public health problem worldwide. It is not only one of the most common types of cancer, but also one of the leading causes of disability and death in women [1]. According to statistics, the diagnosis rate of breast cancer will rank first among malignant tumors in 2020, which highlights its seriousness and universality [2]. The complexity of breast cancer lies in its molecular mechanism of occurrence and development [3]. Because of this complexity, breast cancer shows significant heterogeneity, which means that different breast cancer patients may have different gene and molecular characteristics. This heterogeneity poses challenges at the molecular level for the selection of treatment regimens and disease prognosis [4]. However, with the continuous deepening of molecular research, people have a deeper understanding of the cellular pathways of breast tumor development. These research advances are helpful in identifying new diagnostic markers and developing more effective treatment strategies [5]. Although some prognostic markers have been used to predict the treatment outcome of breast cancer patients, the treatment outcome of a large number of patients is still unsatisfactory. Therefore, further determination of new prognostic markers is crucial to improve the clinical prognosis of breast cancer patients.

Epigenetic modifications have profound effects on the regulation and function of cells, and targeted epigenetic therapy has become an attractive strategy in the field of cancer treatment [6]. Among them, post-translational modifications of histone residues, as a key epigenetic process, regulate chromatin accessibility and thereby regulate gene expression [7]. Essentially, the balance between histone acetyltransferases (HATs) and HDACs is crucial for maintaining normal histone acetylation status. Abnormal HDAC activity is closely associated with the development of various cancers [8]. Histone deacetylases (HDACs) play a key role in gene transcription by deacetylating histone

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lysine residues and remodeling chromatin [9]. Due to its overexpression and abnormal activity in various cancer subtypes, HDAC is considered to be a promising and successful target for anti-cancer drugs, and its effectiveness has been confirmed [10,11]. However, the successful application of HDAC inhibitors in the treatment of solid tumors still faces certain limitations [12].

NEDD9 (neural precursor cell expressed, developmentally down-regulated 9), also known as CasL and HEF1, is a linker protein that plays a key role in multiple kinases such as Src [13], FAK [14], AURKA [15], and integrins [16]. It encodes a multi-domain scaffold protein involved in cell signaling and regulates processes such as cell proliferation, DNA damage response, and migration [16-18]. Although NEDD9 itself is not a carcinogen, more and more studies have shown that it has high levels or activity changes in leukemia, colon cancer, breast cancer and other tumors [16]. Many studies have shown that NEDD9 plays an important role in the metastatic behavior of many kinds of cancers, especially triple negative breast cancer (TNBC) [19,20]. The study found that the level of NEDD9 phosphorylation is significantly increased in MCF-7 estrogen receptor (ER)-positive breast cancer cells [21]. AND the expressions of NEDD9 and E-cadherin correlate with metastasis and poor prognosis in triple-negative breast cancer patients [19]. Some studies also confirmed that NEDD9 stimulates the invasive ability of breast cancer cells by affecting epithelial mesenchymal transformation (EMT) and activating MMP [22]. However, the regulatory mechanism of NEDD9 is still not fully understood. Recently, it has been reported that epigenetic modifications may affect the expression of this gene [20,23].

The development and aggressiveness of cancer cells are determined by both their intrinsic mechanisms and external environmental factors [24]. EMT can induce epithelial cells to transform into mesenchymal cells, promote the movement of cancer cells, and thus promote tumor progression, metastasis, and differentiation [25]. Tumor-associated macrophages (TAMs), derived from peripheral blood mononuclear cells, are recruited to the tumor microenvironment under the influence of factors secreted by cancer cells or cells in the microenvironment, and differentiate into M1 or M2 macrophages [26]. M1 macrophages highly express inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF)-α, which promote inflammatory and immune responses and prevent tumor formation [27]. M2 macrophages highly express arginase 1 (ARG1), producing cytokines, growth factors, and proteases that are crucial in the development of tumors [28]. In addition, M2 macrophages can stimulate tumor angiogenesis, promote cancer cell migration and invasion, inhibit immune response, and remodel the matrix [29].

Several studies have confirmed that NEDD9 promotes the invasiveness of breast cancer cells by influencing the EMT and activating matrix metalloproteinase 14 (MMP14) [22]. The loss of NEDD9 inactivates MMP14 and reduces the migratory ability of breast cancer cells. In oral squamous cell carcinoma, NEDD9 can increase the secretion of MMP9 and accelerate the formation of invadopodia [30]. It has been reported that an elevated protein level of NEDD9 is significantly correlated with lymph node metastasis and tumor-node-metastasis staging, and it indicates a decreased five-year survival rate in patients with triple-negative breast cancer (TNBC) [19]. However, how NEDD9 influences the immune microenvironment leading to tumor immune escape, particularly its effect on macrophage polarization, remains unclear.

In our current research, we have observed a correlation between high expression of NEDD9 and poor prognosis in breast cancer patients. Furthermore, functional experiments have confirmed the essential role of NEDD9 in the progression of breast cancer. We have, for the first time, delved deeper into the mechanism of HDAC4-mediated transcriptional activation of NEDD9. Additionally, we have attempted to clarify the intricate interplay between NEDD9 and breast cancer metastasis, revealing that NEDD9 can activate the FAK/NF-κB/IL-6 signaling pathway and polarize M2 macrophages. Our findings underscore the emerging role of NEDD9 as a regulator of breast cancer metastasis, offering potential for targeting NEDD9 as a novel strategy for preventing breast cancer progression. We also aim to counteract these effects through rational combination therapy.

Method

Patients And Samples

Between March 2007 and January 2017, patients diagnosed with breast cancer who underwent surgery at The First Affiliated Hospital of University of South China were included in this study. Both breast cancer tissues and adjacent normal tissues were obtained from the same patient. Survival time was defined as the time from the date of surgery to the date of last follow-up or death. This study was approved by the ethics committee of The First Affiliated Hospital of University of South China. All patients provided informed consent to participate in this study.

Bioinformatics analysis

The National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) is a national effort to accelerate the understanding of the molecular basis of cancer through the application of large-scale proteome and genome analysis, or proteogenomics. The Kaplan Meier plotter is capable of assessing the correlation between the expression of all genes (mRNA, miRNA, protein, & DNA) and survival in 35k+ samples from 21 tumor types. The core of the integrative level of the ENCODE Encyclopedia is the Registry of candidate cis-Regulatory Elements (cCREs), which integrates all high-quality DNase-seq and H3K4me3, H3K27ac, and CTCF ChIP-seq data produced by the ENCODE and Roadmap Epigenomics Consortia.

Cell culture

The human breast cancer cell lines (MCF7, MDA-MB-231, and HCC1954) and THP-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were authenticated by STR profiling (Suzhou, China) and were tested negative for mycoplasma using the Mycoplasma Detection Kit (Sigma-Aldrich, MP0050, Missouri, USA). The MCF7 and MDA-MB-231 cell lines were cultured in DMEM (CM10017, Macgene, Beijing, China), while HCC1954 and THP1 cells were maintained in RPMI-1640 medium. Both media were supplemented with 10 % fetal bovine serum (FBS, Gibco, Carlsbad, California, USA), 100 U/ml penicillin, and 100μg/ml streptomycin. The cells were incubated at 37 °C in a humidified incubator with 5 % CO2.

Cell transfection

The full-length NEDD9 gene was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, California, USA) to generate the pcDNA3.1-NEDD9 construct. The overexpression vector or empty vector was transfected into cells, followed by selection with G418 (1 μg/ml) for 2 months until visible clones emerged. The surviving cell clones were then expanded and their overexpression efficiency was validated using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The satisfactory clones were identified as stably transfected cells and used for subsequent in vivo experiments.

Cell proliferation assays

The cell proliferation assay was performed using the MTT assay. In brief, 1,500 transfected cells were seeded into a 96-well plate. After a designated period of time, the cell proliferation was evaluated. Each well was then added with 20 μl of MTT (5 mg/ml) and incubated for 4–6 h. The supernatant was then removed, and 100 μl of DMSO was added to each well. The absorbance was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, California, USA).

EdU incorporation assay

To determine the proliferation of cells and nucleic acid, we used the EdU incorporation assay kit (RiboBio, C10310-1, Guangzhou, China) according to the manufacturer's instructions. Briefly, 2×10^4 transfected cells were seeded into 96-well plates, and each well was incubated for 24 h. After incubation with 50 μM EdU for 2.5 h, the cells were washed with PBS, fixed with 4 % paraformaldehyde (PFA) for 30 min, and permeabilized with 0.5 % Triton X-100 for 10 min. Subsequently, the cells were stained with 1 × Apollo dye solution for 30 min in the dark at room temperature and then stained with 1 × Hoechst dye solution for another 30 min. Images were acquired under a fluorescence microscope (Carl Zeiss, Jena, Germany), and positive cells were counted.

