Dysregulation of Transcription Factor EB Contributes to Cell Proliferation, Metastasis and Stemness in Breast Cancer

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Abstract

Purpose: TFEB is a key regulator of autophagy-lysosomal biogenesis pathways, while its dysregulation is highly prevalent in various human cancers, but the specific contribution to breast cancer remains poorly understood. The main purpose of this study is to explore the role of TFEB in breast cancer proliferation, metastasis and maintaining breast cancer stem cells (BCSCs) traits, thus uncovering its underlying mechanism.

Methods: Bioinformatics, western blotting and immunohistochemical staining were applied to analyze the expression of TFEB in breast cancer. Stable down-regulation TFEB cells were established in MCF-7 and MDA-MB-231 breast cancer cell lines. MTT, clone formation, wound healing, transwell and 3D tumor invasion assays were used to evaluate the proliferation, migration and invasion ability of breast cancer cells. Mammosphere formation, immunocytochemical (ICC) staining were used to detect the effect of down-regulating TFEB on breast cancer stem cells.

Results: we demonstrated that higher expression of TFEB was found in breast cancer. TFEB depletion had inhibitory effects on cellular proliferation, migration and invasion of breast cancer cells. Moreover, knockdown TFEB decreased mammosphere formation ability of BCSCs and expression of cancer stem cell markers. Autophagy-lysosomal related proteins were decreased by down regulation of TFEB.

Conclusion: we uncovered a critical role of TFEB in breast cancer proliferation and metastasis, and BCSCs self-renewal and stemness. The underlying mechanisms involve in maintaining BCSCs traits, and dysregulating lysosome functions.

Introduction

Breast cancer is the most common cancer with a high morbidity and lethality rate in females worldwide[1]. Although the prognosis of breast cancer has been significantly improved by effective adjuvant therapies, recurrence and relapse remain a major challenge in the treatment of this disease[2]. In recent years, cancer stem cells (CSCs) were identified and isolated[3] and has received increasing interest. Collective work has revealed that there has a unique population of cells in tumors, which retain the characteristics of stem cells with self-renewal and differentiation capacity, and has been suggested to contribute to chemoresistance and recurrence or relapse[4–6]. Targeting CSCs is important for the development of efficient therapeutic strategies to improve disease prognosis[3, 7].

The lysosome is an acidic organelle that is viewed as the primary degradation compartment of eukaryotic cells[8, 9]. Meanwhile, several studies showed that lysosomes are also a signaling hub that hosts the major nutrient sensors in the cell[10], and participates in many important biological functions for maintaining cellular homeostasis[11, 12]. Lysosome function and dysfunction have been found to play important roles in cancer[13]. Targeting lysosomes could not only trigger apoptotic and lysosomal cell death pathways, but also inhibit cytoprotective autophagy. Furthermore, lysosomes play an important role in cancer drug resistance. Therefore, lysosome became a great therapeutic target in cancer[9].

Recent study showed there is a cell remove networks associated with lysosomal synthesis regulation at the transcriptional level called coordinated lysosomal expression and regulation (CLEAR) gene network[14–16]. This network was regulated by the microphtalmia (MIT) gene family, which includes microphtalmia-associated transcription factor (MITF), TFEB, TFE3 and TFEC[17]. TFEB is a central regulator of the gene network, which can get into the nucleus and bind to the E-box of CLEAR elements, inducing autophagy and lysosomal related genes transcription[18], [19]. However, TFEB showed much wider regulatory activities including metabolism, immunity, angiogenesis and inflammation[20]. Currently, numerous studies have indicated that alterations of TFEB, by mutation or expression changes, strongly link with tumorigenesis[21–24]. The role of TFEB in cancer includes in regulating cancer cell behavior and choreographing tumor microenvironment interaction[25]. Overexpression of TFEB protein in breast tumors is associated with increased mortality[26], but the underlying mechanism is far not clear.

Herein, we aimed to determine whether TFEB regulate the biological behavior of breast cancer cells, and we also explored the role of TFEB in maintaining BCSCs traits. We expected to explore a novel regulatory role of TFEB in breast carcinogenesis and provide a potential therapeutic approach for breast cancer.

Materials And Methods

Clinical samples

All samples were collected from the First Affiliated Hospital of Dalian Medical University (Dalian, China) and were confirmed with histopathological examination. Informed consent was obtained from patients. This research was approved by the medical ethics committee of Dalian Medical University.

Cell culture

The human breast cancer cell lines MDA-MB-231, MCF-7, mammary epithelial cell line MCF-10A and HEK293T cell line were purchased from the Shanghai Obio Technology (Shanghai, China). MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone Laboratories, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, USA). MCF-7 cells were cultured in DMEM/F-12 (Hyclone Laboratories, USA) supplemented with 10% FBS. All cells were cultured in medium supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml) and maintained in incubator with 5% CO_2 at 37°C.

Lentivirus generation and infection

The pLKO.1 shRNA lentivirus system was used to generate shRNA virus against Human TFEB. The shRNA plasmids against human TFEB (#1:GAGACGAAGGTTCAACATCAATTCAAGAGATTGATGTTGAACCTTCGTCTC; #2:GAACAAGTTTGCTGCCCACATTTCAAGAGAATGTGGGCAGCAAACTTGTTC) were purchased from Sigma. The pCDH-CMV-EF1-puro lentivirus system was utilized to generate human GFP-shTFEB virus. Transfection rates were confirmed by fluorescence microscopy. Virus infection was conducted by

incubated virus solution with cells for 48 hours and positive cell population was obtained by puromycin (MCF-7 cells, 1 μg/ml, MDA-MB-231 cells, 6 μg/ml) selection for 7 days.

Immunohistochemistry and Immunocytochemistry

The tumor sections were deparaffinized in xylene and rehydrated with graded ethanol followed by heating for 40 minutes in sodium citrate buffer, blocked with 5% (w/v) BSA for 1 hour at 37°C, 0.3% H_2O_2 solution was used for the blocking of endogenous peroxide activity. The anti-TFEB (1:200, #13372-1-AP, Proteintech, Wuhan) were applied at 4°C overnight. Detection was determined by non-biotin horseradish peroxidase detection system and DAB (di-aminebenzidine) substrate kit (ZLI-9018, ZSGB-BIO, Beijing). For immunocytochemistry, Cells were fixed with 4% paraformaldehyde, then permeabilized in 0.2%Triton-X-100 for 20 minutes at room temperature. Cells were blocked in 5% (w/v) BSA for 1 hour, 0.3% H_2O_2 solution was used for the blocking of endogenous peroxide activity. The anti-ALDH1 (1:400, #15910-1-AP, Proteintech, Wuhan) were applied at 4°C overnight. Detection was the same as immunohistochemistry. Take pictures with Optical microscopy (Nikon Eclipse 80i, Japan).

