Original Article

MiR-301b-3p targets and regulates EBF3 to impact the stem-like phenotype of breast cancer cells through glycolysis

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Background: Cancer stem cells are essential for the development of tumors, their recurrence, metastasis, and resistance to treatment. Previous studies have shown that the silencing of EBF3 promotes the progression of malignant tumors, but its impact on the stem-like phenotype of tumor cells remains unexplored. Therefore, this work aims to investigate the influence of EBF3 on the stem-like phenotype of breast cancer (BC) cells and its underlying molecular mechanisms. Methods: Bioinformatics analysis was utilized to predict EBF3 and miR-301b-3p expression and their binding sites in BC tissues. qRT-PCR was conducted to assess EBF3 and miR-301b-3p expression in BC cells. Cell viability was assessed using CCK-8 assay, while sphere-forming ability was assayed by sphere formation experiments. Western blot analysis was employed to assess the expression of stem cell-related markers and proteins associated with the glycolysis metabolic pathway. ECAR experiments and analysis of glycolysis metabolite production were performed to evaluate cellular glycolysis capacity. Dual-luciferase reporter assays and RIP were utilized to validate the binding relationship between EBF3 and miR-301b-3p. Results: EBF3 was downregulated in BC tissues and cells, and overexpression of EBF3 repressed the glycolysis capacity of BC cells, thereby suppressing stem-like phenotype. Furthermore, miR-301b-3p was identified as a direct target of EBF3, and its expression was increased in BC. Cell experiments revealed that miR-301b-3p suppressed EBF3 expression, thereby promoting the glycolysis capacity and stem-like phenotype of BC cells. Conclusion: miR-301b-3p enhanced glycolysis and promoted the stem-like phenotype of BC cells by targeting EBF3. These findings can offer new therapeutic approaches for BC.

***Key Words*: EBF3, miR-301b-3p, glycolysis, breast cancer, stem-like phenotype**

The most prevalent kind of cancer in the world is breast cancer (BC). According to 2020 statistics, there were 2.26 million cases of BC, with 685,000 deaths. BC is the main reason for women's cancer-related deaths globally.[1] The therapeutic modalities for BC include chemotherapy, radiotherapy, targeted therapy, immunotherapy, as well as pre- and post-operative endocrine therapy.[2] However, even with adjuvant chemotherapy, the five-year survival rate for metastatic BC remains below 30%.[3] Recent advances in BC research, particularly in areas of cancer stem cells (CSCs),[4] cancer metabolism,[5] and the tumor microenvironment,[6] have increased our understanding of resistance and metastasis, highlighting the significance of targeting CSCs.[7]

CSCs have the capacity for self-renewal and multi-lineage differentiation. CSCs possess strong self-renewal capacity and are critical in the development of tumors. Additionally, CSCs can differentiate into varying cell types.[8] Even while BC stem cells (BCSCs) only make up a small portion of the tumor population, they have a profound impact on tumor initiation, recurrence, metastasis, and treatment resistance.[9] Targeting CSCs has emerged as a hotspot in BC treatment, providing a complementary approach to traditional chemotherapy that fails to eradicate tumor dissemination and metastasis.[10,11] In a review by Pan *et al.*,[12] the authors described previous reports on the therapeutic effects of targeting BCSCs. The combination of drugs targeting CSCs and paclitaxel, a drug targeting cancer cells, synergistically enhances cytotoxic effects in BC cells.[13] Zhang *et al.*[14] proposed that by modulating BCSC-associated genes, for instance, salinomycin can induce BCSC differentiation into normal cells, quiescent cells, and mature cancer cells. The mature cancer cells can then be targeted and killed by chemotherapy drugs, achieving the goal of treating BC. Furthermore, recent studies have indicated that tumor stem cells undergo metabolic alterations, including reduced mitochondrial respiration and increased glycolysis activity. For example, Liu *et al.*[15] discovered that overexpression of MnSOD activates the CaMKII/AMPK pathway and enhances glycolysis to promote the stemness of H460 cells derived from non-small cell lung cancer. Chen *et al.*[16] proposed that HBx induces BNIP3L-dependent mitochondrial autophagy, upregulating glycolysis metabolism and increasing the cancer stemness of liver cancer cells *in vivo* and *in vitro*. However, the impact of glycolysis on stem-like phenotype of BC cells is still largely unexplored.

Four members make up the highly conserved family of DNA-binding transcription factors known as early B-cell factors (EBFs). They possess an unusual zinc finger and helix-loop-helix motif and control the expression of many downstream target genes by binding DNA.[17] Studies have shown that EBF3 serves as a suppressor in varying cancers and suppresses metastasis.[18–20] For instance, Tao *et al.*[17] pointed out that increased EBF3 inhibits the proliferation of acute myeloid leukemia cells and enhanced apoptosis. Kim *et al.*[21] demonstrated that EBF3 hampers cell growth and migration of gastric cancer and is an independent prognostic marker. The impact of EBF3 on cancer progression has gradually been reported. However, its effect on the stem-like phenotype of BC cells remains unclear. Therefore, understanding the molecular mechanisms of EBF3 in modulating the stem-like phenotype of BC cells holds significant value for revealing novel strategies for future clinical treatments.

In this project, we confirmed the repressive impact of EBF3 on the stem-like phenotype of BC cells. Further investigation revealed that EBF3 suppressed BC cell stem-like phenotype

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Table 1. Primers used in qRT-PCR

Gene		Sequence
EBF3	Forward Primer	5'-CGAGAAAACCAACAACGGCATC-3'
	Reverse Primer	5'-ATGATTACAGGGTCTGAGGGCG-3'
miR-301b-3p	Forward Primer	5'-CAGTGCTCTGACGAGGTTG-3'
	Reverse Primer	5'-TGTCCCAGATGCTTTGACA-3'
GAPDH	Forward Primer	5'-GACCCCTTCATTGACCTCAAC-3'
	Reverse Primer	5'-CTTCTCCATGGTGGTGAAGA-3'
U6	Forward Primer	5'-CTCGCTTCGGCAGCACATA-3'
	Reverse Primer	5'-AACGATTCACGAATTTGCGT-3'

through the regulation of cellular glycolysis. We validated that the addition of 2-DG reversed the promotion of glycolysis and stem-like phenotype of BC cells by sh-EBF3. Moreover, miR-301b-3p was an upstream regulator of EBF3. Increased miR-301b-3p suppressed expression of EBF3 and enhanced stem-like phenotype of BC cells through glycolysis activation. Our findings generated new insights into the role of miR-301b-3p/EBF3 in BC stem-like phenotype, providing a novel therapeutic avenue for BC.

