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ORIGINAL ARTICLE

Exosomal EPHA2 transfers metastatic potential by stabilizing TGF-βRI and activating the TGF-β/SMAD3 signaling pathway in breast cancer

Liming Liu[1,2,3]*, Yichu Zhang[1,2,3]*, Xiaoxue Li[1,2,3]*, Yueni Mo[1,2,3]*, Lanlan Song[1,2,3], Yidi Jia[1,2,3], Luoming Zhang[1,2,3], Wei Zhou[1,2,3], He Zhang[1,2,3], Hui Guo[1,2,3], Zhiyong Wang[1,2,3], Yanfen Cui[1,2,3], Fei Zhang[1,2,3], Ruifang Niu[1,2,3]

[1]Public Laboratory, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin 300060, China; [2]Tianjin's Clinical Research Center for Cancer, Tianjin 300060, China; [3]Key Laboratory of Breast Cancer Prevention and Therapy, Tianjin Medical University, Ministry of Education, Tianjin 300060, China

ABSTRACT

Objective: Intratumoral heterogeneity refers to the presence of distinct subpopulations of cancer cells within a single tumor, which exhibits variations in phenotypic traits, such as proliferation rate, drug sensitivity, and metastatic potential. Dynamic interactions among heterogeneous cell populations have a critical role in tumor progression. Increasing evidence underscores the importance of intercellular communication among heterogeneous cancer cell subpopulations in driving malignancy. However, the molecular mechanisms governing such cancer cell-to-cancer cell interactions are poorly understood.

Methods: Exosomes were isolated from highly metastatic breast cancer cells (HM-BCCs) and low metastatic breast cancer cells (LM-BCCs). The role of exosome-mediated intercellular communication on metastatic behavior was assessed using wound healing and Transwell assays. Gene knockdown and overexpression strategies, small-molecule inhibitors, and xenograft mouse models were used to elucidate the role of exosomal EPHA2.

Results: Exosomes derived from HM-BCCs considerably enhanced the migratory and invasive capabilities of LM-BCCs *in vitro* and increased the metastatic potential *in vivo*. Mechanistically, EPHA2 was identified as a key protein enriched in exosomes from HM-BCCs and was shown to be transferred to LM-BCCs by these vesicles. Exosomal EPHA2 promoted epithelial-to-mesenchymal transition in LM-BCCs when internalized by stabilizing TGF-βRI and activating the transforming growth factor-β/mothers against decapentaplegic homolog 3 (TGF-β/SMAD3) signaling pathway, thereby facilitating the acquisition of a metastatic phenotype.

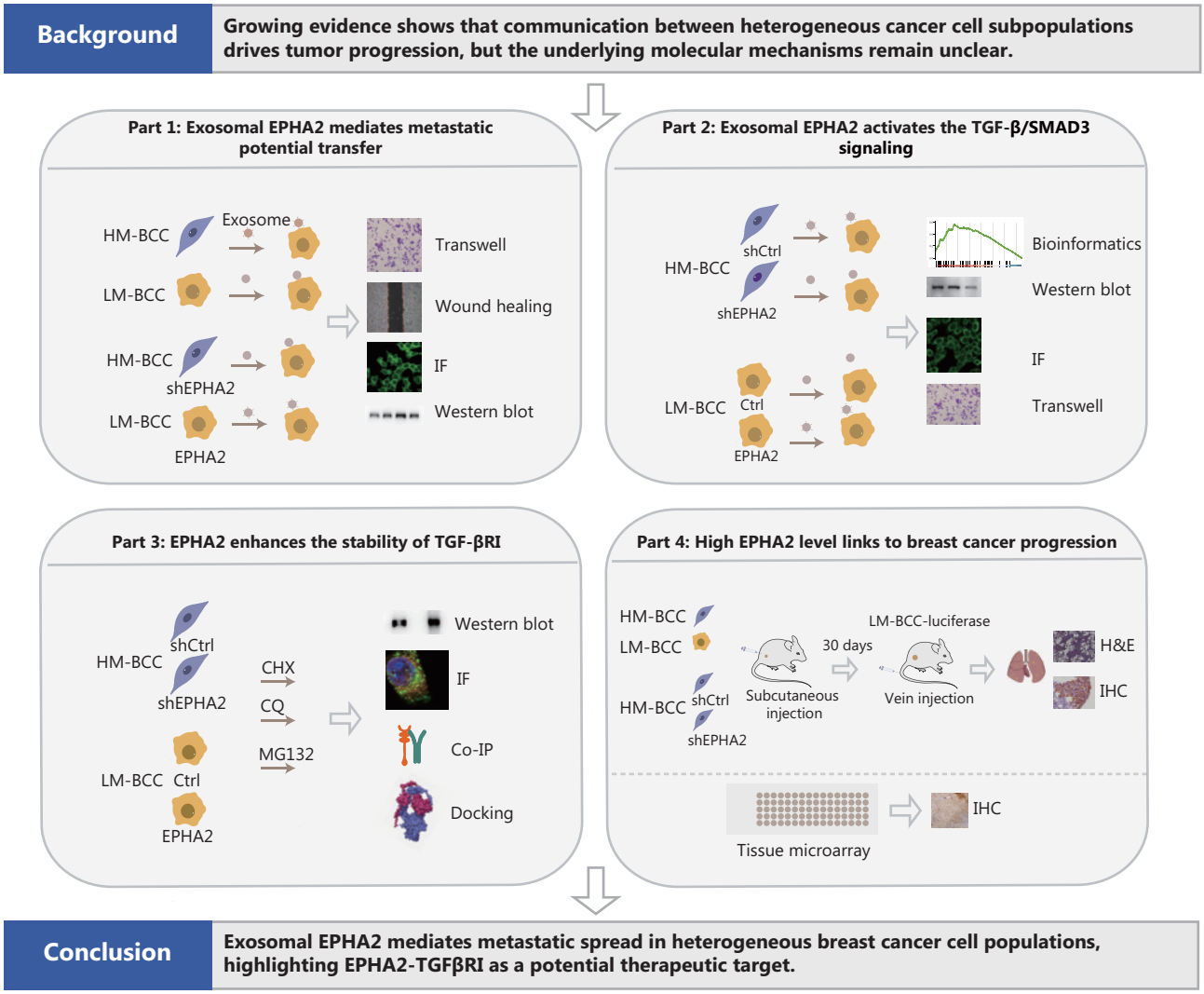
Conclusions: The results underscore the pivotal function of exosomal EPHA2 in mediating the transfer of metastatic potential among heterogeneous breast cancer cell populations. Targeting the EPHA2-TGF-βRI signaling axis may provide a novel therapeutic approach for preventing or limiting breast cancer metastasis.

KEYWORDS Breast cancer; intratumor heterogeneity; exosomes; EPHA2; TGF-βRI

Introduction

Breast cancer is the most prevalent malignancy in women worldwide and continues to pose a substantial threat to women's health. Most breast cancer-related mortalities are due to distant organ metastases[1]. This clinical outcome is driven in part by pronounced intratumoral heterogeneity, wherein the distinct subpopulations of neoplastic cells within a single tumor exhibit divergent proliferative capacities, therapeutic responses, and metastatic potentials[2-4]. Such cellular diversity promotes therapeutic resistance and metastatic dissemination through intricate mechanisms within the tumor microenvironment. Emerging evidence suggests that bidirectional signaling between malignant cells and surrounding stromal compartments drives tumor progression[5-8] because cancer cells reprogram stromal elements to establish pro-metastatic niches and stromal-derived factors reciprocally enhance tumor growth, invasion, and chemoresistance. Notably, recent studies indicate that dynamic interactions among heterogeneous cancer cell subpopulations within a tumor may contribute to malignancy[5,9-11]. However, the mechanisms underlying such heterotypic cancer cell-to-cancer cell communication remain largely unexplored.

*These authors contributed equally to this work.
Correspondence to: Fei Zhang and Ruifang Niu
E-mail: feizhang03@tmu.edu.cn and rniu@tmu.edu.cn
ORCID ID: https://orcid.org/0000-0001-7434-7339 and https://orcid.org/0000-0002-8845-8838
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Study Flowchart **Part 1:** Exosomal EPHA2 secreted by highly metastatic breast cancer cells (HM-BCCs) enhanced metastatic potential to low-metastatic breast cancer cells (LM-BCCs). Exosomes from EPHA2-overexpressing LM-BCCs enhanced migration and invasion in recipient cells, whereas exosomes from EPHA2-knockdown HM-BCCs lost this metastatic-promoting capability. **Part 2:** Exosomal EPHA2 activated the TGF-β/SMAD3 signaling pathway in recipient breast cancer cells, subsequently inducing epithelial-mesenchymal transition (EMT). **Part 3:** Mechanistically, exosomal EPHA2 directly interacted with TGF-βRI in recipient cells, increasing the stability and prolonging TGF-β/SMAD3 pathway activation, thereby reinforcing metastatic signaling. **Part 4:** *In vivo* studies confirmed the pro-metastatic role of exosomal EPHA2. Clinically, metastatic tumors exhibited significantly higher EPHA2 and TGF-βRI expression than non-metastatic lesions, with a strong positive correlation between the protein levels, which supported the functional interplay in breast cancer progression. IF, immunofluorescence; Co-IP, co-immunoprecipitation; H&E, hematoxylin and eosin staining; IHC, immunohistochemistry; Ctrl, control.

Intercellular communication within the tumor microenvironment can occur through contact-dependent and -independent mechanisms. Exosomes have emerged as key mediators of intercellular signaling. Exosomes are small extracellular vesicles (typically 30–200 nm in diameter) that can selectively transport a variety of biomolecules, including nucleic acids, lipids, metabolites, and proteins, to recipient cells[12-14]. Exosome-mediated communication among cancer cells has been increasingly recognized as critical driver of tumor metastasis and therapeutic resistance. For example, highly metastatic liver cancer cells have been shown to transfer miR-92a-3p *via* exosomes to cells with low metastatic potential, thereby enhancing the metastatic capabilities of recipient cells[9]. Similarly, S100A4-enriched exosomes have been implicated in the transfer of metastatic traits among liver cancer cells[10]. Highly metastatic cells can confer metastatic potential to less aggressive counterparts in nasopharyngeal carcinoma through EGFR-rich extracellular vesicles[11]. Our previous

study confirmed that drug-resistant cells secrete EPHA2-enriched exosomes in breast cancer, which promote invasion and metastasis in drug-sensitive breast cancer cells[15]. These findings raise critical questions. Can exosomes modulate the metastatic behavior of breast cancer cells that inherently differ in metastatic potential? If so, what are the underlying molecular mechanisms?

EPHA2 is a key member of the EPH family of receptor tyrosine kinases. EPHA2 binds to the membrane-bound ligand, EphrinA1, on adjacent cells under physiologic conditions, initiating bidirectional signaling that mediates cell-cell communication[16]. Notably, evidence suggests that EPHA2 can participate in non-canonical signaling by forming heterodimeric complexes with other cell surface receptors, thereby modulating diverse physiologic and pathologic processes. For example, EPHA2 has been shown to interact with members of the EGFR family, contributing to activation of EGFR signaling pathways[17,18]. Activation of EGFR signaling has been reported to upregulate EPHA2 expression, thereby reinforcing EPHA2-mediated non-canonical signaling. In addition, EPHA2 can engage in heterotypic interactions with vascular endothelial growth factor receptor 2 (VEGFR2) in lung cancer cells, platelet-derived growth factor receptor alpha (PDGFRA) in glioblastoma, and G protein-coupled receptor class C group 5 member A (GPRC5A) in ovarian cancer, demonstrating functional versatility in mediating signaling cross-talk[19-21]. These receptor interactions involving EPHA2 have been implicated in cancer progression and drug resistance. Furthermore, EPHA2 can be incorporated into exosomes, facilitating long-range intercellular communication. In a recent study we showed that breast cancer cells with high metastatic potential secrete EPHA2-enriched exosomes that promote angiogenesis[5]. Building upon these findings, we hypothesized that the exosome-mediated transfer of EPHA2 between breast cancer cells may contribute to the intercellular dissemination of metastatic traits among heterogeneous tumor cell subpopulations.

In this study we investigated whether exosomes derived from highly metastatic breast cancer cells (HM-BCCs) can confer metastatic potential to low metastatic breast cancer cells (LM-BCCs) and sought to elucidate the underlying molecular mechanism. EPHA2-enriched exosomes from HM-BCCs were shown to enhance the migratory, invasive, and metastatic capacities of LM-BCCs. Mechanistically, exosome-delivered EPHA2 binds to and stabilizes transforming growth factor-β receptor type I (TGF-βRI) in recipient cells, thereby activating the TGF-β/SMAD3 signaling pathway and promoting epithelial-mesenchymal transition (EMT). This process ultimately endows LM-BCCs with an enhanced metastatic phenotype. The findings herein underscored the pivotal role of exosomal EPHA2 in mediating the intercellular transfer of metastatic traits among heterogeneous breast cancer cell subpopulations. These insights into exosome-mediated intratumoral communication suggested that targeting the EPHA2-TGF-βRI signaling axis may represent a promising therapeutic strategy for mitigating breast cancer metastasis.

Materials and methods

Cell lines

Human breast carcinoma cell lines [MDA-MB-231 (MDA-231), BT549, T47D, and MDA-MB-468 (MDA-468)], as well as human embryonic kidney 293T cells (HEK-293T), were obtained from the American Type Culture Collection [ATCC] (Manassas, VA, USA). MDA-231, BT549, and T47D cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA), MDA-MB-468 cells were cultured in DMEM/F12 medium (HyClone, Logan, UT, USA), and HEK-293T cells were cultured in high-glucose DMEM medium (Hyclone). All media were supplemented with 10% fetal bovine serum [FBS] (Hyclone) and 1% penicillin/streptomycin. All cells were maintained in a humidified incubator containing 5% CO_2 at 37°C.

Plasmids construction, establishment of stable cell lines, and siRNA

pCDNA3.1 vector encoding EPHA2 wild-type and truncation mutants (EPHA2-ΔS and EPHA2-ΔL), as well as the construction of pCDH-EPHA2, pLKO.1-shEPHA2, and pLKO.1-shRab27a have been described previously[15]. Lentiviruses were produced in HEK-293T cells by a standard three-plasmid system. Cells were infected by lentivirus for 48 h and selected with 1 μg/mL of puromycin to establish stable knockdown or overexpression cell lines. TGF-βRI siRNAs were synthesized by Genepharma (Suzhou, China) and transfected into cells using Lipofectamine RNAiMax (Thermo Fisher) following the manufacturer's instructions. The sequences are shown in **Table S1**.

Immunofluorescence assay

Cells seeded in 12-well plates with glass coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were incubated overnight at 4°C with indicated antibodies after blocking with 3% BSA. Then, the cells were incubated using indicated secondary antibodies (A32723, A32731, A11035; ThermoFisher, Waltham, MA, USA) at room temperature for 1 h in the dark. Nuclei were counterstained using DAPI. Fluorescent images were obtained utilizing a confocal microscope (Zeiss, Oberkochen, BW, Germany).

Exosome isolation and characterization

FBS was ultracentrifuged at 100,000 × g for 16 h, then filtered using a 0.22-μm filter to prepare exosome-depleted FBS.

Exosomes were isolated from cell culture supernatants, as described previously[5]. In brief, cells were cultured in medium containing 10% exosome-free FBS for 48 h. Next, the medium was collected and sequentially centrifuged to remove cells and debris (300 × g for 10 min, then 3000 × g for 10 min). Next, the supernatant was centrifuged at 10,000 × g for 30 min to remove large vesicles. Finally, exosomes were pelleted by ultracentrifugation at 100,000 × g for 90 min, washed with sterilized PBS, and pelleted again at 100,000 × g for 90 min. The exosome pellets were resuspended in PBS and quantified using a BCA assay (Thermo Fisher). The morphology of isolated exosomes was characterized by transmission electron microscopy (HT7700; HITACHI, Tokyo, Tokyo-to, Japan) at 80 kV. The size distribution and concentration of exosomes were tracked by nanoparticle tracking analysis (NTA) using the NanoSight NS300 device (Malvern, Almelo, Overijssel, The Netherlands). Western blot analysis was performed to detect exosome markers (CD81, Alix, and TSG101) with calnexin (a cytosolic protein) as a negative control to assess exosome purity.