Migration and invasion assays

To assess cell migration, after transfection, 80,000 cells were resuspended in serum-free DMEM and seeded into transwell chambers (pore size 8μm; Corning, New York, USA). A total of 700 μl of culture medium containing 20 % FBS was added to the lower chamber. To assess cell invasion, we used 24-well transwell plates with 8μm pores (Corning, New York, USA, #3422). The upper compartment was seeded with serum-starved cells and placed in a culture medium containing 10 % serum. After culturing for 12 h, the cells were fixed with 90 % ethanol, air-dried, and stained with 1 % crystal violet. Three fields per filter (20x magnification) were randomly selected for counting the migrated cells.

qRT-PCR

To extract total RNA from cells or frozen tissues, we used RNAiso Plus (total RNA extraction reagent) (Takara, 9108, Kyoto, Japan). cDNA was synthesized using the PrimeScript RT reagent kit (Takara, RR037B, Kyoto, Japan). mRNA expression was detected by real-time quantitative PCR (qRT-PCR) using TB Green™ Advantage® qPCR Premix (Takara, 639676, Kyoto, Japan) and the LightCycler480 detection system (Roche, Germany). The relative expression levels of target genes were analyzed using the $2^{-\Delta\Delta Ct}$ method, with β-actin as the normalization control.

Primers sequences are as follows:
NEDD9: ATGGCAAGGGCCTTATATGACA and
TTCTGCTCTATGACGGTCAGG;
HDAC4: GGCCCACCGGAATCTGAAC and
GAACTCTGGTCAAGG GAACTG;
β-actin: GACCTGTACGCCAACACAGT and
CTC AGGAGGAGCAATGATCT.

Western blot analysis

To extract total protein from cells or frozen tissues, we used the RIPA lysis buffer (Beyotime, P0013, Shanghai, China) containing protease inhibitors (PMSF) and phosphatase inhibitors (NaF). The protein concentration was determined using the BCA protein assay kit (Beyotime, P0010, Shanghai, China). The protein samples were separated by 10 % SDS-PAGE electrophoresis and transferred onto a 0.22-μm polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, Massachusetts, USA) using a wet electroblotting system. The membrane was blocked with 5 % non-fat milk in TBST for 1 h at room temperature. Then, the membrane was incubated with primary antibodies overnight at 4 °C and with secondary antibodies for 1 h at room temperature. Finally, the target proteins were visualized using an enhanced chemiluminescence (ECL) detection kit (Vazyme, E423-01, Nanjing, China).

Immunofluorescence staining

5 × 10^5 cells were seeded into 24-well plates and incubated for 24 h. The cells were then treated with the specified drugs or left untreated for another 24 h. The cells were washed with PBS, fixed with 4 % paraformaldehyde (PFA) for 30 min, and permeabilized with 0.5 % Triton X-100 for 10 min. After blocking the cells with 10 % sheep serum PBS for 1 h at room temperature, the primary antibody was added and incubated overnight at 4 °C. The secondary antibody was then added and incubated for 2 h in the dark at room temperature. Finally, the nuclei were stained with DAPI (Beyotime, C1005, Shanghai, China) for 15 min at room temperature. The stained cells were observed and photographed under a fluorescence microscope (ZEISS, Jena, Germany).

ChIP assay

The SimpleChIP Plus Enzymatic Chromatin IP Kit (#9005, CST) was used for chromatin immunoprecipitation (ChIP) analysis. The antibodies used were as follows: anti-IgG (1:100, #2729, CST) and anti-acetyl histone H3 (Lys9, H3K9ac, 1:100, #ab10812, Abcam). The extracted DNA served as a template for the qPCR reactions, and the primers spanned the promoter region of the NEDD9 gene (−3000 to −400).

Luciferase reporter assays

Using genomic DNA as a template, we amplified four distinct regions of the NEDD9 gene promoter from MDA-MB-231 cells. These regions were then digested with MluI and XhoI enzymes and ligated into the pGL3-Basic vector (#E1751, Promega, Wisconsin, US). To assess transcriptional activity, MCF-7 cells were seeded and transfected with the pGL3-nedd9-luc promoter constructs (-2178/-1540/-1190/-666 to +197) along with the PRL-TK (Renilla luciferase) vector using Lipofectamine 2000 (#11668019, Invitrogen). After 24 h, the firefly luciferase activity was measured using the dual-luciferase reporter assay system (#E1910, Promega).

THP-1 polarization

To investigate the interaction between cancer cells and macrophages, we seeded 1 × 10^5 cancer cells into the upper chamber of a Falcon® Cell Culture Inserts (Corning, Corning, NY) and co-cultured them with 1 × 10^6 THP-1 monocytes in the lower chamber for 48 h. In parallel, we also conducted a control experiment in which THP-1 cells were co-cultured with RPMI medium alone. Subsequently, we analyzed the markers of pan-macrophages (F4/80), M1 macrophages (CD86), and M2 macrophages (CD163 and CD206) using qRT-PCR technology with synthetic primers provided by MDBio.

ELISA

To conduct subsequent experiments, we seeded the specified cells into 6-well plates containing serum-free RPMI culture medium, with 2 × 10^5 cells added to each well, and incubated them for 24 h. Subsequently, we centrifuged the conditioned medium at 1000 × g for 20 min at 4 °C and collected the supernatant for detection. For ELISA analysis, we first coated the ELISA plate with diluted IL-6 or IL-6R and incubated it overnight at 4 °C. Then, we washed it 4 times with 0.05 % PBS Tween-20 and incubated it with diluted buffer for 1 h. After another round of washing, we added 100 μl of conditioned medium to each well and incubated it for 2 h. After washing again, we added diluted standards and incubated them for 2 h. Next, we washed the plate 4 times, added diluted detection antibodies, and incubated them for 1 h. After another 5 rounds of washing, we added the substrate solution of 3,3 ',5,5 '-tetramethylbenzidine in the dark and incubated it for 15 min. Finally, we stopped the reaction and detected the absorbance at 450 nm within 15 min.

Animal studies

Forty female BALB/c nude mice (4-6 weeks old) were purchased from GemPharmatech Co., Ltd (Nanjing, China). For subcutaneous

inoculation, 1 × 10^7 MDA-MB-231 cells stably expressing NEDD9 or control vectors were resuspended in 200 μl PBS and injected subcutaneously into the right flank of the mice. When the subcutaneous tumors reached an average size of 50 mm^3, the mice were randomly divided into two subgroups, with each group receiving intravenous injections of 2 mg kg^{-1} Theo-24 or an equal volume of drug-free control every 3 days for a total of 7 injections (n = 10 in each group). The tumor size was measured every 5 days using calipers, and the tumor volume was calculated using the formula: volume = length × (width)^2 / 2. After the experimental period, the mice were sacrificed, and the subcutaneous tumors were weighed and photographed. For the in vivo metastasis assay, 5 × 10^5 MDA-MB-231 cells stably expressing NEDD9 or control vectors were resuspended in 200 μl PBS and injected intravenously into the tail vein of nude mice. After 4 weeks, all mice were sacrificed, and the lungs were harvested for analysis. Histological confirmation of tissue morphology was performed using hematoxylin and eosin (H&E) staining. All animal experiments were approved by the Animal Protection and Utilization Committee of The First Affiliated Hospital of University Of South China.

Immunohistochemistry (IHC)

The tissue was fixed in 4 % paraformaldehyde, dehydrated, paraffin-embedded, dewaxed, and then incubated overnight at 4 °C with the following antibodies: NEDD9 antibody (1:2000, #ab18056, Abcam), Anti-Histone H3 (acetyl K9) (1:2000, #ab10812, Abcam), and Phospho-FAK (Tyr397) (1:2000, #44-624G, Invitrogen). The secondary antibody was incubated for 30 min at 37 °C.

Statistical analysis

All experiments were independently replicated at least three times, and the entire dataset is presented as means ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 9.0 software. The Student's t-test was utilized to assess differences between two independent groups. For comparisons involving multiple groups, one-way ANOVA was performed to evaluate overall group differences, followed by post-hoc Tukey's Honest Significant Difference (HSD) test to identify pairwise significant differences among the groups. The assumptions of normality and homogeneity of variances were verified before applying parametric tests. Specifically, the Shapiro-Wilk test was used to check for normal distribution, and Levene's test was applied to assess equal variances. The choice of these statistical tests was based on the need to ensure robustness and appropriateness for the data structure and experimental design. A p-value < 0.05 was considered indicative of statistical significance, while $p < 0.01$ was deemed to represent highly statistically significant differences.

Result

1. NEDD9 is elevated in breast cancer tissues, and high NEDD9 expression is associated with poor prognosis of breast cancer patients

We first used the UALCAN database to analyze the gene expression of NEDD9. The hierarchical cluster analysis based on the CPTAC database showed that compared with normal tissues, the expression of NEDD9 in breast cancer tissues was up-regulated (Fig. 1A, B; SFigure1), which was consistent with the IHC results in the HPA database (Fig. 1C). In addition, NEDD9 also has high expression in several other cancers (Fig. 1A), such as cervical cancer and endometrial cancer (UCEC), pancreatic cancer (PAAD), head and neck tumors and glioblastoma (GBM). We collected a group of patient tissues to further detect the expression of NEDD9 in human breast cancer tissues and adjacent normal tissues. IHC staining also showed that compared with normal tissues, the expression of NEDD9 protein in breast cancer tissues was up-regulated (Fig. 1D). In addition, compared with normal cells (MCF-10A), NEDD9 is more abundant in most breast cancer cells (Fig. 1E, F), which further supports the tumor promoting effect of NEDD9 in breast cancer. We also studied the clinical significance of NEDD9. First, through IHC staining and GEPIA2.0 database analysis, we found that NEDD9 was also related to the clinical stage of breast cancer (Fig. 1G, H). And through Kaplan Meier analysis based on breast cancer patients, it was found that breast cancer patients with high expression of NEDD9 had a poor survival period after progression (Fig. 1I). In conclusion, these findings indicate that the expression of NEDD9 in breast cancer tissue is increased, which is related to the poor prognosis of breast cancer patients.

