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Secreted LGALS3BP facilitates distant metastasis of breast cancer

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Abstract

Background Patients with estrogen receptor (ER)-positive breast cancer (BC) can be treated with endocrine therapy targeting ER, however, metastatic recurrence occurs in 25% of the patients who have initially been treated. Secreted proteins from tumors play important roles in cancer metastasis but previous methods for isolating secretory proteins had limitations in identifying novel targets.

Methods We applied an in situ secretory protein labeling technique using TurboID to analyze secretome from tamoxifen-resistant (TAMR) BC. The increased expression of LGALS3BP was validated using western blotting, qPCR, ELISA, and IF. Chromatin immunoprecipitation was applied to analyze estrogen-dependent regulation of LGALS3BP transcription. The adhesive and angiogenic functions of LGALS3BP were evaluated by abrogating LGALS3BP expression using either shRNA-mediated knockdown or a neutralizing antibody. Xenograft mouse experiments were employed to assess the in vivo metastatic potential of TAMR cells and the LGALS3BP protein. Clinical evaluation of LGALS3BP risk was carried out with refractory clinical specimens from tamoxifen-treated ER-positive BC patients and publicly available databases.

Results TAMR secretome analysis revealed that 176 proteins were secreted at least 2-fold more from MCF7/TAMR cells than from sensitive cells, and biological processes such as cell adhesion and angiogenesis were associated with the TAMR secretome. Galectin-3 binding protein (LGALS3BP) was one of the top 10 most highly secreted proteins in the TAMR secretome. The expression level of LGALS3BP was suppressed by estrogen signaling, which involves direct ERα binding to its promoter region. Secreted LGALS3BP in the TAMR secretome helped BC cells adhere to the extracellular matrix and promoted the tube formation of human umbilical vein endothelial cells. Compared with sensitive cells, xenograft animal experiments with MCF7/TAMR cells showed increased pulmonary metastasis, which completely disappeared in LGALS3BP-knockdown TAMR cells. Finally, higher levels of LGALS3BP were associated with poor prognosis in ER-positive BC patients treated with adjuvant tamoxifen in the clinic.

Conclusion TAMR secretome analysis identified secretory proteins, such as LGALS3BP, that are involved in biological processes closely related to metastasis. Secreted LGALS3BP from the TAMR cells promoted adhesion of the cells to the extracellular matrix and vasculature formation, which may support metastasis of TAMR cells.

Keywords Secretome, Tamoxifen-resistant breast cancer, LGALS3BP, Metastasis

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Introduction

Breast cancer (BC) is the most prevalent cancer in women and is one of the leading causes of cancer death in women worldwide [1]. Approximately 70% of BCs express ERα and depend on ER signaling for tumor growth and progression [2]. By blocking estrogen from binding to the ER, tamoxifen has proven effective and remains the frontline treatment for patients with ER-positive BC, especially in premenopausal women [3]. One of the greatest challenges with ER-positive BC is a delayed pattern of distant recurrence up to 20 years after diagnosis, which is unlike most other cancers whose recurrence risk remains stable beyond 5 years after diagnosis [4]. Meta analyses with large cohorts showed that ER-positive BC patients who had been administered with endocrine therapy for 5 years experienced distant recurrence at a steady rate for 20 years from the time of diagnosis, reaching a total of 30% recurrence and a mortality rate of more than 25% [5]. In order to overcome the long-term recurrence of ER-positive BC, clinical trials have been conducted to test other treatment options. Whereas a clinical trial testing extended tamoxifen treatment demonstrated marginal improvement, clinical trials testing endocrine therapy with adjuvant treatment options such as everolimus, an mTOR inhibitor, or palbociclib, a CDK4/6 inhibitor, failed to improve disease recurrence [6–8]. In this respect, the development of new treatment options to defend against the distant metastasis of therapy-resistant ER-positive BC is urgently needed.

The search for drivers of metastatic recurrence has led researchers to focus more on the BC secretome. Proteins secreted from cancer cells mediate interactions with the tumor microenvironment (TME) to enhance distant metastasis [9]. The serum protein portraits in patients with BC provided a biomarker signature that was able to predict the risk of developing distant recurrence [10]. Prolonged chemotherapy reshapes the secretory profiles of cancer, and further aggravates tumor recurrence and therapy resistance [9, 11]. Plasma proteomics analysis of patients with tamoxifen-resistant BC revealed several differentially expressed proteins that had potential values as biomarkers for predicting the response to tamoxifen treatment [12]. Recently, Fu et al. reported that ectopic high FOXA1 expression in endocrine-resistant MCF7 cells resulted in the upregulation of a set of genes encoding secretory proteins, which predicted distant metastasis and poor outcomes for patients with ER-positive BC [13]. The previous methods for identifying secretory proteins used either predefined antibodies or genetic screening [10, 12, 13]. Each of these approaches had drawbacks: the use of predefined antibodies was limited to known targets, while genetic screening did not involve protein isolation. Technical advances were thus required for more comprehensive BC secretome analysis.

Owing to recent advances in protein labeling techniques, researchers are now able to identify secretory proteins globally through experimentation. One of these labeling techniques, termed 'iSLET (in situ secretory protein labeling via endoplasmic reticulum-anchored TurboID)' achieves isolation of secretory proteins specifically by tethering TurboID, a biotin-labeling protein, to the endoplasmic reticulum resident protein Sec61b [14]. In TurboID-expressing cells, biotin-labelled proteins traffic via transport vesicles and eventually to the extracellular space, where they can be selectively sorted and identified via liquid chromatography–tandem mass spectrometry (LC-MS/MS). This secretome-specific labeling technique has allowed successful isolation of secretomes from hepatocytes, liver, and pancreatic ductal adenocarcinoma (PDAC) [14–17]. In the present study, we applied the iSLET system to uncover secretory proteins specifically expressed in the tamoxifen-resistant BC cells. We aimed to identify secretory proteins that are associated with metastatic dissemination of tamoxifen-resistance and examine whether targeting these proteins would provide therapeutic options to overcome the distant recurrence of tamoxifen-resistant breast cancer cells.

Materials and methods

Cell lines and cell culture

The tamoxifen-resistant (MCF7/TAMR-1 and MCF7/TAMR-8), and parent MCF7 subline (MCF7/S0.5), and the tamoxifen-resistant and parent T47D sublines (T47D/TR-1 and T47D/S2) were obtained from Ximbio (UK). MCF7/S0.5 cells were maintained in phenol-red-free Dulbecco's modified Eagle's medium (DMEM): F12 (1:1) containing 1% fetal bovine serum (FBS), 2 mM GlutaMax™ (Gibco-Invitrogen), and 6 ng/ml insulin. T47D/S2 cells were maintained in phenol-red-free RPMI: F12 (1:1) containing 2% FBS, 2 mM GlutaMax™, and 8 μg/ml insulin. The tamoxifen-resistant cells were maintained in media supplemented with 1 μM tamoxifen (3412, Tocris, UK). Whenever estradiol (E2) was treated, the cells were adapted to media containing 5% charcoal-stripped FBS (Gibco-Invitrogen) for one passage before performing experiments. HEK293T cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FBS. Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Cambrex and maintained in M199 media supplemented with endothelial cell growth supplement (20 μg/mL; Sigma), 5 units/mL heparin, and 20% FBS. All the cells were maintained under 5% CO_2 in humidified air at 37 °C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell growth.

