UBE2S is associated with malignant characteristics of breast cancer cells

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Running title; UBE2S and breast cancer

Abstract

Ubiquitination is essential for various biological processes, such as signal transduction, intracellular trafficking and protein degradation. Accumulating evidence has demonstrated that ubiquitination plays a crucial role in cancer development. In this report, we examine the expression and function of ubiquitin conjugating enzyme E2S (UBE2S) in breast cancer. Immunohistochemical analysis revealed that UBE2S is highly expressed in breast cancer. The depletion of UBE2S by siRNA induced disruption of the actin cytoskeleton and focal adhesions. Interestingly, phosphorylation FAK at Tyr397, which is important for the transduction of integrin-mediated signaling, was significantly reduced by UBE2S knockdown. We also show that UBE2S knockdown suppressed the malignant characteristics of breast cancer cells, such as migration, invasion and anchorage-independent growth. Our results indicate that UBE2S could be a potential target for breast cancer treatment.

Introduction

Ubiquitination is a reversible biochemical process that attaches ubiquitin to substrate proteins to regulate multiple cellular functions.[1,2] The process of ubiquitination is mediated by three types of enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase. Ubiquitin proteins are first attached to the active cysteine of E1 and then transferred to the active cysteine of E2 by thioester linkage. E3 ligases mediate the transfer of ubiquitin on the active cysteine of E2 to a specific lysine of specific target proteins.[3,4] Depending on the types of ubiquitination, substrate proteins are destined for degradation or modulated their functions for numerous biological activities. Polyubiquitinated proteins are generally targeted to the 26S proteasome for degradation, whereas monoubiquitinated proteins are associated with various cellular functions, such as endocytosis, chromatin remodeling, DNA repair and signal transduction.[5,6]

Accumulating evidence has demonstrated that ubiquitination is associated with the progression of cancer.[7] There are more than 600 E3 ligases in humans, some of which regulate the expression of tumor-suppressor or tumor-promoting proteins. p53 is one of the most frequently mutated genes in cancer, and reduced levels of p53 protein can promote cancer initiation. Mdm2 is an E3 ligase for p53 and is one of the major regulators of p53 expression.[8,9] Overexpression of Mdm2 is observed in a variety of cancers, such as breast carcinoma, oral squamous cell carcinoma, glioma, lymphoma and leukemia.[10] COP1 and Pirh1 are also E3 ligases for p53 and they are overexpressed in several cancers.[11-15] There are additional E3 ligases whose mutation or dysregulation are associated with cancer progression. For example, germline mutations of BRCA1 gene are predictors for the risk of ovarian and breast cancers.[16,17] Von Hippel-Lindau (VHL) E3 ligase was originally identified as a tumor suppressor, and somatic mutations of VHL gene are related to the development of clear cell renal carcinoma.[18]

In addition to E3 ligases, E2 conjugating enzymes are associated with cancer progression.[19] One of the most studied E2 is UBE2C because of its association with cancer. UBE2C is required for the degradation of mitotic regulators in cooperation with anaphase-promoting complex/cyclosome (APC/C).[20,21] High expression of UBE2C is found in many human cancers of the brain, lung, cervix, colon, liver, thyroid, breast and nasopharynx.[22-25] Depletion of UBE2C from cancer cells significantly reduced proliferation and induced cellular apoptosis. Transgenic mice overexpressing UBE2C were prone to developing carcinogen-induced lung tumors and a broad spectrum of spontaneous tumors.[26] These results clearly indicate that UBE2C is involved in cancer development and progression. UBE2S, also known as E2-EPF, is essential for the

elongation of ubiquitin chains to target substrate proteins to the 26S proteasome.[27-29] Once UBE2C attaches ubiquitin onto the target proteins, UBE2S promotes the elongation of ubiquitin chains for the degradation. Recent studies have shown that UBE2S is also overexpressed in cancers. High expression of UBE2S was observed in cervical, breast and kidney cancers[30-33], but the physiological role of UBE2S in cancer still remains uncertain. In this report, we show that UBE2S depletion suppressed migration, spreading and invasion of breast cancer cells.

Results

UBE2S is expressed in breast cancer tissues

We first generated purified anti-UBE2S antibody to examine the expression level of protein in breast cancer cells as well as tissues. Immunoblot analysis with the antibody detected single bands at the expected molecular weight. Although UBE2S was highly expressed in breast cancer cell lines, a reduced expression of UBE2S was observed in human mammary epithelial cells (MBE) (Fig. 1A). Immunostaining and cell fractionation analysis revealed that UBE2S was localized to both the nucleus and cytoplasm (Fig. 1B and 1C). We next examined the expression of UBE2S in breast cancer tissues using the antibody. The antibody specifically stained cells and UBE2S expression was assessed and scored (Fig. 1D). We found that UBE2S expression was increased in breast cancer tissues compared with normal tissues (Fig. 1E). The association between UBE2S expression and clinicopathological variables is shown in table 1.

UBE2S depletion disrupts organization of actin cytoskeleton and focal adhesions

We next examined the effect of UBE2S depletion using siRNAs. Two luminal cell lines, T47D and MCF7, and two basal cell lines, BT20 and MDA-MB-231 cell lines, were transfected with two siRNAs targeting different regions of the gene. We noticed that the morphology of these cells was significantly changed by UBE2S depletion. The cells became significantly round in the absence of UBE2S (Fig. 2A). We speculated that the morphological changes were induced by the disruption of actin cytoskeletal organization. Immunostaining for actin cytoskeleton and vinculin, a component of focal adhesions, demonstrated a disrupted organization of the actin cytoskeleton and focal adhesions by UBE2S knockdown (Fig. 2B). Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is critical for the organization of focal adhesions and the actin cytoskeleton (34,35). We thus checked the expression and activity of FAK in the absence of UBE2S. To detect the activity of FAK, we used an antibody that specifically detects

phosphorylation at Tyr397. UBE2S knockdown did not affect expression level of FAK but significantly reduced the phosphorylation of FAK on Tyr397 (Fig. 2C). We used other luminal and basal cell lines (MDA-MB-453 and Hs578t), but we did not observe changes in cellular morphology and FAK phosphorylation.