MTT assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT, #M1020-500T, Solarbio) cell metabolism assay. Cells were seeded in 96-well plates at 3000 cells in 200 μl culture medium, followed by three days. Only culture medium was used as a blank control. 10 μl of MTT were added to each well and incubated for 4 hours at 37°C. After removing medium, 100 μl of dimethyl-sulfoxide (DMSO, Sigma) were added to each well. Cell viability was assessed using the microplate reader (Enspire2300, Bio-Rad) at 490 nm absorbance.

Colony formation assay

Cells were trypsinized, counted, and transferred into 6 well plates with 500 cells/well and cultured for 14 day, followed by staining with 0.1% crystal violet for 20 min. Cell colonies were recorded by digital camera.

Wound healing assay

Equal numbers of cells were plated in 6 well plates. After being confluent, monolayer cells were wounded with a sterile pipette tip to generate a crossing cell-free space. Followed by washing twice with PBS. Cells were then incubated in medium for 24 hours and 48 hours, cell migration was observed and images were recorded respectively. For quantification, the migration rates were compared.

Transwell migration assay

Cells were prepared into cell suspension with a density of 2×10^4 cells/well with culture medium. Transwell chambers (8.0 mm pores, Corning) were placed into a 24 well plates. 100 μl of cell suspension was added to the upper chamber, while cell conditioned medium was added to the lower chamber for co-incubation for 24 hours. Set 3 parallel wells for each group. The filter membrane was taken out, and the cells of the upper compartment surface were wiped with a cotton swab. The cells were fixed with 4%

paraformaldehyde for 30 minutes and stained with 0.1% crystal violet. Five fields were randomly selected under light microscope to count the number of cells in the sub compartment surface of the filter membrane.

Three-dimensional (3D) spheroid invasion assay

The cell suspension was mixed with carboxymethyl cellulose. Cells spheroids were generated by hung-drop non-treated plates (#TCD000035, BIOFIL) at 800 cells/drop for 24 hours. Spheroids were collected with PBS containing 10% FBS and transfer to in a 15 ml tube, following by spheroids sink down by gravity for 10 min. After removal of medium, spheroids were embedded in collagen matrix (900 µl Type I collagen, 10×DMEM, 2M NaOH) in 5%CO_2, 37°C for 30 min until matrix polymerization. Culture mediums were filled and spheroids were further cultured for another 24 hours. Spheroids images were recorded in the indicated time points and area was measured with Image J software. The invasive performance was assessed by Invasion Index calculated as the ratio of (Area end - Area initial)/Area initial.

Mammosphere formation assay

Different groups of cells were inoculated at a density of 4×10^4 cells/well in ultra-low attachment well plates (Corning, NY, USA) and grown in serum-free DMEM/F-12 medium (Hyclone Laboratories, USA) supplemented with 20µg/ml b-FGF (#100-18B, PEPROTEH), 20µg/ml EGF (#AF-100-15, PEPROTEH), 2% B27 (Life Technologies, American). After 7-10 days culture, the number and diameter of mammosphere were calculated under light microscope (Nikon Eclipse 80i, Japan).

Lyso-Tracker Live Staining and Imaging

Make a dilution of the Lyso-Tracker Red (C1046, Beyotime) to a final working concentration in cell culture medium warm to 37°C. Cover the cells with prewarmed (37°C) probe-containing cell culture medium, incubate for 30 min. Replace the labeling solution with fresh medium. Observe the cells using a fluorescent microscope (Nikon Eclipse 80i, Japan).

Western blotting

Cells were harvested after twice rising with PBS, lysed using RIPA buffer (50mM Tris, pH7.5, 120nM NaCl, 1%Triton-X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 5mM EDTA) with protease inhibitor cocktail (MedChemExpress, HY-K001). Nuclear-cytosol extraction kit (R0050, Solarbio, Beijing) were used to separate nuclear and cytosol proteins. Samples were electrophoresed on 10% SDS-polyacrylamide gels, and transferred to PVDF membranes. The membranes were blocked for 1 hour with 3% (w/v) BSA, incubated with the primary antibodies overnight at 4°C and the secondary antibodies for 1h at room temperature. Antigen antibody complexes were detected by Odyssey CLx image system (LI-COR). Antibodies against TFEB (#13372-1-AP), ALDH1A1 (#15910-1-AP), LAMP1 (#21997-1-AP), CD133(#18470-1-AP) were purchased from Proteintech. Antibodies against TFEB (#4240) was purchased from Cell Signaling Technology. Antibodies against CTSD (ab75852), ATP6V1A (ab199326) were purchased from Abcam. Antibodies against LAMP2 (WL02761), LC3I/II (WL01506), P62 (WL02385),

SOX2 (WL03767), NANOG(WL03273) were from Wanleibio. Secondary antibodies of goat anti-rabbit IgG (#A23920) and goat anti-mouse (#A23710) were purchased from Abbkine.

TFEB expression profiles in Oncomine database

The expression of TFEB in breast cancer samples.were downloaded from the Oncomine public database(http://www.oncomine.org/) from Curtis breast[27].

Statistical analysis

In this part, statistical software Image J and GraphPad Prism7.0 were used for data analysis. All experiments were performed independently in triplicate. The results are presented as the mean ± S.E.M. Statistical analysis was performed using a two-way Student's t-test. Statistical significance was set as * $p < 0.05$, ** $p < 0.01$.

Results

TFEB is upregulated in human breast cancer

To investigate the expression level of TFEB in breast cancer, we first systematically examined the expression of TFEB in breast cancer and normal breast samples. From the Oncomine public database, we performed a TFEB expression analysis from Curtis breast[27]. TFEB expression was significantly higher in breast cancer tissues than in normal tissue (Figure 1a). Among the 12 breast neoplasm, the different expression of TFEB in breast phyllodes tumor (p=0.094) was not statistically significant. The rest were statistically significant (p<0.05). The expression of TFEB in breast cancer clinical samples were further confirmed by Immunohistochemistry. The expression of TFEB was examined in 47 breast cancer specimens and 25 normal adjacent breast tissues are displayed in (Table 1) TFEB was highly expressed in tumor than adjacent normal breast tissues (Figure 1b I, II). Then we determined the expression level of TFEB in normal mammary epithelial cell line (MCF-10A) and two breast cancer cell lines (MCF-7, MDA-MB-231). It showed that TFEB expression was highly expressed in two breast cancer cell lines (Figure 1c). Consider the possible function of TFEB in BCSCs, we further validated the expression of TFEB in BCSCs, MCF-7 cells were cultured in serum-free stemness enrichment medium for 7 days. The expression of TFEB was evaluated by western blot. Higher level of TFEB (T-TFEB) expression were found in the BCSCs than normal cultured cells (WT) (Figure 1d). Meanwhile, in order to distinguish the location of overexpressed TFEB protein, we carried out nuclear and cytosolic protein separation, and found that TFEB were both highly expressed in the nucleus (N-TFEB) and cytosol (C-TFEB, Figure 1d). Taken together, we speculate that TFEB may serve as an oncogene in breast cancer tumorigenesis.