Materials and Methods

Bioinformatics. mRNA (Normal: 113, Tumor: 1113) and miRNA (Normal: 104, Tumor: 1103) expression data were available for download at The Cancer Genome Atlas (TCGA) database. Differential analysis was conducted on miRNAs to identify significantly differentially expressed miRNAs (DEmiRNAs) using the criteria |logFC|>1 and padj<0.05. Target gene EBF3 was determined based on previous studies.[22] The upstream miRNAs of EBF3 were predicted using starbase (https://starbase.sysu.edu.cn/index.php) and miRDB (http://mirdb.org/) databases. Intersection of DEmiRNAs with the predicted upstream miRNAs was obtained and analyzed using the UpSet R package. Pearson correlation analysis was conducted to screen the upstream gene, miR-301b-3p. GSEA was used for the enrichment analysis of EBF3.

Cell culture. Human breast epithelial cells 184A1, BC cells (MDA-MB-468, MDA-MB-231, and sk-br-3), and embryonic kidney cells 293T were obtained from ATCC (Manassas, VA). 184A1 cell line was cultured in MEBM medium (Lonza, Basel, Switzerland), MDA-MB-468 and MDA-MB-231 cells were kept in Leibovitz's L-15 medium (Procell, Wuhan, China), sk-br-3 cells were in McCoy's 5A medium (Procell), and 293T cells were in DMEM (Gibco, Grand Island, NY). The culture media were added with 10% fetal bovine serum (FBS) and final concentrations of 0.1 mg/ml streptomycin and 100 U/ml penicillin (Solarbio, Beijing, China). Cells were cultured at 37°C with 5% CO_2.

Cell transfection. Plasmids vector pcDNA3.1 for overexpressing EBF3 (oe-EBF3), knocking down EBF3 (sh-EBF3) (5'-GCACAACAATTCCAAACACGG-3'), and miR-301b-3p mimic, and negative controls, were accessed from Ribobio (Guangzhou, China). Cells were plated in a 24-well plate (approximately 2×10^4 cells/well) and transfected with the corresponding plasmids by Lipofectamine 3000 (Invitrogen, Carlsbad, CA). Transfection efficiency was assessed after 48 h.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated from cultured cells by TRIzol (Invitrogen), and cDNA was synthesized by PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Kusatsu, Japan). qPCR was done on the ABI 7500 Sequence Detection System with SYBR® Green Realtime PCR Master Mix (Toyobo, Osaka, Japan). GAPDH was utilized as an internal reference for EBF3, and U6 for miR-301b-3p. Relative levels of target genes were calculated by $2^{-\Delta\Delta CT}$. Primers used are listed in Table 1.

CCK-8 assay. BC cells transfected with oe-EBF3, sh-EBF3, miR-301b-3p mimic, and treated with glycolysis inhibitor 2-deoxy-D-glucose (2-DG) (MCE, Monmouth Junction, NJ), 3-Bromopyruvate (3-BP) (Merck, Darmstadt, Germany) were plated into a 96-well plate (100 µl/well medium). At 0, 24, 48, 72, and 96 h of incubation, each well was treated with 10 µl of CCK-8 solution, followed by a 2-h incubation. Absorbance at 450 nm wavelength was assessed by a microplate reader.[23]

Sphere formation assay. Single-cell suspensions were plated into a 96-well ultra-low attachment plate (1,000 cells/well) in serum-free medium containing 2% B-27 (Invitrogen), 20 ng/ml EGF (MCE), 20 ng/ml bFGF (Invitrogen), and 10 µg/ml heparin (Sigma, St. Louis, MO). After 10 days-culture, the spheres were observed and pictured with an inverted microscope, and the longest diameter was computed using Image-Pro Plus software.[24]

Western blot (WB). Cellular proteins were isolated using pre-cooled RIPA lysis buffer containing a protease inhibitor (Beyotime, Shanghai, China). Protein concentration was quantified by a BCA protein assay kit (Beyotime). Equal amounts of protein were separated by 10% SDS-PAGE gel electrophoresis and transferred onto a PVDF membrane. The membrane was sealed with 5% skim milk for 1 h and then incubated at 4°C overnight with primary antibodies: rabbit anti-human CD133, CD44, Oct-4, HK2, PFKP, PKM2, and GAPDH antibodies (Abcam, Cambridge, UK). Following rinsing, the membrane was further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Abcam), for 2 h. Protein bands were assessed with an ECL kit (Millipore, Billerica, MA) and a fluorescence and chemiluminescence imaging system (Clinx, Shanghai, China).[22]

Extracellular acidification rate (ECAR) measurement. ECAR was assessed by a Seahorse XFe96 analyzer (Seahorse, Billerica, MA). BC cells transfected with the respective plasmids were seeded into an XFe96 cell culture microplate (2×10^4 cells/well) and incubated with a base medium containing 96 mM glutamine (pH 7.4). Glucose (10 mM), oligomycin (1 mM), and 2-DG (50 mM) were added in order, and ECAR was assessed by Seahorse XFe96 analyzer.[25]

Pyruvic acid, lactate, and ATP levels. The Pyruvic Acid Kit (Cat. No. BC2205), Lactate Kit (Cat. No. BC2230), and ATP Kit (Cat. No. BC0300) were all purchased from Solarbio. The levels of the relevant biomarkers were measured according to the instructions provided with the kits.