2. NEDD9 overexpression facilitates breast cancer cell proliferation, migration, and invasion in vitro

To delve into the question of whether NEDD9 can modify the tumor biology of breast cancer cells, we conducted a series of in vitro experiments using the MCF7, HCC1954, and MDAMB-231 cell lines. Within these experiments, NEDD9 was either overexpressed or depleted. The efficiency of overexpression was confirmed by qRT-PCR and western blot (Fig. 2A). The MTT assays revealed that NEDD9 overexpression boosted the proliferation of breast cancer cells (Fig. 2B). Furthermore, EdU assays indicated that NEDD9 overexpression led to an increase in DNA synthesis activity (Fig. 2C). Transwell assays revealed that NEDD9 overexpression significantly heightened the migration and invasion capabilities of MCF7, HCC1954, and MDAMB-231 cells (Fig. 2D). Epithelial-mesenchymal transition (EMT) is a crucial mechanism for cancer cell migration and invasion. Therefore, we further examined the impact of NEDD9 on the expression of EMT markers. Western blot assays (Fig. 2E) showed that NEDD9 overexpression led to a decrease in the expression of the epithelial marker E-cadherin and an increase in the expression of the mesenchymal markers Fibronectin, Ncadherin, and Vimentin, emphasizing the significant role of NEDD9 in regulating EMT in breast cancer cells. Additionally, we observed significant morphological changes from polygonal to fibroblast-like shapes (Fig. 2F) following NEDD9 overexpression. In summary, these results suggest that elevated expression of NEDD9 promotes the proliferation, migration, and invasion of breast cancer cells in vitro.

3. HDAC4 inhibition increases histone acetylation at NEDD9 promoter and promotes NEDD9 transcription

Recently, it has been reported that epigenetic modifications are involved in the regulation of NEDD9 gene expression, such as miR145-5p and miR-363-3p [20,23]. High levels of miR-107 have also been shown to reduce NEDD9 levels in breast cancer cells, reducing cell migration and proliferation [31]. Concurrently, by utilizing the ENCODE and UCSC databases, we observed that histone acetylation modification in the NEDD9 promoter region was more prominent compared to other epigenetic modifications (SFigure 2A, B). Moreover, histone acetylation modification in the NEDD9 promoter region was more intense in breast cancer cells compared to normal cells (SFigure 2C). Therefore, we speculate that changes in histone acetylation in the NEDD9 promoter region may affect NEDD9 expression in breast cancer. To further determine the regulatory mechanism of NEDD9, we treated NEDD9 knockdown cells with different histone deacetylase inhibitors, including type 1 and type 2 HDAC family inhibitors trichostatin A (TSA) and type 3 HDAC family (Sirtuins) inhibitors nicotinamide (NAM) [32,33]. We found that TSA treatment reversed the inhibitory effect of knockdown on NEDD9, while NAM treatment had minimal effects on the recovery of NEDD9 knockdown (Fig. 3A, B). Given that HDAC family members regulate different biological pathways and have different functions, we next attempted to determine whether specific HDAC family members are responsible for upregulating NEDD9. siRNA was transfected into breast cancer cells to knock out HDACs (class I HDAC1, 2, 3 and 8, class II HDAC4, 5, 6 and 7).

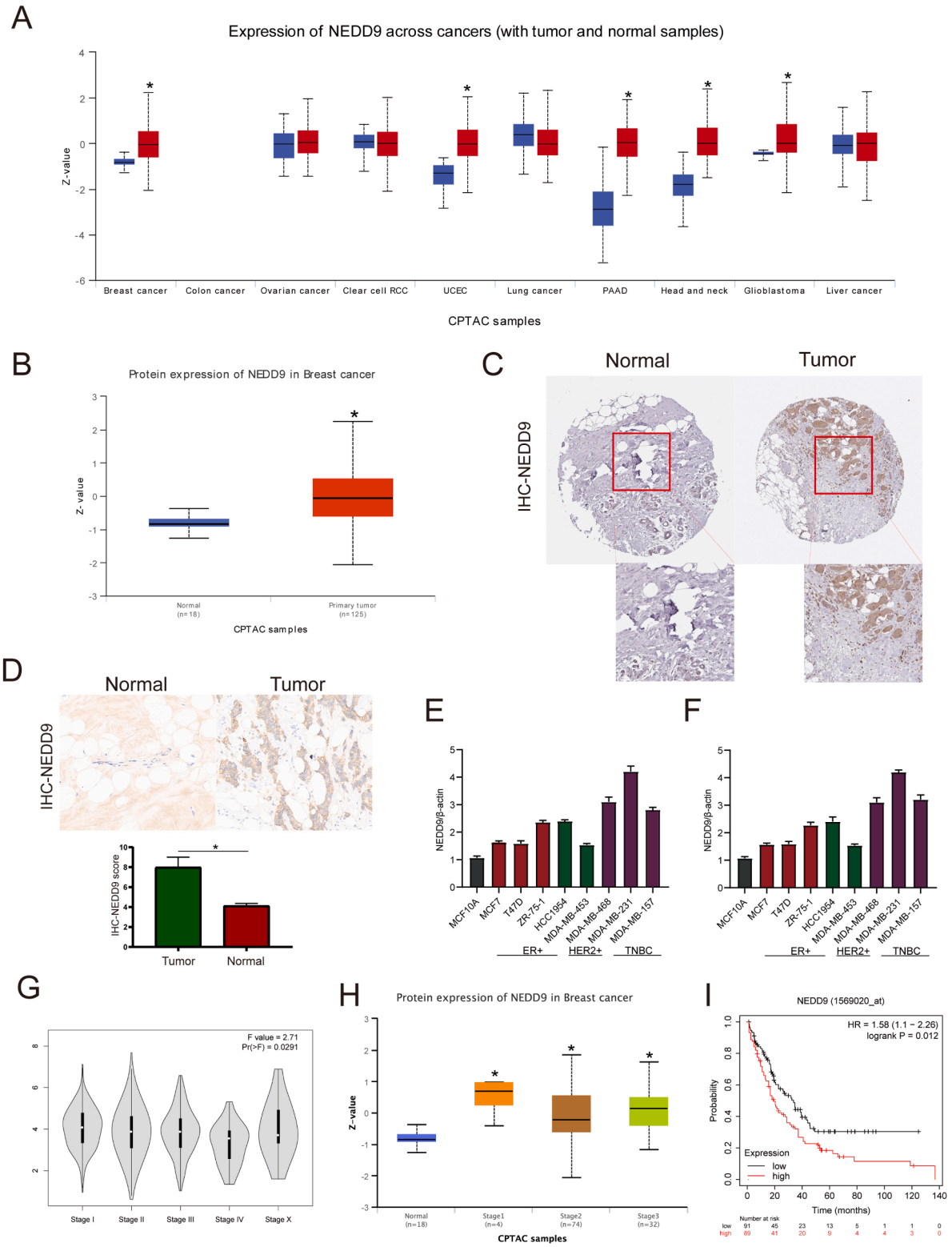

Fig. 1. NEDD9 is elevated in breast cancer tissues, and high NEDD9 expression is associated with poor prognosis of breast cancer patients. (A) Analysis of NEDD9 protein levels in diverse tumor and adjacent non-cancerous tissues based on the CPTAC database. Blue: adjacent non-cancerous tissue. Red: tumor tissue. (B) Evaluation of NEDD9 protein levels in breast tumors and normal tissues using the CPTAC database. Blue: adjacent non-cancerous tissue. Red: Breast tumors. (C) HIC results for NEDD9 in breast tumors and normal tissues were analyzed using the HPA database. Normal: para-carcinoma tissue; Tumor: tumor tissue. (D) NEDD9 protein levels in normal breast tissue and breast tumor tissue were analyzed using immunohistochemistry techniques. Scale, 100 μm. Normal: para-carcinoma tissue; Tumor: tumor tissue. (n=6). Normal human breast cells (MCF10A) and breast cancer cells (MCF7, T47D, ZR-75-1, HCC1954, MDA-MB-453, MDA-MB-468, MDA-MB-231, MDA-MB-231) were evaluated using QRT-PCR (E) and Western blot (F) to determine NEDD9 mRNA and protein levels in MDA-MB-157). (n=3). The GEPIA2.0 database (G) and CPTAC database (H) were utilized to assess the expression level of NEDD9 across different stages of breast cancer. (I) The impact of NEDD9 expression level on prognosis was analyzed based on the KM database. Black: Low NEDD9 expression. Red: High expression of NEDD9. All the data analysis *$P < 0.05$.