Table 1
The expression of TFEB in breast cancer samples

	n	Expression of TFEB				
		-	+	++	+++	Positive%
Normal	25	18	6	1	0	28.00
Tumor	47	8	15	16	8	82.98
p-value		<0.05				

Down regulation of TFEB inhibits proliferative potential in breast cancer cells

Our results above showed a higher expression level of TFEB in breast cancer. Therefore, we speculated that manipulation of TFEB expression may interfere with the oncogenic properties of breast cancer cells. Down regulation of TFEB expression was conducted by generating stable knockdown cell line in MCF-7 and MDA-MB-231 cells via lentivirus packaging system. Western blotting showed that silenced TFEB by using shRNA-TFEB (shTFEB#1 and #2) was successfully used to establish stable breast cancer cell lines, and significantly suppressed its protein expression (Figure2a, b; *p <0.01*).

To further show the manipulated effects of the TFEB on proliferation of breast cancer cells, MTT assay was applied. Compared with the control group (Ctrl), down regulated TFEB (shTFEB#1 and #2) significantly inhibited cell proliferation in MCF-7 cells (Figure 2c, p *<0.01*) and MDA-MB-231cells (Figure 2d, p *<0.01*). Additionally, the capability of colony formation in MCF-7 cells and MDA-MB-231 cells were examined. The results showed that down regulation of TFEB remarkably suppressed the colony forming capacity as compared to controls in both cell lines (Figure 2e, f, p *<0.01*). These results suggested that down regulation of TFEB inhibited the cell proliferation and colony forming capacity of breast cancer cells.

Down regulation of TFEB inhibits breast cancer migration and invasion

To investigate the function of TFEB in breast cancer cell migration and invasion, the effect of down regulation of TFEB on metastasis was evaluated by wound healing assay, transwell migration assay, and three-dimensional (3D) spheroid invasion assay. The wound healing assay indicated that decreased motility was observed in TFEB knockdown cells as compared to control cells after 48 hours in MCF-7 cells (Figure 3a, p *<0.05*), while in MDA-MB-231 cells decreased motility were observed both after 24 hours and 48 hours (Figure 3b, p *<0.05*). In transwell migration assays, on the other hand, showed that migration capacities were also significantly decreased in cells down regulation of TFEB (Figure 3c; *p <0.05*). Moreover, three-dimensional culture assay was applied to evaluate breast cancer cells invasive ability. Compared with the control group, the invasion ability in down regulated TFEB cells were also

remarkably reduced, invasion index was calculated (Figure3d, p *<0.05*). These data clearly indicate that down regulation of TFEB inhibits migration and invasion in breast cancer cells.

Down regulation of TFEB decreases breast cancer stem-like cells properties

To study the regulatory effects of slienced TFEB in BCSCs, mammosphere formation assay was performed. We found that looser and smaller spheres were formed in down regulated TFEB cells as compared to the control cells (Figure 4a). The number and diameter of spheres were evaluated from three replicate wells. Down regulation of TFEB significantly decreased the number (Figure 4b; *p <0.05*) and diameter (Figure 4b; *p <0.01*) of spheres both in MCF-7 and MDA-MB-231cells. To further study the inhibitory effects of TFEB on BCSCs, immunocytochemistry staining of ALDH1were performed. As ALDH1 acts as a BCSCs marker, its expression was significantly decreased in down regulated TFEB cells as compared to control cells (Figure 4d). Western blotting analysis further confirmed that protein expression of ALDH1, breast cancer stem cells surface marke CD133, and stem cell marker SOX2, NANOG were significantly decreased (Figure 4e, f). All together, these data suggest that modulation of TFEB in breast cancer cells reduced the self-renewal ability and stemness of BCSCs.

Down regulation of TFEB inhibits lysosomal biogenesis and autophagy in breast cancer cells

The emerging role of lysosome in cancer biology makes lysosome a therapeutic target, and TFEB acts as a main regulator of lysosomal biogenesis and autophagy, we hypothesize that lysosomal function may be reduced by TFEB knockdown in breast cancer cells. First, we dyed lysosome by using Lyso-Tracker Red. The results showed that compared with the control group (Ctrl), the number and brightness of lysosomes in the knockdown group (shTFEB#1 and shTFEB#2) were significantly reduced (Figure 5a). Western blotting analysis further confirmed the decreased expression of lysomal related proteins. The expression of lysosome associated membrane protein-1 (LAMP-1) and -2, and lysosomal protease Cathepsin D, V-ATPase components ATP6V1A in breast cancer were significantly decreased in TFEB knockdown groups compared to control groups (Figure 5b). In addition, the expression of autophagy associated proteins LC3I/II and P62 were detected. The results showed that down regulation of TFEB distinctly reduced the expression of autophagy transporter P62, but autophagy-induced protein LC3II/I ratio was not statistically significant reduced (Figure 5c). Taken together, the defective lysosomal activity may partially explain the role of TFEB in breast cancer cells.

Discussion

Although dysregulation of TFEB is highly prevalent in various human cancers[25], its specific contribution to breast cancer remains poorly understood. Thus, the current study sought to investigate the role of TFEB in the regulation of breast cancer growth and potential underlying mechanisms. We found that TFEB promotes breast cancer cell proliferation, migration and invasion, and the underlying mechanisms involve in regulating BCSCs stemness and self-renewal ability, and dysregulating lysosomal activities.

The existence of TFEB contributes to breast cancer cell proliferation and metastasis, thus play a critical role in breast cancer cell stemness.