UBE2S knockdown suppresses cell spreading, migration and invasion

Organization of the actin cytoskeleton and focal adhesions is essential for cell attachment to the extracellular matrix (ECM) and cell spreading. To evaluate cell attachment to the ECM, cells transfected with siRNAs were seeded onto the surface of coverslips coated with fibronectin and the numbers of attached cells were counted at different time points. We found that cell attachment to the fibronectin-coated surface was reduced by UBE2S depletion (Fig.3A). To examine cell spreading, siRNA-transfected cells were seeded onto the fibronectin coated surface, and spread cells were counted 60 min later. As shown in Fig. 3B, cell spreading was suppressed by UBE2S knockdown.

We next investigated the migration and invasion of the cells in the absence of UBE2S using transwell chambers. Three luminal cell lines (MCF7, T47D, MDA-MB-453) and three basal cell lines (BT20, MDA-MB-231, Hs578t) were transfected with siRNAs and 72 h later, cells were placed on the upper surface of the filter and allowed to migrate to the bottom surface, which was coated with fibronectin. Twenty hours later, cells that migrated to the bottom surface were quantified. Cell migration was significantly suppressed by UBE2S depletion (Fig. 4A). To examine cell invasion, we used matrigel-coated transwell chambers. siRNA-transfected cells were seeded on the matrigel-coated chamber, and cells that invaded to the lower surface of the filter were counted. As shown in Fig. 4B, UBE2S depletion suppressed invasion of these cell lines (Fig. 4B).

UBE2S knockdown promotes anoikis

Anchorage-independent growth is one of the major characteristics of tumor cells. To determine whether UBE2S depletion affects the anchorage-independent growth of breast cancer cells, siRNA-transfected cells were cultured on agar; 2 weeks later, colony formation was evaluated. MDA-MB-453 cells did not form colonies, thus we used other 5 breast cancer cell lines. The proliferation of cells on the agar was significantly reduced by UBE2S knockdown (Fig. 5A). Anoikis is a form of cell apoptosis that is induced by the detachment of cells from the extracellular matrix. We speculated that the suppression of colony formation by UBE2S knockdown was partly mediated by the promotion of

anoikis. Cells transfected with siRNAs were cultured in suspension for 48 h, and apoptotic cells were then detected via TUNEL assay. As shown in Fig. 5B, UBE2S knockdown promoted anoikis of breast cancer cells.

Discussion

Despite significant advances in the techniques used for screening and therapy, breast cancer remains a leading cause of cancer-related deaths in women worldwide. In this report, we examined the expression of UBE2S in breast cancer tissues by immunohistochemical analysis. Consistent with previous findings, we found that the expression of UBE2S was increased in breast cancer tissues. High expression of UBE2S has also been reported in other cancers, such as renal cell carcinoma and cervical carcinoma.[31,32] These previous reports and our study clearly indicate that UBE2S can be used as a marker for multiple cancers.

UBE2S is involved in the degradation of proteins by APC/C during mitosis. Once APC/C in combination with UBE2C primes the lysine residues of substrates with ubiquitin, UBE2S promotes elongation of ubiquitin chains via K11-mediated attachment.[27,28] Ubiquitin chains generated by UBE2S promote degradation of substrate proteins by the proteasome pathway and promote exit from mitosis. In addition to its important functions in mitosis, we found that UBE2S is associated with regulating the actin cytoskeleton and focal adhesions. The depletion of UBE2S induced changes in cellular morphology and significantly disrupted the formation of actin stress fibers and focal adhesions. The actin cytoskeleton plays a critical role in numerous cellular functions, such as cell migration and spreading. We found that both migration and spreading were delayed in the absence of UBE2S. These results suggest that UBE2S is important not only for mitotic progression but also for cell migration and spreading as well as actin cytoskeletal organization.

A number of proteins are associated with the regulation of actin cytoskeletal organization and cell migration. Among these proteins, FAK is one of the most studied proteins, and its inhibitors are being investigated in clinical trials for cancer treatment.[34] FAK is also known to activate survival signals to prevent anoikis. We found that the phosphorylation of FAK at Tyr397 was reduced in the absence of UBE2S. Suppression of Tyr397 phosphorylation was observed using two different siRNAs. Thus, it is unlikely that the reduced phosphorylation was induced by off-target effects of siRNAs. Although the exact molecular mechanisms of Tyr397 phosphorylation are still not clear, it is believed that integrin binding to ECM promotes dimerization of FAK for the phosphorylation of Tyr397.[36,37] Src family kinases, which are non-receptor tyrosine

kinases, are recruited to the phosphorylated Tyr397, where they phosphorylate other tyrosine residues of FAK for the activation of downstream signals.[38,39] Therefore, a decrease in Tyr397 phosphorylation via UBE2S knockdown may suppress numerous signals for cell spreading, migration and invasion. Further analysis is required to determine whether the functions of FAK or other signal proteins are affected by UBE2S knockdown to suppress cell migration and invasion.

In summary, we have shown that UBE2S is highly expressed in breast cancer tissues, and depletion of UBE2S induced disruption of the actin cytoskeleton and focal adhesion organization. In addition, UBE2S depletion suppressed malignant characteristics of cancer cells, such as migration, invasion and anchorage-independent growth. Further investigation to identify target proteins of UBE2S may give novel insight into the regulation of actin cytoskeletal organization and cell migration.

Materials and Methods

Cells and antibodies

MCF7 cells were cultured in DMEM supplemented with 10% FBS and 0.01 mg/ml of insulin. Hs578t cells were cultured in RPMI -1640 supplemented with 10% FBS. Other cell lines were cultured in DMEM with 10% FBS. To generate anti-UBE2S antibody, the C-terminus of UBE2S (amino acids 158-222) with a GST tag was produced in bacteria, and recombinant protein was purified using glutathione agarose beads (Sigma-Aldrich, St. Louis, MO, USA). The protein was mixed with Freund's adjuvant (Sigma-Aldrich) and injected into a rabbit four times every two weeks. To purify the anti-UBE2S antibody, we used HiTrap NHS-activated HP columns (GE Healthcare BioScience, Uppsala, Sweden) coupled with recombinant GST-tagged UBE2S. Anti-FAK, anti-phospho-FAK (Tyr397) and anti-vinculin antibodies were obtained from BD Biosciences (San Jose, CA, USA).