TAMR secretome analysis

MCF7/S0.5, TAMR-1, and TAMR-8 cells were infused with adenovirus encoding Sec61b-TurboID at an MOI of 5 for 24 h and then further incubated with 50 μM biotin (B4639, Sigma) for 16 h. After washing with phosphate-buffered saline (PBS), the cells were further cultured in serum-free media for 24 h. The culture media was collected and concentrated with Amicon Ultra-15 10 K by centrifugation. Peptide samples were prepared by exchanging with bicarbonate buffer containing 4 M urea in 50 mM ammonium bicarbonate. Details on the peptide preparation and subsequent LC-MS/MS were as described previously [14]. Gene ontology term analysis was performed using DAVID 2021 software (https://david.ncifcrf.gov/).

Western blotting, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence assay

Western blotting was carried out as described previously using specific antibodies against Galectin-3 binding protein (LGALSBP) (10281-1-AP, ProteinTech Group), streptavidin protein-HRP (21126, Thermo Scientific), and β-actin (sc-47778, Santa Cruz Biotechnology) [18]. The band intensity of each protein was quantified by UVITEC (UK). To analyze the secreted proteins, the culture media were precipitated with 20% trichloroacetic acid (TCA), and the resulting pellets were further processed for western blotting. The amount of soluble human LGALS3BP protein was measured using a commercially available ELISA kit (DGBP30B, R&D Systems). Immunofluorescence staining was performed with an LGALSBP antibody. The nuclei were stained with 40,6-diamidino-2-phenylindole (DAPI). Images were analyzed using a confocal microscope (Leica, Germany).

Quantitative real-time polymerase chain reaction (qPCR) and chromatin immunoprecipitation (ChIP) assay

cDNA was synthesized from total RNA using Moloney-Murine Leukemia Virus reverse transcriptase and random primers (Thermo Scientific). qPCR was performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific) with specific primers (Table S1). The relative mRNA level of the target gene was analyzed by the Eq. 2^{-DCt} (DCt = Ct of target gene minus Ct of β-actin). The ChIP assay was conducted with specific primers as previously described (Table S1) [19].

Establishment of stable LGALS3BP knockdown MCF7/TAMR-8 sublines

A lentiviral vector encoding LGALS3BP (pLKO.1-shLGALS3BP) was constructed by conventional DNA recombination techniques. To obtain the lentivirus particles, the pLKO.1-shLGALS3BP, the lentiviral packaging plasmids (psPAX2), and the envelope plasmid (pMD2.G) were cotransfected into HEK293T packaging cells using Lipofectamine 2000 (Thermo Scientific). The lentivirus was transduced into MCF7/TAMR-8 cells with hexadimethrine bromide, and stable clones that LGALS3BP was knocked down were obtained by 2 to 3 weeks of puromycin selection. The knockdown of LGALS3BP was subsequently confirmed by western blotting.

Cell adhesion assay and tube formation assay

For the adhesion assay, the cells were seeded onto fibronectin-coated dishes. The cells were allowed to adhere for 2 h and then non-adherent cells were removed by washing with PBS. After the adherent cells were stained with 0.5% crystal violet, images were taken. For quantification, the cells were dissolved in 100% methanol and the absorbance at 570 nm was assessed by spectrophotometry. For the tube formation assay, HUVECs were seeded in Matrigel (Corning) coated 24-well plates and incubated in serum-free DMEM: F12 (1:1) media or conditioned media for 3 h. Images of tube formation were taken at 100X magnification. Quantitative determination was performed by manually counting the number of enclosed structures (meshes) in quadruplicate experiments as detailed in DeCicco-Skinner, et al. [20]. The neutralizing antibodies against LGALS3BP (clones #67 and #84) were kind gifts from Dr. Chang (Asan Medical Center, South Korea) [21]. For the adhesion assay, the cells were preincubated with 1 μg/mL anti-LGALS3BP antibodies for 10 min. For the tube formation assay, the conditioned media were preincubated with 1 μg/mL anti-LGALS3BP antibodies for 1 h prior to the seeding of HUVECs. The recombinant LGALS3BP protein (2226-GAB) was purchased from R&D Systems.

Xenograft experiments

The xenograft experiments were performed in accordance with the guidelines of the Seoul National University Animal Care and Use Committee (SNU-210906-2-3 and SNU-240124-3). All experiments with mice were performed in a blind and randomized fashion. Five-week-old female athymic (nu/nu) BALB/c mice were obtained from Japan SLC Inc. After 5 days of acclimatization, the mice were randomized based on the body weight and then injected on the fourth mammary fat pad with 5×10^6 cells mixed 1:1 with Matrigel (Corning, 356231). When the tumor volume reached approximately 50 mm^3, the tumor diameter was measured with a caliper two times a week. The tumor volumes were estimated using the following formula: tumor volume (cm^3) = (length x $width^2$) x 0.5. Immunohistochemistry was performed using specific antibodies against CD31 (102501, Biolegend), and Fibronectin (ab2413, Abcam, UK).