TFEB is one of the wildly studied cancer associated transcription factor. It is a master regulator of the autophagy-lysosomal biogenesis[28], which is dysregulated in various types of malignant tumors. Importantly, TFEB could act as an oncogene by promoting cancer in human tumor, including colorectal cancer[21], breast cancer[26, 29], pancreatic cancer[30], non-small cell lung cancer[24] and renal cell carcinoma[22]. In colorectal cancer and non-small cell lung cancer, TFEB knockdown could significantly inhibit cell proliferation and migration. It was also reported that higher TFEB expression predicts higher risk of recurrence or death[24], [21]. In this paper, we showed that TFEB gene was elevated expression in breast cancer tissues based on analysis of the database. In addition, the expression level of TFEB was associated with poor survival. We further validate the expression of TFEB in breast cancer tissues, breast cancer cell lines and BCSCs-enriched breast cancer cell line. Then we confirmed the inhibitory effects of TFEB knockdown in the proliferation, migration, and invasion potential in luminal type MCF-7 cells and triple negative cell line MDA-MB-231 cells. Our study serves as a proof-of-concept that TFEB may play a role in breast cancer tumorigenesis and development.

An additional mechanism that may contribute to the poor prognosis in breast cancer is tumor-associated macrophages (TAMs), the study showed that TFEB controls TAM gene expression and function through multiple autophagy-lysosomal dependent and independent pathways[31]. In our study, we discussed another factor for tumor recurrence and drug resistance, cancer stems cells. Growing evidence suggests that cancer recurrence and/or relapse may be initiated and maintained by remaining CSCs from either residual tumors or those with intrinsic resistance to adjuvant therapy[3, 32]. In addition, autophagy-lysosomal function can exert effects on the initiation, proliferation and progression of tumors and CSCs[33, 34]. Our results showed silenced TFEB expression decreased mammosphere formation capacity and resulted in lower expression of stem cell markers ALDH1 and SOX2, CD133, NANOG in MCF-7 and MDA-MB-231 cells. ALDH1, a well-established breast cancer stem cell marker, is an intracellular enzyme that oxidizes aldehydes and retinol. In CSCs, it has been shown to lead to a more aggressive phenotype, treatment resistance, and a worse prognosis on patients[35]. CSC can express several distinct transcription factors and surface markers to maintain its stemness, such as transcriptional factors KLF4, OCT4, SOX2, and NANOG, surface markers CD133, CD138, CD44, Lgr5, CD34, and TNFRSF16[36] Nanog, Sox2, and Oct4 are transcription factors all essential to maintaining the pluripotent embryonic stem cell phenotype.[37] NANOG is a key transcription factor in embryogenesis and tumorigenesis, and is overexpressed in most CSCs[38].Furthermore, autophagy can sustain the activity of CSCs, which can facilitate breast cancer cell growth and can lead to resistance to drug treatment. The inhibition of autophagy can decrease the drug resistance of breast CSCs, as well as reduce the ability of breast CSCs to form tumors[39, 40]. We confirmed that knockdown TFEB could significantly inhibit expression of autophagy related proteins. Therefore, further research is required to elucidate the complex association between autophagy and BCSCs. Moreover, mammosphere formation is a typical property of CSCs that reflects the self-renewal potential of stem cells. By using serum free cultural medium supplemented with EGF, b-FGF and B27, mammosphere formation assay is kind of starvation status for cultured cells. After

knocking down TFEB, even with the existence of growth factors, such as EGF and b-FGF, the breast cancer cells still could not grow properly, the mammosphere formation ability were strikingly reduced. These results indicate that TFEB plays an essential role in BCSCs self-renewal, which may due to the decreased lysosomal function and autophagy of cells. However, how TFEB affects EGF and b-FGF pathways needs further study.

In recent years, studies of TFEB mostly focus on autophagy and lysosomal related, whose basic helix-loop-helix(b-HLH) leucine zipper structure makes it have specific transcriptional regulation characteristics and dominate the coordination of lysosomal gene network expression[14]. Whereas the depletion of TFEB could inhibit the expression of lysosomal genes and affect intracellular clearance system in multiple cell types[41]. Alexandra[26] et al. report that in early breast carcinomas exists intense expression of TFEB and lysosomal biogenesis. They also report that acidity can trigger TFEB overexpression and verified TFEB is a sound target to develop lysosomal interference. Additional important findings were the direct association of TFEB with HIF-2α and lysosomal markers, with increased angiogenic activity in the invading tumor front[26]. Another study showed that TFEB can positively regulate angiogenesis[42]. In this study, we showed that TFEB was essential for the development of a migratory phenotype in breast cancer and was positively correlated with lysosomal activity. LAMP-1 and -2 are major protein components of the lysosomal membrane[43], which play an important role in cancer cell migration[44]. Cathepsin D (CTSD), belongs to the family of lysosomal aspartic protease, whose most general and basic function is intracellular catabolism in lysosomal compartments[45]. CTSD was suggested as a tumor marker, and associated with progression of breast cancer[46]. The ATP6V1A gene encodes the A subunit in the structural domain V1 of V-ATPases on the membrane of lysosomes, which is important for the maintenance of pH values on both sides of the lysosomal membrane[47]. V-ATPase could be a target for treating multidrug-resistant cancer[48]. The expression of these lysosomal related proteins was decreased by down regulating TFEB expression, which implies lysosomal activity may partly explain the regulatory effect of TFEB on breast cancer cells.

Our results suggest a possibility of down regulation of TFEB with considerable impact on triple negative breast cancer. By disrupting lysosomal function, and generating a secretome comprising exosomes with unique cargo and soluble hydrolases that degrade the extracellular matrix, could increase MDA-MB-231 survival and invasion[49]. But the specific mechanism for triple-negative breast cancer remains to be further explored.

Mechanistic target of rapamycin complex 1 (mTORC1) and extracellular signal-regulated kinase 2 (ERK2, also known as MAPK1), both master controllers of cellular growth, are the main protein kinases known to phosphorylate TFEB under nutrient-rich conditions in most cell types[50]. mTORC1 mediated TFEB phosphorylation can occur at the lysosomal membrane[51]. It remains unclear whether ERK2 phosphorylates TFEB in the lysosomal surface or in other subcellular compartments. In CSCs research, the function of lysosomal can be regulated through the mTORC1 pathway to improve the function of stem cells[52]. Wnt/β-catenin signaling pathway involves in regulating development and stemness of healthy stem cells as well as cancer stem cells[53]. Wnt/β-catenin signaling pathway was enriched in the

TFEB overexpression gastric cancer tissues. It could trigger the aggregation of β-catenin in nucleus and activate its transcription[54]. Moreover, Wnt activation has been observed in breast malignancies and contributes to tumor recurrence[55]. Given the contribution of TFEB in BCSCs above, we speculate that mediation of β-catenin transcription maybe another explanation of by which TFEB controls BCSCs self-renewal and stemness.