Exosome uptake analysis

Purified exosomes were labeled with a PKH26 Red Fluorescent Cell Linker Kit (Merck, St. Louis, MO, USA) according to the manufacturer's protocol. Briefly, exosomes were resuspended in 100 μL of diluent C and mixed with 100 μL of diluted-PKH26 dye solution for 5 min, then serum was added to stop the staining. PKH26-labeled exosomes were collected by ultracentrifugation and co-incubated for 24 h with cells prestained by DiO (Servicebio, Wuhan, China). The cells were observed under a confocal microscope after fixation with 4% paraformaldehyde and staining with DAPI.

Western blot analysis

Western blot analysis was performed as described previously[22]. Protein samples were separated using SDS-PAGE, then transferred onto PVDF membranes. The membranes were blocked with 5% milk and immunoblotted with primary antibodies overnight at 4°C, then incubated with HRP-conjugated secondary antibodies. Protein bands were detected using an ECL kit (Millipore, Billerica, MA, USA). The following primary antibodies were used: anti-EPHA2 (6997; CST, Danvers, MA, USA); anti-EPHA2 (398832; Santa Cruz Biotechnology, Inc., Santa Cruz, Dallas, CA, USA); anti-TGF-βRI (235578; Abcam, Cambs, Cambridge, UK); anti-TGF-βRII (TA808048; ZSGB-Bio, Beijing, China); anti-p-Smad3 (52903; Abcam); anti-Smad3 (9523; CST); anti-twist (175430; Abcam); anti-snail (216347; Abcam); anti-vimentin (8978; Abcam); anti-E-cadherin (1416; Abcam); anti-CD81 (56039; CST); anti-Alix (92880; CST); anti-TSG101 (136111; Santa Cruz Biotechnology, Inc.); anti-calnexin (23954; Santa Cruz Biotechnology, Inc.); anti-Rab27a (55667; Abcam); anti-p-Src (6943; CST); anti-Src (2110; CST); anti-p-Akt (2965; CST); anti-Akt (9272; CST); anti-β-actin (A1978; Sigma-Aldrich); and anti-mCherry (213511; Abcam).

Wound healing and Transwell assays

Wound healing and Transwell assays were performed as described previously[23]. Cells were grown to approximately 80–90% confluence in 6-well plates for the wound healing assay and a linear wound was created in the monolayers using a sterile 200-μL pipette tip. Detached cells were removed by washing with PBS. Cells were cultured in appropriate medium with treatments as indicated for 24 or 48 h. The width of the wound gap was analyzed using a bright-field microscope.

Cells suspended in serum-free medium were plated in the upper chamber for the Transwell assay and medium containing 10% FBS was added to the lower chamber. Cells that migrated to the underside of the membrane were stained with 0.1% crystal violet after 24 or 48 h, imaged, and counted with ImageJ software. The Transwell membrane was pre-coated with Matrigel (Corning, Corning, New York, USA) for invasion assays.

Quantitative real-time PCR (qRT-PCR) and RNA sequencing (RNA-Seq)

qRT-PCR was performed as described previously[24]. Briefly, RNA was isolated using TRIzol reagent (ThermoFisher) and quantified using a Nanodrop spectrophotometer (ThermoFisher). cDNA was synthesized using HiScript II QRT SuperMix (Vazyme, Nanjing, China). qRT-PCRs were performed using AceQ qPCR SYBR Green Master Mix (Vazyme). β-actin served as the internal control. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were listed in **Table S2**.

Total RNA was extracted using Trizol and sent to Novogene (Beijing, China) for RNA-Seq. The raw data were processed using standard workflows by Novogene. The top 100 upregulated genes were selected based on *P* values and subjected to the Gene Set Cancer Analysis [GSCA] (https://guolab.wchscu.cn/GSCA) database. The correlation between these top upregulated genes and EMT pathway activity in breast cancer was calculated.

CCK8 and colony formation assay

Cell viability was assessed by CCK8 and colony formation assays as previously described[23]. Cells were plated in 96-well plates, treated as indicated, then incubated with 10% CCK8 (V/V) reagent (Vazyme, Nanjing, China) for 2-4 h at 37°C to perform the CCK8 assay. Absorbance at 450 nm were measured. Cells were plated in 6-well plates and grown for 10–14 d

to perform the colony formation assay. The colonies were fixed with methanol, stained with 0.1% crystal violet, and counted using a microscope.

Tissue microarray and immunohistochemistry (IHC)

Human breast cancer tissue microarrays (HBreD055CD01) were purchased from Shanghai SuperChip Biotech (Shanghai, China). IHC was described previously[5]. In brief, the slides were dewaxed using xylene and rehydrated in graded alcohol-to-water. Antigen retrieval was carried out by heating slides in citrate buffer. Endogenous peroxidase activity was blocked with 3% H_2O_2. Then, the slides were incubated with antibodies [Luciferase (185924; Abcam), EPHA2 (6997; CST), TGF-βRI (235578; Abcam)] overnight at 4°C. The slides were incubated with HRP-conjugated secondary antibodies (PV-6000; ZSGB-Bio) for 1 h at room temperature after washing. Signals were developed using a DAB chromogen (ZSGB-Bio) and slides were counterstained with hematoxylin. Staining intensity and percentage of positive cells were evaluated to compare protein expression between samples.

Co-immunoprecipitation (Co-IP)

Co-IP were performed as described previously[25]. Cells were lysed using IP buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and protease inhibitor cocktail] for 1 h at 4°C. Then, the lysates were centrifuged at 12,000 rpm (14,000 g) for 20 min at 4°C and supernatants were incubated with antibodies [EPHA2 (6997; CST), TGF-βRI (235578; Abcam), mCherry (213511; Abcam)] overnight at 4°C with gentle rotation. Protein A/G beads were added to the lysates the next day and incubated for 4 h at 4°C. The beads were then washed and the bound proteins were eluted by boiling in SDS buffer for subsequent analysis by western blot.

In vivo metastasis assay

The *in vivo* metastasis model was established as described previously[5]. Four-week-old female SCID mice were obtained from Charles River (Beijing, China). A total of 5×10^6 cells (LM-BCCs, including T47D; HM-BCCs, including MDA-231; and control or EPHA2-knockout MDA-MB-231 cells) were inoculated subcutaneously into mice (6 mice per group). In addition, a total of 1×10^6 luciferase-labeled T47D cells were injected into mice *via* the tail vein 30 d post-injection. Mice were monitored and at the end of the experiment, bioluminescent imaging was performed to detect metastatic tumor signals. Mice were then euthanized and lung tissues were harvested, fixed in 4% formaldehyde, and embedded in paraffin. All experimental operations followed the protocol approved by Tianjin Medical University Cancer Institute and Hospital (Approval Number: NSFC-AE-2022238) in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Gene set enrichment analysis (GSEA)

GSEA was conducted using The Cancer Genome Atlas (TCGA) data through the clusterProfiler R package to investigate pathways associated with EPHA2. In summary, mRNA expression data were first acquired from the TCGA data portal (available at https://portal.gdc.cancer.gov/) and normalized to transcripts per million (TPM) values. Subsequently, Pearson correlation coefficients were computed and input into the clusterProfiler R package for GSEA. The gene sets used in this analysis were sourced from the MSigDB database (accessible at https://www.gsea-msigdb.org/gsea/msigdb).

Protein-protein docking

Structural modeling was performed with AlphaFold3 using the EPHA2 and TGF-βRI protein sequences. Protein-protein docking was carried out using HDOCK software, wherein both proteins were treated as rigid bodies and the docking interface was defined across the entire surfaces. A total of 100 conformations were generated during docking and the structure with the most favorable (most negative) energy was selected based on the scoring function. Interaction analysis between the resulting complex was subsequently performed using Discovery Studio Visualizer v24 and the interactions were visualized with PyMol 3.0 software.

Statistical analysis

The data were analyzed using GraphPad Prism7 software. The Kaplan-Meier analysis was used to determine the correlation between gene expression and prognosis of patients. Unpaired Student's *t*-test and one- or two-way ANOVA were used to compare the statistical significance between different groups. The correlation was determined by Pearson analysis. All data are expressed as the mean ± SD. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{ns}P > 0.05$ indicate no statistical significance. All experiments were repeated three times.

Results

Exosomes mediate transfer of metastatic potential from HM-BCCs to LM-BCCs

Tumors consist of heterogeneous populations of cancer cells that differ in metastatic potential, proliferative capacity, and resistance to therapy. Communication among these

heterogeneous subpopulations has a key role in promoting tumor progression. LM-BCCs (MAD-468 and T47D) were co-cultured with HM-BCCs (MAD-231 and BT549; **Figure S1A**) to determine whether HM-BCCs can transfer metastatic traits to LM-BCCs, then Transwell migration assays were performed. LM-BCCs were labeled in these co-culture experiments with GFP to distinguish LM-BCCs from HM-BCCs. The results demonstrated that increasing the number of co-cultured HM-BCCs led to a considerable enhancement in the migratory capacity of GFP-labeled LM-BCCs (**Figure 1A**).

Conditioned media (CM) were subsequently collected from breast cancer cell lines and applied to LM-BCCs in wound-healing assays. CM derived from HM-BCCs promoted wound closure in LM-BCCs, which enhanced migratory activity more effectively than CM from LM-BCCs or fresh medium (**Figure S1B**). We hypothesized that LM-BCCs may be responsible for the observed transfer of metastatic traits between breast cancer cells given that exosomes are well-established mediators of intercellular communication. Exosomes were isolated from the culture supernatants of the breast cancer cell lines and characterized to test this hypothesis. Transmission electron microscopy (TEM) revealed the presence of typical cup-shaped vesicles and NTA confirmed a size distribution between 30 and 200 nm, consistent with exosomal morphology (**Figure 1B**). Western blot analysis demonstrated that the isolated vesicles were enriched in exosomal markers (CD81, Alix, and TSG101) with minimal contamination from cytosolic proteins, indicating successful purification of exosomes (**Figure 1C**). HM-BCC-derived exosomes (HM-Exos) were labeled with the red fluorescent dye, PKH26, and added to LM-BCC cultures to assess the uptake of these exosomes by recipient cells. Confocal microscopy showed efficient internalization of PKH26-labeled HM-Exos by LM-BCCs (MDA-468 and T47D; **Figures 1D and S1C**). Transwell migration and invasion assays, as well as wound-healing assays, were performed to evaluate the functional impact of exosomes. HM-Exos significantly enhanced the migration and invasion of LM-BCCs compared to LM-BCC-derived exosomes (LM-Exos; **Figures 1E and S1D**). Rab27a, which is a key GTPase involved in exosome secretion, was silenced in MDA-231 cells to directly implicate exosomes in this intercellular communication (HM-BCCs; **Figure 1F**). Co-culture of Rab27a-silenced HM-BCCs with LM-BCCs resulted in a marked reduction in the ability of HM-BCCs to promote LM-BCC migration, indicating that exosome secretion is critical for this effect (**Figures 1G and S1E**). Collectively, these results provided compelling evidence that exosomes mediate the transfer of metastatic potential from HM-BCCs to LM-BCCs.

Exosomal EPHA2 mediates metastatic potential transfer between breast cancer cells

We previously demonstrated that EPHA2 is overexpressed in HM-BCCs and the secreted exosomes and that this enrichment confers pro-angiogenic properties in the context of breast cancer[5]. Building on these findings, we hypothesized that EPHA2 may serve as a key mediator of the exosome-driven transfer of metastatic traits between breast cancer cell subpopulations. To test this hypothesis, EPHA2 expression was modulated in the cell models. Specifically, stable EPHA2-knockdown HM-BCCs and EPHA2-overexpressing LM-BCCs were established *via* lentiviral transduction. Successful modification of EPHA2 expression was confirmed by western blot and RT-qPCR analyses (**Figures 2A and S2A**). The intrinsic impact of EPHA2 on cellular migratory and invasive behaviors was first investigated. EPHA2 overexpression in LM-BCCs significantly enhanced cell migration, which was consistent with our hypothesis, whereas EPHA2 knockdown in HM-BCCs markedly reduced this capability (**Figure S2D**). Notably, modulation of EPHA2 expression had a minimal effect on cell proliferation in both cell types (**Figure S2B and C**), suggesting that EPHA2 primarily influences invasive rather than proliferative behavior. Collectively, these results indicated that elevated EPHA2 expression is associated with a more metastatic phenotype in breast cancer cells.

Whether exosome-associated EPHA2 is responsible for the transfer of metastatic potential between breast cancer cells was investigated next. Exosomes were isolated from donor cells with varying levels of EPHA2 expression and subsequently applied to recipient LM-BCCs. Exosomes enriched in EPHA2 significantly enhanced the migratory capacity of LM-BCCs compared to exosomes derived from EPHA2-deficient cells (**Figures 2E, F and S2E, F**). Treatment of LM-BCCs with EPHA2-enriched exosomes resulted in a marked increase in EPHA2 protein levels within the recipient cells. In contrast, exosomes derived from EPHA2-silenced HM-BCCs failed to elevate EPHA2 expression in LM-BCCs (**Figure 2B-D**). These findings demonstrated that EPHA2 is indeed transferred *via* exosomes and that EPHA2 presence is essential for the pro-migratory effect conferred by HM-BCC-derived exosomes on LM-BCCs.

EPHA2-enriched exosomes generated from HEK-293T cells, which lack the confounding influence of breast cancer-specific signaling pathways, were utilized to validate the role of exosomal EPHA2. EPHA2 was overexpressed in HEK-293T cells and exosomes were isolated and characterized by TEM, NTA, and western blot analysis (**Figure 2G and H**). These analyses confirmed that the vesicles were similar in size and marker profile to breast cancer-derived exosomes and contained elevated levels of EPHA2. EPHA2-enriched HEK-293T exosomes induced a marked increase in EPHA2 protein expression when applied to LM-BCCs (**Figure 2I**), indicating efficient delivery of the protein. These EPHA2-enriched exosomes considerably enhanced the migratory and invasive abilities of LM-BCCs compared to control exosomes (**Figure 2J and K**). Collectively, these findings provided strong evidence that exosomal EPHA2 is a critical mediator of the metastatic potential transfer from HM-BCCs to LM-BCCs.