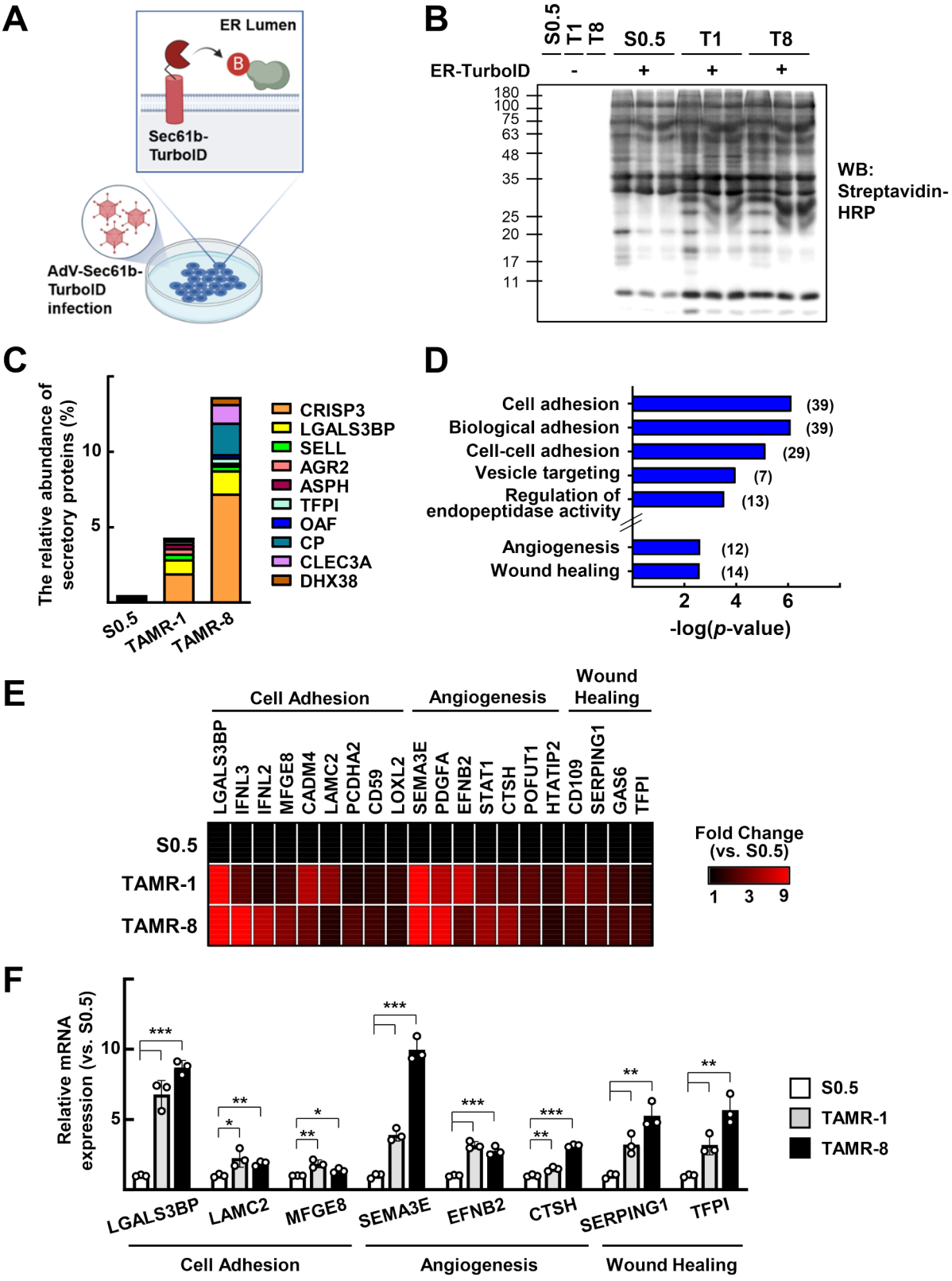

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Fig. 1 Isolation and characterization of the secretome from the MCF7/TAMR cells. (**A**) Schematic representation of the TAMR cell-specific secretory protein labeling using the Sec61b-TurboID system. ER: Endoplasmic reticulum, AdV: Adeno-virus (**B**) The culture supernatants of the AdV-Sec61b-TurboID infected MCF7/TAMR cells and those of the parental cells were collected, concentrated, and subjected to western blotting (WB) using streptavidin-HRP. (**C**) The relative abundance of the top 10 secretory proteins upregulated in terms of biotinylated spectral counts in the MCF7/TAMR cells. (**D**) GO Biological process analysis for the 176 upregulated genes in the MCF7/TAMR (fold change ≥ 2). Bar graph showing the most enriched biological process based on *p*-value. The number of annotated genes in each ontology term is shown in parenthesis. (**E**) Heat map representation of differentially regulated genes associated with "cell adhesion", "angiogenesis" and "wound healing" biological processes. The full names of the genes are annotated in Table S3. (**F**) Total RNA extract obtained from the MCF7/TAMR cells and their parental cells were subjected to qPCR analysis. Data presented as mean ± SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$, and ***, $P < 0.001$

Immunohistochemistry of human BC tissues

Studies using human BC samples were approved by the institutional review board (IRB) of Seoul National University Bundang Hospital (IRB No. B-2209-778-301), and informed consent was waived by the IRB. All experiments and procedures were performed in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Patients with recurrent ER-positive BC who had been treated with adjuvant tamoxifen after surgery and had two different tumor tissue samples—one from the primary tumor and the other from the metastatic tumor—were selected from the pathology archives of Seoul National University Bundang Hospital (2012–2021). A total of 16 patients were included in this study. Patient details and clinicopathologic information were previously reported [22]. Immunohistochemistry was performed on tissue sections using a specific antibody against LGALS3BP (10281-1-AP, ProteinTech Group).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software. Statistically significant differences between the two groups were determined using the unpaired Student's t-test and the nonparametric Mann–Whitney U test (two-tailed) for in vitro and in vivo studies, respectively. Statistical analyses of multiple groups were performed using a two-way ANOVA followed by a Bonferroni post-test correction. Data are reported as the mean ± SD and $P < 0.05$ was considered significant.

Results

Analysis of the secretome obtained from tamoxifen-resistant BC cells

In order to delineate the tamoxifen-resistant BC-specific secretome (TAMR secretome), we adopted the iSLET system [14]. TurboID labels proteins within the endoplasmic reticulum lumen with biotin, which enables us to identify Sec61b-TurboID expressing cell-specific secretory proteins (Fig. 1A). The tamoxifen-resistant and their parent cells were transduced with the adenovirus encoding Sec61b-TurboID and the resulting secreted proteins were analyzed by LC-MS/MS. The MCF7/TAMR-1 and MCF7/TAMR-8 cells showed a similar pattern of secretory proteins but distinct from that of MCF7/S0.5 control cells (Fig. 1B). Among the 1,003 proteins identified globally, 176 proteins were secreted at least 2-fold more from the MCF7/TAMR cells than from the sensitive cells (Fig. S1A and Table S2). The top 10 proteins enriched in the tamoxifen-resistant cells included Cysteine Rich Secretory Protein 3 (CRISP3), Galectin-3 binding protein (LGALS3BP), and Ceruloplasmin (CP) (Fig. 1C). The relative abundance of the top 10 proteins corresponded to 5% and 13% of the total secretory proteins in terms of biotinylated spectral counts from the TAMR-1 and TAMR-8 cells, respectively, which may indicate the richness of these secretory proteins. Gene Ontology analysis of the 176 commonly enriched proteins in TAMR compared to parental MCF/S0.5 showed that biological processes such as cell adhesion, angiogenesis, and wound healing were associated with the secretome (Fig. 1D, Fig. S1B and S1C). The proteins in these ontology terms were presented in the Heat map and their increases in the MCF7/TAMR cells were confirmed by qPCR analysis (Fig. 1E and F).

Secretion of LGALS3BP is enhanced in the tamoxifen-resistant BC cells

LGALS3BP was previously reported as a key component in inducing cellular adhesion in various cancer types [23]. Thus, we carried out further studies to examine the role of secreted LGALS3BP in cell adhesion and metastasis of tamoxifen-resistant BC cells. First, we validated the secretory property of LGALS3BP in the tamoxifen-resistant MCF7 and T47D cells. As shown in Fig. 2, the expression of LGALS3BP was largely increased in the tamoxifen resistant cells (Fig. 2B). Secretion of the LGALS3BP protein was validated by western blotting of the TCA-precipitated cell culture supernatants and ELISA assay (Fig. 2B and C). An immunofluorescence study also showed the increased expression of LGALS3BP in the cytosol of the tamoxifen-resistant cells (Fig. 2D).

Estrogen signaling regulates the expression of LGALS3BP in BC cells

Since the expression of LGALS3BP was largely increased in the tamoxifen-resistant BC cells in general, we questioned whether estrogen signaling was involved in the regulation of this gene. We observed that the protein and