Conclusion

In summary, overexpressed TFEB were found in breast cancer. Down regulation of TFEB striking suppressed breast cancer cell proliferation, migration and invasion ability. At the same time, silenced TFEB decreased BCSCs mammosphere formation ability and showed a lower expression level of CSCs related markers. Herein, we uncovered a critical role of TFEB in BCSCs self-renewal and stemness, and the molecular mechanisms may partially by down regulating lysosomal activity. To further assess the impact of TFEB in breast cancer and its underlying mechanism may serve as a novel therapeutic approach for breast cancer.

Abbreviations

TFEB: transcription factor EB; CSCs: cancer stem cells; CLEAR: coordinated lysosomal expression and regulation; MIT: microphtalmia; MITF: microphtalmia-associated transcription factor; BCSCs: breast cancer stem cells; 3D: Three-dimensional; TAMs: tumor-associated macrophages; b-HLH: basic helix-loop-helix; CTSD: Cathepsin D; mTORC1: mechanistic target of rapamycin complex 1; ERK2: extracellular signal-regulated kinase 2;

Declarations

Author contributions

Ningwei Fu and Ning Fan: Conceptualization, methodology, software, formal analysis, writing - Original Draft; Wenchao Luo: Data curation, software, formal analysis; Lijia Lv:Methodology, software, visualization; Jing Li: software,visualization; Jun Mao: Supervision, resources; Feifei Li: Methodology, formal analysis; Xujiao Wang and Xin Zhang: Methodology, data curation, software ; Fei Xu: Supervision, visualization; Yan Li: Writing - review & editing, resources, visualization; Wei Ma: Conceptualization, methodology, software, formal analysis, visualization, writing - review & editing, funding acquisition.

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Competing Interests

Authors declare that there is no competing interests.

Consent for publication

Not applicable.

Data availability statement

Data available on reasonable request from the corresponding authors.

Ethics approval

This research was approved by the medical ethics committee of Dalian Medical University.

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Conflict of interest

Authors declare that there is no conflict of interests.

Data availability statement

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Figures

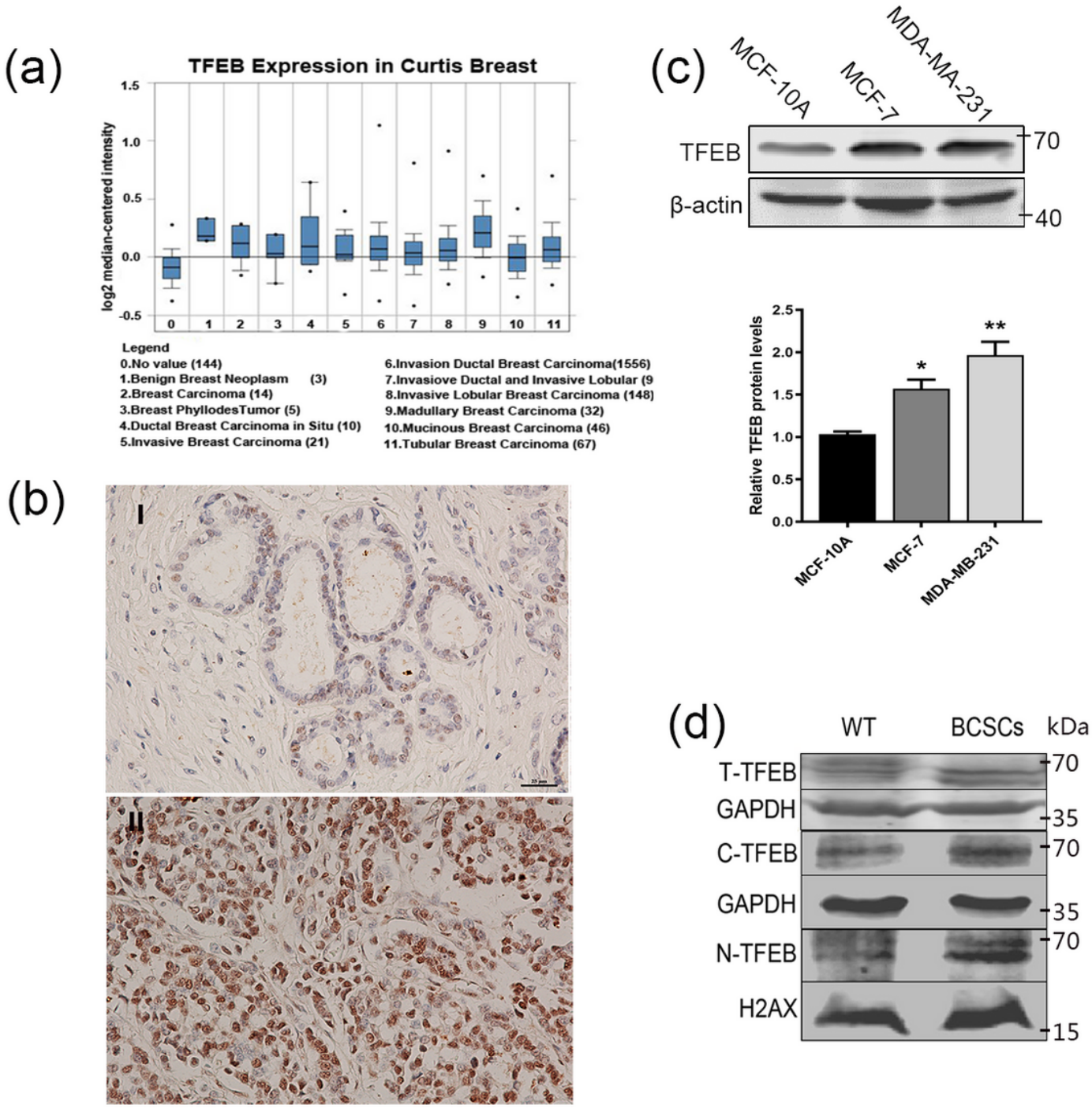

Figure 1

TFEB is upregulated in Breast cancer. (a) TFEB gene expression is highly up-regulated in variety breast cancers compared with that in normal breast tissues. Microarray data analyses of TFEB gene expression in human breast cancer and normal breast tissues (Curtis are plotted). The Student's t test was conducted using the Oncomine software (www.oncomine.org). The boxes represent the 25th to 75th percentiles. The horizontal lines represent the medians. The whiskers represent the 10th and 90th percentiles, and the asterisks represent the end of the ranges. Among the 12 breast neoplasm, the different expression of TFEB in breast phyllodes tumor (p=0.094) was not statistically significant. The rest were statistically

significant ($p<0.05$). (b) Representative immunohistochemical analyses of TFEB in human cancer adjacent normal tissues (I) and breast cancer tissues by IHC(II), scale bar = 25 μm. (c) Expressions of TFEB were detected in normal mammary epithelial cell (MCF-10A) and breast cancer cell lines (MCF-7, MDA-MB-231) by Western blot analysis, the quantitative results were showed on the bottom. (d) Western blotting results showed that TFEB(T-TFEB) was expressed in WT (normal cultural medium) and BCSCs (serum-free DMEM/F12 supplemented with 2% B27, 20 μg/ml EGF and 20 μg/ml b-FGF). Cytosolic(C-TFEB) protein and nuclear (N-TFEB) protein were extracted. Western Blotting showed higher expression of TFEB in both nuclear and cytoplasmic.