Immunohistochemical analysis

Tissue microarrays were stained with anti-UBE2S (1:100) overnight at 4°C and incubated with HRP-labeled rabbit secondary antibody. Tissues microarrays for breast cancer were obtained from US Biomax (Rockville, MD, USA). The catalog numbers of the tissue microarrays that we used are T086c, T087, T088a and BC08013a. Staining intensity was scored as 0 (no staining), 1 (mild), 2 (moderate), and 3 (strong).

siRNA transfection

siRNA sequences used to knockdown UBE2S are 5'-AGGGCUACUUCCUGACCAATT-3' (siUBE2S-1) and 5'-CCAUCAAGUGCCUGCUGAUTT-3' (siUBE2S-2). The sequence of control siRNA targeting luciferase is 5-CUUACGCUGAGUACUUCGATT-3'. 20 nM of siRNAs were transfected using RNAi/Max (Invitrogen) according to the manufacturer's protocol.

Immunofluorescence analysis

Cells cultured on cover glass were transfected with siRNAs, and 72 h later, cells were fixed with 4% paraformaldehyde for 20 min. The cells were blocked with 7% FBS in PBS for 30 min and then incubated with primary antibodies. After washing with PBS, the cells were incubated with FITC-conjugated anti-rabbit antibody (Invitrogen). Rhodamine-conjugated phalloidin was used to stain actin fibers. Images were acquired using a FV1000 confocal microscope (Olympus, Tokyo, Japan).

Preparation of cytoplasmic and nuclear fractions

The subcellular fractionation was done using protocol for REAP nuclear/cytoplasmic fractionation. To separate cytoplasmic and nuclear proteins, cells were washed twice with ice-cold phosphate buffered saline (PBS) and collected with ice-cold PBS (1000 μl per 10 cm diameter dish) into 1.5 mi microcentrifuge tube. The tube was allowed to pop-spin for 10 sec and supernatant was discarded. The cell pellet was triturated 5 times using 0.1% NP40-PBS. This was considered as whole cell lysate. The remainder in the tube was allowed to pop-spin again for 10 sec. The supernatant was collected in a new tube as cytoplasmic fraction. The pellet was resuspended with ice-cold 0.1% NP40-PBS and pop-spinned for 10 sec. The supernatant was discarded and the resulting pellet was considered as nuclear fraction.

Cell Attachment Assay

Cells were seeded onto a 24-well plate coated with fibronectin at a density of 1×10^5 cells per well. After 20 min, 40 min, 60 min and 120 min, unattached cells were removed by tapping the plate and rinsing the wells with PBS twice. Attached cells were counted in five representative high-power fields. The data are presented as the average of the results from three independent experiments.

Cell Spreading Assay

Cells were seeded onto a 24-well plate coated with fibronectin at a density of 1×10^5 cells per well and fixed 1 h later. Spread and nonspread cells were counted in five

representative high-power fields. Nonspread cells were defined as small round cells with few or no membrane protrusions, whereas spread cells were defined as large cells with extensive visible lamellipodia. The results represent the percentage of spread cells in five high-power fields. The data are presented as the average of the results from three independent experiments.

Migration assay

Cell migration was assessed using 24-well Boyden chambers (8 μm pore size, 6.5 mm membrane diameter). The lower surface of the filter was coated with fibronectin to promote cell migration. $5x10^4$ cells were seeded onto the upper surface of the chamber and 20 h later, the cells were fixed with 100% ethanol and stained with 0.5 % crystal violet. Cells that migrated to the lower surface of the chambers were counted under a microscope at 10X magnification. Five independent fields were randomly selected in each experiment, and three independent experiments were performed.

Invasion assay

To measure cell invasion using 24-well Boyden chambers (8 μm pore size, 6.5 mm membrane diameter), the filter was pre-coated with Matrigel, and $1.5x10^5$ cells were seeded onto the upper surface of the chamber. After 20 hours, the cells were fixed with 100% ethanol and stained with 0.5% crystal violet. Cells that invaded the lower surface of the filters were surveyed under a microscope at 10X magnification and five fields were randomly selected. Three independent experiments were performed.

Colony formation assay

Cells (1×10^4) were mixed with 0.36% agar in DMEM supplemented with 10% FBS and overlaid onto a 0.72% agarose layer in 6-well plates. After 2 weeks of incubation, colonies in five randomly selected fields were counted. Three independent experiments were performed.

Anoikis assay

siRNA-transfected cells were mixed with 1.68% methyl cellulose in medium and incubated for 48 hours. After incubation, the cells were collected via centrifugation and subjected to TUNEL assay using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's protocol.

Statistical Analysis

Data are expressed as the mean ± SD. Comparisons between the groups were performed using unpaired *t* tests. A chi-square test was performed for the immunohistochemical analysis. *P* values of <0.05 were considered statistically significant.

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Figure legends

Figure 1. UBE2S is expressed in breast cancer cells and tissues. (A) Expression of UBE2S in the indicated cell lines was examined by immunoblot analysis. (B) BT20 cells cultured on fibronectin-coated glass coverslips were fixed and stained with anti-UBE2S antibody (scale bar=20μm). (C) Cytoplasmic and nuclear fractions were immunoblotted with the indicated antibodies. (D) Tissue microarray was stained with anti-UBE2S antibody. Representative images of score0, score1, score2 and score3 (E) Semiquantitative scoring of the image intensity of each tissue was performed. The graph indicates the percentage of score2- and score3-positive samples.