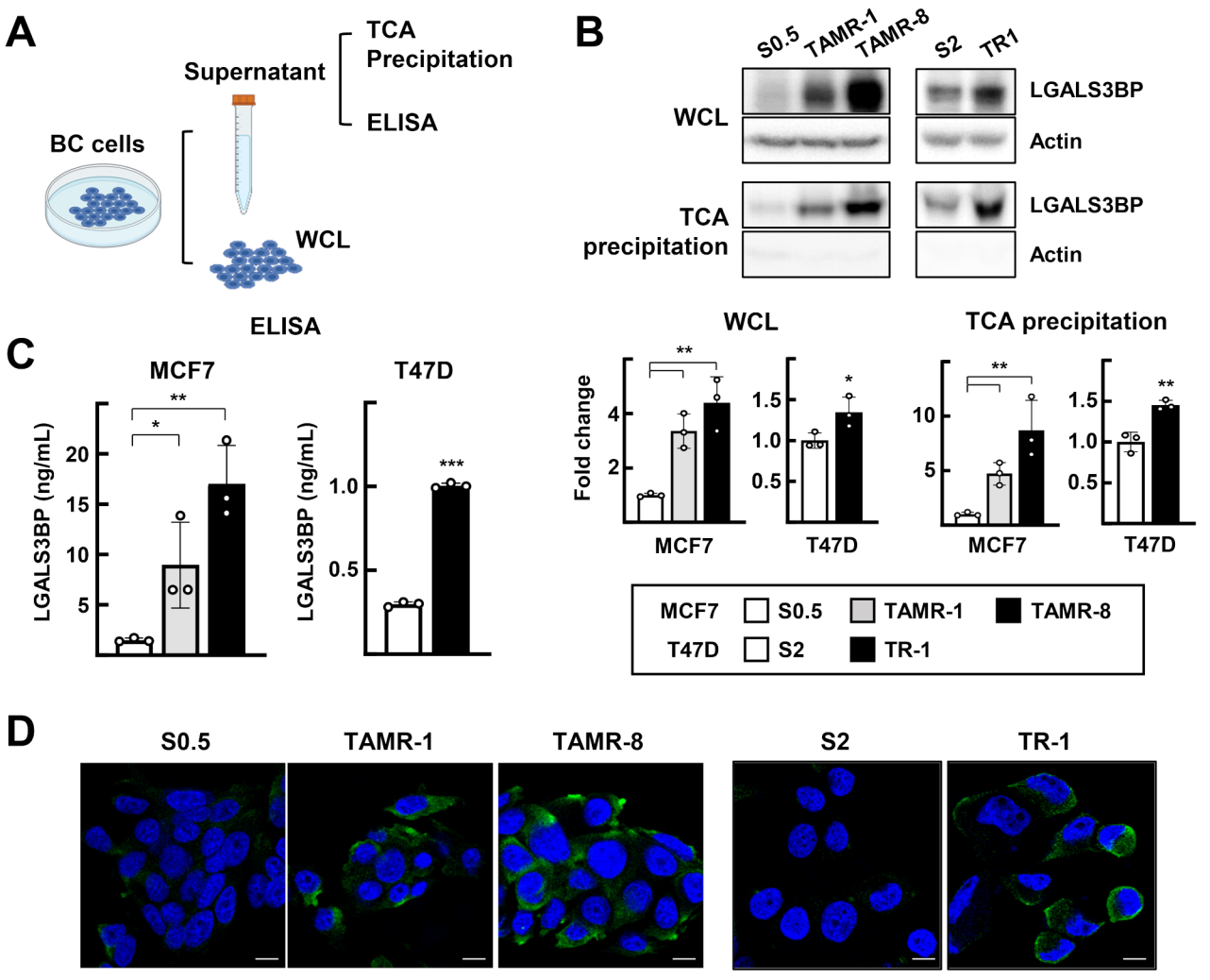

Fig. 2 Secretion of LGAL3BP increased from the MCF7/TAMR and T47D/TR cells. (**A**) Brief experimental scheme for Fig. 2. (**B**) Whole cell lysates obtained from the MCF7/TAMR and T47D/TR cells and their parental cells were subjected to western blotting (upper). The proteins in the culture supernatants were TCA precipitated and immunoblotted with anti-LGALS3BP antibodies (lower). Band intensity was quantified and normalized to the intensity of the corresponding β-actin band. (**C**) The amounts of LGALS3BP in the culture supernatants were measured by ELISA. (**D**) Expression of LGALS3BP was visualized by green immunofluorescence. The nuclei were stained with DAPI. Scale bars: 20 μm. Data presented as mean ± SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$, and ***, $P < 0.001$

mRNA expression levels of LGALS3BP were reduced after estradiol (E2) treatment in the MCF7/TAMR-1 and MCF7/TAMR-8 cells (Fig. 3A, B and Fig. S2A). Moreover, the reduced level of LGALS3BP was recovered by tamoxifen treatment, indicating that the expression of LGALS3BP was regulated by estrogen signaling (Fig. 3C, D and Fig. S2B). Transcription factor binding site prediction tools, CiiiDER and JASPAR, revealed a potential estrogen response element (ERE) upstream of the LGALS3BP transcription start site (Fig. 3E) [24, 25]. The results of the ChIP assay showed ER binding at the upstream region of LGALS3BP containing the ERE in response to E2 (Fig. 3E). The ER binding was twice as high in the MCF7/S0.5 cells than in the resistant cells. On the contrary, the levels of the transcription activation markers, both H3 lysine 4 trimethylation (H3K4me3) and H3 lysine 27 acetylation (H3K27ac), in this region were higher in the MCF7/TAMR-8 cells compared to the MCF7/S0.5 cells. E2 treatment resulted in a decrease in the levels of H3K4me3 and H3K27ac binding in this region, which was more substantial in the MCF7/TAMR-8 cells (Fig. 3E). These results indicated that estrogen signaling suppressed the expression of LGALS3BP.

Similarly, LGALS3BP secretion was increased in Let-R1, the MCF7 subline resistant to an aromatase inhibitor, letrozole. At both protein- and mRNA-levels in the MCF7/Let-R1 cells, the expression of LGALS3BP was increased when compared to their control cells (Fig. S3A, B and C). The increased mRNA and protein expression of LGALS3BP in the Let-R1 cells were decreased by E2