Dual-luciferase reporter gene assay. Cells were plated in a 24-well plate and co-transfected with pmirGLO-EBF3-3'-UTR-WT and pmirGLO-EBF3-3'-UTR-MUT luciferase reporter vectors constructed with Lipofectamine 2000 (Invitrogen), as well as mimic-miR-301b-3p/mimic-NC. Following 48 h of transfection, the fluorescence activity was assessed with a Dual-Luciferase Reporter Assay Kit (MCE).[26]

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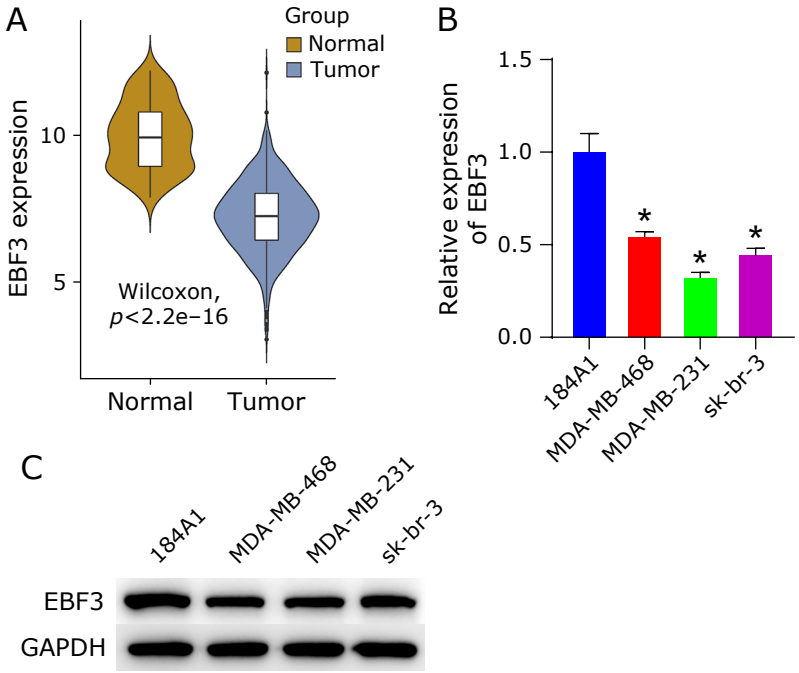

Fig. 1. Low expression of EBF3 in BC tissues and cells. (A) EBF3 expression in BC tissues. (B, C) EBF3 mRNA and protein expression in human breast epithelial cells and human BC cells detected by qRT-PCR and WB. * indicates $p<0.05$.

RNA immunoprecipitation (RIP). The sk-br-3 cells were transfected with miR-301b-3p mimic or NC mimic. RIP was performed with RIP Kit (Enzyme-linked, Shanghai, China). The cell lysate was incubated with magnetic beads coupled with Ago2 or IgG at 4°C for 4 h. Precipitates were harvested for further analysis by qRT-PCR.[27]

Statistical analysis. For bioinformatics analysis, differential analysis was conducted using the "edgeR" package. Pearson correlation analysis was performed, and abnormally distributed data were analyzed by the Wilcoxon test. In experimental analysis, data were presented as mean ± SD. Prism software was utilized for data analysis. The *t* test was utilized to analyze data between two groups, and one-way ANOVA was utilized to analyze data from multiple groups (>2). A $p<0.05$ was deemed statistically significant.

Results

Downregulation of EBF3 in BC. Previous research has found that EBF3 is a repressor that represses the malignant progression of cancer cells.[22] To dissect the modulatory role of EBF3 in BC, we analyzed EBF3 expression in BC tissues using bioinformatics analysis. We found a significant downregulation of EBF3 in BC tissues (Fig. 1A). qRT-PCR further examined EBF3 expression in BC cells. EBF3 expression was significantly lower in BC cells than in normal breast epithelial cells (Fig. 1B). The WB uncovered that the expression of EBF3 protein in BC cells was considerably lower than that in normal breast epithelial cells (Fig. 1C). These results indicated a specific downregulation of EBF3 in BC.

EBF3 inhibits the stem-like phenotype of BC cells. The impact of EBF3 on BC cell stem-like phenotype was assayed. Pearson correlation analysis reported a substantial negative correlation between EBF3 expression in BC and the tumor stemness index mRNAsi (mRNAsi based on previous research[28]) (Fig. 2A). To examine the possible role of EBF3 in the stem-like phenotype of BC cells, we transfected sh-EBF3 into MDA-MB-468 cells and oe-EBF3 into MDA-MB-231 cells. Transfection efficiency was verified by qRT-PCR, which presented decreased expression of EBF3 in sh-EBF3-transfected MDA-MB-468 cells and increased expression of EBF3 in oe-EBF3-transfected MDA-MB-231 cells (Fig. 2B and C). Furthermore, WB also demonstrated that sh-EBF3 reduced the expression of EBF3 protein in MDA-MB-468 cells, while oe-EBF3 enhanced the expression of EBF3 protein in MDA-MB-231 cells (Fig. 2D). The CCK-8 detection revealed that sh-EBF3 remarkably increased the proliferation ability of MDA-MB-468 cells (Fig. 2E), while oe-EBF3 substantially reduced the proliferation ability of MDA-MB-231 cells (Fig. 2F). The influence of EBF3 on the sphere-forming ability of BC cells was evaluated using sphere formation experiments. sh-EBF3 notably enhanced sphere-forming ability of MDA-MB-468 cells (Fig. 2G), whereas oe-EBF3 markedly reduced sphere-forming ability of MDA-MB-231 cells (Fig. 2I). WB of stem cell markers (CD133, CD44, and Oct-4) showed substantially increased expression in sh-EBF3-treated MDA-MB-468 cells and decreased expression in oe-EBF3-treated MDA-MB-231 cells (Fig. 2H and J). Our analysis indicated that overexpression of EBF3 could suppress the stem-like phenotype of BC cells.