Figure 2. Depletion of UBE2S induces disruption of actin stress fibers and focal adhesion formation. (A) Representative images of cells transfected with siRNAs. (B) BT20 and MDA-MB-231 cells cultured on fibronectin-coated glass coverslips were transfected with siRNAs and 72 h later, cells were fixed and immunostained for vinculin and F-actin (scale bar=20 μm). (C) Expression of phosphorylated FAK and Src in siRNA-transfected cells were examined by immunoblot analysis.

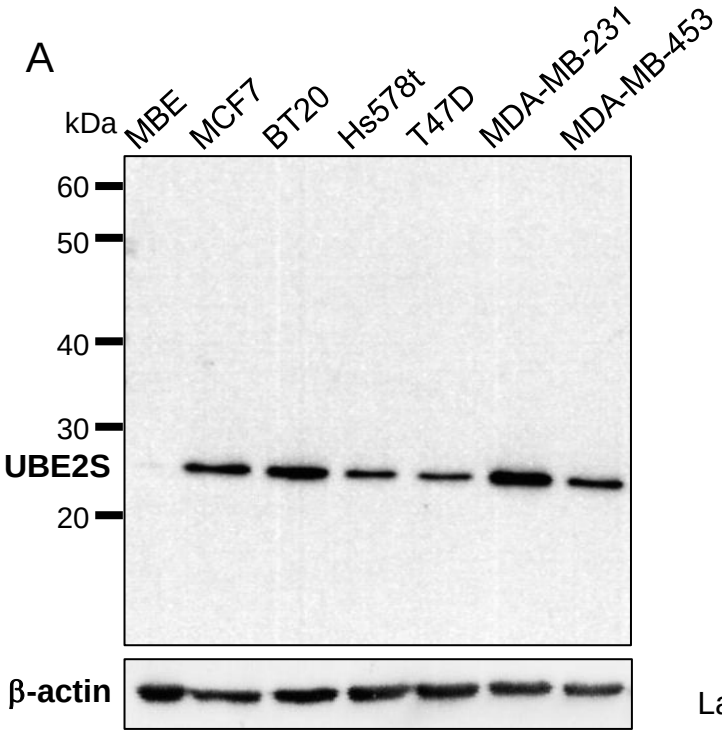
Figure 3. Cell attachment and spreading were suppressed by UBE2S depletion. (A) siRNA-transfected cells were seeded onto the fibronectin-coated surface and the number of attached cells was counted at the indicated time points. (B) siRNA-transfected cells were seeded onto the fibronectin-coated surface and fixed 60 min later. Representative pictures are shown and the graph shows the percentage of spread cells (**$P<0.01$).

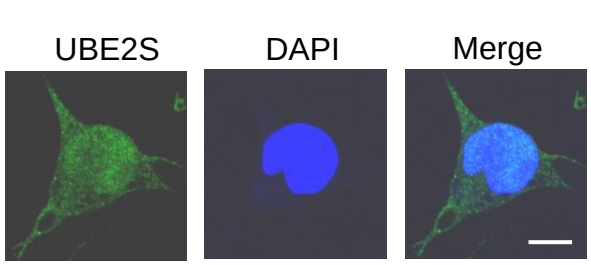
Figure 4. Cell migration and invasion were suppressed by UBE2S knockdown. (A) Cells were subjected to a migration assay. The graph indicates the average number of

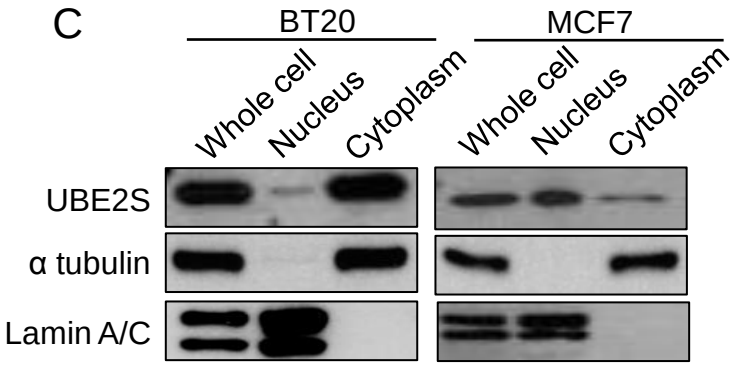
migrated cells per field (**P<0.01). (B) Cells were subjected to an invasion assay. The graph indicates the average number of invaded cells per field (**P<0.01).

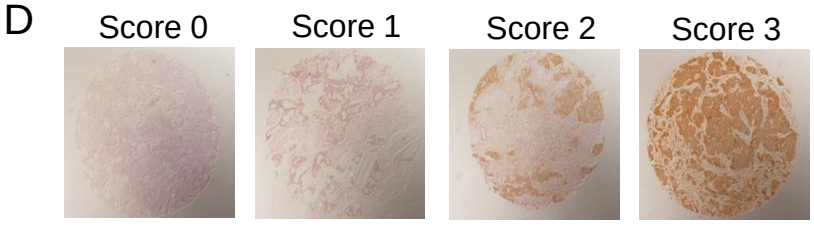
Figure 5. UBE2S knockdown induced anoikis. (A) siRNA-transfected cells were cultured in soft agar for 2 weeks and then pictures were taken. The representative images of colonies are shown. The graph shows the average number of colonies per field (**P<0.01). (B) siRNA-transfected cells were cultured in suspension for 48 h and then subjected to TUNEL assay. Representative images of the TUNEL assay are shown. The graphs indicate the percentage of cells survived (*P<0.05).