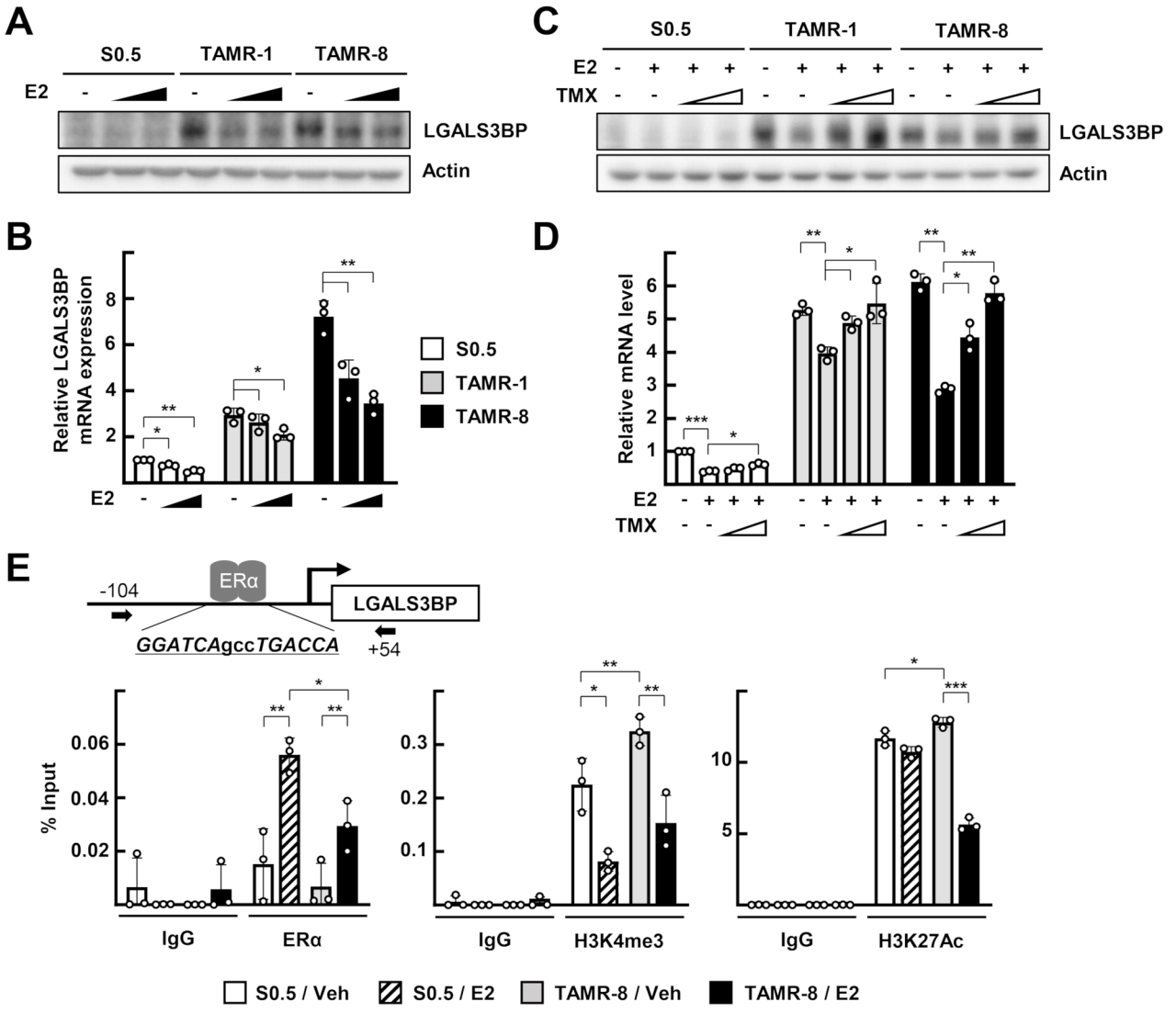

Fig. 3 Expression of LGAL3BP is regulated by E2 in the MCF7/TAMR cells. (**A** and **B**) The MCF7/TAMR cells and their parental cells were treated with 1 and 10 nM of E2 for 24 h. Whole cell lysates or total RNA were obtained and subjected to western blotting (A) or qPCR analysis (B), respectively. *, $P < 0.05$ and **, $P < 0.01$ ($n = 4$ for WB and $n = 3$ for qPCR). (**C** and **D**) The MCF7/TAMR cells and their parental cells were treated with 0.5 and 5 μM tamoxifen for 4 h prior to treatment with 10 nM E2. The cells were further incubated for an additional 24 h. At the end of incubation, whole cell lysates or total RNA were obtained and subjected to western blotting (C) or qPCR analysis ($n = 3$) (D), respectively. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$. (**E**) MCF7/S0.5 and MCF7/TAMR-8 cells were treated with 10 nM E2 or vehicle (100% EtOH). After 24 h, the cells were subjected to ChIP-qPCR analysis. Data were normalized as percentages relative to 1 μg input. Data presented as mean ± SD ($n = 3$). *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$

treatment (Fig. S3D and E). These results suggest that the induction of LGALS3BP may be a shared trait in the endocrine therapy resistant cells.

LGALS3BP supports the adhesion of TAMR cells and tube formation of HUVECs

To determine the role of secreted LGALS3BP in MCF7/TAMR cells, we established MCF7/TAMR-8 sublines, shLG, lacking LGALS3BP using letrovirus delivery system (Fig. S4A). Knockdown of LGALS3BP in MCF7/TAMR-8 cells did not affect proliferation of the cells (Fig. S4B). Previously, it was reported that LGALS3BP mediates the adhesion of cancer cells to neighboring cancer cells and to the ECM [26, 27]. Similarly, we observed that the MCF7/TAMR cells attached to the culture plates much faster than did the sensitive cells after seeding (Fig. S4C). A cell adhesion assay using fibronectin-coated cell culture plates showed that adherence capabilities of the MCF7/TAMR-1 and MCF7/TAMR-8 cells were significantly higher than that of the control cells (Fig. 4A). The shLG cells lost the adherence ability to the level of the control cells, but it was recovered by supplementing recombinant LGALS3BP protein in the culture media (Fig. 4A and Fig. S4D). Moreover, pre-incubating MCF7/