EBF3 inhibits stem-like phenotype of BC cells through glycolysis pathway. To probe into the mechanism of EBF3 in regulating the stem-like phenotype of BC cells, we performed GSEA of EBF3 and found great enrichment in the glycolysis/gluconeogenesis metabolic pathway (Fig. 3A). Glycolysis facilitates stemness of cancer cells.[24,29] Therefore, we examined the expression of glycolysis pathway-related proteins in BC cells. WB demonstrated that oe-EBF3 considerably repressed HK2, PFKP, and PKM2 levels in MDA-MB-231 cells (Fig. 3B). Seahorse assay was employed to assess metabolic changes in EBF3-overexpressing MDA-MB-231 cells. ECAR revealed a notable reduction in the glycolysis capacity of EBF3-overexpressing MDA-MB-231 cells (Fig. 3C). Subsequently, we measured levels of glycolysis metabolites to investigate the influence of oe-EBF3 on the metabolic profile of BC cells. Levels of pyruvic acid, lactate, and ATP were considerably lower in the oe-EBF3 group than in the control group (Fig. 3D). To further validate the impact

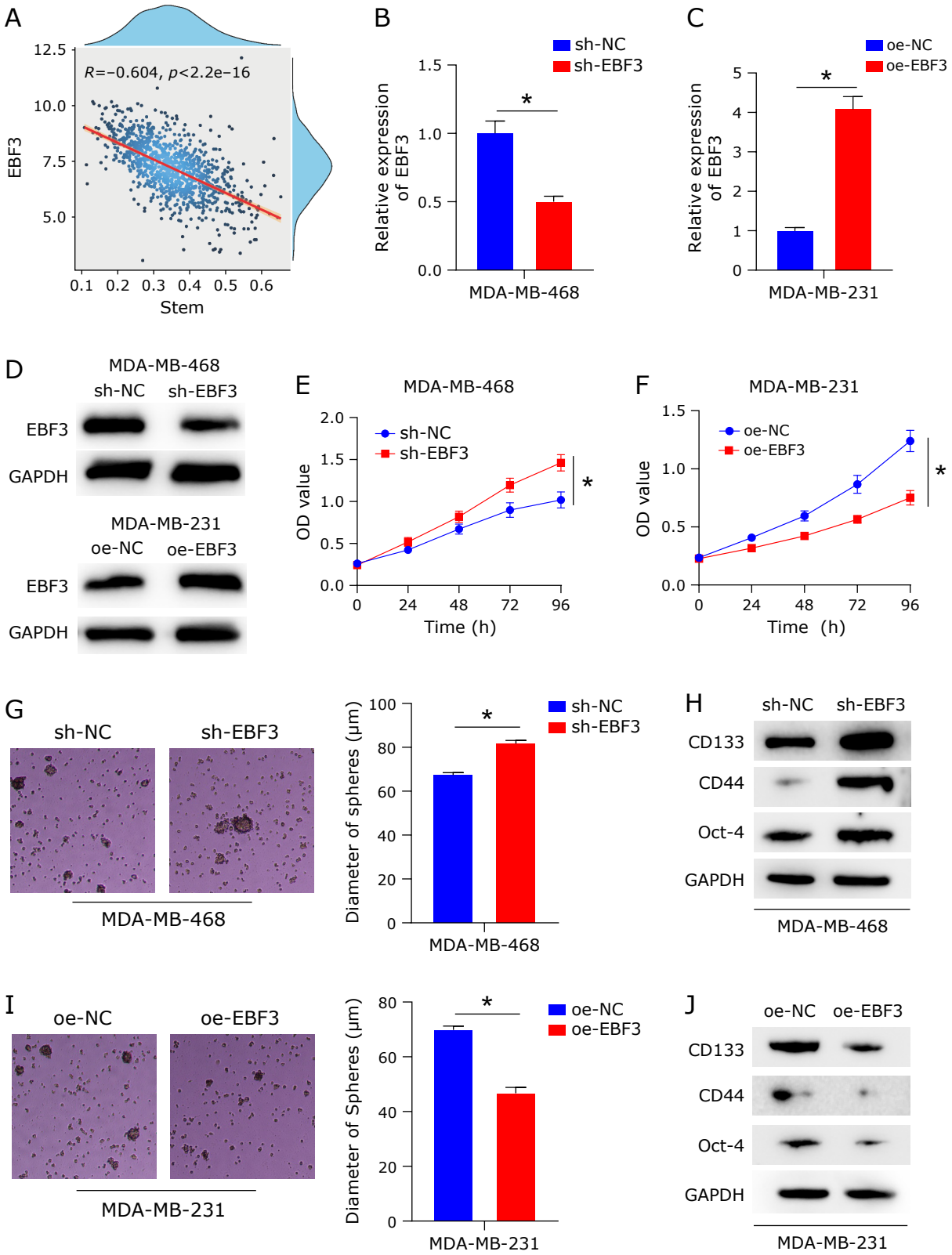

Fig. 2. EBF3 inhibits the stem-like phenotype of BC cells. (A) Correlation between EBF3 and tumor stemness index (mRNAsi). (B, C) Transfection efficiency detected by qRT-PCR. (D) EBF3 protein expression analyzed by WB. (E, F) Cell viability detected by CCK-8 assay. (G, I) Sphere formation assay tested sphere-forming ability. (H, J) WB detected stem cell markers. * indicates $p<0.05$.