Figure 1









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Breast Cancer	119	17	26	45	31

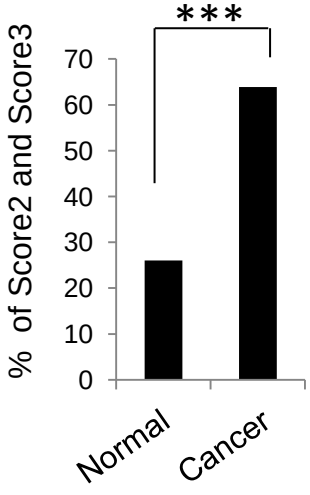
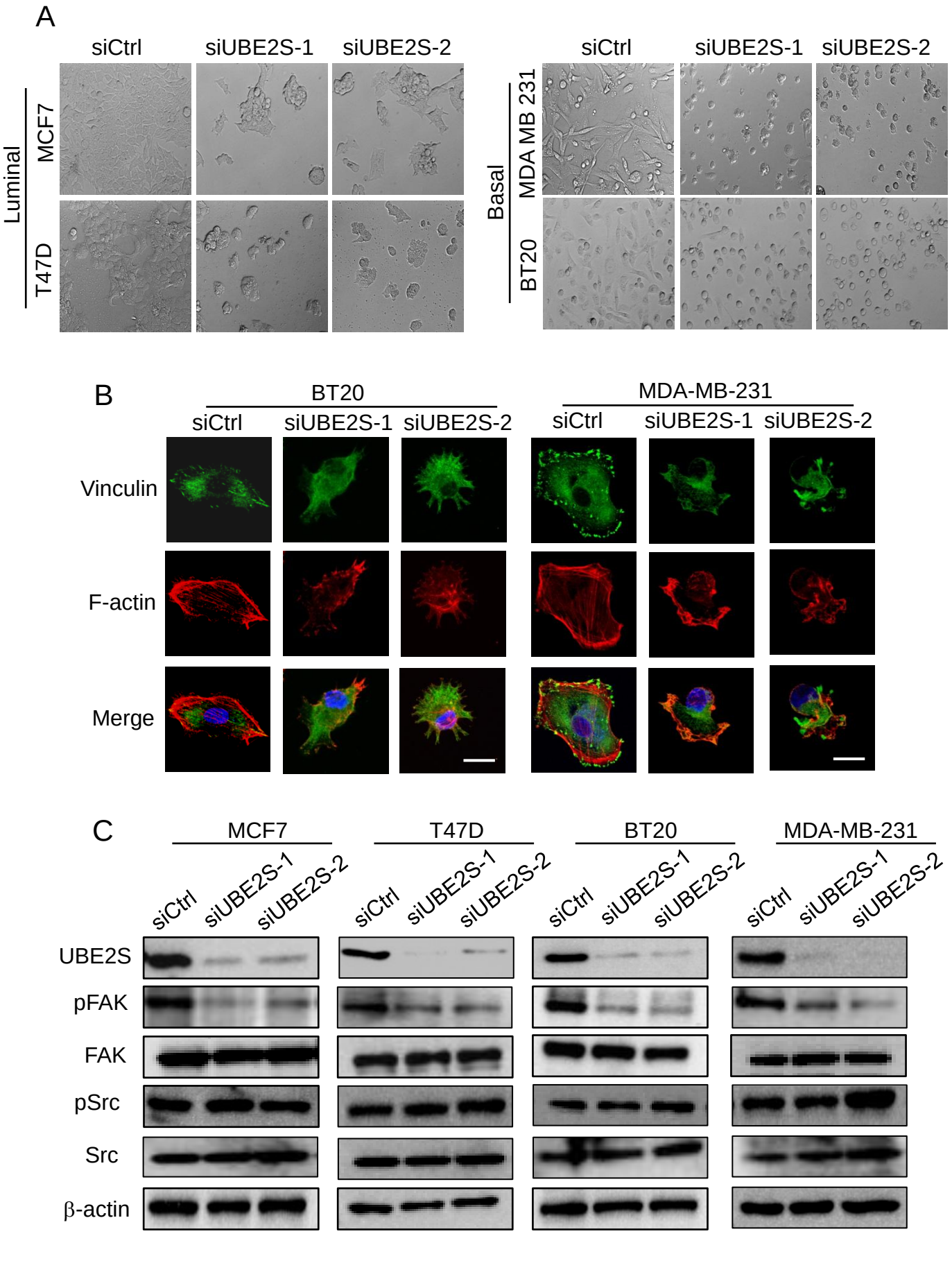


Figure 2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Vinculin
F-actin
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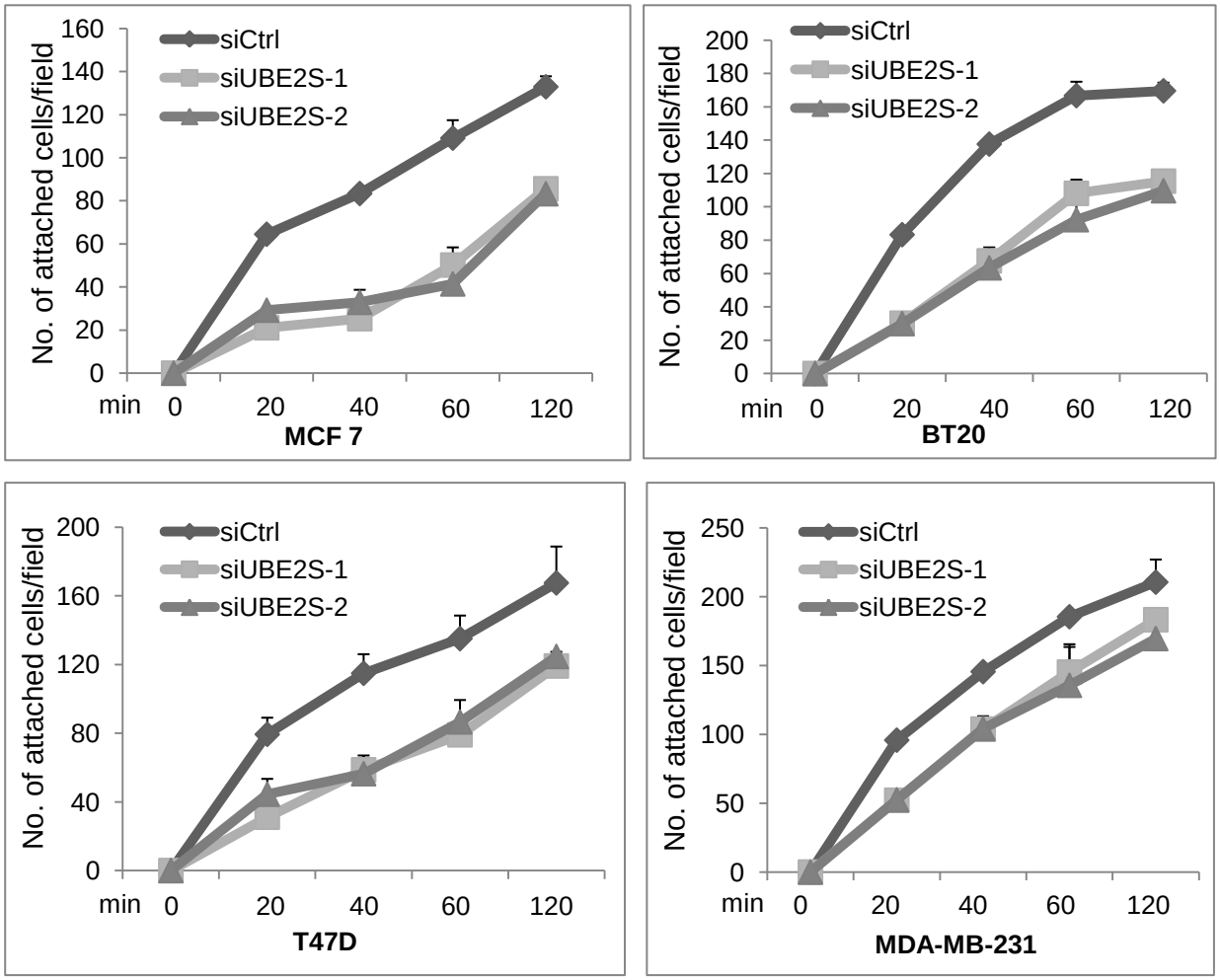
C
MCF7
T47D
BT20
MDA-MB-231
UBE2S
pFAK
FAK
pSrc
Src
β-actin