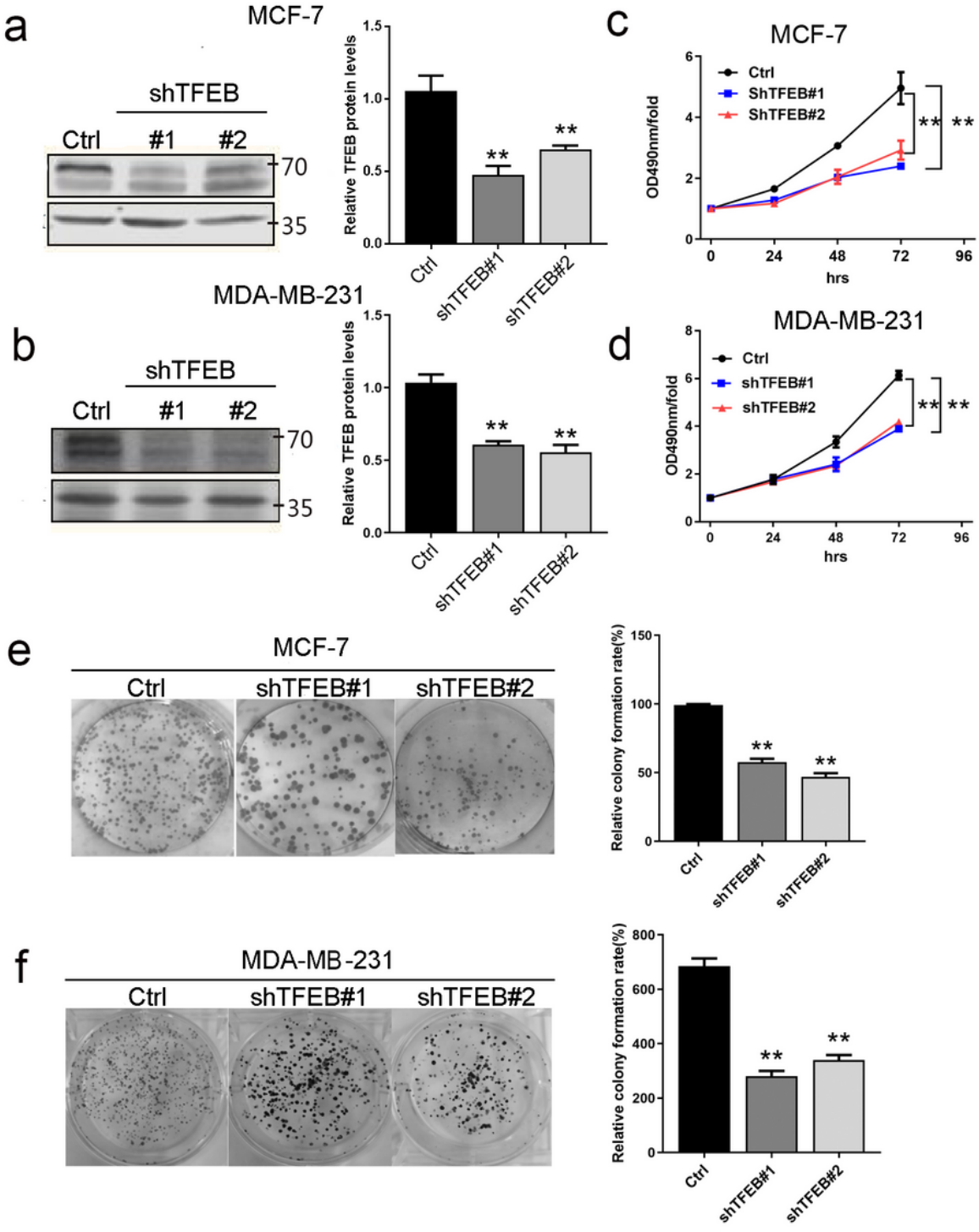

Figure 2

Down regulation of TFEB inhibits cell proliferative potential in Brest Cancer. (a) The efficacy of shRNA mediated stable knockdown against TFEB was detected by western blotting in MCF-7 and MDA-MB-231 cells, the quantitative results were showed on the right. (c, d) MTT assays showed that down-regulation of TFEB could inhibit cell proliferation rate in MCF-7 and MDA-MB-231 cells. (e, f) Compared to the

control group, down-regulation of TFEB reduces the number of colonies in MCF-7 and MDA-MB-231 cells. Data were obtained from 3 independent experiments, *p <0.05, **p <0.01.