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of EBF3 on the glycolysis capacity of BC cells, we set up sh-NC + PBS, sh-EBF3 + PBS, sh-EBF3 + glycolysis inhibitor (2-DG or 3-BP) treatment groups. The CCK-8 detection uncovered that sh-EBF3 considerably increased the proliferation ability of MDA-MB-468 cells, while the addition of 2-DG or 3-BP attenuated the promoting effect of sh-EBF3 on the proliferation ability of cells (Fig. 3E). The sphere-forming ability of cells was assessed by sphere formation experiments. The results demonstrated that sh-EBF3 significantly promoted the sphere-forming ability of MDA-MB-468 cells, while the addition of 2-DG or 3-BP attenuated promoting effect conferred by sh-EBF3 on the sphere-forming ability of BC cells (Fig. 3F). WB results also demonstrated that the addition of glycolysis inhibitors 2-DG or 3-BP weakened the upregulation of sh-EBF3 knockdown on BC cells and the expression of stem cell markers (CD133, CD44, and Oct-4) (Fig. 3G). Here, we unearthed that EBF3 may inhibit BC cell stem-like phenotype by inhibiting glycolysis.

miR-301b-3p targets and binds to EBF3. To delineate the mechanism of EBF3 in BC progression, we predicted upstream regulatory genes of EBF3 and intersected the predicted results with upregulated DEmiRNAs, resulting in 11 DEmiRNAs (Fig. 4A). Pearson correlation analysis was conducted between EBF3 and these 11 DEmiRNAs, revealing a notable negative correlation of hsa-miR-301b-3p with EBF3 (Fig. 4B). In the bioinformatics database, the predicted binding sequences of hsa-miR-301b-3p and EBF3 were found, indicating the presence of binding sites between them (Fig. 4C). Therefore, we believed that hsa-miR-301b-3p was an upstream regulatory gene of EBF3. Next, we analyzed the hsa-miR-301b-3p level in BC tissues. Bioinformatics analysis uncovered considerably higher miR-301b-3p levels in BC tissues (Fig. 4D). qRT-PCR was conducted to validate miR-301b-3p level in BC cells, confirming bioinformatics analysis results (Fig. 4E). To verify the binding relationship between miR-301b-3p and EBF3, a dual-luciferase reporter assay was performed. Luciferase activity of the EBF3-WT group was remarkably reduced in 293T cells overexpressing miR-301b-3p, while there was no great difference in the luciferase activity of the EBF3-MUT group (Fig. 4F), confirming the binding relationship of miR-301b-3p with EBF3. Additionally, the results of the RIP experiment showed significant enrichment of EBF3 in the miR-301b-3p-mimic group (Fig. 4G). Our study demonstrated that EBF3 was a direct downstream target of miR-301b-3p.

miR-301b-3p downregulates EBF3 to promote BC cell stem-like phenotype by mediating glycolysis. Earlier research has demonstrated the role of miR-301b-3p in promoting cell proliferation and migration in various cancers.[30–32] To validate the regulation of miR-301b-3p on EBF3, we constructed cell groups based on the MDA-MB-231 cell line: mimic-NC + oe-NC, miR-301b-3p-mimic + oe-NC, and miR-301b-3p-mimic + oe-EBF3. qRT-PCR was used to assess the influence of miR-301b-3p on EBF3 expression. Overexpression of miR-301b-3p considerably inhibited EBF3 level compared to the control, while further upregulation of EBF3 reversed this inhibitory effect (Fig. 5A). The results of WB also showed that compared with the control group, overexpression of miR-301b-3p strikingly inhibited the protein expression of EBF3, and further overexpression of EBF3 could reverse the inhibitory effect of miR-301b-3p overexpression on EBF3 protein expression (Fig. 5B). The CCK-8 detection demonstrated that overexpression of miR-301b-3p greatly increased the proliferation ability of MDA-MB-231 cells, while EBF3 overexpression could reverse the promoting effect of miR-301b-3p overexpression on cell proliferation (Fig. 5C). Cell sphere-forming ability was assessed using sphere formation experiments, which revealed that the group with miR-301b-3p overexpression exhibited significantly higher cell sphere-forming ability than the control group, while further overexpression of EBF3 weakened the stimulatory effect of overexpression of miR-301b-3p on cell sphere-forming ability (Fig. 5D). WB examined levels of glycolysis-related proteins. Upregulation of miR-301b-3p considerably promoted HK2, PFKP, and PKM2 levels in cells, while further overexpression of EBF3 attenuated this promoting effect (Fig. 5E). In addition, ECAR measurement was conducted to investigate the influence of miR-301b-3p overexpression on the glycolysis capacity of BC cells. Results indicated that the upregulation of EBF3 weakened the promotive effect of miR-301b-3p overexpression on glycolysis (Fig. 5F). Furthermore, we measured the levels of pyruvic acid, lactate, and ATP in the cells to investigate the impact of miR-301b-3p overexpression on BC cell metabolism. Levels of pyruvic acid, lactate, and ATP were remarkably elevated in the group with miR-301b-3p overexpression than the control, suggesting that overexpression of miR-301b-3p greatly enhanced the metabolic capacity of BC cells, while further overexpression of EBF3 attenuated this promoting effect (Fig. 5G). Finally, we assessed the expression of stem cell markers. WB presented that upregulation of miR-301b-3p upregulated CD133, CD44, and Oct-4 expression, while overexpression of EBF3 weakened this upregulating effect (Fig. 5H). These findings unveiled that miR-301b-3p downregulated EBF3 to mediate the glycolysis pathway and promote BC cell stem-like phenotype.

Discussion

BC is a common malignancy that primarily affects the epithelial tissue of mammary glands, significantly impacting the physical and mental health of women and even posing a threat to their lives. The incidence of BC accounts for approximately 7–10% of all malignant tumors in the body.[33] Due to the absence of treatments that are successful across all subtypes, BC has become a global health concern. As the primary source of cancer cell heterogeneity, mounting data shows that CSCs are essential for tumor development, metastasis, recurrence, and therapy resistance.[34] The transcription factor EBF3 serves as a tumor repressor and is critical in tumor cell growth, proliferation, and apoptosis.[35] For instance, in esophageal squamous cell carcinoma, downregulation of EBF3 expression and induction of cell proliferation and metastasis were observed through the inhibition of EBF3 by miR-23b-3p.[22] Our study yielded similar results, demonstrating a significant downregulation of EBF3 in BC cells and the ability of EBF3 overexpression to suppress BC cell activity. Additionally, database analysis revealed a negative correlation between EBF3 and the stemness index. Further functional cell experiments confirmed that EBF3 considerably inhibited BC cell stem-like phenotype. Our research reported the inhibitory effect of EBF3 on the BC cell stem-like phenotype, filling a gap in the study of EBF3 in the BC stem-like phenotype.