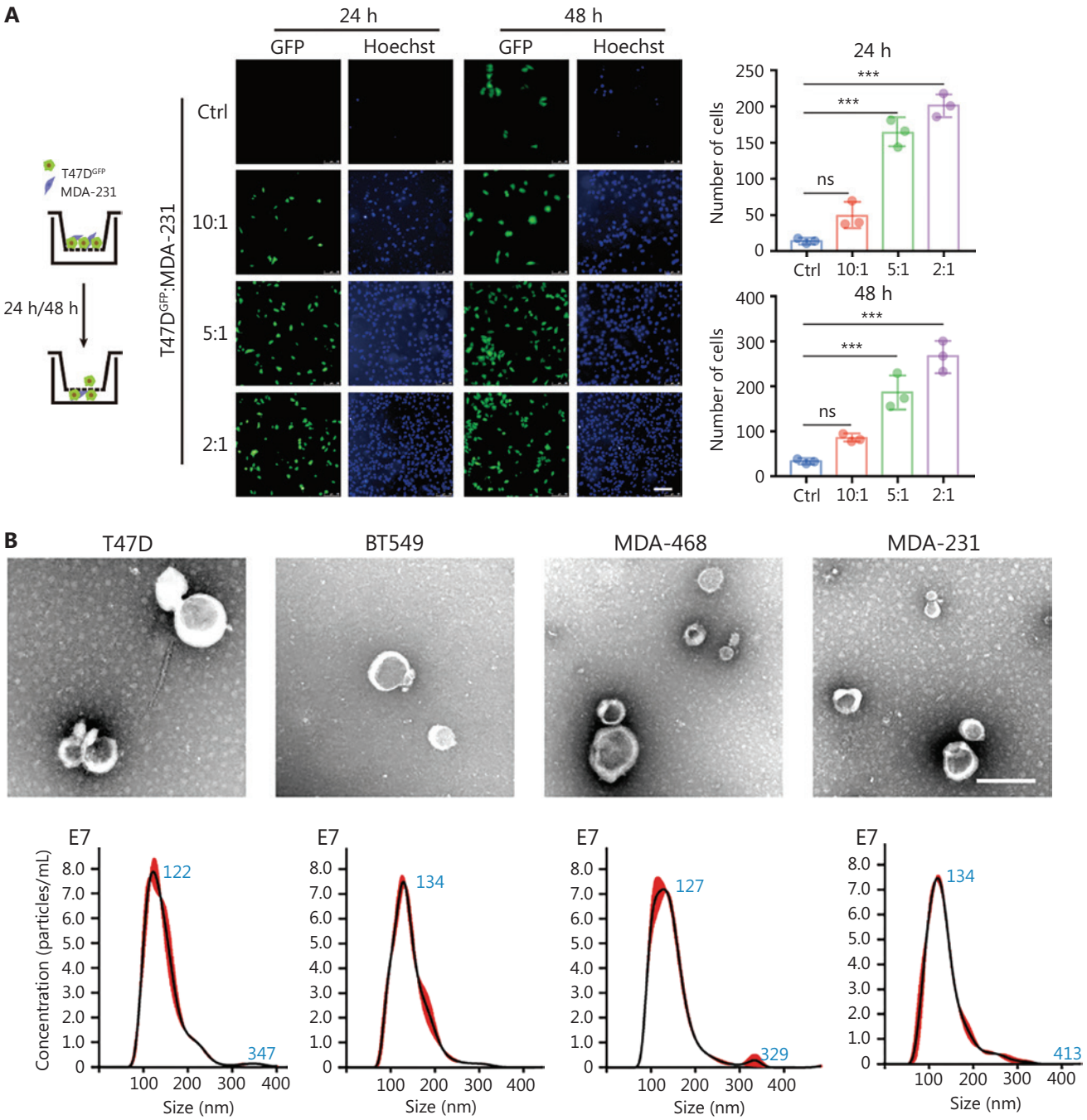


Figure 1 Continued

Exosomal EPHA2 promotes EMT in breast cancer cells

The mechanism by which exosome-delivered EPHA2 enhances the metastatic potential of recipient cells was investigated next. We hypothesized that exosomal EPHA2 induces EMT in LM-BCCs given that EMT is a key process by which epithelial cancer cells acquire migratory and invasive properties. To test this hypothesis, transcriptomic analysis was performed on T47D cells treated with exosomes derived from EPHA2-overexpressing HEK-293T cells and these cells were compared to cells treated with control exosomes.

Analysis of the top 100 upregulated genes in LM-BCCs treated with EPHA2-rich exosomes using the GSCA

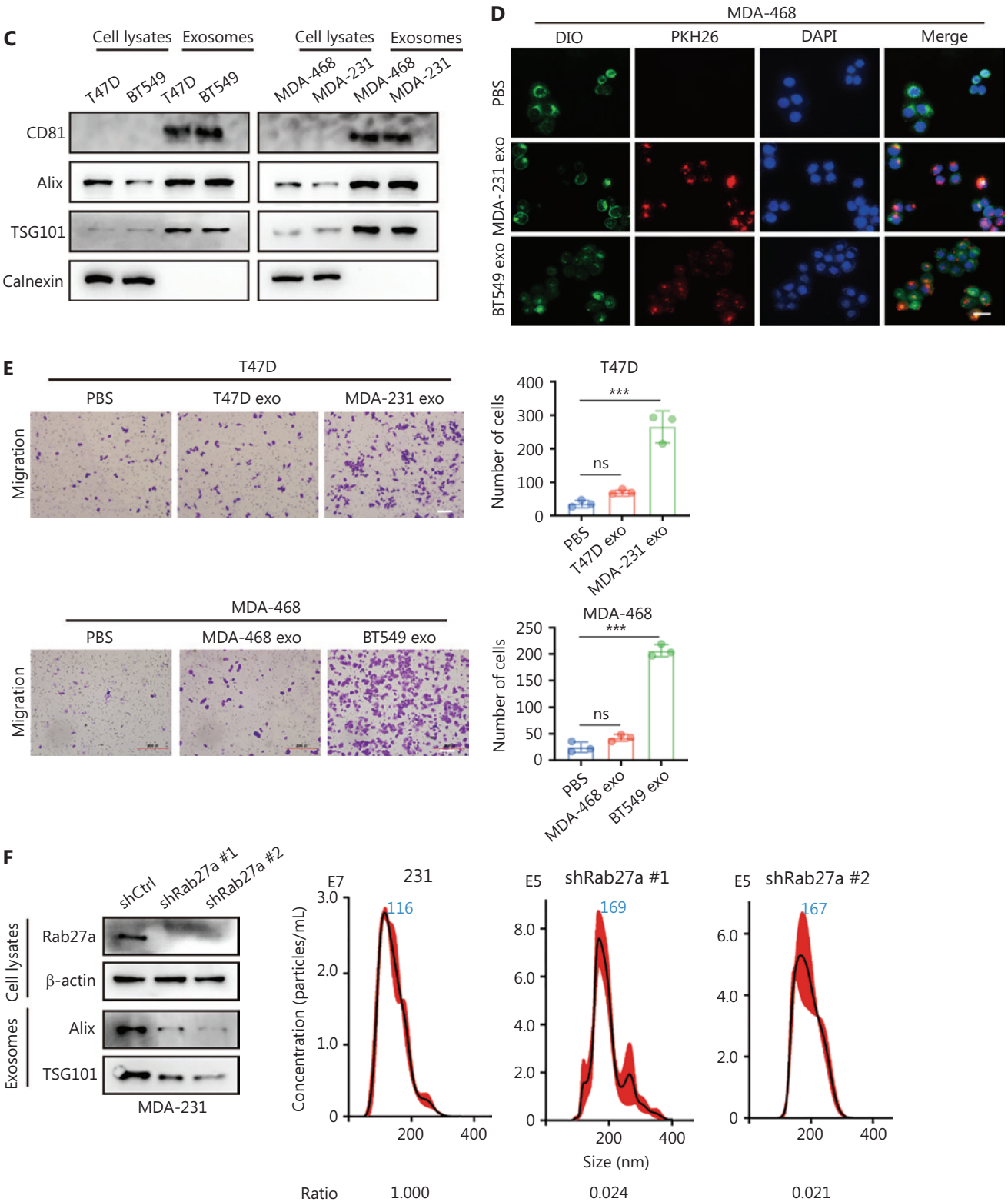


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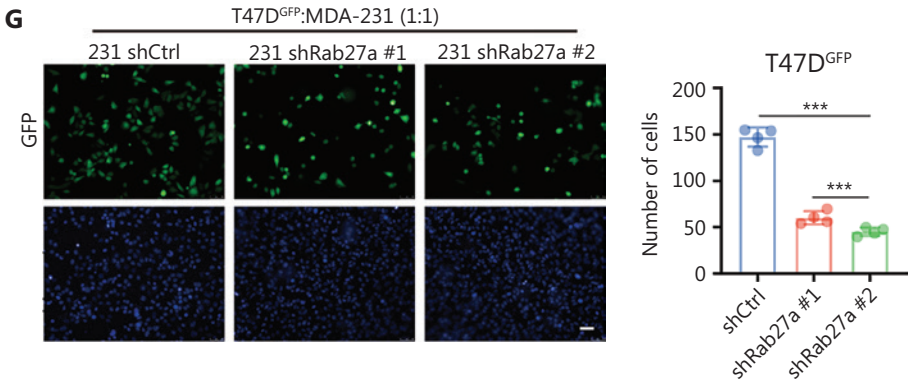


Figure 1 Exosomes derived from HM breast cancer cells enhance breast cancer cell migration and invasion. (A) The migration ability of GFP-labeled T47D cells were enhanced when co-cultured with HM breast cancer MDA-231 cells compared to control as measured by the Transwell assay. Scale bar: 50 μm. (B) Transmission electron microscopic pictures showed the morphology of exosomes derived from HM and LM breast cancer cells. Scale bar: 200 nm. Nanoparticle tracking analysis (NTA) of exosomes derived from HM and LM breast cancer cells. (C) Protein (100 ug) from indicated cells and exosomes were analyzed by western blot. Alix, TSG101, and CD81 were exosomal markers; calnexin was the negative control. (D) Exosomes labeled by PKH26 were endocytosed into MDA-468 cells. Cell membrane was stained by DiO and the nucleus was stained by DAPI. Scale bar: 5 μm. (E) HM-Exos enhanced the migration ability of T47D and MDA-468 cells compared to LM-Exos and control. Scale bar: 100 μm. (F) Western blot and NTA analysis showing that Rab27a silencing reduced the amounts of exosomes from MDA-231 cells. (G) MDA-231 cells with Rab27a silencing lost the migration-promoting effect on GFP-labeled T47D cells. Scale bar: 50 μm. Data are expressed as the mean ± SD. ***P < 0.001 and ^{ns}P > 0.05 indicate no statistical significance, n = 3. Exo, exosome; Ctrl, control.

platform (https://guolab.wchscu.cn/GSCA)[26] revealed activation of the EMT pathway (**Figure 3A**). GSEA showed a positive correlation between EPHA2 expression and EMT-associated gene signatures (**Figure 3B**). The expression of canonical EMT markers in our co-culture models was assessed to validate these transcriptomic findings. LM-BCCs treated with HM-Exos exhibited elevated expression of mesenchymal markers, including vimentin, twist, and snail, and a concomitant reduction in the epithelial marker, E-cadherin, compared to cells treated with LM-Exos or PBS (**Figure 3C and D**). Similarly, exosomes isolated from EPHA2-overexpressing LM-BCCs induced upregulation of mesenchymal markers and downregulation of E-cadherin in recipient LM-BCCs (**Figure 3E and F**). In addition to these molecular changes, treatment with EPHA2-enriched exosomes induced morphologic alterations characteristic of EMT (**Figure 3G**).

The induction of EMT by exosomal EPHA2 was further validated through immunofluorescence staining of key EMT markers. LM-BCCs treated with EPHA2-enriched exosomes, including HM-Exos or EPHA2-overexpressing cells, exhibited a marked decrease in E-cadherin and a concomitant increase in vimentin expression (**Figure 3H and I**). In contrast, LM-BCCs exposed to exosomes with low EPHA2 levels, such as LM-Exos or EPHA2-silenced HM-BCCs, showed no significant changes in these markers (**Figure 3H and I**). In addition, phalloidin staining of F-actin revealed that treatment with EPHA2-enriched exosomes induced pronounced formation of actin stress fibers in LM-BCCs (**Figure 3J**). This cytoskeletal reorganization is commonly associated with EMT and increased cell motility. Collectively, these findings suggested that exosomal EPHA2 from HM-BCCs promotes EMT in LM-BCCs, thereby contributing to the acquisition of an invasive phenotype.

Exosomal EPHA2 activates the TGF-β/SMAD3 signaling pathway in breast cancer cells

Pathways known to regulate EMT were focused on to elucidate the signaling mechanisms underlying EPHA2-induced EMT. TGF-β signaling is one of the most potent inducers of EMT in cancer and has a critical role in tumor progression and metastasis [27]. Notably, the GSEA of TCGA data revealed a positive correlation between high EPHA2 expression and activation of the TGF-β signaling pathway (**Figure 4A**). Therefore, whether exosome-delivered EPHA2 activates TGF-β/SMAD signaling in LM-BCCs was investigated. As anticipated, the treatment of LM-BCCs with HM-Exos resulted in a considerable increase in phosphorylated SMAD3, which is a key transcription factor downstream of TGF-β signaling, compared to treatment with LM-Exos or PBS (**Figure 4B, C**). By contrast, the phosphorylation levels of SRC and AKT (representative kinases from other signaling pathways) remained largely unchanged upon HM-Exo treatment (**Figure 4B**). Notably, HM-Exos not only activated SMAD3 but also increased the total protein level

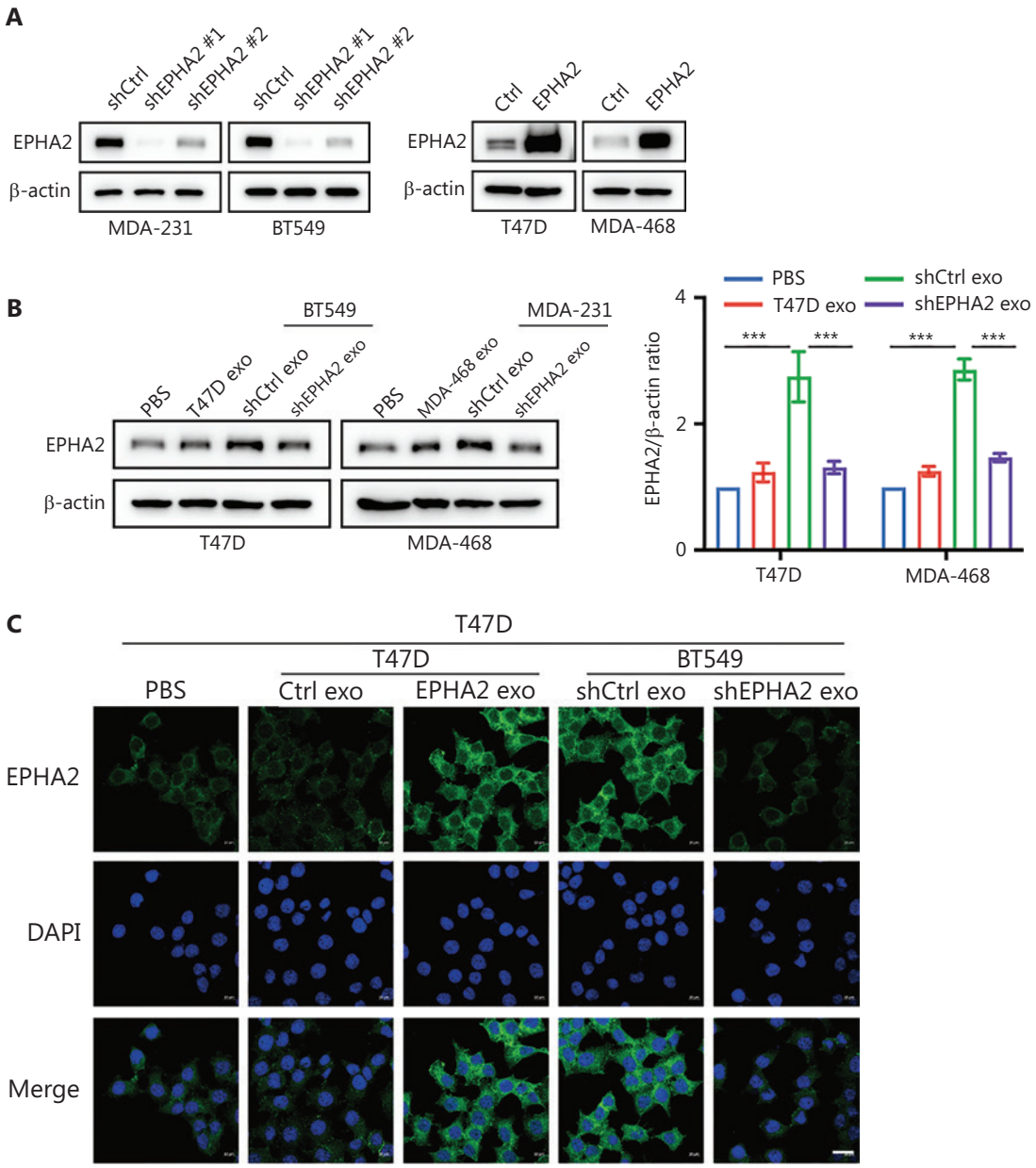


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of TGF-βRI in LM-BCCs, whereas the level of transforming growth factor-beta receptor type II (TGF-βRII) remained relatively unaffected (**Figure 4B**). These findings suggested a potential mechanism in which exosome-delivered EPHA2 stabilizes TGF-βRI, thereby promoting TGF-β/SMAD3 signaling and EMT induction.