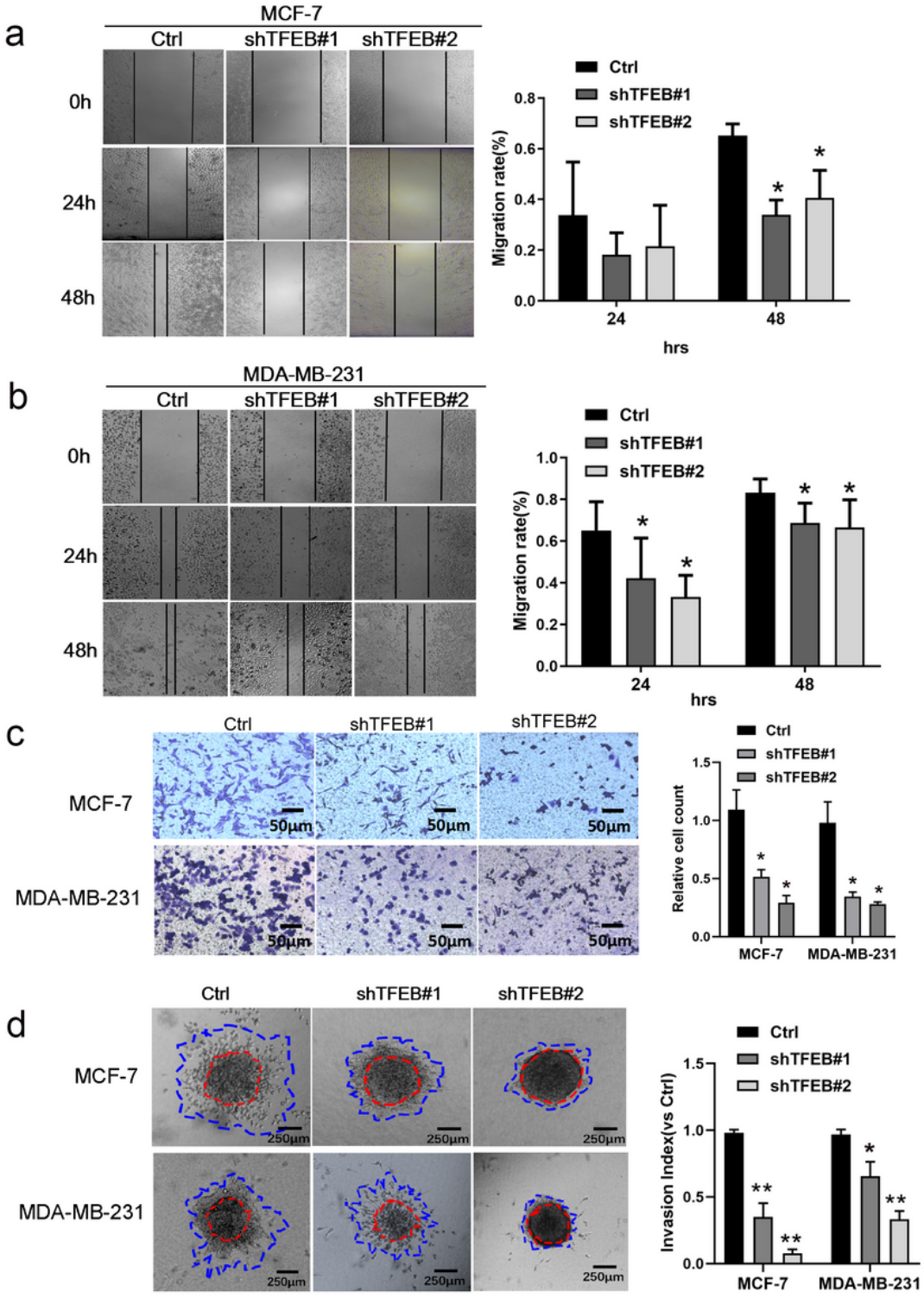

Figure 3

Reduced TFEB expression inhibits BC migration and invasion. (a, b) The wound healing assay was performed in control (Ctrl) and shTFEB (shTFEB#1 and shTFEB#2) treated MCF-7 and MDA-MB-231 cells. At 24h and 48h after wounding, the migration rate compared to control is shown on the right. (c)

Transwell based migration assay showed down-regulation of TFEB significantly decreased the migration ability of cells. scale bar=50 μm. (d) Three-dimensional (3D) spheroid invasion assay. 3D based tumor spheroids invasion after being embedded into a collagen matrix, down regulation of TFEB decreased the invasive ability of breast cancer cells. scale bar=250 μm. Data were obtained from 3 independent experiments, *p <0.05, **p <0.01.