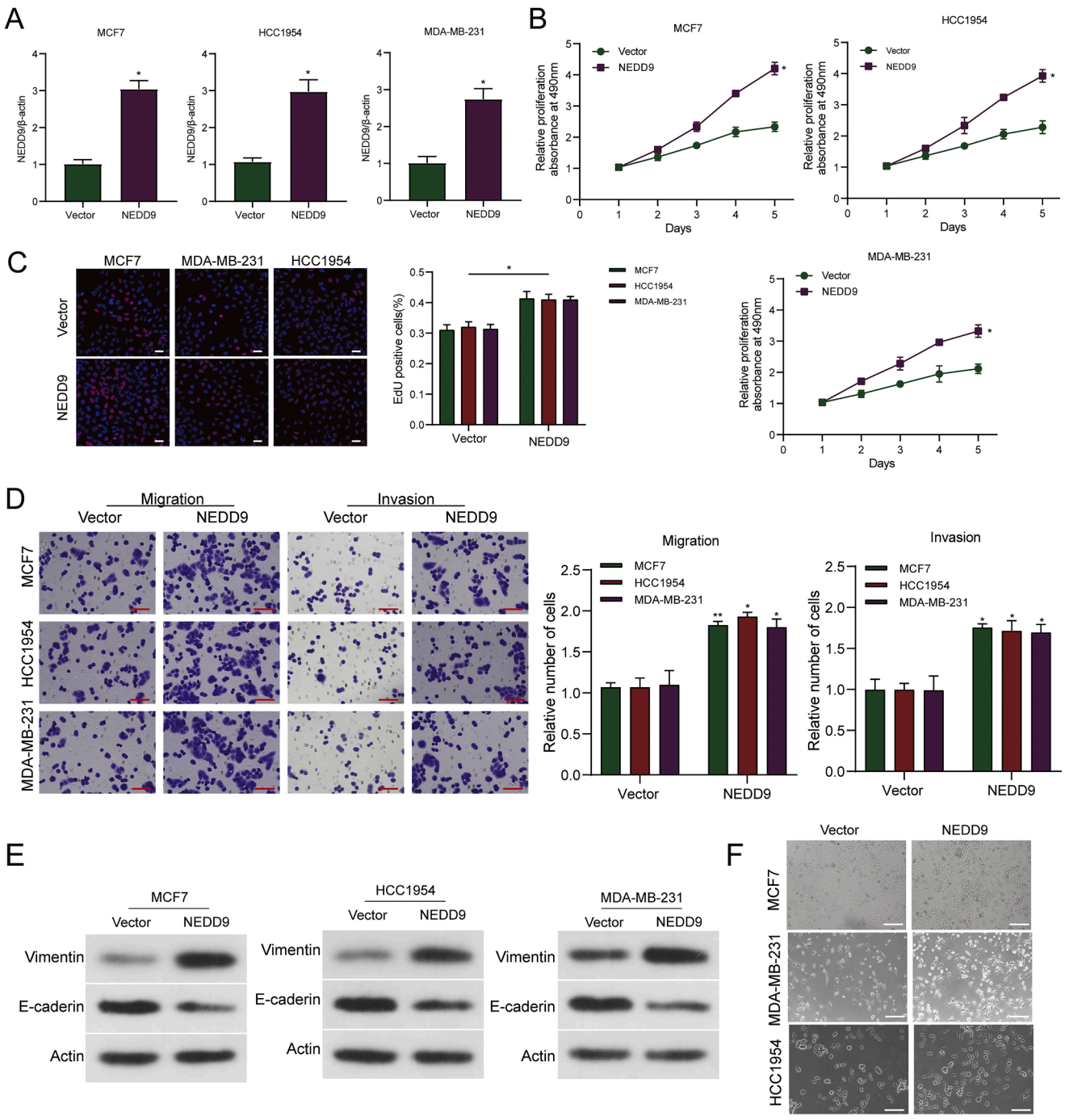

Fig. 2. NEDD9 overexpression facilitates breast cancer cell proliferation, migration, and invasion in vitro.(A) The overexpression of NEDD9 in three breast cancer cells (MCF7, HCC1954, and MDA-MB-231) was confirmed using QRT-PCR and Western blot analysis. (B) The overexpression of NEDD9 was induced in three breast cancer cell lines (MCF7, HCC1954, and MDA-MB-231), and cell viability was assessed using the MTT assay. (C) The overexpression of NEDD9 was introduced in three breast cancer cell lines (MCF7, HCC1954, and MDA-MB-231), and cell proliferation was evaluated using EdU staining. Scale, 100 μm. (D) The overexpression of NEDD9 was achieved in three breast cancer cell lines (MCF7, HCC1954, and MDA-MB-231), and the migration and invasion capabilities of the cells were determined using Transwell assays. Scale, 100 μm. (E) The overexpression of NEDD9 was induced in three breast cancer cell lines (MCF7, HCC1954, and MDA-MB-231), and the expression levels of EMT markers were analyzed using Western blot assays. (F) The overexpression of NEDD9 was established in three breast cancer cell lines (MCF7, HCC1954, and MDA-MB-231), and the cell morphology was observed under an optical microscope. Scale Bar:100 μm. In all the results, Vector: control group; NEDD9: overexpression NEDD9 group. All the results are presented as means ± SD from three independent experiments. n=3, $^*P < 0.05$.

Among them, only HDAC4 silencing resulted in increased expression of both NEDD9 gene and protein (Fig. 3C, D). In addition, the specific HDAC4 inhibitor tasquinimod also increased NEDD9 protein levels (Fig. 3E). Consistent with previous results, we observed increased H3K9 acetylation levels in the NEDD9 promoter when HDAC4 was knocked out (Fig. 3F). At the same time, we found low expression of HDAC4 in breast cancer through UALCAN database analysis, and the same result was obtained in HPA database, which was consistent with our experimental results (SFigure 3). The pGL3-nedd9-luc reporter system was then used to measure promoter activity, further confirming that HDAC4 regulates NEDD9 transcription. According to our ChIP assay results, four different NEDD9 promoter fragments were cloned into the pGL3 basic