However, when EPHA2 expression was silenced in HM-BCCs, the resulting HM-Exos lost the ability to activate SMAD3 or upregulate TGF-βRI levels in recipient LM-BCCs. Consequently, these exosomes failed to alter the expression of EMT markers (**Figure 4D, E**). These findings confirmed that EPHA2 in HM-Exos is essential for activating the TGF-β/SMAD3 signaling pathway and for initiating EMT-related changes in LM-BCCs. Furthermore, immunofluorescence staining for SMAD3 revealed clear nuclear accumulation of SMAD3 in LM-BCCs treated with HM-Exos or exosomes derived from EPHA2-overexpressing LM-BCCs, indicating the activation and nuclear translocation of SMAD3. By contrast, LM-BCCs treated with EPHA2-deficient exosomes from LM-BCCs or EPHA2-knockdown HM-BCCs exhibited predominant cytoplasmic retention of SMAD3 (**Figure 4F**). These results demonstrated that exosomal EPHA2 effectively

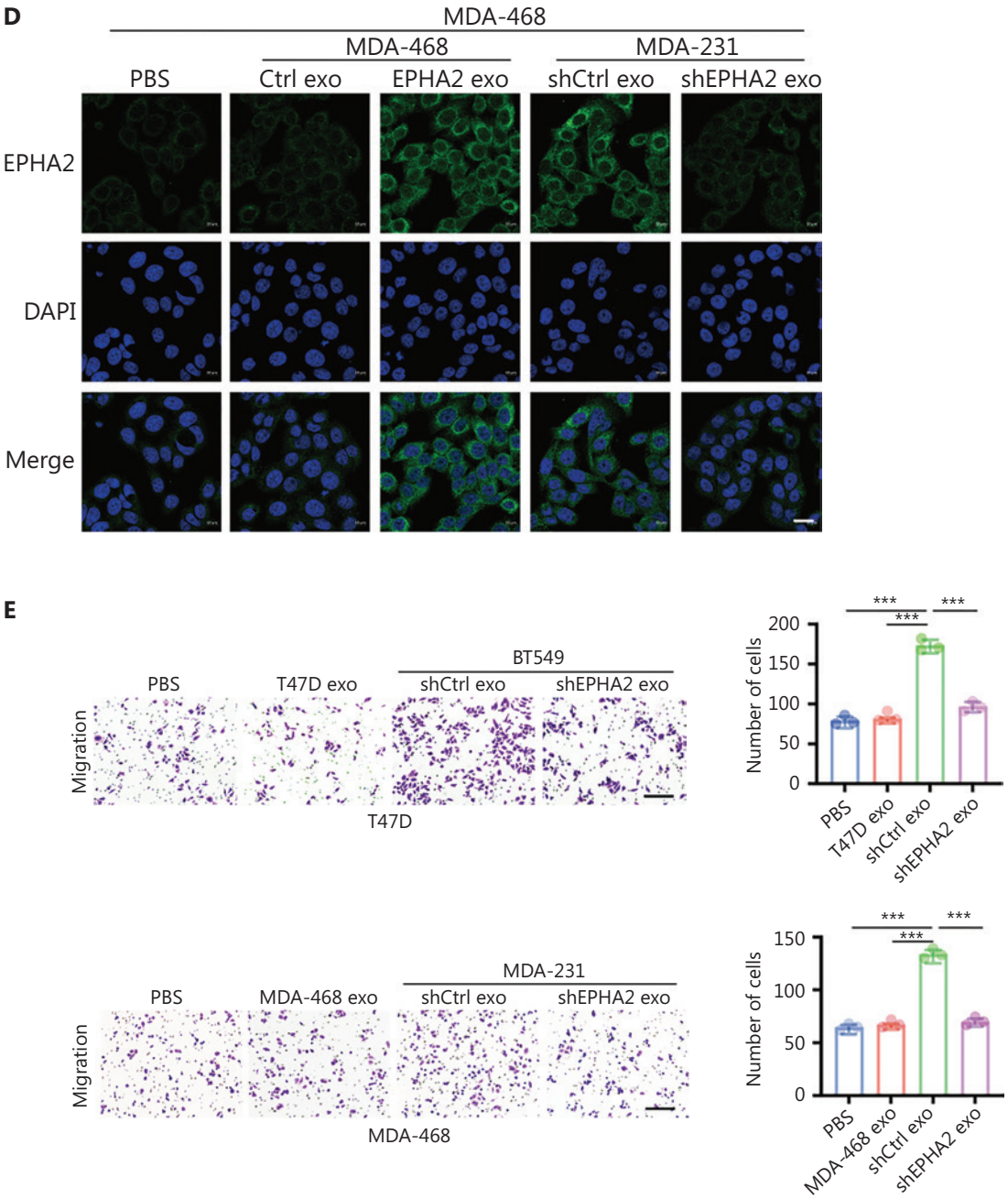


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activates the canonical TGF-β/SMAD3 signaling pathway in recipient breast cancer cells.

Pharmacologic and genetic inhibition approaches were used to determine whether TGF-β signaling is functionally required for EPHA2-enriched exosomes to promote metastasis-associated traits. Pre-treatment of LM-BCCs with the TGF-βRI inhibitor, SB431542, attenuated the migration-promoting effect of EPHA2-enriched exosomes (**Figure 4G**). SB431542 blocked SMAD3 phosphorylation and prevented the EMT marker alterations typically induced by EPHA2-enriched exosome treatment (**Figure 4H, I**). In parallel, the genetic silencing of TGF-βRI in LM-BCCs similarly reduced the ability of EPHA2-enriched exosomes to enhance cell migration and abolished exosome-induced SMAD3 activation and EMT-associated protein expression (**Figure 4J-L**). Collectively, these results indicated that TGF-β/SMAD3 signaling is required for the functional effects of exosomal EPHA2 and this signaling axis is critical for EMT induction in LM-BCCs.

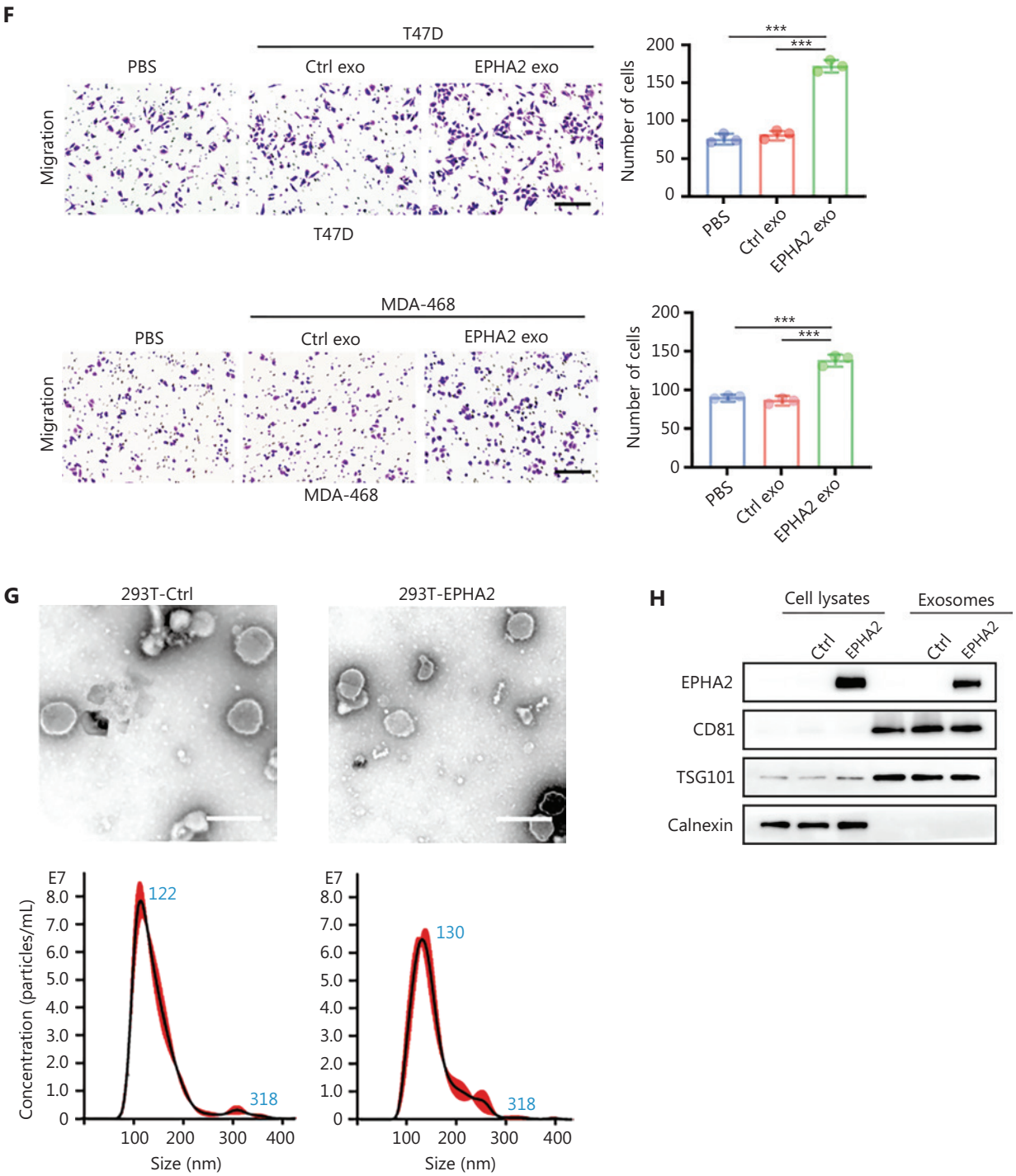


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EPHA2 enhances the stability of TGF-βRI in breast cancer cells

These findings suggested that exosomal EPHA2 elevates TGF-βRI levels and activates TGF-β signaling in recipient cells. Next, the mechanism by which EPHA2 regulates TGF-βRI abundance was determined. We hypothesized that EPHA2 stabilizes the TGF-βRI protein by preventing degradation. Accordingly, protein stability assays were performed. EPHA2-knockdown and control HM-BCCs were treated with cycloheximide to inhibit new protein synthesis and TGF-βRI levels were monitored over time. TGF-βRI degraded considerably

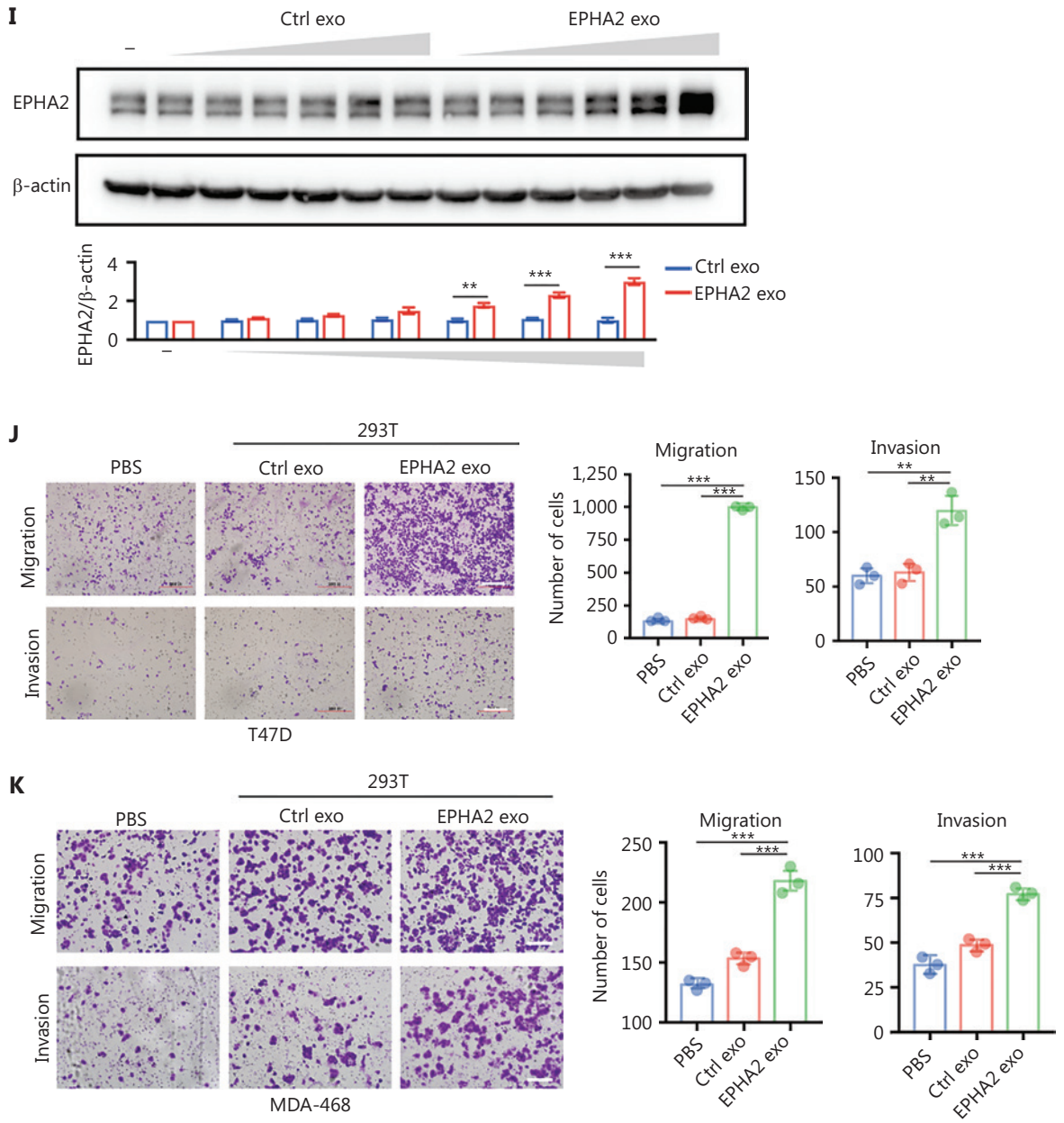


Figure 2 Exosomal EPHA2 promotes breast cancer cell migration and invasion. (A) Western blot analysis of EPHA2 expression in MDA-231 and BT549 cells infected with lentivirus expressing EPHA2-specific shRNAs or T47D and MDA-468 cells infected with lentivirus expressing EPHA2. β-actin was used as the loading control. (B) HM-Exos enhanced the protein levels of EPHA2 in T47D and MDA-468 cells compared to exosomes from HM breast cancer cells with EPHA2 silencing, LM-Exos, and PBS. (C, D) IF assays were performed to detect the level of EPHA2 in MDA-468 and T47D cells treated with exosomes from indicated cells. Scale bar: 10 μm. (E, F) Transwell assays analyzed the migration ability of T47D and MDA-468 cells treated with indicated exosomes. Scale bar: 200 μm. (G) Transmission electron microscopy showed the morphology of exosomes derived from HEK-293T cells expressing EPHA2 or vector. Scale bar: 200 nm. Nanoparticle tracking analysis (NTA) of exosomes derived from HEK-293T cells expressing EPHA2 or vector. (H) Western blot analysis of the expression of indicated protein in HEK-293T exosomes and cells transfected with EPHA2 or vectors. β-actin was used as the loading control. (I) Western blot analysis of EPHA2 expression in T47D cells treated with exosomes derived from HEK-293T cells expressing EPHA2 or vector. (J, K) Exosomes from HEK-293T cells expressing EPHA2 promoted the migration and invasion capacity of T47D and MDA-468 cells compared with control exosomes and PBS. Scale bar: 100 μm. Data are expressed as the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{ns}P > 0.05$ indicate no statistical significance, $n = 3$. Exo, exosome; Ctrl, Control.