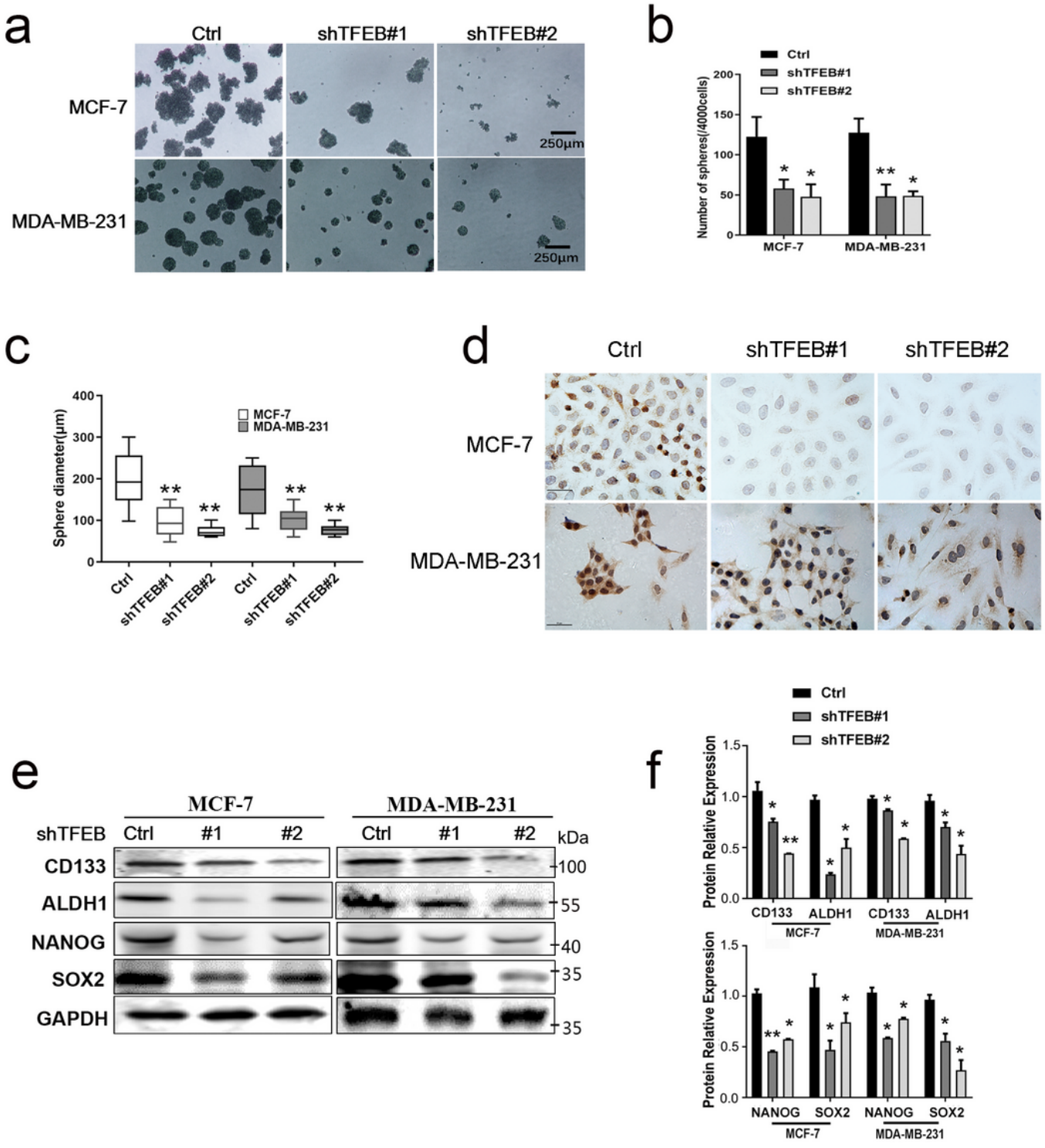

Figure 4

The properties of BCSCs were decreased by TFEB knockdown. (a) Representative images of mammospheres are shown. After transfection with TFEB, cells were cultured in ultra-low attachment plates with serum-free medium for 7d. Mammospheres were collected and evaluated, scale bar=250μm. Down regulation of TFEB decreased the number. (b) and diameter (c) of mammospheres both in MCF-7 and MDA-MB-231 cells. Mammospheres were counted from 3 replicate wells. (d)Immunocytochemistry staining confirmed lower ALDH1 protein expressions in silenced TFEB cells. scale bar=25 μm . (e) Well established BCSCs marker ALDH1 and stem cell marker SOX2, CD133, NANOG were down-regulated after silencing TFEB. (f) The quantitative results were analyzed by Image J, and GAPDH was used as an endogenous control. Data were obtained from 3 independent experiments, *p <0.05, **p <0.01.

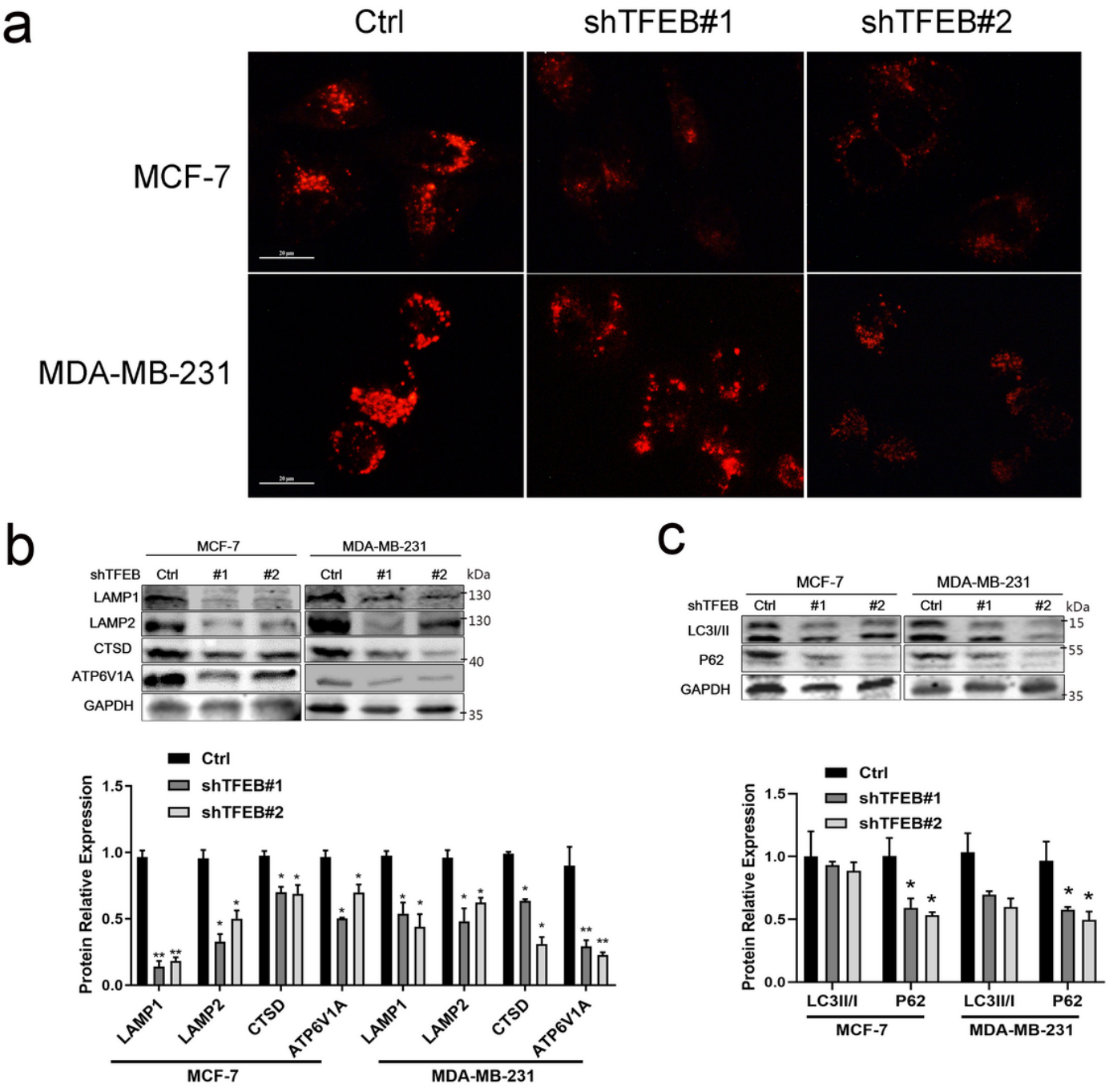

Figure 5

Reduced TFEB expression inhibits breast cancer cells lysosome biogenesis and autophagy. (a) Microscopy of breast cancer cells stained with Lyso-Tracker Red. Compared to the control group (Ctrl), the number and brightness of lysosomes in TFEB konckdown group (shTFEB#1 and shTFEB#2) were reduced. scale bar=20 μm. (b) Protein levels of lysosomal associated proteins were evaluated by Western blotting analysis. Lysosomal membrane proteins LAMP1, LAMP2 and lysosomal functional protein CTSD, ATP6V1A were down regulated depending on the reduced TFEB. The quantitative results were shown on the bottom. (c) Western blotting results show that autophagy induced protein LC3II/I was not statistically significant. The expression of autophagy transporters protein P62 are decrease in TFEB knockdown groups. The quantitative results were shown on the bottom. Data were obtained from 3 independent experiments, *p <0.05, **p <0.01.