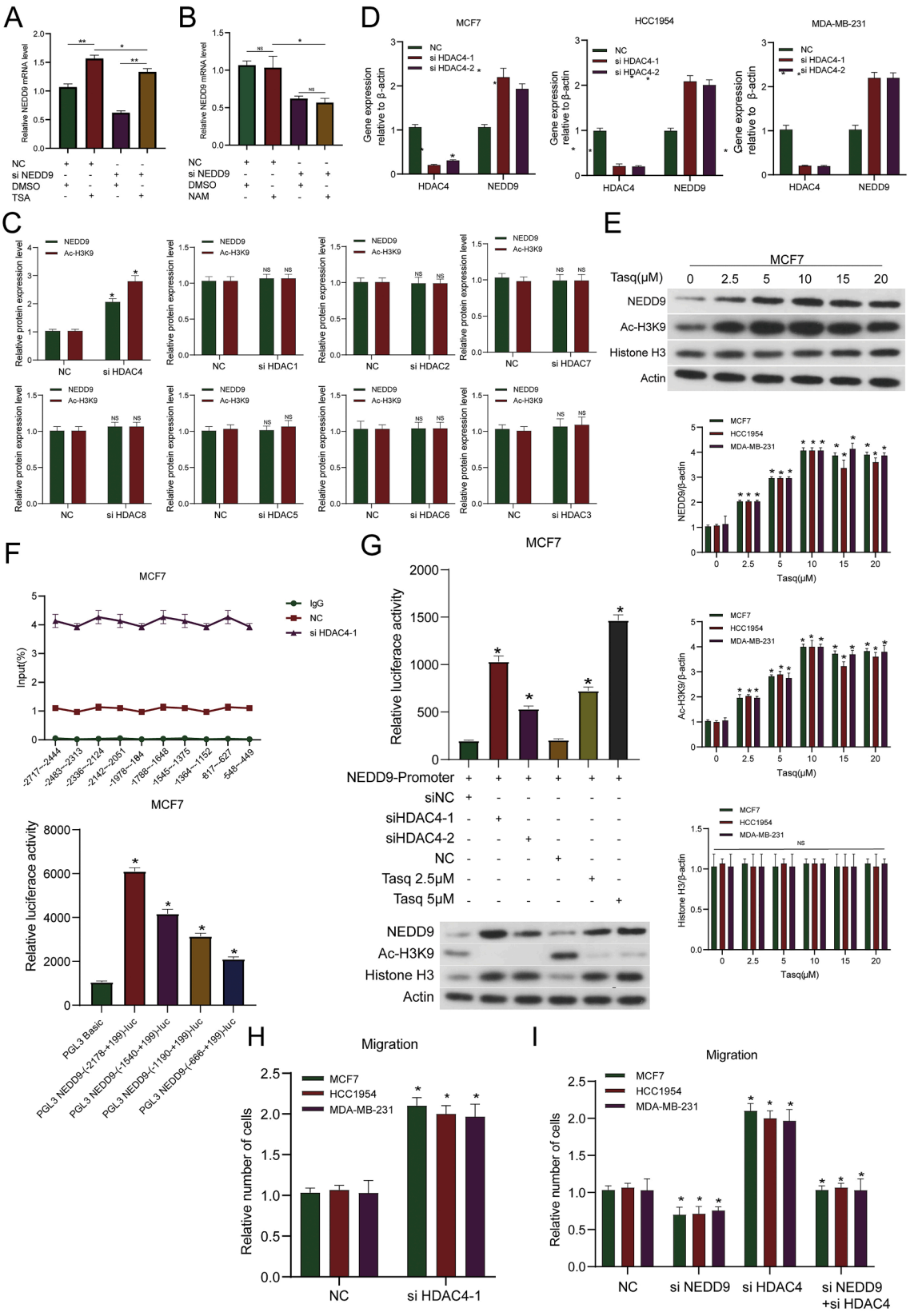

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Fig. 3. HDAC4 inhibition increases histone acetylation at NEDD9 promoter and promotes NEDD9 transcription.(A) The relative mRNA levels of NEDD9 were analyzed in NEDD9 knockout cells, with or without TSA treatment (100nM).(B) The relative mRNA levels of NEDD9 were evaluated in NEDD9 knockout cells, with or without NAM treatment (500μM).(C) siRNA interference technology was utilized to knockout HDACs (Class I HDAC1, 2, 3 and 8, Class II HDAC4, 5, 6 and 7), and protein expression was subsequently detected by Western blot.(D) siRNA interference was employed to knockout HDAC4, and mRNA expression was detected by QRT-PCR.(E) Breast cancer cells (MCF7, HCC1954 and MDAMB-231) were treated with tasquinimod at varying concentrations. After 12 h, protein expression was detected by Western blot.(F) Breast cancer cells were transfected with siHDAC4, and histone acetylation in the NEDD9 promoter region was determined by CHIP assay.(G) Breast cancer cells were treated with siHDAC4 and tasquinimod individually. The NEDD9 promoter luciferase activity and related protein expression were then measured.(H) Breast cancer cells (MCF7, HCC1954 and MDAMB-231) were transfected with siHDAC4, and the cell migration capacity was evaluated using the Transwell assay.(I) Breast cancer cells (MCF7, HCC1954, and MDAMB-231) were transfected with siHDAC4 or/and siNEDD9, and the cell migration capacity was measured using the Transwell method . Scale Bar:100 μm. NC: control group; si NEDD9: knockdown NEDD9 group. DMSO: control group; TSA: type 1 and type 2 HDAC family inhibitors; NAM: type 3 HDAC family (Sirtuins) inhibitors. All the results are presented as means ± SD from three independent experiments. n=3, $^{*}P < 0.05$.

fluorescent reporter vector (Fig. 3G). As expected, in MCF-7 cells, siRNA or tasquinimod inhibition of HDAC4 stimulated NEDD9 transcription using the pGL3NEDD9-luc system (Fig. 3G). The Transwell assay was used to detect cell motility. As expected, HDAC4 knockdown promoted breast cancer cell invasion (MCF7, HCC1954 and MDAMB-231) in vitro (Fig. 3H, I). In summary, these findings support the hypothesis that inhibiting HDAC4 increases histone acetylation in the NEDD9 promoter region, promotes NEDD9 transcription, and promotes breast cancer metastasis.

4. NEDD9 activates the FAK/NF-κB signaling pathway and reveals new therapeutic opportunities

It has been reported that NEDD9 promotes tumor metastasis by increasing FAK phosphorylation at Tyr397 (p-FAK-397Y) [13,34]; NF- κ B signaling pathway is involved in the progression of many diseases by regulating iron prolapse, such as polycystic ovary syndrome [35], colorectal cancer [36], glioblastoma [37], pancreatic cancer [38], etc. The latest study reports that the connectomes coordinate the progression of breast cancer metastasis to the brain by inducing NF-κB activation through FAK signal transduction [39]. Next, we examined whether NEDD9 activates the FAK/NF-κB signaling pathway to regulate breast cancer. Western blot results showed that overexpression of NEDD9 led to increased expression of p-FAK, NF-κB, and NF-κB downstream molecules (Twist1, IL-6, and Survivin) in breast cancer cells (MCF7, HCC1954, and MDAMB-231) (Fig. 4A), and the protein and gene levels in related pathways increased more significantly after HDAC4 knockout (Fig. 4B, C), indicating that NEDD9 activates FAK/NF-κB signaling pathway. κ B signal pathway. IF detection also showed that the expression of p-FAK and NF-κB in breast cancer cells (MCF7, HCC1954 and MDAMB-231) was increased- κ B underwent nuclear transfer (Fig. 4D). These data suggest that NEDD9 is involved in activation of the FAK/NF-κB signaling pathway.

5. NEDD9 promotes mammary tumor progression and TAM polarization

Therefore, we would like to investigate the effect of NEDD9 on breast tumor progression and TAM polarization. First, in vivo experiments, female BALB/c nude mice (4-5 weeks, 16-20 g) were injected with MDAMB-231/4175 cells (1×10^6 cells per mouse) through the tail vein to generate a metastatic model simulating lung metastasis of breast cancer. After surgical resection of lung tissue, the number of lung metastatic nodules was counted and examined using H&E staining. It was found that the number of lung metastatic nodules significantly increased after overexpression of NEDD9 (Fig. 5A, B). Immunohistochemical staining revealed an increase in NEDD9 and vimentin in the TME, but a decrease in E-cadherin (Fig. 5C; SFigure 4 A), along with an enrichment of angiogenesis (CD31) and macrophages (F4/80). M2 macrophages promote tumor progression, while M1 macrophages inhibit tumor progression [40]. CD163-positive M2 macrophages are more abundant in NEDD9-promoted tumors (Fig. 5D; SFigure 4 B), suggesting that NEDD9 has an effect on the recruitment and polarization of tam. Previous studies have reported that IL-6 plays an important role in regulating various cellular functions, such as cell proliferation, metastasis, vascular permeability, metabolism, and immune cell infiltration [41,42].Next, the ELISA detection results in cell experiments showed that NEDD9 knockdown led to a decrease in il-6 secretion, while NEDD9 overexpression promoted il-6 secretion (Fig. 5E; SFigure 4 C). Therefore, we further evaluated the use of IL-6R monoclonal antibodies to confirm that the enhancement of macrophage behavior induced by NEDD9 was mediated by IL-6. The results showed that the addition of IL-6 neutralizing antibody could eliminate the enhancement effect of NEDD9 overexpression cell supernatant on THP-1 migration (Fig. 5F). Further monitoring of THP-1 polarity co-cultured with NEDD9-overexpressing cells showed that the M2 markers (CD163 and CD206) were up-regulated compared to THP-1 co-cultured with control MDA-MB-231 cells or RPMI medium (Fig. 5G). Consistent with this, compared to control cells (THP-1/control), cells overexpressing NEDD9 (THP-1/NEDD9) induced higher levels of M2 markers Arginase-1 and IL-10 in polarized THP-1 cells (Fig. 5H; SFigure 4 D). In addition, pretreatment of MDA-MB-231 (control and NEDD9) cells with IL-6R monoclonal antibody and subsequent co-culture with THP-1 cells (Fig. 5I) induced CD86 expression in polarized m1-like macrophages. However, IL-6 pretreatment did not significantly increase the differentiation of cd86-positive M1 macrophages. In addition, when THP-1 cells were co-cultured with IL-6-treated cancer cells, the m2-like polarization exhibited by CD163 expression was induced, while when THP-1 cells were co-cultured with IL-6R monoclonal antibody-treated cancer cells, CD163 expression was inhibited (Fig. 5I). Therefore, breast cancer cells overexpressing NEDD9 promoted the polarization of m2 like macrophages in vitro. IL-6R monoclonal antibody antagonizes NEDD9 function to enhance M1 polarity, while IL-6-stimulated NEDD9 action promotes M2

6. NEDD9 promotes tumor growth and breast cancer metastasis through the FAK/NF-κB signaling pathway in vivo

We further evaluated the function of NEDD9 in breast cancer using a nude mouse xenotransplantation model. Stably overexpressing NEDD9 or control MDA-MB-231 cells were injected into the flank of BALB/c nude mice. The overexpression of NEDD9 resulted in increased tumor volume and tumor weight (Fig. 6A, B). Additionally, the tumors of mice injected with histone deacetylase activator (Theophylline) were smaller and lighter compared to those treated with DMSO (Fig. 6A, B), indicating that activating HDAC4 in vivo and reducing NEDD9 can inhibit the growth of breast cancer. There was no significant weight loss during the treatment period (Fig. 6C), excluding drug-related toxicity. HE staining was used to evaluate tumor morphology (Fig. 6D). Immunohistochemical analysis showed that the expression changes of p-FAK, NF-κB, and its target genes were consistent with the results of in vitro experiments (Fig. 6D). These findings suggest that NEDD9 functions in vivo through the FAK/NF-κB signaling pathway, promoting tumor growth. Furthermore, we also studied the effect of NEDD9 on breast cancer metastasis in vivo using a lung metastasis model. Stably overexpressing NEDD9 or control MDA-MB-231 cells were injected into the