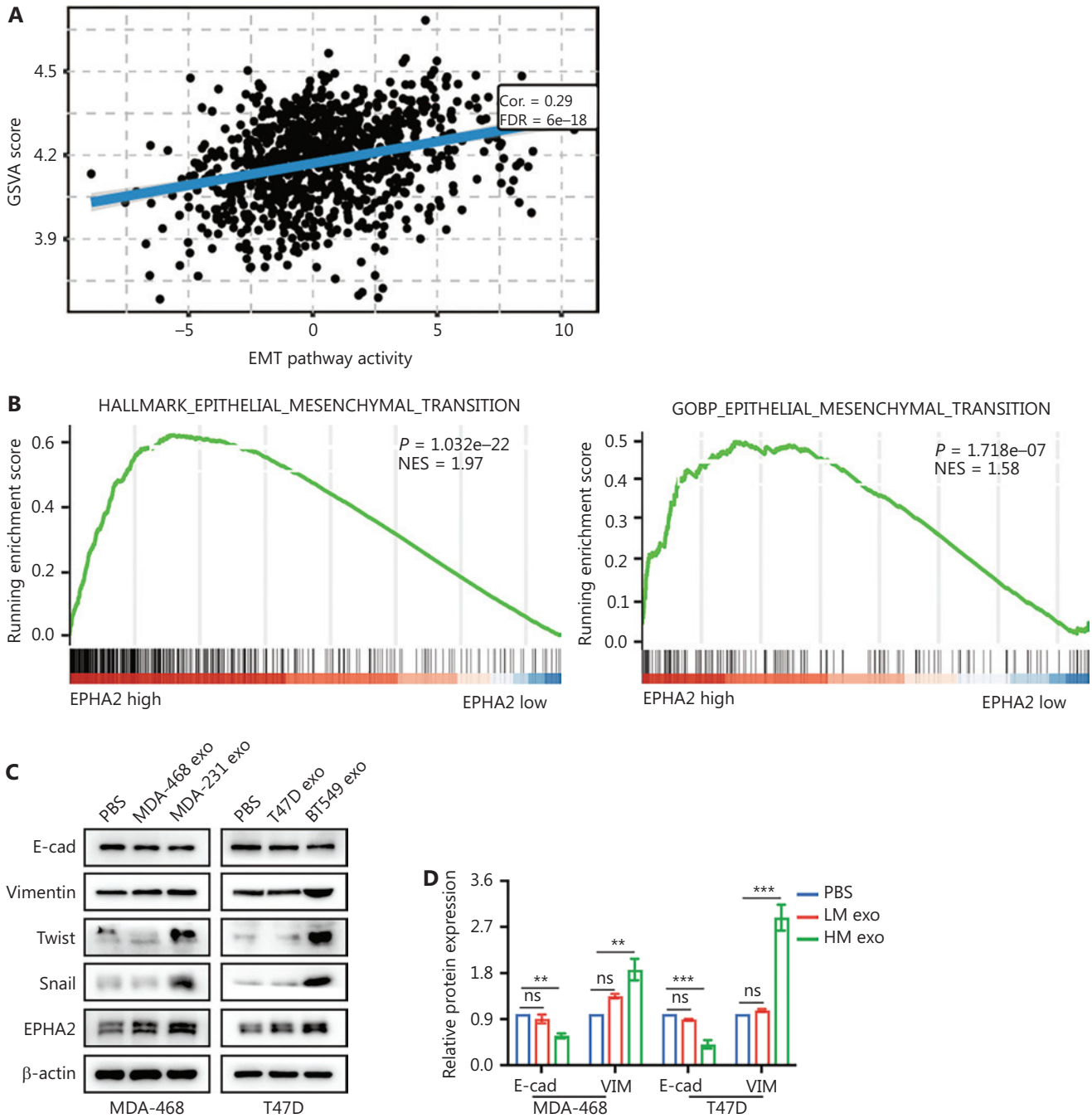


Figure 3 Continued

faster in EPHA2-knockdown cells than control cells, indicating a reduced half-life of the receptor in the absence of EPHA2 (**Figure 5A, B**). Conversely, pre-treatment of LM-BCCs with EPHA2-enriched exosomes markedly delayed TGF-βRI degradation compared to untreated controls, indicating enhanced protein stability in the presence of EPHA2 (**Figure 5C**). These results support the conclusion that EPHA2 contributes to stabilization of TGF-βRI in cells.

Next, we investigated the protein degradation pathway through which EPHA2 stabilizes TGF-βRI. Most proteins are degraded through the ubiquitin–proteasome system or the lysosomal pathway in eukaryotic cells. EPHA2-knockdown HM-BCCs were treated with the proteasome inhibitor, MG132, or the lysosome inhibitor, chloroquine (CQ), and TGF-βRI levels were assessed to distinguish between these mechanisms. CQ treatment substantially restored TGF-βRI levels in

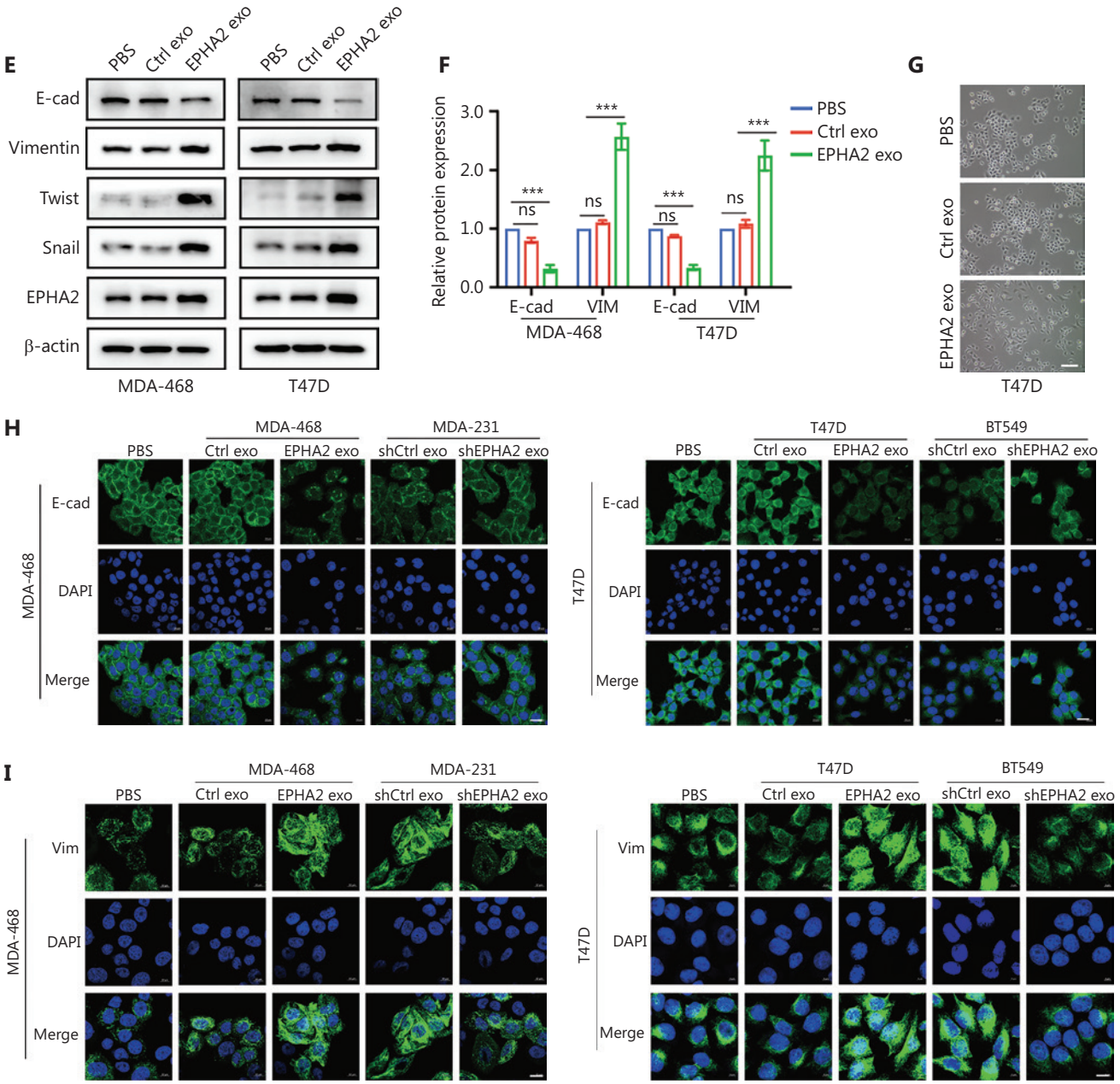


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EPHA2-knockdown HM-BCCs, whereas MG132 had minimal effect (**Figure 5D, E**), indicating that EPHA2 primarily protects TGF-βRI from lysosomal degradation. Furthermore, the treatment of LM-BCCs with CQ after exposure to EPHA2-enriched exosomes did not further elevate TGF-βRI levels compared to exosome treatment alone (**Figure 5F**), suggesting that EPHA2-enriched exosomes had already maximally stabilized TGF-βRI by inhibiting lysosomal turnover.

We examined whether EPHA2 stabilizes TGF-βRI through direct binding, given that both EPHA2 and TGF-βRI are transmembrane receptors. Protein-protein docking analysis provided structural support for direct EPHA2-TGF-βRI interaction (**Figure 5G**). Co-IP assays involving MDA-231 cells, which express high endogenous EPHA2, and in EPHA2-overexpressing T47D cells showed that EPHA2 co-precipitated with TGF-βRI in both settings (**Figure 5H, I**). To further define the interaction, Co-IP experiments using EPHA2 truncation mutants in T47D cells revealed that wild-type EPHA2 and the EPHA2-ΔS mutant, but not the ΔL mutant, co-precipitated TGF-βRI (**Figure 5J**). Furthermore, immunofluorescence

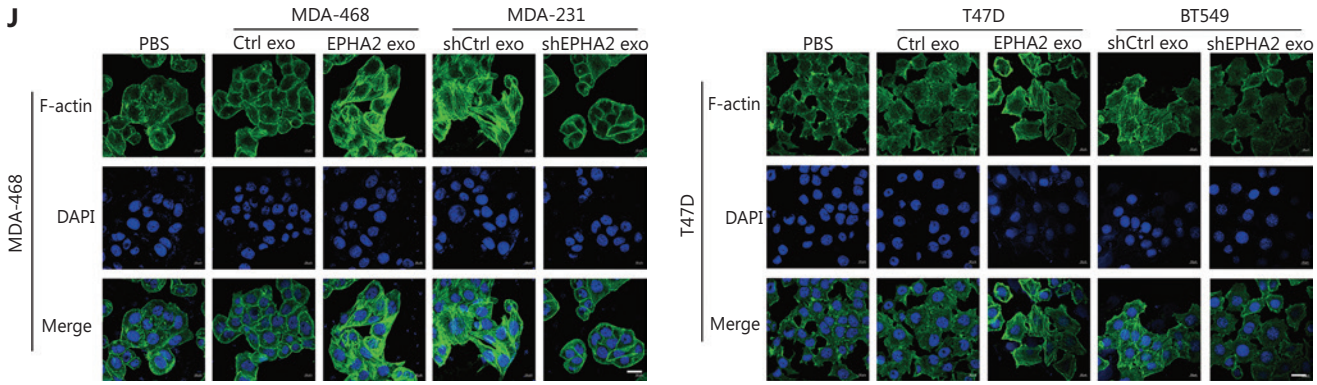


Figure 3 Exosomal EPHA2 promotes EMT of breast cancer cells. (A) Analyzing the top 100 upregulated genes in RNA-Seq data using GSCA (https://guolab.wchscu.cn/GSCA) showed that EMT pathway was activated in T47D cells by exosomes derived from HEK-293T cells expressing EPHA2 compared to control exosomes. (B) Gene set enrichment analysis (GSEA) showed that the expression of EPHA2 significantly correlated with the EMT process based on the TCGA database. (C–F) Western blot analyzing the expression of indicated proteins in T47D and MDA-468 cells treated with exosomes derived from breast cancer cells (C, D) or HEK-293T cells expressing EPHA2 or vector (E, F). (G) The morphology of T47D cells treated with exosomes derived from HEK-293T cells expressing EPHA2 or vector. Scale bar: 50 μm. (H–J) IF assays were performed to detect the level of E-cadherin, vimentin, and F-actin in MDA-468 and T47D cells treated with exosomes from indicated cells. Scale bar: 10 μm. Data are expressed as the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{ns}P > 0.05$ indicate no statistical significance, $n = 3$. Exo, exosome; Ctrl, Control, E-cad, E-cadherin; VIM, Vimentin.

assays demonstrated that EPHA2 knockdown increased the co-localization of TGF-βRI with the lysosomal marker, lysosome-associated membrane glycoprotein 2 (LAMP2) (**Figure 5K**), which was consistent with enhanced lysosomal degradation in the absence of EPHA2. Together, these findings support a model in which EPHA2 directly binds to TGF-βRI and protects the model from lysosomal trafficking, thereby stabilizing TGF-βRI at the membrane and sustaining activation of the TGF-β/SMAD3 signaling pathway.

High expression of EPHA2 and TGF-βRI is associated with breast cancer progression and poor prognosis

A xenograft tumor model was established by subcutaneously injecting LM-BCCs, HM-BCCs, or HM-BCCs stably expressing control shRNA or EPHA2-targeting shRNA to elucidate the role of exosomal EPHA2 *in vivo*. Luciferase-labeled LM-BCCs were intravenously injected after primary tumor formation to assess metastatic colonization of the lungs (**Figure 6A**). Histologic analysis revealed that mice bearing EPHA2-knockdown HM-BCC tumors developed considerably fewer lung metastases compared to mice with control HM-BCC tumors, as shown by H&E staining and IHC analysis of lung tissue (**Figure 6B**). Moreover, mice with HM-BCC tumors exhibited a markedly higher frequency and number of metastatic lesions than mice with LM-BCC tumors (**Figure 6B**). These *in vivo* findings aligned with our *in vitro* data and further supported the conclusion that exosomal EPHA2 enhances the metastatic potential of breast cancer cells.

A bioinformatic analysis was performed using the TCGA breast cancer dataset to evaluate the clinical relevance of EPHA2 and TGF-βRI in human breast cancer. High EPHA2 expression was significantly associated with reduced overall survival (OS) and distant metastasis-free survival (DMFS) compared to low EPHA2 expression, as shown in **Figure 6C**. Similarly, elevated TGF-βRI expression correlated with worse OS and DMFS outcomes (**Figure 6D**). These results highlighted the potential prognostic value of EPHA2 and TGF-βRI, also underscored their pivotal roles in breast cancer progression and metastasis.

IHC analysis was performed on a breast cancer tissue microarray to further validate these findings at the protein level. EPHA2 protein expression was considerably elevated in metastatic lesions compared to primary tumors and TGF-βRI levels were higher in metastatic samples relative to non-metastatic samples (**Figure 6E, F**). In addition, a positive correlation between EPHA2 and TGF-βRI expression was noted across tissue samples (**Figure 6G**), supporting the notion that these proteins may functionally interact or act within the same signaling pathway. Our *in vivo* and clinical data indicated that elevated EPHA2 and TGF-βRI expression is associated with increased metastatic potential and worse prognosis in breast cancer, which is consistent with the mechanistic role of exosomal EPHA2 in promoting metastasis.