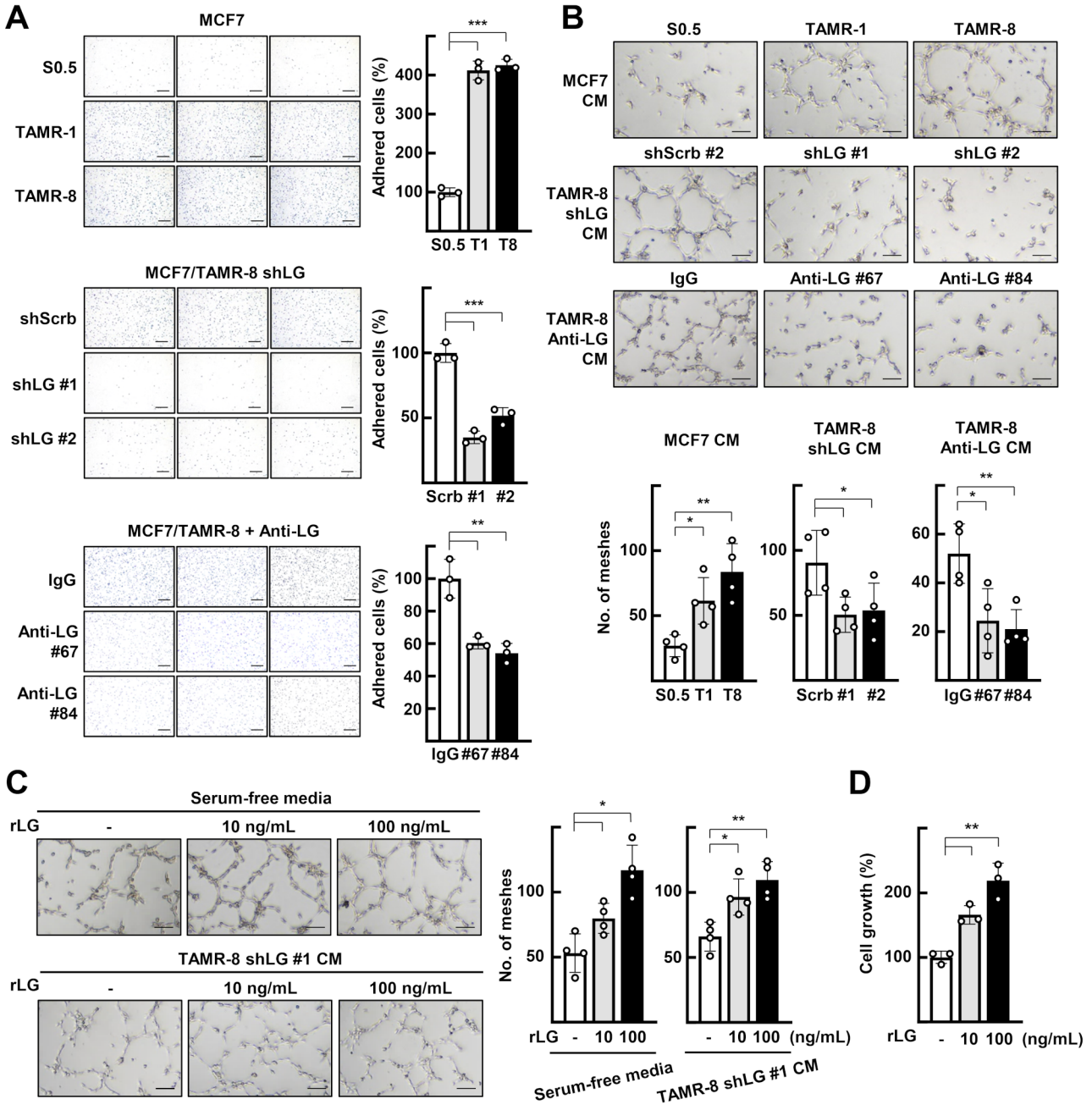

Fig. 4 LGALS3BP enhances cell adhesion of MCF7/TAMR cells and tube formation of HUVEC. (**A**) Attachment of the MCF7/TAMR, shLG, and their matched control cells was evaluated by staining with crystal violet 2 h after seeding (upper). Or MCF7/TAMR-8 cells were pre-treated with 1 μg/mL of anti-LGALS3BP antibodies or IgG control for 10 min before the adhesion assay was performed (lower). Adherent cells were quantified by dissolving them in MeOH before the absorbance at 570 nm was detected. Scale bars = 500 μm. (**B**) HUVECs were cultured in conditioned media obtained from MCF7/TAMR, shLG, and their matched control cells (upper). Or HUVECs were cultured in MCF7/TAMR-8 conditioned media treated with 1 μg/mL of anti-LGALS3BP antibodies or IgG control (lower). Tube formation was quantified by counting the number of meshes formed. Scale bars = 100 μm. (**C**) HUVECs were cultured in serum-free or conditioned media obtained from shLG #1 cells and treated with recombinant LGALS3BP protein as indicated. Tube formation was quantified by counting the number of meshes formed. Scale bars = 100 μm. rLG, recombinant LGALS3BP (**D**) HUVECs were treated with recombinant LGALS3BP protein as indicated. The cell growth was determined using MTT assay. Data presented as mean ± SD. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ ($n = 3$ for A and D and $n = 4$ for B and C)

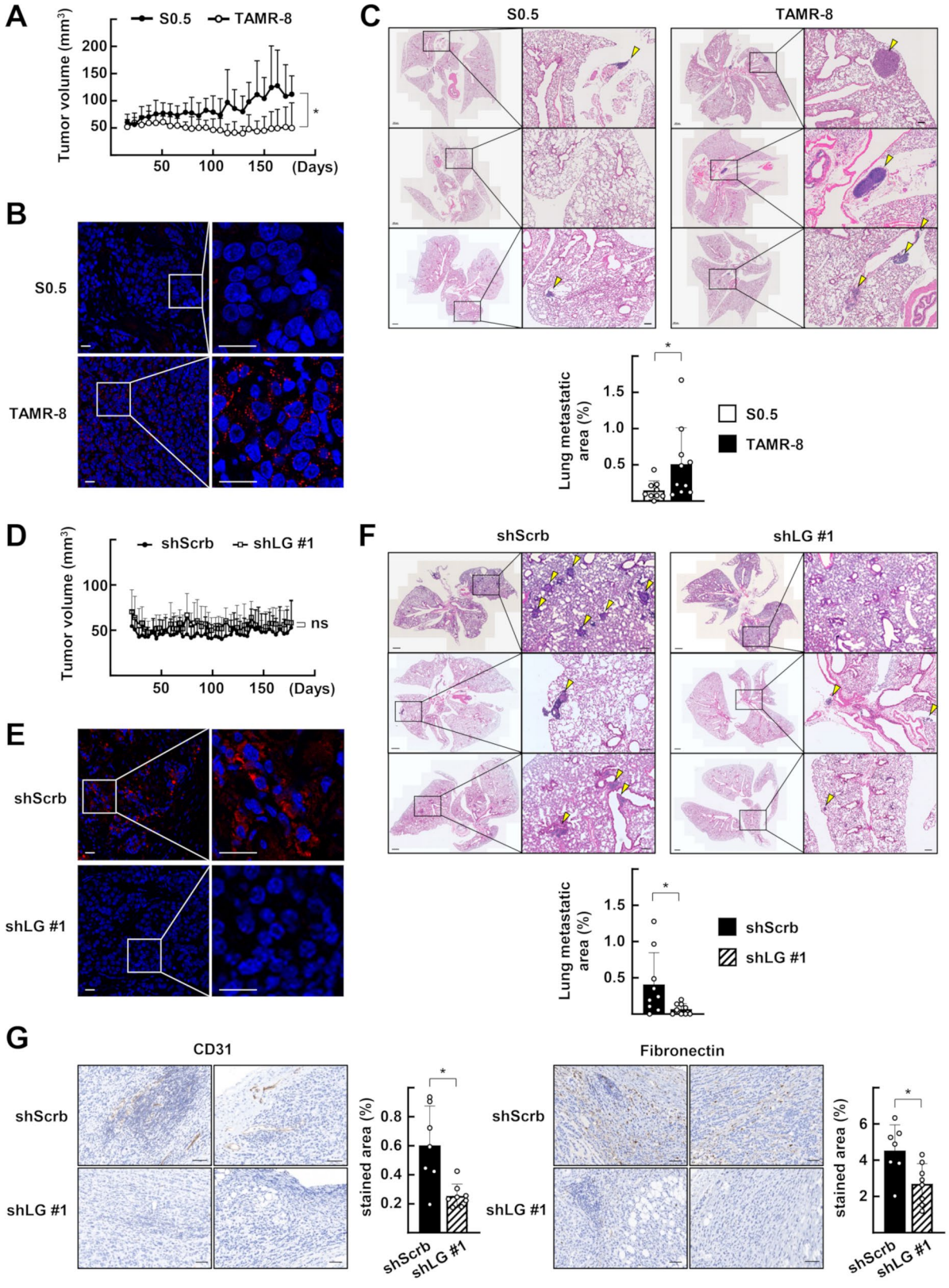

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Fig. 5 Depletion of LGALS3BP suppresses pulmonary metastasis of the MCF7/TAMR-8 in xenograft experiments. (**A**) MCF7/S0.5 or TAMR-8 cells were inoculated into the fourth mammary fat pads of female athymic nude mice. When the tumor volume reached approximately 50 mm^3, the tumor diameter was measured with a caliper ($n = 9$–10). (**B**) Representative images of LGALS3BP immunofluorescent staining in the primary tumor sections. Scale bars = 20 μm. (**C**) The spontaneous lung metastasis developed from the MCF7/TAMR cells and their parental cell xenografts. Hematoxylin and eosin staining of lung sections are shown. Arrowheads indicate metastatic lesions. Scale bars = 800 μm for full images and 200 μm for inserts. The area of metastatic lesions in the lungs were quantified using ImageJ. Data presented as mean ± SD ($n = 9$–10). *, $P < 0.05$. (**D**) MCF7/TAMR-8-shScramble or MCF7/TAMR-8-shLG cells were inoculated into the fourth mammary fat pads of female athymic nude mice. When the tumor volume reached approximately 50 mm^3, the tumor diameter was measured with a caliper. ($n = 9$) (**E**) Representative images of LGALS3BP immunofluorescent staining in the primary tumor sections. Scale bars = 20 μm. (**F**) The spontaneous lung metastasis developed from the shLG and its control cell xenograft. Hematoxylin and eosin staining of lung sections are shown. Arrowheads indicate metastatic lesions. Scale bars = 800 μm for full images and 200 μm for inserts. The area of the metastatic lesions in the lung was quantified using ImageJ. Data presented as mean ± SD ($n = 9$). *, $P < 0.05$. (**G**) Immunohistochemistry of CD31 or fibronectin of shScrb and shLG primary tumors are shown. Scale bars = 100 μm. At least two images of 2 mm^3 in size from each primary tumor sections were quantified using ImageJ. Data presented as mean ± SD ($n = 7$–8). *, $P < 0.05$

TAMR-8 cells with the neutralizing LGALS3BP antibodies significantly reduced the adhesion to fibronectin-coated culture plates (Fig. 4A).