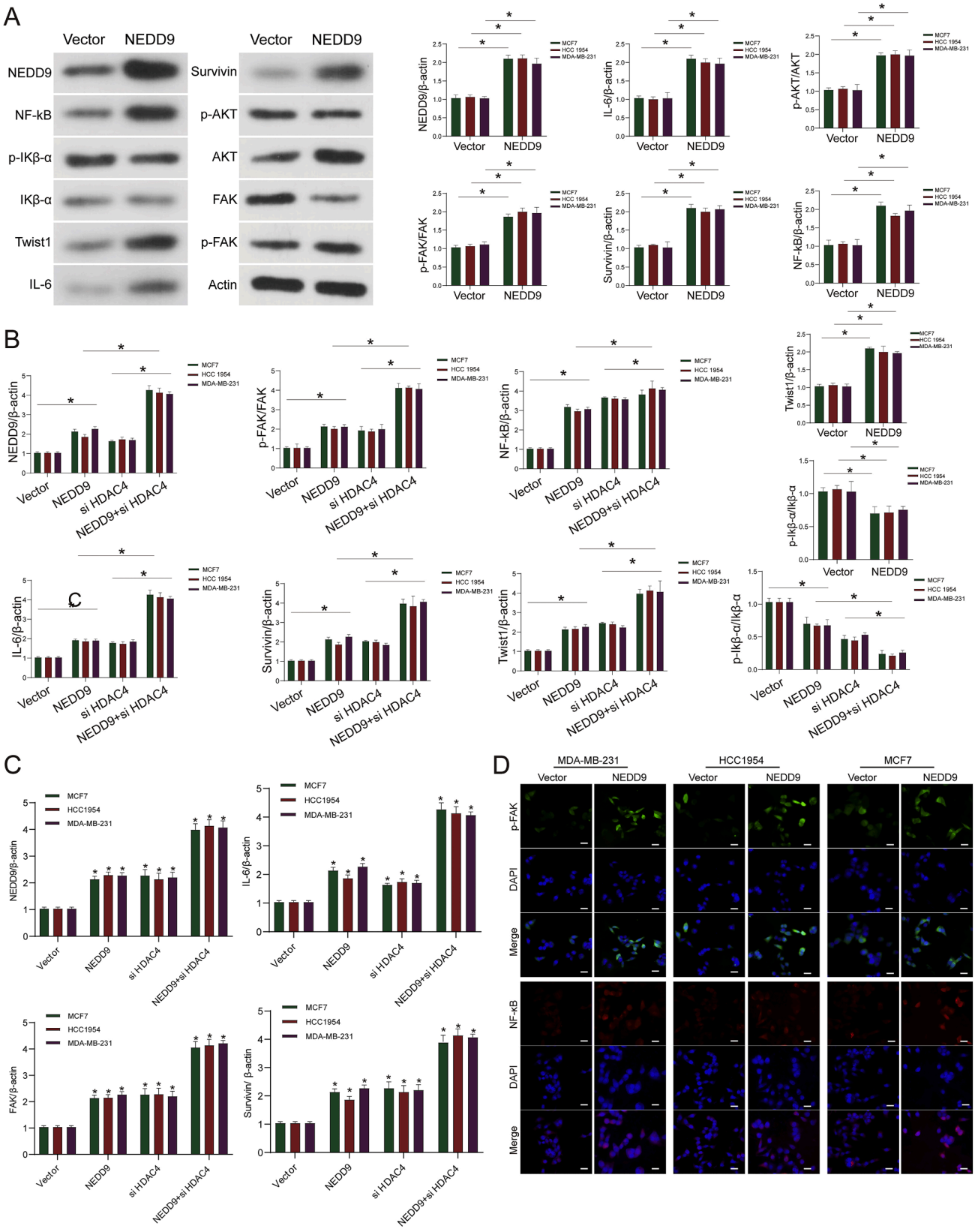

Fig. 4. NEDD9 activates the FAK/NF-κB signaling pathway and reveals new therapeutic opportunities.(A) The overexpression of NEDD9 was confirmed by Western blot analysis. (B) The expression of NEDD9 was upregulated, and siRNA-mediated knockdown of HDAC4 was also performed. The expression of related proteins was then evaluated by Western blot. (C) The overexpression of NEDD9 was implemented, along with siRNA-mediated knockdown of HDAC4. mRNA expression was then determined by quantitative real-time PCR (QRT-PCR). (D) Cellular immunofluorescence was used to assess the overexpression of NEDD9, related protein expression, and cell localization. Scale Bar:10 μm. NC: control group; si HDAC4: knockdown HDAC4 group. Vector: control group; NEDD9: overexpression NEDD9 group. All the results are presented as means ± SD from three independent experiments. n=3, *$P < 0.05$.

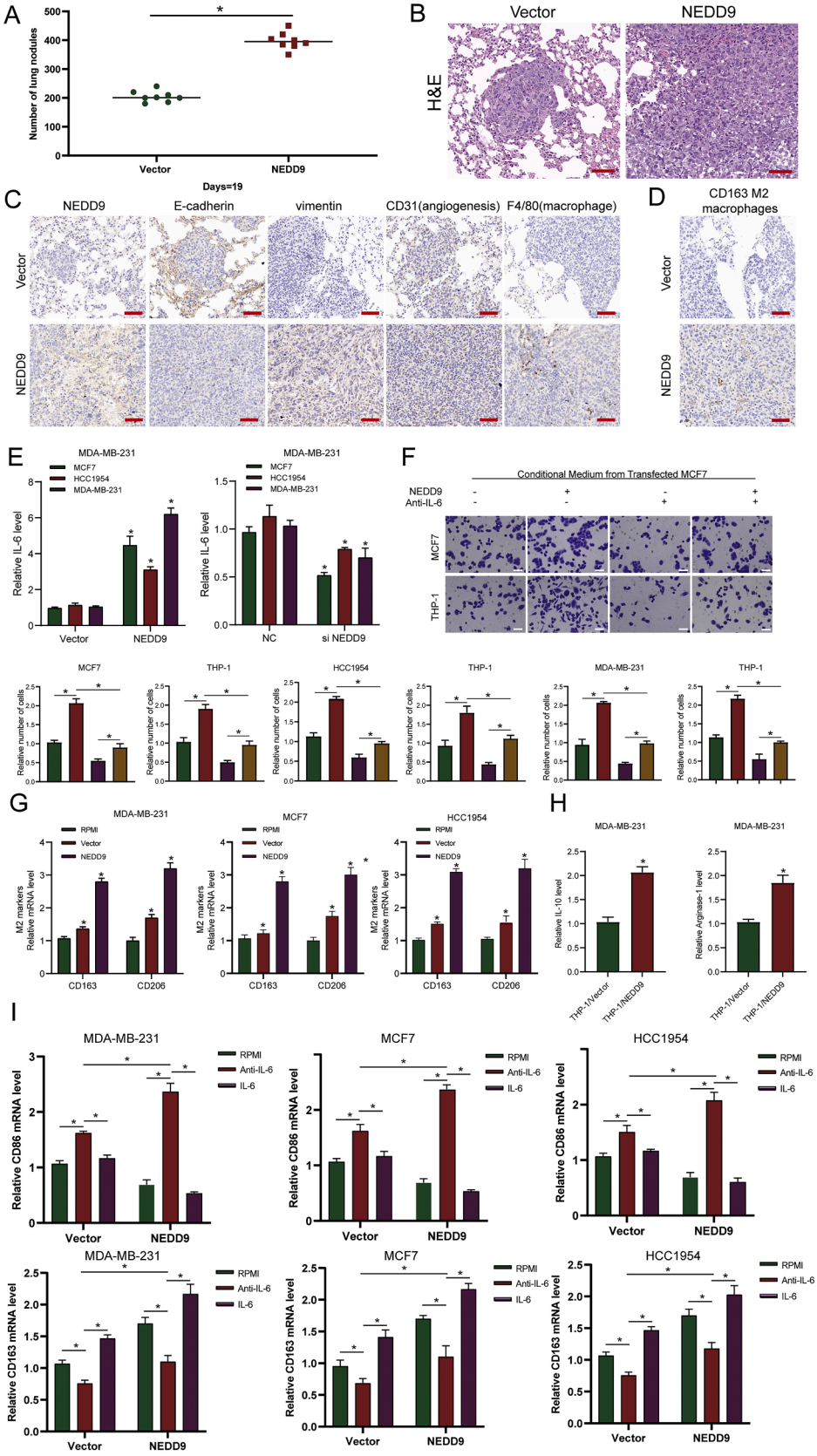

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Fig. 5. NEDD9 promotes mammary tumor progression and TAM polarization.(A) The number of metastatic nodules in the lungs of BalB/c female nude mice was counted. (B) Hematoxylin and eosin (H&E) stained lung sections were used to visualize metastatic nodules in the lungs of BalB/c female nude mice. Scale Bar: 2 mm. (C) Immunohistochemistry (IHC) sections of lung metastasis nodules from BalB/c female nude mice were used to assess the expression of various proteins. Scale Bar: 50 μm.(D) Immunohistochemistry (IHC) sections of lung metastasis nodules from BalB/c female nude mice were used to detect the expression of M2 macrophage marker proteins. Scale Bar: 50 μm.(E) Enzyme-linked immunosorbent assay (ELISA) was used to measure interleukin-6 (IL-6) expression in the medium following overexpression or siRNA knockout of NEDD9. (F) Breast cancer cells overexpressing NEDD9 or breast cancer cell culture medium overexpressing NEDD9 were treated with THP-1 cells, followed by neutralization with an IL-6 monoclonal antibody. Cell migration ability was then assessed using the Transwell method. Scale, 100 μm. (G) Breast cancer cell culture medium overexpressing NEDD9 was treated with THP-1 cells, and the expression of M2 macrophage marker protein was determined by quantitative real-time PCR (QRT-PCR).(H) Breast cancer cells with control or overexpression of NEDD9 were co-cultured with THP-1 cells, and the expression of marker protein in M2 macrophages was measured using ELISA.(I) Breast cancer cells with control or overexpression of NEDD9 were co-cultured with THP-1 cells, treated with IL-6 or an IL-6 monoclonal antibody, and the expression of M1 and M2 macrophage marker genes was assessed by quantitative real-time PCR (QRT-PCR).. Scale bar:100 μm. RPMI: blank control group; Vector: control group; NEDD9: overexpression NEDD9 group; Anti-IL6: Anti-IL6 treatment group; IL6: IL6 treatment group. All the results are presented as means ± SD from three independent experiments. n=3, $^{*}P < 0.05$.

tail vein of BALB/c nude mice. The number of lung metastases and metastatic foci increased in mice overexpressing NEDD9 (Fig. 6E); immunohistochemical analysis showed that the expression changes of p-FAK, NF-κB, and its target genes were consistent with the results of in vitro experiments (Fig. 6F; SFigure 5). After treatment with Theophylline, the number of metastatic nodules and the expression of p-FAK and NF-κB in lung tissues were significantly reduced, along with a significant decrease in IL-6 expression. Overall, these results suggest that NEDD9 promotes tumor growth and breast cancer metastasis in vivo through the FAK/NF-κB signaling pathway, but HDAC4 can weaken this process.