To dissect the relationship of EBF3 with BC cell stem-like phenotype, we conducted a GSEA pathway enrichment analysis on the EBF3 gene, revealing its involvement in the regulation of glycolysis/gluconeogenesis metabolic pathways. Aerobic glycolysis (Warburg effect) is the basis for various biological characteristics of cancer cells.[36] Zhou *et al.*[37] found that ZEB1 enhances glycolysis by transcriptionally activating PFKM, promoting hepatocellular carcinoma occurrence and metastasis. Hou *et al.*[38] reported that miR-30d inhibits aerobic glycolysis and pancreatic tumor formation by directly targeting RUNX1, which binds to SLC2A1 and HK1 promoters and manipulates their expression. We found that EBF3 overexpression suppressed glycolysis capacity in MDA-MB-231 cells, leading to decreased expression of glycolysis products such as pyruvic acid, lactate, and ATP. Moreover, some investigations reported the role of glycolysis in cancer cell stemness and its molecular modulatory mechanisms. For example, Gao *et al.*[39] discovered that CD44ICD promotes BC stem cell characteristics through PFKFB4-mediated glycolysis. Zhu *et al.*[40] revealed that ETV4 promotes glycolysis

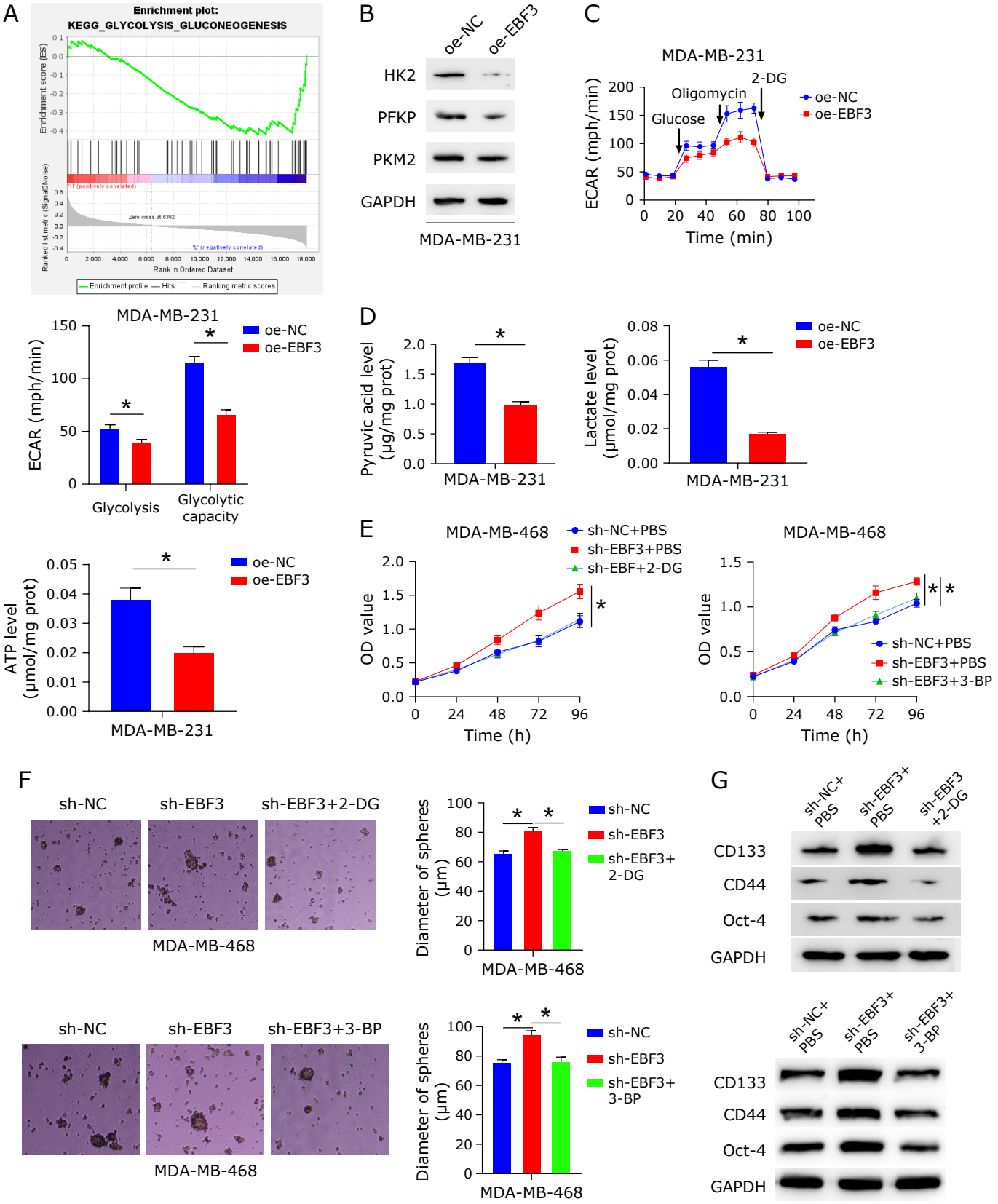

Fig. 3. EBF3 represses stem-like phenotype of BC cells through cellular glycolysis. (A) GSEA pathway enrichment analysis results. (B) WB detected proteins related to glycolysis pathway. (C) BC ECAR detection in different treatment groups. (D) Levels of pyruvic acid, lactate, and ATP in BC cells of different groups. (E) Cell viability assessed via CCK-8. (F) Sphere formation assay tested sphere-forming ability. (G) WB detected stem cell markers. * indicates $p<0.05$.