Figure 3

A


No. of attached cells/field
siCtrl
siUBE2S-1
siUBE2S-2
min 0 20 40 60 120
MCF 7
BT20
T47D
MDA-MB-231

B

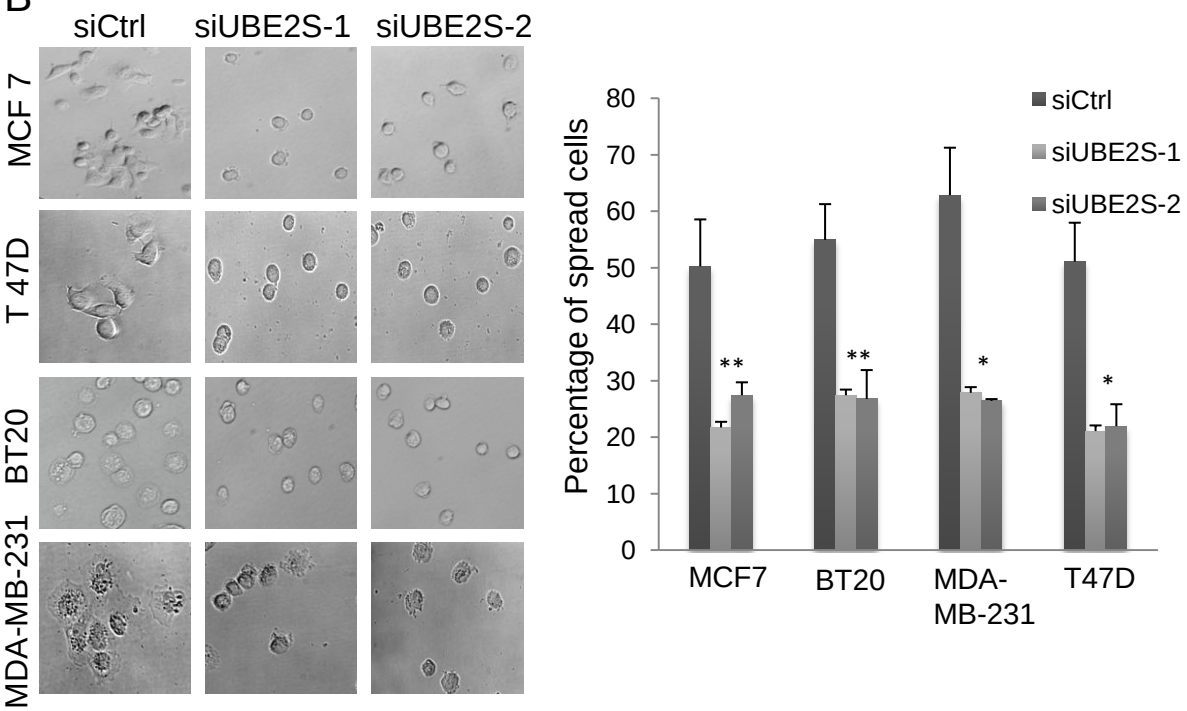

siCtrl
siUBE2S-1
siUBE2S-2
MCF 7
T 47D
BT20
MDA-MB-231
Percentage of spread cells
MCF7
BT20
MDA-MB-231
T47D
**
**
*
*