Also, LGALS3BP has been shown to promote angiogenesis in cancers such as BC and melanoma [28, 29]. As expected, compared with the conditioned media from the control cells, the conditioned media from the MCF7/TAMR cells increased the tube formation of HUVECs by more than twice (Fig. 4B). The conditioned media from either the shLG cells or the neutralizing LGALS3BP antibody-treated parental cells showed significantly reduced activity in inducing tube formation when compared with that of the parental cells (Fig. 4B). Supplementation of recombinant LGALS3BP to the serum-free or the conditioned media from the shLG cells promoted tube formation of the HUVECs (Fig. 4C). Furthermore, the recombinant LGALS3BP treatment increased HUVEC cell growth, suggesting the role of LGALS3BP in new vessel formation (Fig. 4D). These data support the hypothesis that the secreted LGALS3BP from MCF7/TAMR cells functions in the adhesion of MCF7/TAMR cells to the ECM and angiogenesis of endothelial cells.

Pulmonary metastasis of the TAMR cells lacking LGALS3BP is less than that of parental cells in xenograft experiments

Next, we performed xenograft experiments with MCF7/S0.5 and MCF7/TAMR-8 cells by inoculating the cells in the mammary fat pads of athymic (nu/nu) BALB/c mice. These cells grew slowly, which is known as one of the characteristics of ER-positive BC cells, and reached approximately 100 mm^3 after 180 days of inoculation for both cell lines (Fig. 5A and Fig. S5A) [30]. An immunofluorescence study showed that LGALS3BP was expressed in primary xenograft tumors derived from MCF7/TAMR-8 cells but not in those derived from MCF7/S0.5 cells, indicating that these cells maintained the initial expression level of LGALS3BP (Fig. 5B and Fig. S5B). Surprisingly, we observed pulmonary metastasis in the MCF7/TAMR xenograft, but not in the control xenograft model at 180 days after tumor cell inoculation despite the slow growth rate (Fig. 5C). The loss of LGALS3BP in MCF7/TAMR-8 cells did not affect tumor growth or primary tumor size (Fig. 5D and E, Fig. S5C and D). However, pulmonary metastasis was not observed in the shLG xenografts (Fig. 5F). Next, vasculature of the primary xenograft tumors of shScrb and shLG was examined by immunohistochemistry. Both CD31- and fibronectin-stained areas were significantly reduced in the shLG primary tumors compared with those of the shScrb (Fig. 5G and Fig. S5E). These data suggest that the secreted LGALS3BP plays a critical role in the tumor angiogenesis and metastasis of TAMR BC cells.

LGALS3BP expression could predict the clinical breast cancer recurrence

To examine the clinical relevance of our findings, we performed immunohistochemistry on the paired primary and recurrent tumor tissue samples collected from ER-positive BC patients who had been treated with adjuvant tamoxifen and experienced relapse thereafter [22]. Although most tissue samples from both the primary tumors and the recurrent tumors were positive for LGALS3BP, we noticed a significant difference in the staining intensities between the primary tumor and the recurrent tumor. The recurrent tumor tissue samples exhibited strong LGALS3BP staining which showed significant increase compared to their matched primary tumor tissue samples (Fig. 6A). The large-scale public database analysis of tamoxifen-treated patients with ER-positive BC also supported the association between LGALS3BP expression and BC recurrence (Fig. 6B). Both the Kaplan-Meier plotter database which integrates multiple GEO datasets (GSE17705, GSE12093, GSE9195, GSE2990, and GSE45255), and the GSE9893 database analysis demonstrated poor relapse-free and distant-metastasis-free survival, respectively, in patients with ER-positive BC with high LGALS3BP expression. Taken together, these findings indicate that high LGALS3BP expression could be related to the long-term recurrence of ER-positive BC, which may be associated with tamoxifen resistance.