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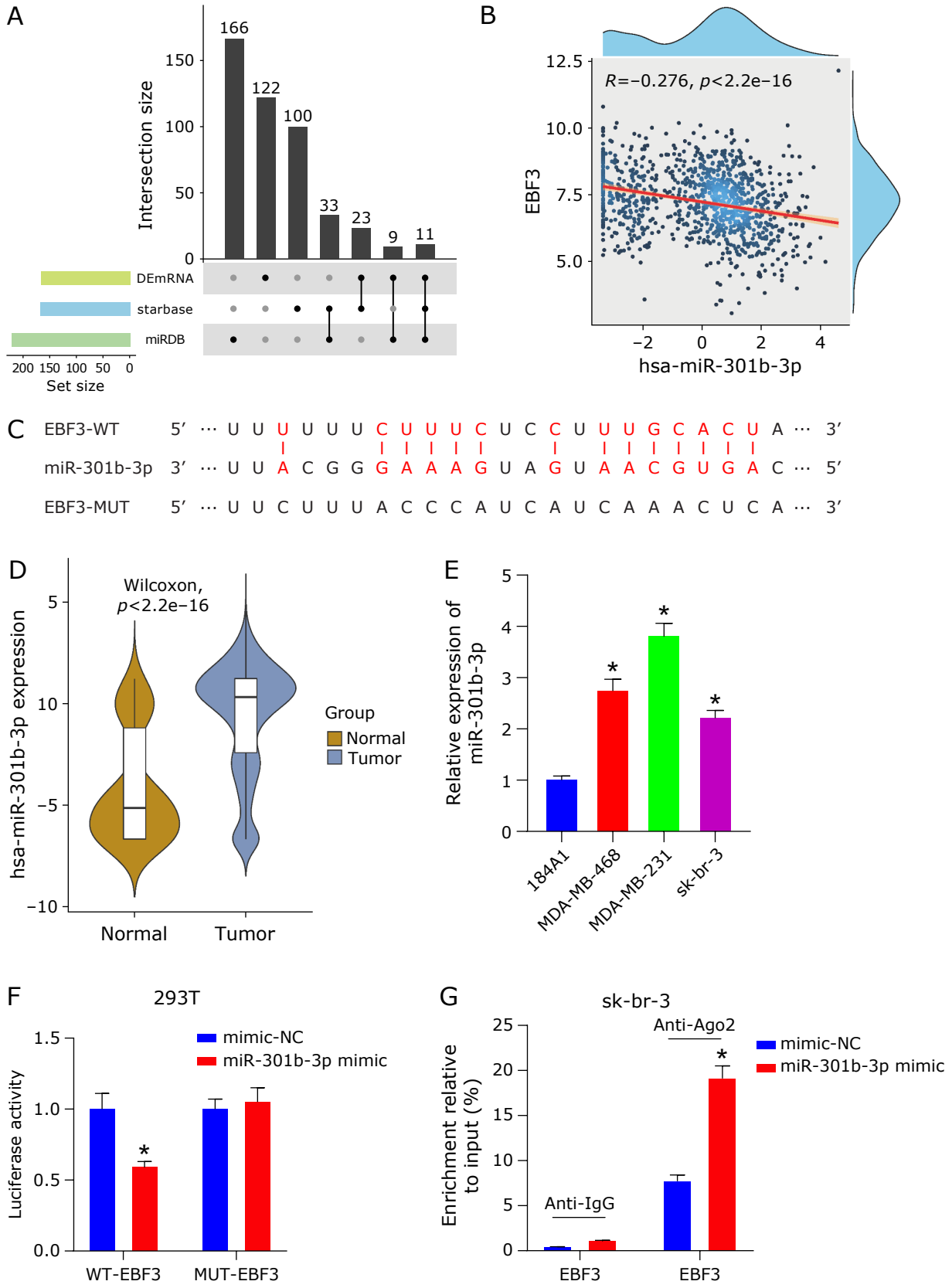

Fig. 4. miR-301b-3p modulates EBF3. (A) UpSet plot showing the intersection of predicted upstream regulatory genes of EBF3 and upregulated DEmiRNAs. (B) Correlation analysis of EBF3 and miR-301b-3p. (C) Display of binding sites of miR-301b-3p into EBF3. (D) Analysis of miR-301b-3p expression in BC tissues using bioinformatics. (E) miR-301b-3p expression in breast epithelial cells and BC cells assayed by qRT-PCR. (F, G) Dual-luciferase reporter and RIP assays validated binding relationship. * indicates $p<0.05$.