Figure 4

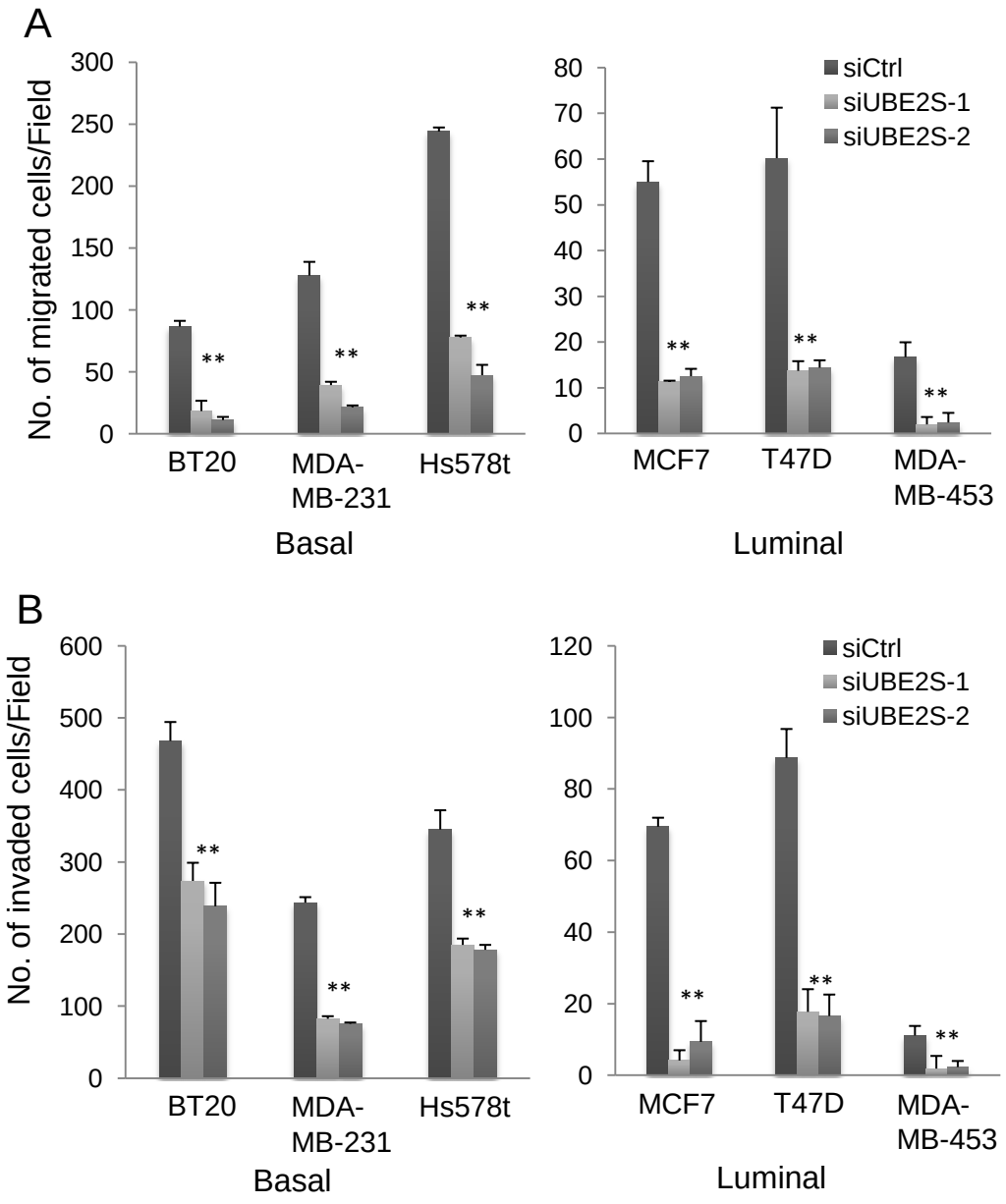
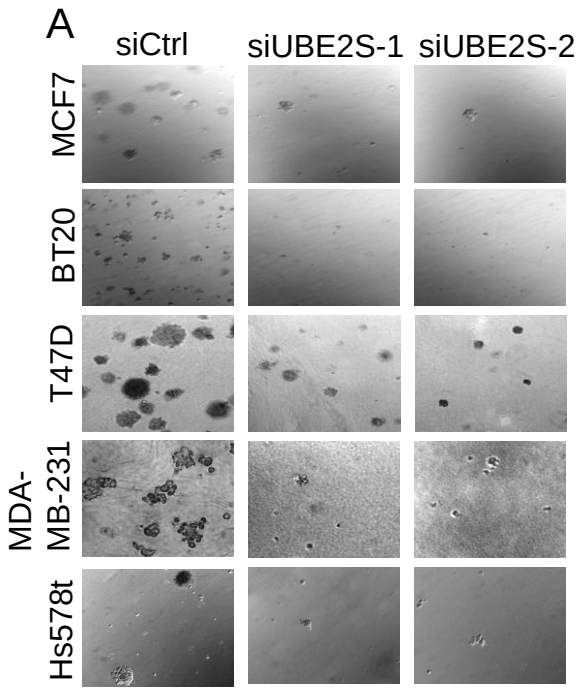
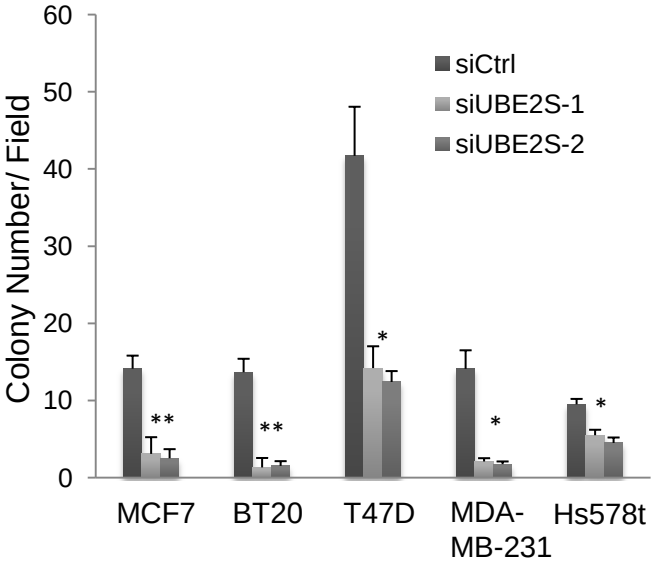
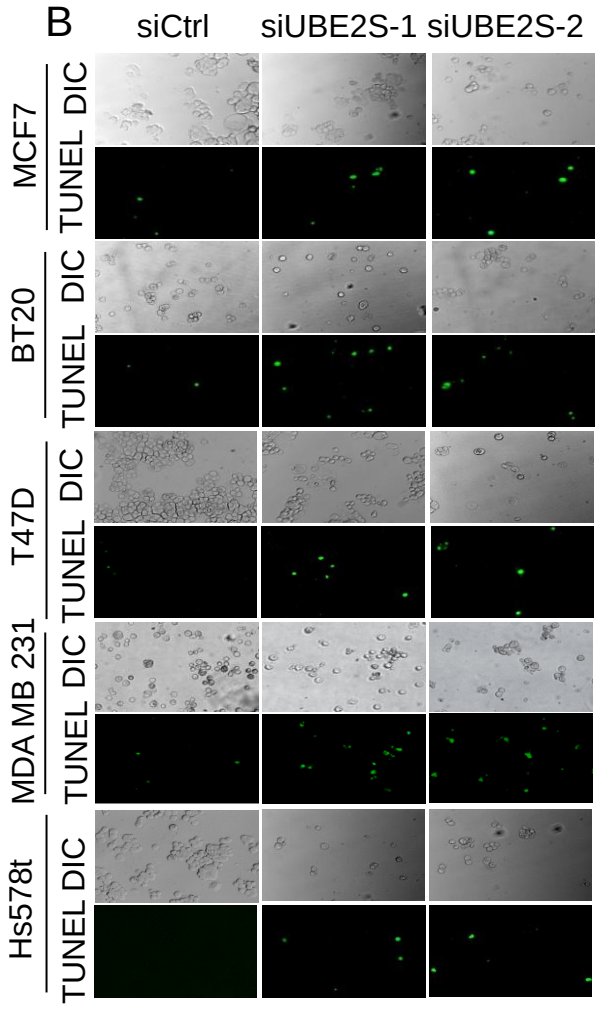