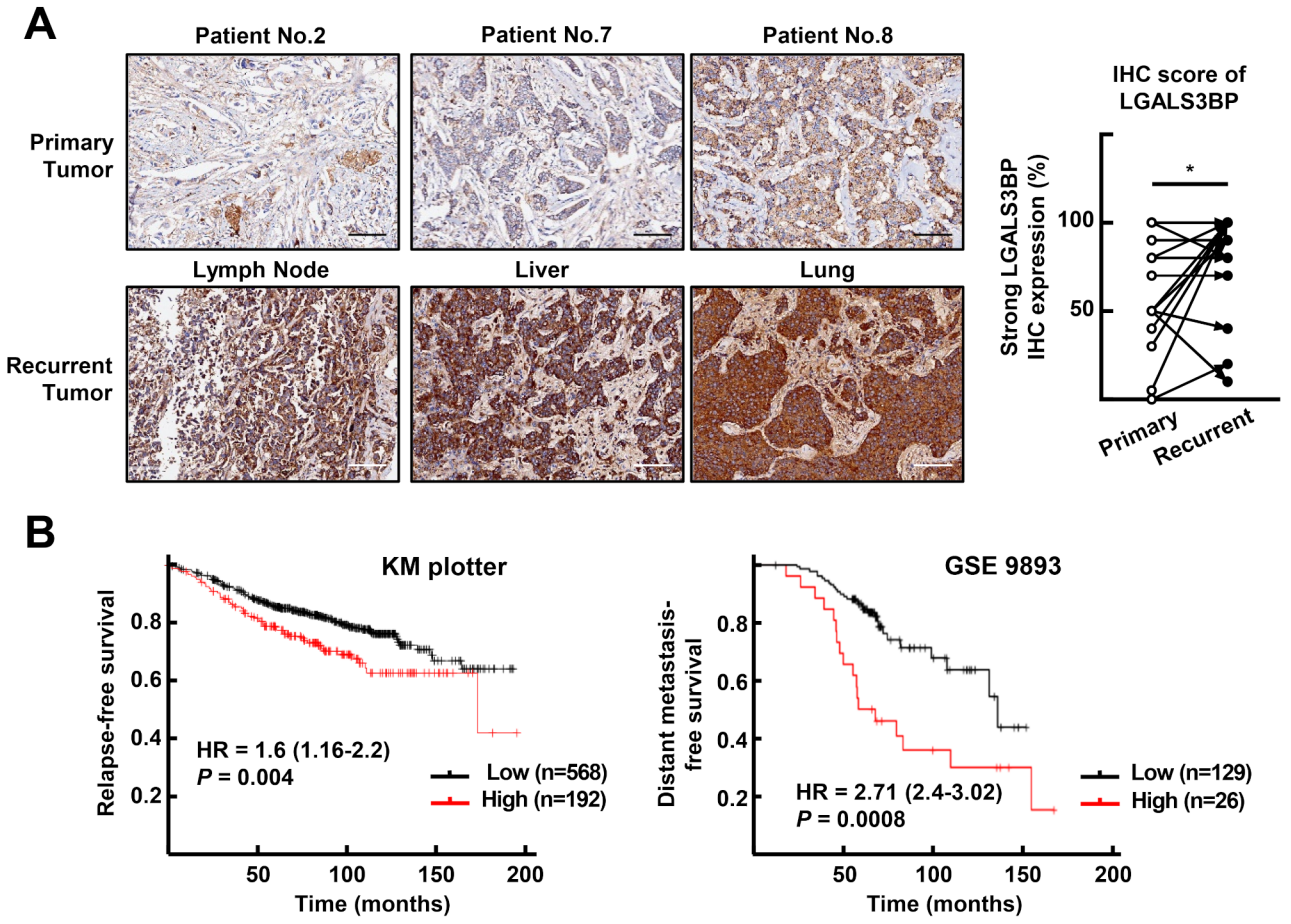

Fig. 6 Expression level of LGALS3BP is enhanced in the relapsed BC in clinic. (**A**) The expression level of LGALS3BP was evaluated by IHC in a total of 16 sets of primary and relapsed BC samples that obtained from patients with ER-positive BC. All patients received tamoxifen as adjuvant treatment [22]. Representative images for immunohistochemical staining of LGALS3BP in the human breast cancer specimens. LGALS3BP staining intensity was scored by a board-certified pathologist. The comparison was made between primary tumor and matching relapsed tumor. Scale bars = 100 μm. *$P < 0.05$. (**B**) Kaplan-Meier analyses (log-rank tests) for relapse-free and distant-metastasis free survival of patients with ER-positive BC treated with tamoxifen were performed using KM plotter (GSE17705, GSE12093, GSE9195, GSE2990, and GSE45255) and GSE9893 dataset. High and low groups were defined based on maximal cut-off values

Discussion

Here, we have successfully uncovered a profile of secretory proteins specifically expressed in the tamoxifen-resistant BC using the iSLET system. The proteins identified in the TAMR secretome had specialized functions in biological processes such as cell adhesion and angiogenesis which could facilitate distant metastasis (Fig. 1D). The TAMR secretome profile showed close similarity to the previously reported secretomes obtained from tamoxifen-resistant BC cells. A secretome analysis of the endocrine-resistant FOXA1-high MCF7 cells discovered gene products such as Golgi membrane protein 1, anterior gradient 2, and Dickkopf-1, which were found in our TAMR secretome [13]. In particular, the secretomes from the tamoxifen-resistant BC cells from our study and others shared common enriched biological processes such as ECM remodeling and wound healing [13, 31]. The cell type-specific labeling technique had also been applied in vivo to profile the secretome of PDAC cells. The results from this study showed that the secretome of the highly metastatic PDAC subtype was enriched with ECM constituents such as secreted protein acidic and cysteine rich, vascular endothelial growth factor C, and serpin family G member 1, which were also found in our TAMR secretome [17]. These results suggest that the secretome of TAMR BC cells may play a role in the ECM remodeling and cell adhesion that support metastasis. Further studies employing in vivo secretome analysis at different points of tamoxifen resistance progression would help to expand our understanding of the development of long-term and long-distance metastasis in patients with BC treated with tamoxifen.

LGALS3BP was one of the most upregulated proteins in the TAMR secretome (Fig. 2). LGALS3BP expression is reportedly increased in several types of cancers, such as triple-negative BC and PDAC, and LGALS3BP

functions in metastasis, invasion, and immune suppression during the progression of these diseases [21, 32, 33]. LGALS3BP secreted from the TAMR cells increased the adhesion of these cells to the ECM and induced proliferation and tube formation of the HUVECs (Fig. 4). Recently, it was reported that LGALS3BP secreted from retinal microglia and endometrial cancer increased both proliferation and tube formation of endothelial cells via the activation of Phosphatidylinositol 3-Kinase/Protein Kinase B (PI3K/AKT) signaling [34, 35]. Whether the PI3K/AKT signaling was involved in the action of LGALS3BP from tamoxifen-resistant breast cancer cells remains to be addressed. Next, LGALS3BP knockdown almost completely abrogated the pulmonary metastasis of TAMR cells in vivo (Fig. 5). These observations suggest that secreted LGALS3BP has a critical role in the pulmonary metastasis of TAMR cells. However, whether the secreted LGALS3BP affects tamoxifen-sensitivity of the tumor cells has not been evaluated in this study. Additional investigations on the role of LGALS3BP in tamoxifen-resistance is remained for better understating of significance of the TAMR secretome. Recently, the efficacy of anti-LGALS3BP antibody-drug conjugates was tested for their ability to induce cytotoxic effects on patient-derived xenograft tumors of adenoid cystic carcinoma and neuroblastoma [36, 37]. Considering the abundance of LGALS3BP expressed in tamoxifen-resistant BC cells, anti-LGALS3BP antibody-drug conjugates with neutralizing functions could reduce metastatic dissemination and attenuate residual tumors at the same time.

The long-term recurrence of ER-positive BC is attributed first to the increased metastatic potential of BC and second to the dormant characteristic of ER-positive BC, in which the tumor remains stable for years [4]. Mounting evidence suggests that the upregulation of adhesion molecules contributes to the awakening of dormancy, the final step in distant recurrence. For example, forced expression of E-cadherin was sufficient to awaken ER-positive BC xenograft cells from dormancy [30]. Similarly, the overexpression of VCAM-1 in xenograft BC cells promoted the growth of dormant micrometastases in bone [38]. Interestingly, a long-term treatment with tamoxifen or estrogen deprivation caused dormancy in MCF7 and T47D cells. Prolonged endocrine therapy in these cells induced the expression of adhesion molecules such as CD109 and integrin alpha-E and awoke them from dormancy, implicating the role of estrogen signaling in the awakening of ER-positive BC dormancy [39]. We also found that the expression of LGALS3BP was regulated in an estrogen-dependent manner through an estrogen response element present in the upstream promoter of LGALS3BP in TAMR cells (Fig. 3). Further, LGALS3BP was upregulated in the MCF7 cells resistant to letrozole, an aromatase inhibitor (Figure S3). LOXL2 and MFGE8, components of the TAMR secretome found in this study, were negatively correlated with histological ER expression in human BC patients (Fig. 1E) [40, 41]. Together, long-term treatment with tamoxifen induced the expression of cell adhesion molecules such as LGALS3BP in the TAMR secretome, which may support the awakening of dormant BC cells. Therefore, a therapeutic strategy to block the action of the cell adhesion proteins induced during prolonged tamoxifen treatment could prevent the reawakening of dormant ER-positive BC cells and distant metastasis, and thereby rescue BC patients from long-term disease conditions.

Conclusion

In this study, we identified proteins specifically secreted from tamoxifen-resistant breast cancer cells using a comprehensive secretome analysis. Among the many proteins within the TAMR secretome, increased LGALS3BP secretion increased adhesion of the TAMR cells to ECM and especially vasculature formation of HUVECs. Knockdown of LGALS3BP in tamoxifen-resistant breast cancer cells reduced pulmonary metastasis of these cells, suggesting a role of LGALS3BP in distant metastasis of TAMR cells.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Author contributions

SSK and MOL designed the study, interpreted the results, and wrote the manuscript (conceptualization, validation, writing). SSK, NLK, GYL, MYP, SH and JEK conducted the in vitro and in vivo experiments (investigation). IP, JK, JSK and HWR conducted peptide preparation and LC-MS/MS for TAMR secretome analysis (investigation, resources, analysis). SYP collected appropriate human breast cancer samples for analysis and conducted IHC (resources, investigation). SSK performed bioinformatic analysis of the clinical dataset (analysis). MOL supervised the research (supervision). All authors approved the final version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval

The animal experiments were performed in accordance with the guidelines of the Seoul National University Animal Care and Use Committee (SNU-210906-2-3 and SNU-240124-3).

Competing interests

The authors declare no competing interests.

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