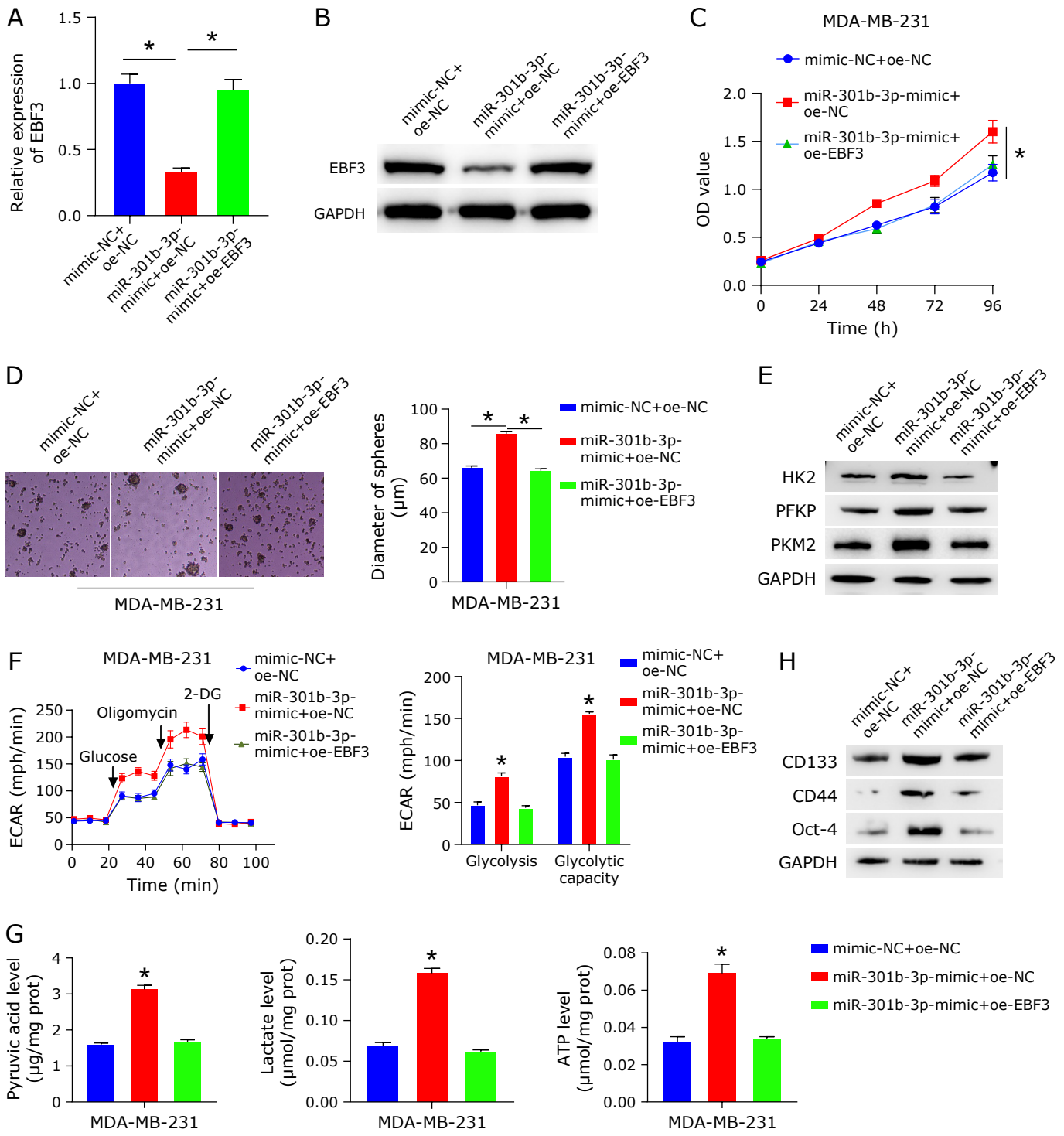

Fig. 5. EBF3 downregulated by miR-301b-3p promotes glycolysis and stem-like phenotype of BC cells. (A, B) EBF3 mRNA and protein expression in BC cells detected by qRT-PCR and WB. (C) Cell viability assayed by CCK-8. (D) Sphere formation assay tested sphere-forming ability. (E) WB detected proteins related to glycolysis pathway. (F) ECAR detection. (G) Levels of pyruvic acid, lactate, and ATP in BC cells in different groups. (H) WB detected stem cell markers. * indicates $p<0.05$.

metabolism by enhancing the expression of HK2 and LDHA, thereby promoting BC cell stemness. However, the regulatory role of EBF3 in BC cell stem-like phenotype still requires further investigation. Our study provided experimental evidence to demonstrate that EBF3 knockdown considerably enhanced glycolysis in BC cells, promoting their stem-like phenotype, while the addition of 2-DG attenuated the promoting effect of EBF3 knockdown on glycolysis and cell stem-like phenotype of BC cells. This indicated that EBF3 inhibited BC cell stem-like phenotype by suppressing glycolysis. Therefore, EBF3 may be a possible target in BC treatments.

miRNAs typically modulate mRNA expression.[41] The modu-

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latory role of miRNAs in cancer progression has been extensively investigated. Wang *et al*.[42] revealed that miR-106b-5p facilitates lung metastasis in BC by targeting CNN1. Chouman Sulidankazha *et al*.[43] reported that miR-146a represses cell proliferation through the targeting of SOX7 in pancreatic cancer. Therefore, through bioinformatics analysis, we predicted the upstream regulatory genes of EBF3 and identified miR-301b-3p, which presented a significant negative correlation with EBF3. The high level of miR-301b-3p has been previously demonstrated in BC,[30] which is consistent with our findings. Furthermore, Lu *et al*.[44] revealed that miR-301b-3p facilitates BC occurrence and development through the targeting of HOXA5. Xiong *et al*.[45] reported that miR-301b-3p promotes colorectal cancer cell proliferation and migration through the targeting of HOXB1. However, investigations regarding miR-301b-3p on cancer cell stem-like phenotype are rare. This study demonstrated through validation and functional experiments that miR-301b-3p could target EBF3 to enhance BC cell glycolysis ability, thus facilitating BC cell stem-like phenotype.

In conclusion, our work unveiled expression patterns and roles of miR-301b-3p and EBF3 in BC cells, confirming that miR-301b-3p facilitated glycolysis in BC cells through inhibition of EBF3, which promoted their cell stem-like phenotype. It provided research support for the development of BC-targeted drugs. However, this research also had some deficiencies, and we lacked *in vivo* experiments to prove accuracy. We will increase animal experiments to provide a better theoretical basis for BC-targeted therapeutic strategies.

Author Contributions

Conceptualization & Methodology: JH and XM. Formal Analysis & Supervision: LM and SG. Collection and assembly of data: WZ and SG. Software & Visualization: JH and XM. Writing – original draft: LM, WZ, and SG. Writing – review & editing: JH and XM. All the authors read and approved the final manuscript.

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Ethics Approval and Consent to Participate

Not applicable.

Data Availability Statements

The data that support the findings of this study are available on request from the corresponding author.

Conflict of Interest

No potential conflicts of interest were disclosed.

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