A
No. of migrated cells/Field
300
250
200
150
100
50
0
**
**
**
BT20
MDA-MB-231
Hs578t
Basal
80
70
60
50
40
30
20
10
0
siCtrl
siUBE2S-1
siUBE2S-2
**
**
**
MCF7
T47D
MDA-MB-453
Luminal
B
No. of invaded cells/Field
600
500
400
300
200
100
0
**
**
**
BT20
MDA-MB-231
Hs578t
Basal
120
100
80
60
40
20
0
siCtrl
siUBE2S-1
siUBE2S-2
**
**
**
MCF7
T47D
MDA-MB-453
Luminal

Figure 5


A
siCtrl
siUBE2S-1
siUBE2S-2
MCF7
BT20
T47D
MDA-MB-231
Hs578t


Colony Number/ Field
60
50
40
30
20
10
0
siCtrl
siUBE2S-1
siUBE2S-2
**
**
*
*
*
MCF7
BT20
T47D
MDA-MB-231
Hs578t


B
siCtrl
siUBE2S-1
siUBE2S-2
MCF7
DIC
TUNEL
BT20
DIC
TUNEL
T47D
DIC
TUNEL
MDA MB 231
DIC
TUNEL
Hs578t
DIC
TUNEL

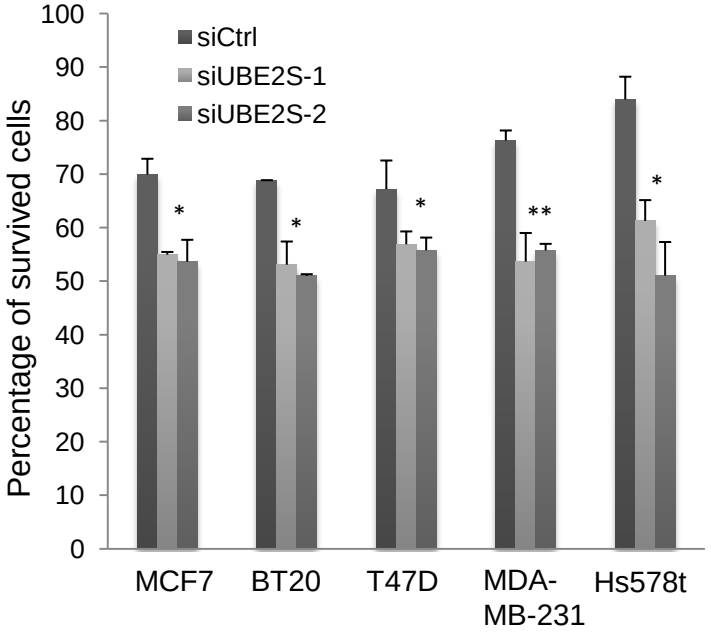

Percentage of survived cells
100
90
80
70
60
50
40
30
20
10
0
siCtrl
siUBE2S-1
siUBE2S-2
*
*
*
**
*
MCF7
BT20
T47D
MDA-MB-231
Hs578t

Table 1

UBE2S expression in Normal and Breast Cancer Patient's tissue lesions as evaluated by tissue microarray based immunohistochemistry

Characteristics	No. of Patients	UBE2S Expression ,No. (%)		
		Low	High	p Value
All Study Subjects				
Normal/NAT	23	17 (73.9)	6 (26.1)	
Cancer	119	43 (36.1)	76 (63.9)	< 0.001[a]
Cancer Patients				
Age,y				
≥55	30	7 (23.3)	23 (76.7)	0.0585[b]
<55	89	37 (41.6)	52 (58.4)	
Pathology diagnosis				
Invasive ductal carcinoma	90	22 (24.4)	68 (75.6)	<0.001[c]
Medullary carcinoma	13	7 (53.8)	6 (46.2)	0.0171[d]
Invasive lobular carcinoma	8	8 (100)	0	
Mucinous carcinoma	4	4 (100)	0	
Neuroendocrine carcinoma	2	2 (100)	0	
Aprocrine carcinoma	2	0	2 (100)	
Clinical Stage				
I - IIa	51	29 (56.9)	22 (43.1)	
IIb - IV	66	19 (28.8)	47 (71.2)	0.002[e]
Unknown	2	2 (100)	0	

Low expression means score 0/1; high expression means score 2/3

[a]Normal vs cancer

[b]Patient's age(y) ≥55 vs <55

[c]Invasive ductal carcinoma vs invasive lobular carcinoma

[d]Medullary carcinoma vs mucinous carcinoma

[e]Clinical Stage I – IIa vs IIb - IV