Discussion

Tumor heterogeneity is a hallmark of cancer and is characterized by the co-existence of multiple phenotypically

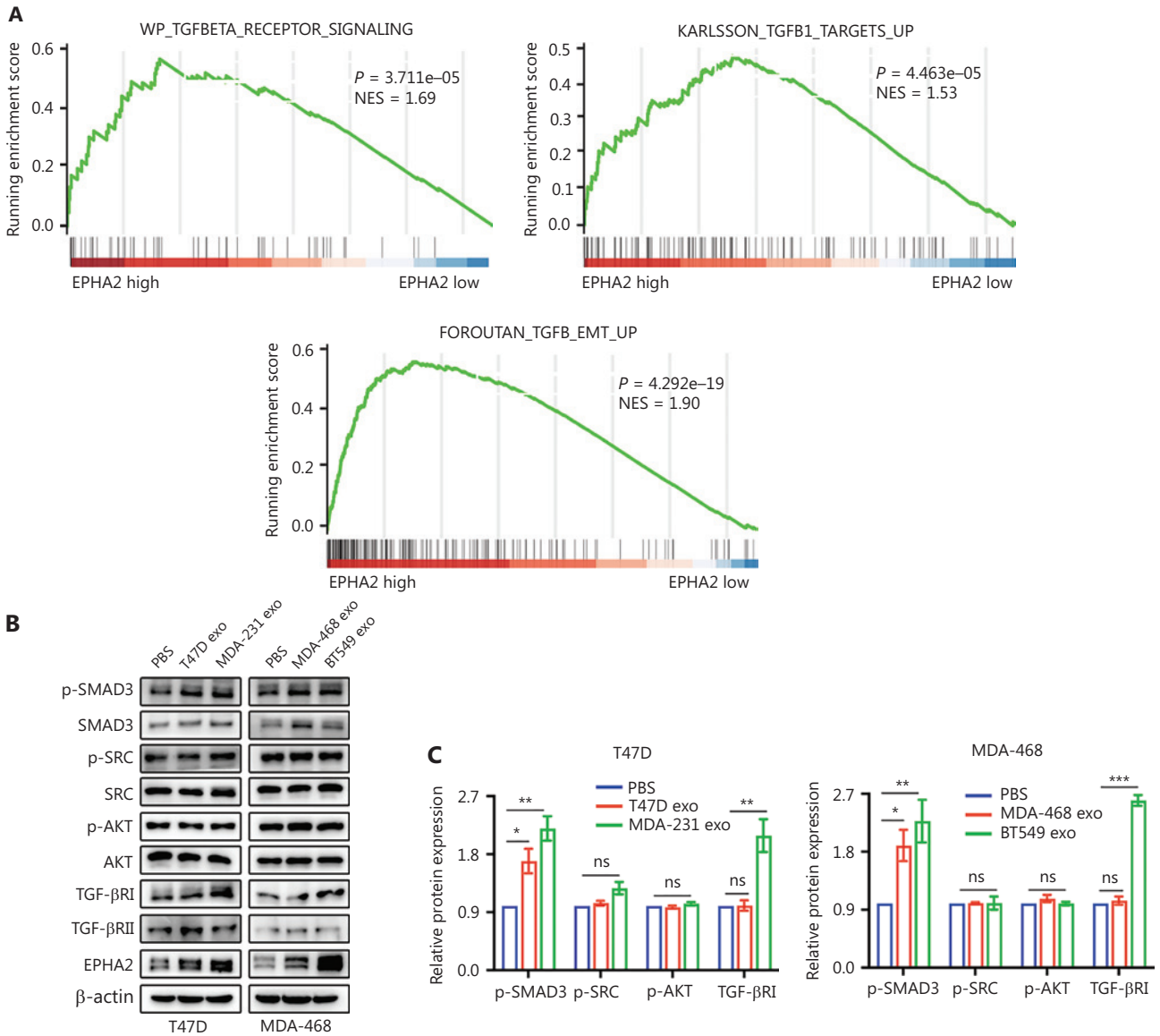


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distinct subpopulations of tumor cells within the same tumor. Emerging evidence has shown that communication among heterogeneous tumor cell subpopulations is a critical driver of cancer progression and metastasis, and exosomes facilitate the delivery of bioactive molecules that can modulate the behavior of recipient cells. However, the role of exosome-mediated transfer of metastatic traits between heterogeneous breast cancer cell populations is poorly understood. In the current study exosomes derived from HM-BCCs were shown to confer metastatic potential to less metastatic breast cancer cells (LM-BCCs). This effect is linked to the enrichment of EPHA2 in HM-BCC-derived exosomes. Exosomal EPHA2 was shown to be efficiently delivered to LM-BCCs, where exosomal EPHA2 becomes functionally expressed. Mechanistically, exosome-delivered EPHA2 directly interacts with and stabilizes the TGF-βRI in recipient cells, thereby activating the TGF-β/SMAD3 signaling pathway and promoting EMT, ultimately enhancing the metastatic capacity of LM-BCCs (**Figure 7**). Notably, this exosome-mediated EPHA2-TGF-βRI-SMAD3 signaling axis represents a previously undescribed mechanism driving metastatic progression. Overall, the findings identified exosomal EPHA2 as a critical mediator of metastatic trait transfer among heterogeneous breast cancer cells, offering novel insights for targeted cancer therapies.

The present study supports the emerging concept that exosome-mediated communication among heterogeneous

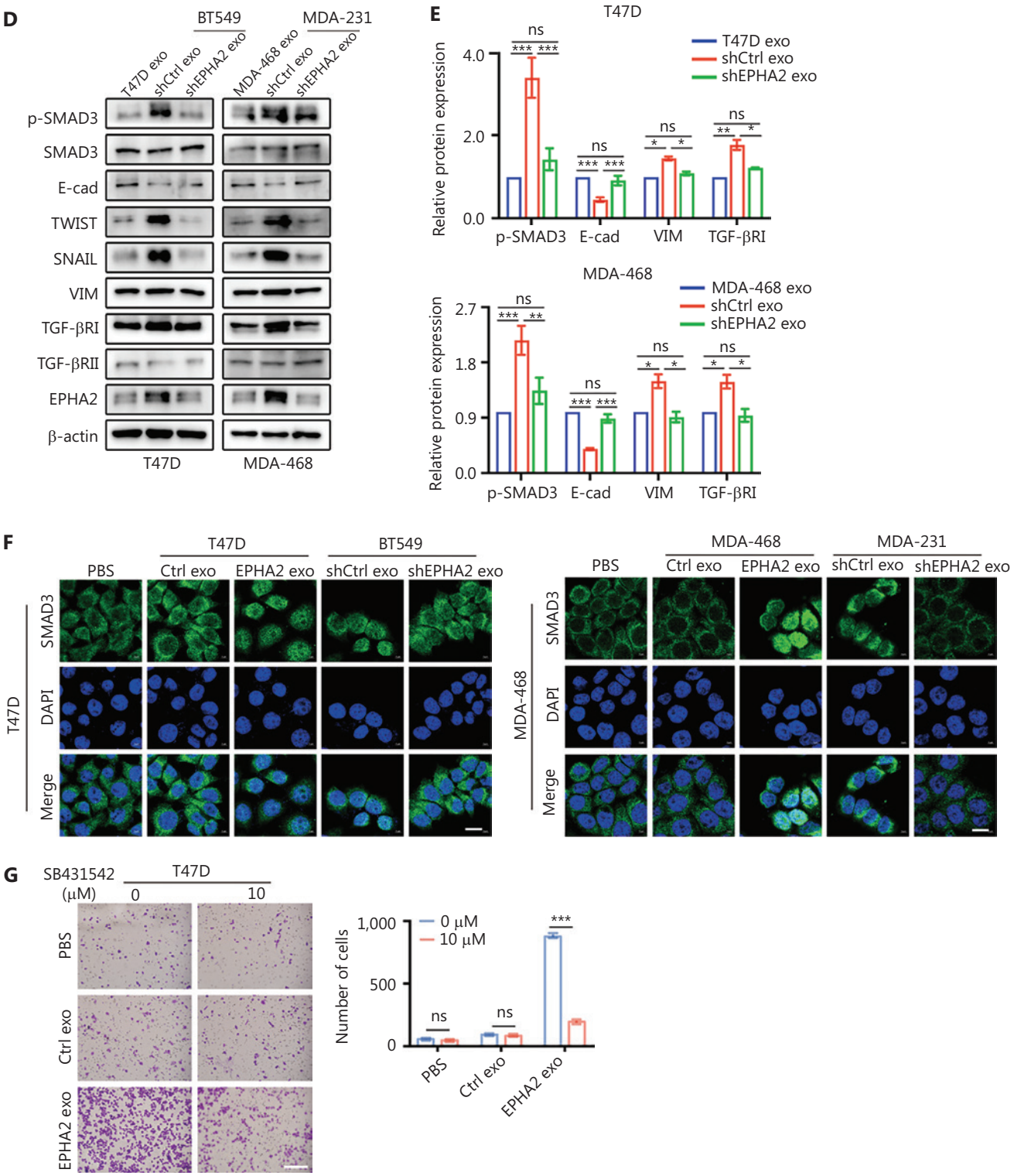


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tumor cell subpopulations is a critical driver of cancer progression[28-30]. Although the importance of tumor-stroma interactions in cancer development is well-recognized, relatively little attention has been given to the functional interplay between phenotypically distinct cancer cells within the same tumor. Herein the migratory and invasive capacities of LM-BCCs

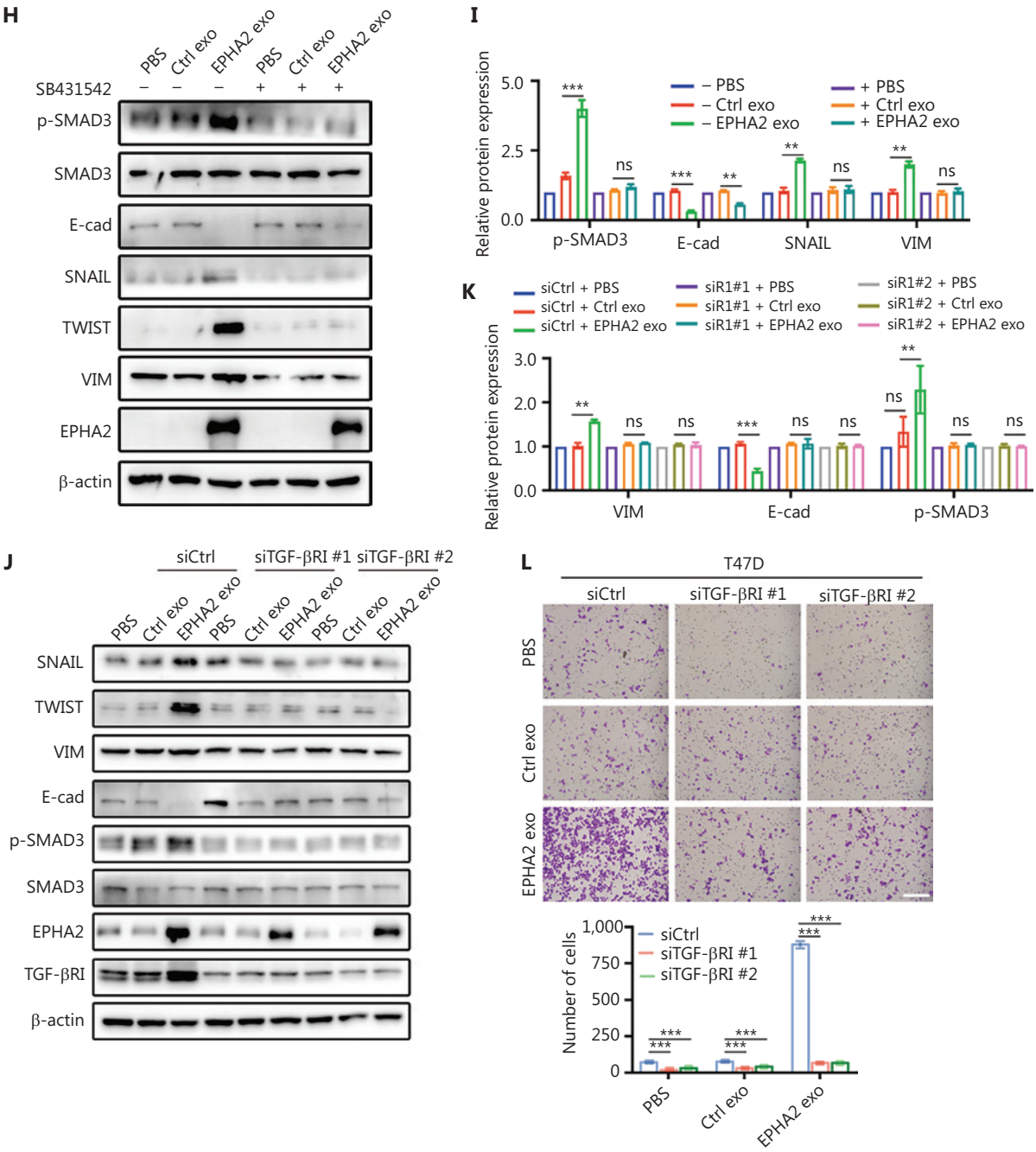


Figure 4 Exosomal EPHA2 promotes EMT of breast cancer cells through TGF-β/SMAD3 signaling. (A) Gene set enrichment analysis (GSEA) showed that the expression of EPHA2 significantly correlated with TGF-β signaling based on the TCGA database. (B, C) Western blot analysis showed the expression of TGF-βRI, TGF-βRII, EPHA2, phosphorylated and total SMAD3, phosphorylated and total SRC, phosphorylated and total AKT in T47D, and MDA-468 cells treated with PBS or exosomes derived from T47D and MDA-231 cells. (D, E) Western blot analysis analyzed the expression of indicated proteins in T47D and MDA-468 cells treated with exosomes derived from T47D, shCtrl BT549, and shEPHA2 BT549 cells. (F) IF images of SMAD3 staining in T47D and MDA-468 cells treated with exosomes from Ctrl T47D, EPHA2-overexpressed T47D, shCtrl BT549, and shEPHA2 BT549 cells. Scale bar: 10 μm. (G) Transwell assays showed inhibiting TGF-βRI by 10 μM SB431542 blocked the pro-migration effect of EPHA2-enriched exosomes in T47D cells. Scale bar: 100 μm. (H, I) Western blot showed the expression of indicated proteins in T47D cells pre-treated with 10 μM SB431542 and subsequently treated with exosomes derived from control T47D and EPHA2-overexpressed T47D cells. (J, K) siTGF-βRI in T47D cells blocked the pro-migration effect of EPHA2-enriched exosomes by Transwell assays. Scale bar: 100 μm. (L) Western blot analysis of indicated proteins in TGF-βRI-silenced T47D cells treated with EPHA2-enriched and control exosomes. Data are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{ns}P > 0.05$ indicate no statistical significance, $n = 3$. Exo, Exosome; Ctrl, Control; E-cad, E-cadherin; VIM, Vimentin.