Discussion

Breast cancer is the most common malignant tumor among women in the world. It is a highly heterogeneous disease characterized by different molecular characteristics. Clinically, according to the status of hormone receptors (ER and PR) and HER2 (ERBB2), it can be divided into three main subtypes: lumen ER positive and PR positive, and further subdivided into lumen A and B, HER2 positive and triple negative breast cancer (TNBC) [43]. So far, in the fight against breast cancer, the screening program is the most important help to reduce the mortality caused by this disease. Conventional chemotherapy and radiotherapy (often including targeted drugs) are the main systemic treatments for patients with BC, but many patients experience drug resistance, recurrence, and secondary metastasis [44]. While there is evidence suggesting that NEDD9 promotes metastasis in various cancers, its specific role within different subtypes of breast cancer remains somewhat contentious. On one hand, studies indicate that upregulation of NEDD9 correlates with morphological changes in breast cancer cells towards a fibroblast-like phenotype, enhanced cell invasiveness, and in vivo lung metastasis; conversely, knocking down NEDD9 in vitro and in vivo can inhibit the invasive phenotype of breast cancer cells. Additionally, NEDD9′s role in the activation of the FAK/NF-κB signaling pathway, which promotes breast cancer metastasis and M2 polarization of macrophages, highlights its importance in tumor progression. This process not only enhances the immunosuppressive tumor microenvironment but also contributes to chemotherapy resistance. Therefore, a deeper understanding of NEDD9′s multifaceted roles in breast cancer is essential for developing more effective treatment strategies. Future research should aim to uncover the precise mechanisms by which NEDD9 operates and explore new therapies targeting NEDD9, to improve outcomes for breast cancer patients. For example, combining NEDD9 inhibitors with chemotherapy could offer a complementary therapeutic strategy, enhancing anticancer efficacy while reducing side effects. Furthermore, given NEDD9′s pivotal role in EMT, targeting NEDD9 may help prevent or slow down distant metastasis in late-stage cancers, opening new avenues for preventive and therapeutic approaches. Lastly, since NEDD9 is closely linked with immune evasion mechanisms, high NEDD9-expressing tumor cells can alter the tumor microenvironment, suppressing the anti-tumor activity of immune cells. Thus, NEDD9 inhibitors might synergize with immune checkpoint inhibitors and other immunotherapies, enhancing the success rate of immunotherapy, particularly in patient populations that initially respond poorly to immunotherapy. In summary, a thorough understanding of NEDD9's role in breast cancer and its relationship with TAM polarization will lay the groundwork for developing more precise and effective clinical strategies.. In this study, we confirmed that NEDD9 can be regulated by HDAC4 transcription and regulates the expression of FAK/NF-κB signaling pathway promotes breast cancer metastasis and M2 polarization of macrophages (Fig. 7).

Nearly 20 years ago, changes in the expression of NEDD9 were identified as a contributing factor to the metastasis of many different types of cancer. However, although previous studies have found that the increased expression of NEDD9 promotes metastasis, other studies have shown that NEDD9 has an anti-metastasis effect in breast cancer metastasis [45,46]. We demonstrate that NEDD9 changes the shape of breast cancer cells into fibroblast like morphology, increases cell invasiveness, and promotes lung metastasis in vivo. In contrast, knockdown of NEDD9 in vitro and in vivo can inhibit the invasive phenotype of breast cancer cells. In addition, some studies have shown that HDAC regulates events related to the progression of breast cancer, including self-renewal and expansion, invasion and metastasis of stem cells [47]. We demonstrated that inhibiting HDAC4 increased histone acetylation of the NEDD9 gene promoter, consistent with previous reports [13,34].

We report that FAK signals can activate NF-κB, promoting breast cancer metastasis [39]. Similarly, the role of FAK/NF-κB signaling in hepatocellular carcinoma has been reported [48]. Our findings align with previous reports demonstrating that up-regulated NEDD9 can activate FAK phosphorylation [13,34]. As a scaffold protein, NEDD9 contains multiple protein interaction domains, allowing it to dock with chaperone proteins. It is reasonable to speculate that NEDD9 provides a binding site for FAK, enhancing its phosphorylation, which then activates the FAK/NF-κB signaling pathway and regulates breast cancer cell migration. In line with these observations, we demonstrate that overexpression of NEDD9 leads to increased expression of phosphorylated FAK, NF-κB, and downstream NF-κB molecules (Twist1, IL6, and Survivin) in breast cancer cells. Therefore, our findings suggest that NEDD9 promotes breast cancer cell metastasis by activating the FAK/NF-κB signaling pathway.

The inflammatory microenvironment plays a crucial role in cancer progression [49]. Immunotherapy is a promising new treatment option for breast cancer [50]. Breast cancer cells that overexpress NEDD9 secrete higher levels of the inflammatory cytokine IL-6. IL-6 affects TAM polarity [51-53], and it regulates the fate of CSCs and the TME [54]. Therefore, immunotherapy interventions targeting cytokines may modify the TME. Serum IL-6 levels increase with cancer advancement and are associated with poor survival rates in various cancers [55]. IL-6 drives metastasis and differentiation in breast cancer [56]. Aggressive cancer cells release large amounts of IL-6, stimulating angiogenesis and immune evasion [57]. EGFR signals trigger IL-6 production through NF-kB activation [58]. Our findings suggest that overexpression of NEDD9 activates the FAK/NF-κB signaling pathway, leading to increased IL-6 expression. This affects M2 macrophage polarization and promotes immune escape. Furthermore, systemic administration of