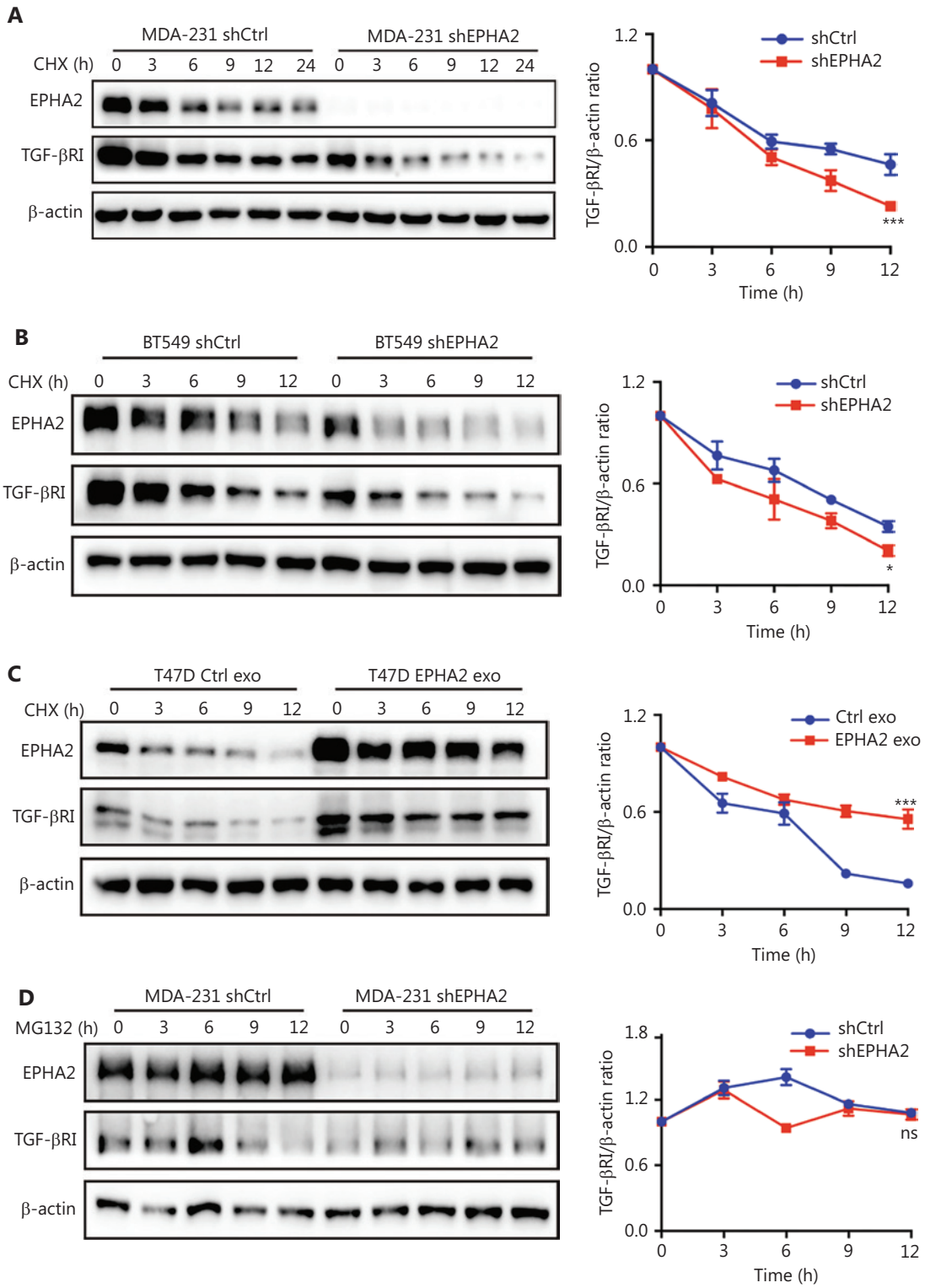


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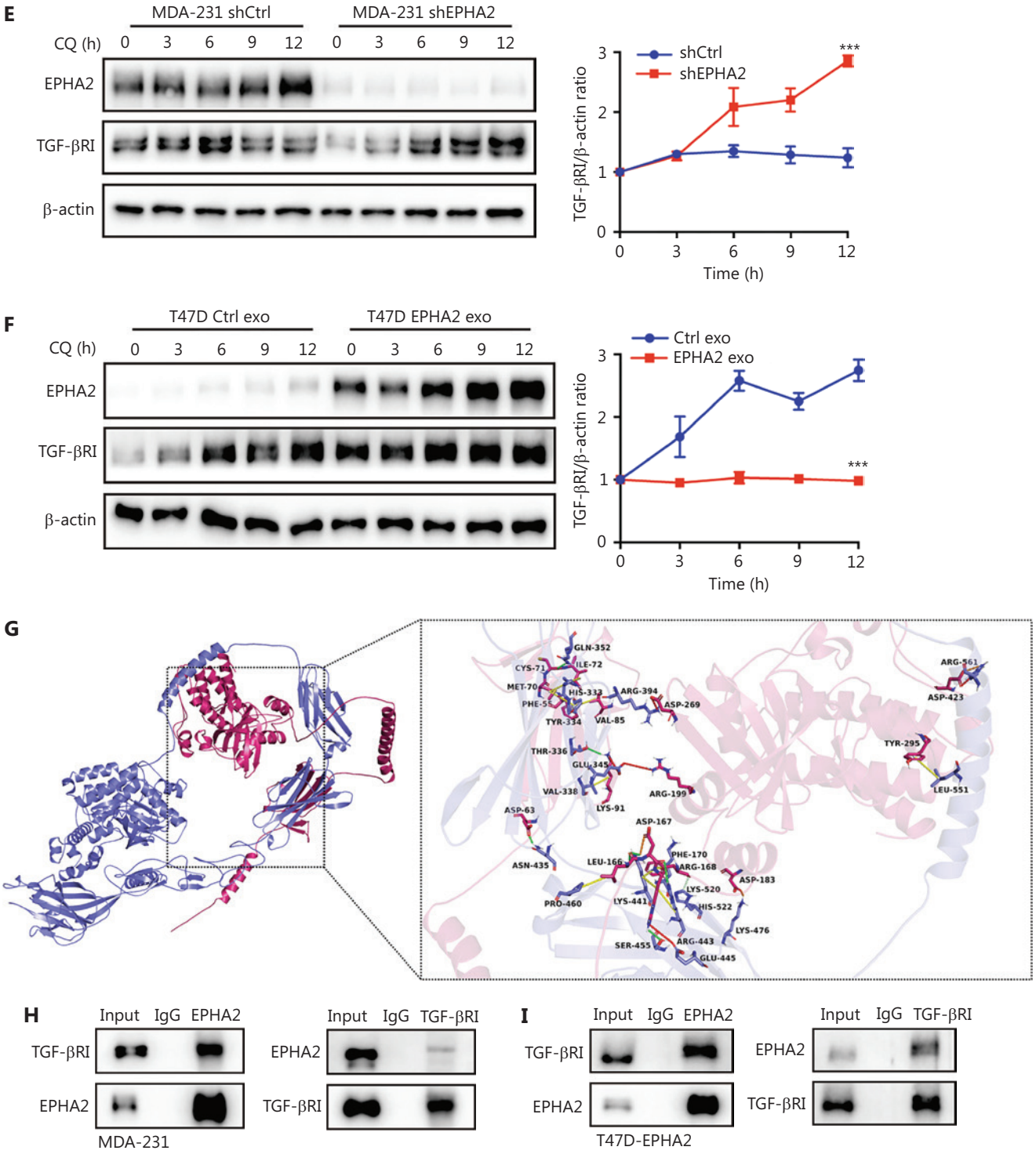


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were shown to be significantly enhanced when co-cultured with HM-BCCs or exposed to CM from HM-BCCs, indicating that HM-BCCs promote metastatic traits in recipient cells through non-contact mechanisms. Moreover, inhibition of exosome secretion from HM-BCCs abrogated the ability to enhance LM-BCC invasion, suggesting that exosomes serve as a key vehicle for this communication. This finding is consistent with recent reports showing that highly metastatic

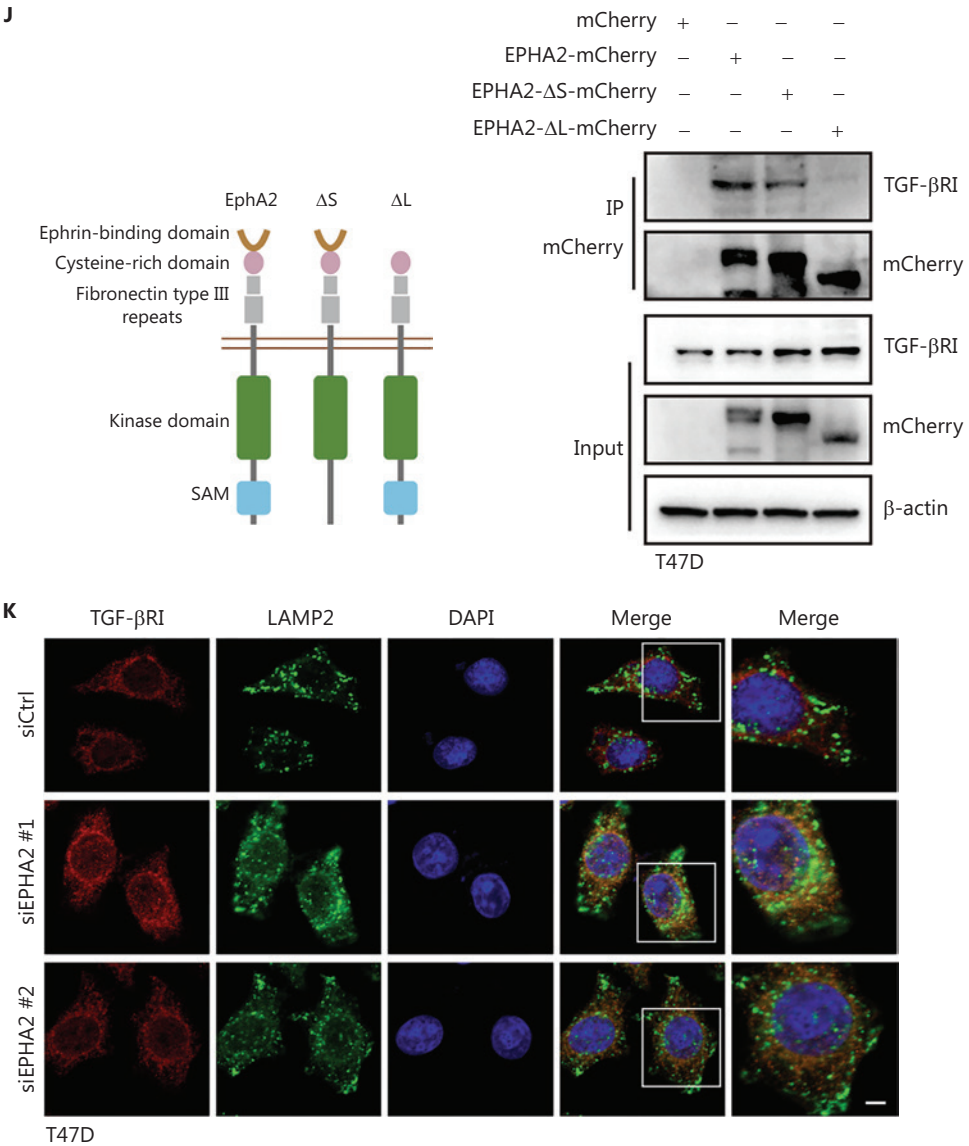


Figure 5 EPHA2 regulates TGF-βRI stability through inhibition of lysosome-mediated degradation. (A, B) Western blot analysis of TGF-βRI level in control and EPHA2-silenced MDA-231 (A) and BT549 (B) treated with 50 μg/mL of cycloheximide (CHX). Grayscale analysis was performed using Image J software and Grayscale values were normalized to the value at the 0-h timepoint. (C) Western blot analysis of TGF-βRI level in T47D cells pre-treated with exosomes from EPHA2-overexpressing or control cells and subsequently treated with 50 μg/mL of CHX. The relative protein expression values were normalized to the control group at the 0-h timepoint. (D, E) Western blot analysis of TGF-βRI level in control and EPHA2-silenced MDA-231 cells treated with 50 μM choroguine (CQ) (D) or 10 μM MG132 (E). The relative protein expression values were normalized to the control group at the 0-h timepoint. (F) Western blot analysis of TGF-βRI level in T47D cells pre-treated with exosomes from EPHA2-overexpressing or control cells and subsequently treated with 50 μM CQ. The relative protein expression values were normalized to the control group at the 0-h timepoint. (G) Molecular docking analysis of the binding pattern of EPHA2 and TGF-βRI. (H, I) Co-immunoprecipitation (Co-IP) analysis of the interaction between EPHA2 and TGF-βRI in MDA-231 (H) and EPHA2-overexpressed T47D cells (I). (J) Schematic representation of full-length EPHA2 and its mutants tagged with mCherry (left). Co-IP analysis of the interaction between TGF-βRI and the indicated EPHA2 mutants in T47D cells (right). (K) IF images of SMAD3 staining in T47D and MDA-468 cells treated with exosomes from Ctrl T47D, EPHA2-overexpressed T47D, shCtrl BT549, and shEPHA2 BT549 cells. Scale bar: 10 μm. Data are expressed as the mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{ns}P > 0.05$ indicate no statistical significance, $n = 3$.

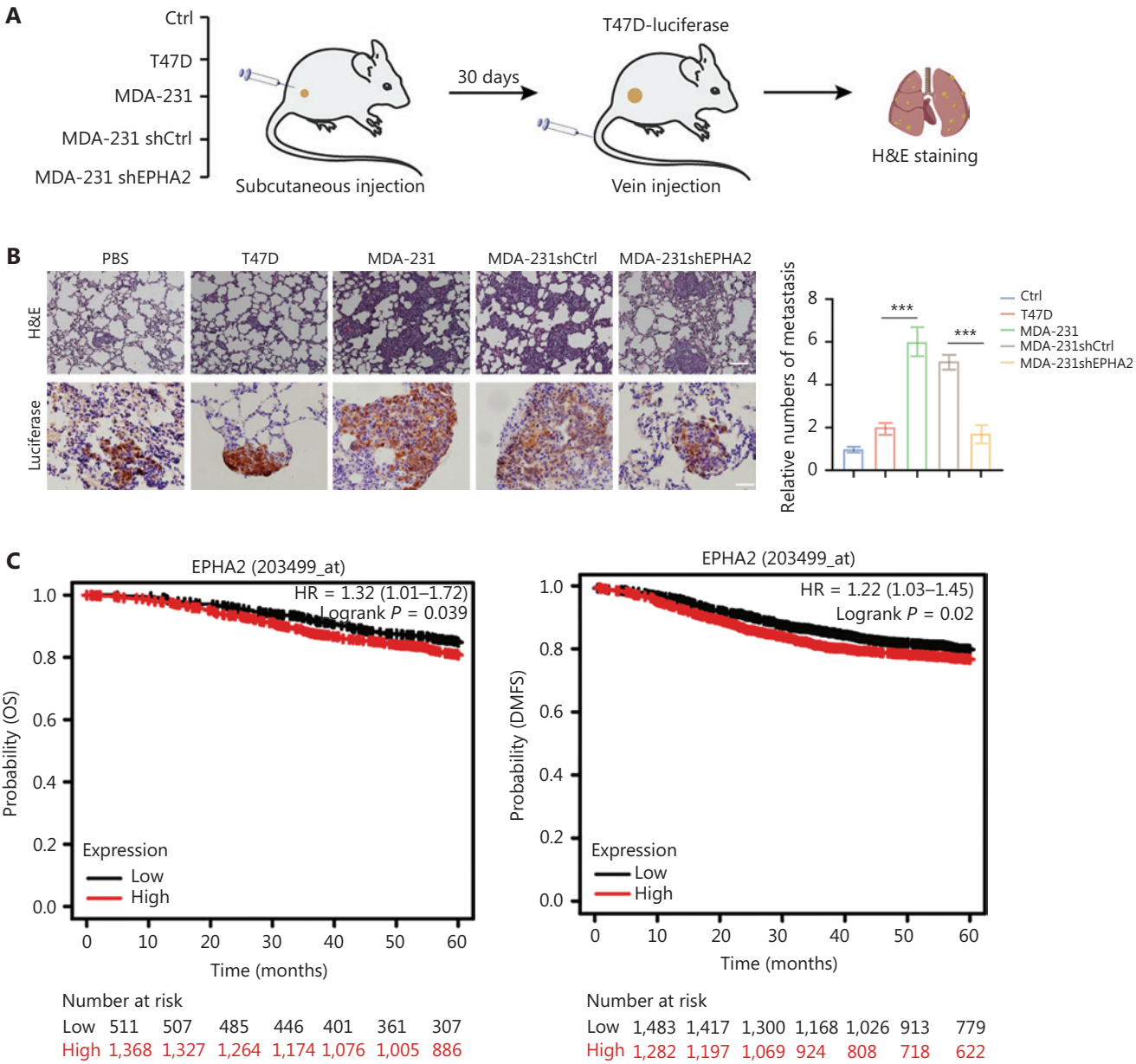


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hepatocellular carcinoma cells enhance the metastatic capacity of less aggressive cells through exosome-mediated transfer[9]. Notably, low metastatic breast cancer cells can promote the lung colonization of highly metastatic cells by releasing exosomes[31]. These findings suggested that exosome-mediated interactions among heterogeneous cancer cell populations may be a widespread mechanism contributing to tumor evolution. Such synergistic cooperation may provide an adaptive advantage, wherein subpopulations with distinct traits, can support each other to overcome the various barriers of metastasis.