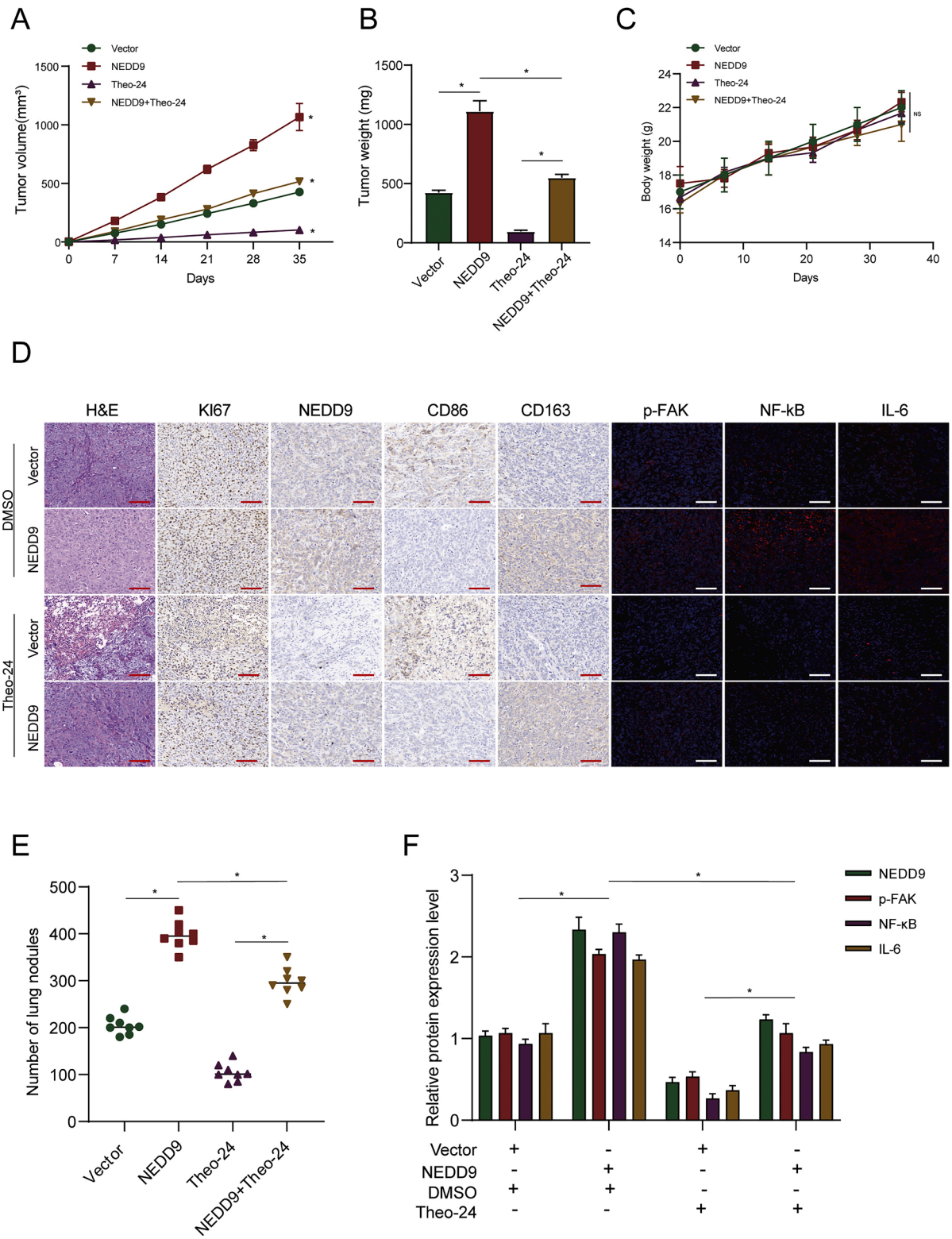

Fig. 6. NEDD9 promotes tumor growth and breast cancer metastasis through the FAK/NF-κB signaling pathway in vivo. (A-C) Control or NEDD9 overexpressing breast cancer cells were implanted into the lateral wall of nude mice. Following treatment with Theo-24 or DMSO, tumor mass, volume, and mouse body weight were evaluated. (D) Hematoxylin and eosin (H&E) staining was performed to visualize the histological morphology of the implanted tumor. The expression level of related proteins in tumor tissues was assessed by immunohistochemistry (IHC). Scale, 100 μm. (E) Control or NEDD9 overexpressing breast cancer cells were injected intravenously into nude mice. Following treatment with Theo-24 or DMSO, the number of pulmonary metastatic nodules was determined. (F) Breast cancer cells with control or NEDD9 overexpression were injected into the whole body of nude mice via tail vein. Following treatment with Theo-24 or DMSO, the expression level of related proteins in tumor tissues was assessed by immunohistochemistry (IHC). Vector: control group; NEDD9: overexpression NEDD9 group; DMSO: control group; Theo-24: histone deacetylase activator. All the results are presented as means ± SD from three independent experiments. n=6, $^{*}P < 0.05$.

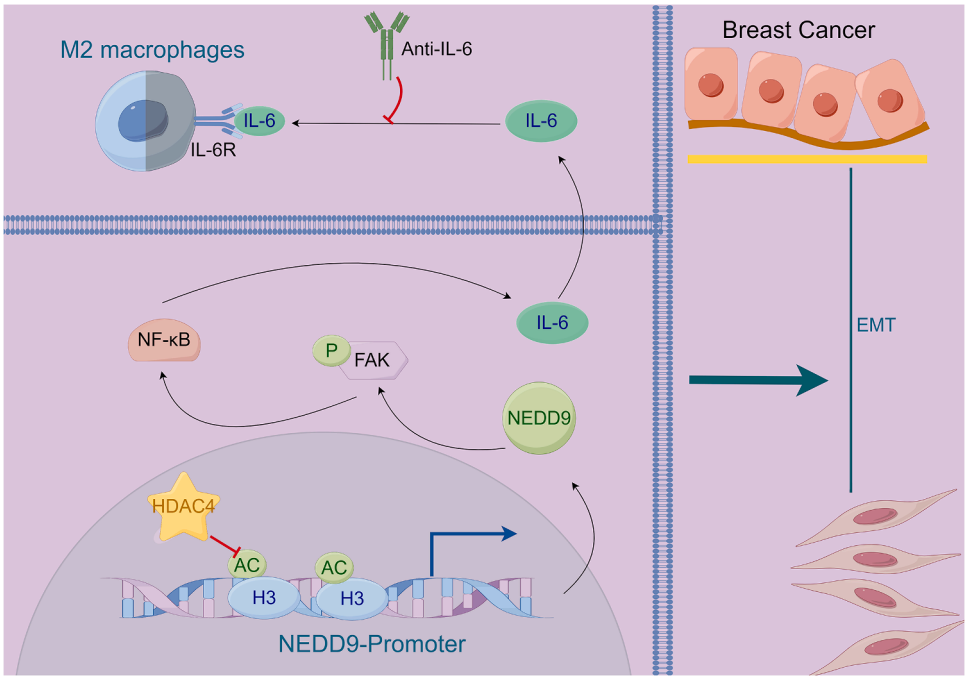

Fig. 7. NEDD9 is transcriptionally regulated by HDAC4 and promotes breast cancer metastasis and macrophage M2 polarization via the FAK/NF-κB signaling pathway.

IL-6/IL-6R antagonists and HDAC activators may reduce breast cancer metastasis and promote immune cell invasion.

Macrophages, key components of the immune system, exhibit complex roles in breast cancer, presenting as either pro-inflammatory M1 or anti-inflammatory M2 polarized states. M1 macrophages attack tumor cells directly and enhance the immune response through the secretion of cytokines such as TNF-α and IL-1β [59]; whereas M2 macrophages establish an immunosuppressive environment conducive to tumor growth, invasion, and metastasis by secreting IL-10 and TGF-β [60]. TAMs, predominant in the breast cancer microenvironment, have their polarization influenced by various tumor-secreted factors. In their M2-polarized state, TAMs promote tumor vasculature by secreting angiogenic factors like VEGF, while simultaneously suppressing immune surveillance through immunosuppressive molecules such as PGE2 and Arginase 1, leading to resistance against chemotherapy, radiation, and immunotherapy. Understanding the polarization status of TAMs in breast cancer is critical for the development of targeted therapies. Shifting TAMs from an M2 to an M1 polarization state could enhance the efficacy of tumor immunotherapies, mitigate therapy resistance, and improve patient outcomes. Future research will focus on elucidating the mechanisms that govern TAM polarization in breast cancer and exploring innovative treatments designed to reprogram TAM function, aiming for more precise and effective clinical strategies.

Overexpression of NEDD9 activates the FAK/NF-κB/IL6 signaling pathway, significantly enhancing chemotherapy resistance in tumor cells. This pathway not only supports cell survival and proliferation but also promotes the production of inflammatory mediators, protecting cancer cells from the cytotoxic effects of chemotherapeutic drugs. Targeting NEDD9, by inhibiting this signaling cascade, holds promise for restoring chemotherapy sensitivity in cancer cells, thereby enhancing treatment efficacy. Consequently, NEDD9 inhibitors may become powerful tools in overcoming chemotherapy resistance and improving patients' responsiveness to chemotherapy.

NEDD9 plays a critical role in the EMT, a fundamental step for tumor cells to acquire migratory and invasive capabilities, which is also a key aspect of tumor metastasis. Through regulating extracellular matrix remodeling, expression of cell adhesion molecules, and cytoskeletal rearrangement, NEDD9 facilitates the migration and invasion of tumor cells. Considering that metastasis is one of the major clinical challenges in late-stage cancers, targeting NEDD9 may offer an effective approach to prevent or slow down distant dissemination of cancer, paving the way for new strategies in the prevention or treatment of metastatic diseases.

Expression levels of NEDD9 are tightly associated with tumor immune evasion mechanisms. Tumor cells with high NEDD9 expression can alter the tumor microenvironment, including modulating the polarization of TAMs, thus suppressing the antitumor activity of immune cells. Targeting NEDD9 could potentially reshape the tumor microenvironment, reducing the generation of immunosuppressive factors and enhancing the efficacy of immunotherapies. This implies that NEDD9 inhibitors may synergize with immune checkpoint inhibitors and other immunotherapies, increasing the success rate of immunotherapy, particularly in patient populations who have shown poor initial responses to immunotherapy.

Given that monotherapies can sometimes face the development of drug resistance, combining NEDD9 inhibitors with Theo-24 may offer a complementary therapeutic strategy. Theo-24, with its anti-inflammatory and immunomodulatory properties, theoretically could potentiate the effects of NEDD9 inhibitors, collectively inhibiting tumor growth and metastasis while mitigating the side effects of chemotherapy and immunotherapy. The synergistic effect of such combination therapy aims to overcome the limitations of single-agent drugs, providing more optimized treatment options for patients.

In summary, as a multifunctional target, NEDD9 inhibitors, when used in conjunction with conventional chemotherapy, immunotherapy, and potential combinatorial therapies, demonstrate substantial potential in overcoming treatment resistance and improving patient outcomes. Further research will be instrumental in elucidating the optimal application strategies for NEDD9 inhibitors and their exact positioning in the comprehensive treatment of cancer.In the context of breast cancer metastasis and immune modulation, our study has delved into the role of NEDD9, comparing it with established markers like TWIST1, SNAIL, and ZEB1, all known drivers of EMT. Unlike these markers, which mainly concentrate on the transcriptional regulation of EMT genes, NEDD9′s influence extends beyond gene expression, encompassing downstream signaling cascades and interactions with the extracellular matrix. This comprehensive impact positions NEDD9 uniquely as a central node

integrating diverse aspects of the metastatic cascade.

Our investigation further challenges prevailing paradigms by unveiling novel dimensions of NEDD9′s role in immune modulation. Particularly, we have gathered evidence indicating that NEDD9 modulates the polarization of TAMs toward an immunosuppressive phenotype, contrasting with the conventional emphasis on cytokines and chemokines in this context. This revelation paves the way for fresh explorations into the intricate dynamics between tumor cells and the immune system, potentially fostering the development of innovative immunotherapeutic approaches. By integrating these perspectives, our study not only consolidates NEDD9′s significance in breast cancer research but also propels the field forward with novel insights and therapeutic prospects.

CRediT authorship contribution statement

Wenhong Liu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Guanghua Luo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors confirm that there are no conflicts of interest.

Availabilsity of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The present study obtained ethical approval from the First Affiliated Hospital of University of South China, The Ethics Committee of the First Affiliated Hospital of the University of South China, and all participants provided their informed consent.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

All the authors agreed to publish it.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2024.101059.

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