The findings showed that EPHA2 is selectively enriched in exosomes and exosomal EPHA2 facilitates the intercellular transfer of invasive and metastatic traits within heterogeneous breast cancer populations. Consistently, a recent study showed that EPHA2 is enriched in exosomes derived from triple-negative breast cancer cells and contributes to breast cancer metastasis[32]. This observation is aligned with our previous study, which demonstrated that exosomal EPHA2 promotes metastasis in a different breast cancer model. Similar findings have been reported in other tumor types, including pancreatic and renal cancers[33,34]. These findings collectively

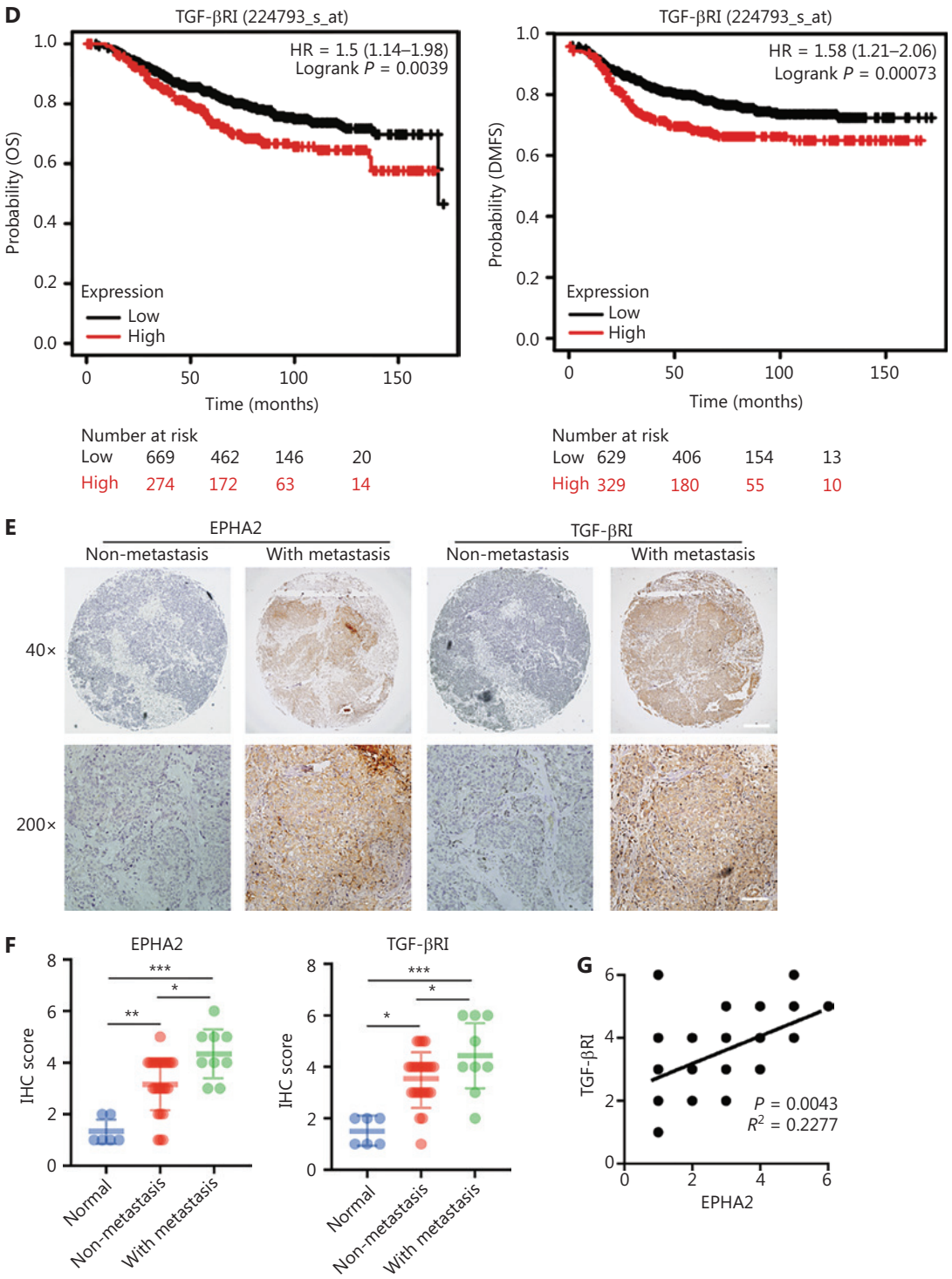


Figure 6 EPHA2 and TGF-βRI are associated with the progression and poor prognosis in breast cancer. (A) Schematic diagram of the design of the animal experiment. (B) H&E and IHC staining using luciferase antibody showed that the metastases in mice lung tissue were increased in the MDA-231 cells group compared to EPHA2-silenced MDA-231 cells, T47D cells, and control groups. Scale bar: 50 μm. (C, D) TCGA database analysis showed breast cancer patients with high EPHA2 or TGF-βRI expression had a poor survival rate. (E) IHC staining to analyze the levels of EPHA2 and TGF-βRI in breast cancer tissue microarray. Scale bar: 500 μm (upper), 100 μm (lower). (F) The EPHA2 scores in the paracancer (normal), non-metastatic, and metastatic groups. (G) IHC staining showed the expression of EPHA2 was positively correlated with TGF-βRI in breast cancer tissues. Data are expressed as the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001, n = 3. Ctrl, Control; H&E, Hematoxylin and Eosin staining; IHC, Immunohistochemistry.

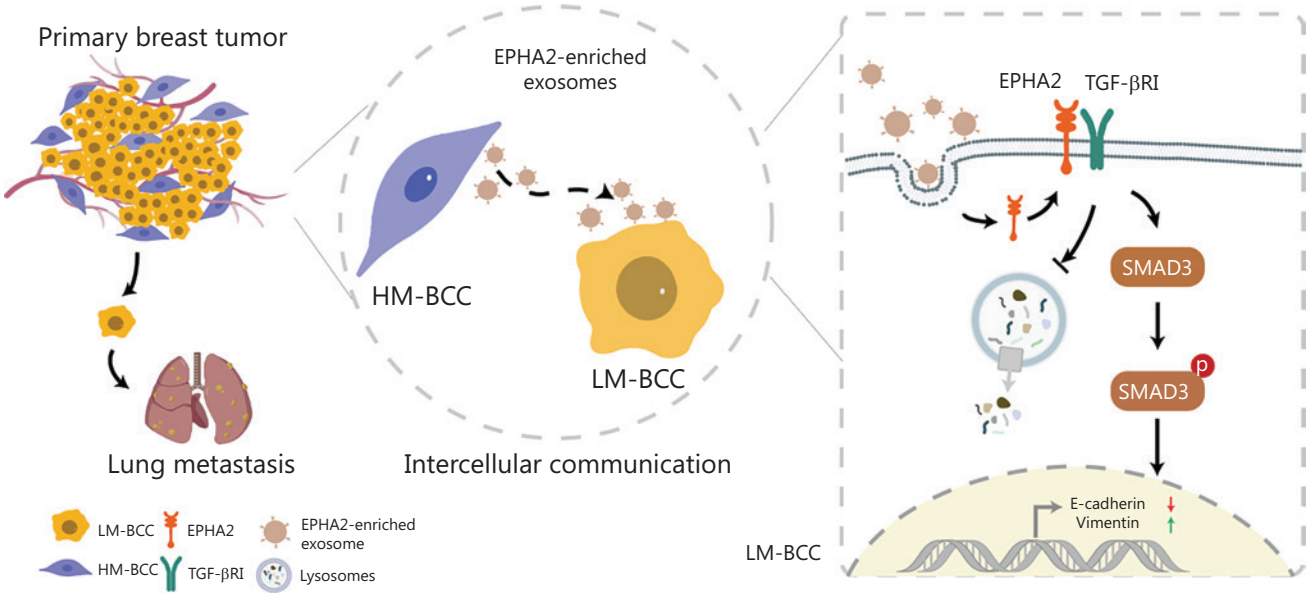


Figure 7 Exosomal EPHA2 mediates the transfer of metastatic potential through activation of the TGF-β/SMAD3 signaling pathway in breast cancer. Highly metastatic breast cancer cells (HM-BCCs) can transfer their aggressive phenotype to low-metastatic breast cancer cells (LM-BCCs) through exosome-mediated communication. Mechanistically, EPHA2 is highly enriched in both HM-BCCs and the secreted exosomes. Upon uptake by recipient LM-BCCs, exosomal EPHA2 directly interacts with and stabilizes TGF-β receptor I (TGF-βRI), sustaining the expression and leading to prolonged activation of the TGF-β/SMAD3 signaling pathway. This sustained signaling induces epithelial-mesenchymal transition (EMT) in LM-BCCs, thereby enhancing the metastatic potential. These findings reveal exosomal EPHA2 drives metastatic potential transfer between heterogeneous breast cancer cells, identifying the EPHA2-TGF-βRI axis as a promising therapeutic target for preventing breast cancer metastasis.

indicated that elevated levels of exosomal EPHA2 are associated with cancer progression and poor patient prognosis, supporting the notion that exosomal EPHA2 serves as a key mediator of intercellular communication that drives malignant progression. EMT is a critical process by which epithelial cells lose cell-cell adhesion and acquire mesenchymal characteristics, thereby gaining motility and invasive potential. EMT has a central role in early metastatic dissemination from the primary tumor site[35]. Although elevated EPHA2 expression has been linked to EMT in multiple cancer types, it is unclear whether EPHA2 can induce EMT through exosome-mediated delivery. The current study provides novel evidence that exosomal EPHA2 is indeed capable of inducing EMT in recipient cells, uncovering a new mechanism by which EPHA2 contributes to cancer progression *via* the exosomal route.

The mechanistic investigations have uncovered a novel pathway by which exosomal EPHA2 exerts pro-metastatic functions. Recent studies have highlighted exosomes as key mediators of TGF-β signaling. Exosomal circRNAs sequester miRNAs to upregulate TGF-β1 in colorectal cancer, directly promoting cancer cell invasion[36]. Exosomes are internalized by hepatic Kupffer cells in pancreatic cancer, inducing TGF-β release that activates stromal cells to establish a pro-fibrotic pre-metastatic niche[37]. Similarly, in breast cancer, exosomal LAP-TGF-β1, the predominant form of exosomal TGF-β1, remodels the pulmonary vascular niche to support lung metastasis in breast cancer[38]. In this study EPHA2-enriched exosomes were shown to markedly enhance TGF-β signaling in recipient cells, as evidenced by elevated SMAD3 phosphorylation and nuclear translocation following EPHA2-enriched exosome treatment. Conversely, EPHA2 silencing in HM-BCCs abolished the ability of the exosomes to activate SMAD3 and modulate EMT markers in recipient cells. Mechanistically, exosomal EPHA2 exerted an effect by physically binding to TGF-βRI and preventing lysosomal degradation, thereby increasing TGF-βRI protein stability. The interaction was evidenced by Co-IP assays showing that EPHA2 formed a complex with TGF-βRI. Moreover, an *in silico* protein-protein docking analysis predicted a plausible binding interface between EPHA2 and TGF-βRI, further confirming the interation between EPHA2 and TGF-βRI. In addition, EPHA2 and TGF-βRI were validated to be co-elevated and positively correlated in clinical breast cancer tissues, although the findings were limited by the sample size, they suggests a potential functional association between these two protiens in breast cancer. Future studies should involve multi-center cohorts and longitudinal follow-up to strengthen

these conclusions. To our knowledge, this is the first study to demonstrate that EPHA2 directly binds to TGF-βRI and enhances TGF-βRI stability.

The mechanism by which exosomal EPHA2 exerts effects is highly intriguing. EPHA2 traditionally functions through canonical forward or reverse signaling, which is mediated by an interaction with the membrane-bound ligand, Ephrin-A1, on neighboring cells[39,40]. The findings revealed that EPHA2 mediates long-distance intercellular signaling *via* exosomes, extending the reach beyond neighboring cells. However, the mechanism by which EPHA2 functions upon delivery to recipient cells requires further investigation. In theory, when Ephrin-A1 is expressed on the surface of the recipient cell and the exosome-delivered EPHA2 is not internalized, EPHA2 may function by binding to the membrane-bound Ephrin-A1, thereby activating a reverse signaling pathway. Once internalized and integrated into the recipient cell membrane, exosomal EPHA2 may function as a receptor by binding to Ephrin-A1 on neighboring cells and participating in canonical EPHA2-Ephrin signaling. Another possibility exists. EPHA2, once integrated into the recipient cell membrane, could interact with other receptors, generating ligand-independent positive signaling effects. For example, the novel EPHA2-TGF-βRI axis, which was described in the current study, promotes cancer progression. The findings herein cannot rule out the possibility that exosome-delivered EPHA2 promotes cancer progression through pathways beyond the pathways described above. Furthermore, we propose that exosome-delivered EPHA2 may drive cancer progression in multiple ways, although the specific mechanisms remain to be explored in future studies.

Conclusions

The current study demonstrated that EPHA2-enriched exosomes mediate the transfer of invasive and metastatic potential between heterogeneous breast cancer cell populations. Specifically, exosome-delivered EPHA2 binds to and stabilizes TGF-βRI in LM-BCCs, leading to the activation of the canonical TGF-β/SMAD3 signaling pathway, induction of EMT, and acquisition of metastatic traits by recipient cells. These findings suggested that exosomal EPHA2-mediated intercellular communication is a key mechanism driving breast cancer progression. Therefore, targeting the EPHA2-TGF-βRI axis emerges as a promising therapeutic strategy against metastatic breast cancer. Moreover, the current findings, together with our recent reports[5,15], collectively demonstrated that the metastatic status of cancer patients can be monitored by a minimally invasive measurement of exosomal EPHA2 in peripheral blood, providing a robust rationale for its clinical translation. Therefore, targeting the EPHA2-TGF-βRI axis represents a promising therapeutic strategy for breast cancer.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Conceived and designed the analysis: Liming Liu, Yichu Zhang, Xiaoxue Li, Yueni Mo, Fei Zhang, Ruifang Niu.
Collected the data: Liming Liu, Yueni Mo, Yichu Zhang, Xiaoxue Li, Lanlan Song, Yidi Jia, Luoming Zhang, Wei Zhou, He Zhang, Hui Guo, Zhiyong Wang, Yanfen Cui.
Contributed data or analysis tools: Yueni Mo, He Zhang, Zhiyong Wang, Yanfen Cui.
Performed the analysis: Liming Liu, Yueni Mo, Yichu Zhang, Xiaoxue Li, Lanlan Song.
Wrote the paper: Liming Liu, Fei Zhang, Ruifang Niu.

Data availability statement

The data generated in this study are available upon request from the corresponding author.

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