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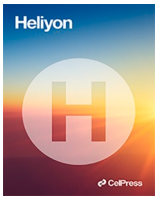

Research article

QSOX2 Upregulated in triple-negative breast cancer exacerbates patient prognosis by stabilizing integrin β1

A-In Kim [a,b], Ji Hoon Oh [a,b,1], Je-Yoel Cho [a,b,*]

[a] Department of Biochemistry, Brain Korea 21 Project and Research Institute for Veterinary Science, Seoul National University College of Veterinary Medicine, Seoul, 08826, Republic of Korea
[b] Comparative Medicine Disease Research Center, Seoul National University, Seoul, 08826, Republic of Korea

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ABSTRACT

Breast cancer (BC) remains a significant global health threat, with triple-negative breast cancer (TNBC) standing out as a particularly aggressive subtype lacking targeted therapies. Addressing this gap, we propose Quiescin Q6 sulfhydryl oxidase 2 (QSOX2) as a potential therapeutic target, a disulfide bond-forming enzyme implicated in cancer progression. Using publicly available datasets, we conducted a comprehensive analysis of QSOX2 expression in BC tumor and non-tumor tissues, assessing its specificity across different molecular subtypes. We further explored correlations between QSOX2 expression and patient outcomes, utilizing datasets like TCGA and METABRIC. In addition, we performed in vitro experiments to evaluate QSOX2 expression in BC cell lines and investigate the effects of QSOX2 knockdown on various TNBC cellular processes, including cell proliferation, apoptosis resistance, migration, and the epithelial-to-mesenchymal transition (EMT). Our results reveal significantly elevated QSOX2 expression in BC tumor tissues, particularly in TNBC, and establish an association between high QSOX2 expression and increased patient mortality, cancer progression, and recurrence across various BC subtypes. Notably, QSOX2 knockdown in TNBC cell lines reduces cell proliferation, enhances apoptosis, and suppresses migration, potentially mediated through its influence on the EMT process. Furthermore, we identify a significant link between QSOX2 and integrin β1 (ITGB1), suggesting that QSOX2 enhances ITGB1 stability, subsequently exacerbating the malignancy of TNBC. In conclusion, elevated QSOX2 expression emerges as a key factor associated with adverse patient outcomes in BC, particularly in TNBC, contributing to disease progression through various mechanisms, including the modulation of ITGB1 stability. Our findings underscore the potential of targeting QSOX2 as a therapeutic strategy for improving patient prognoses not only in TNBC but also in other BC subtypes.

1. Introduction

Despite medical advances and research, breast cancer (BC) remains a global threat to women's health. In 2020, over 2.3 million new BC cases were diagnosed, with more than 685,000 recorded deaths [1]. BC is categorized into three subtypes based on molecular

* Corresponding author. Department of Biochemistry, College of Veterinary Medicine Seoul National University, Gwanak-ro1, Gwanak-gu, Seoul, Republic of Korea.
E-mail address: jeycho@snu.ac.kr (J.-Y. Cho).
[1] Current address: Department of Biological Sciences, Keimyung University College of Natural Sciences, Daegu 42601, Republic of Korea.

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Abbreviations

AKT	Protein Kinase B
BC	Breast cancer
CCLE	Cancer Cell Line Encyclopedia
CHX	Cycloheximide,
CRC	Colorectal cancer
E2F1	E2F transcription factor 1
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
GPNMB	Glycoprotein non-metastatic B
HER2	Human epidermal growth factor receptor 2
ITGB1	Integrin β1
ITGB3	Integrin β3
ITGB4	Integrin β4
LCM	Laser capture microdissection
NSCLC	Non-small cell lung cancer
PDI	Protein disulfide isomerase
PI	propidium iodide,
PR	Progesterone receptor
QSOX2	Quiescin Q6 sulfhydryl oxidase 2
SEM	Standard error of the mean
SRB	Sulforhodamine B
TCGA	The Cancer Genome Atlas
TNBC	Triple negative breast cancer
VEGF	Vascular endothelial growth factor

receptors: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Notably, triple-negative breast cancer (TNBC), often termed 'basal' [2], lacks all three of these receptors and constitutes 15–20% of all BC cases [3]. Regrettably, TNBC stands as the most aggressive BC subtype, characterized by its elevated metastatic tendency, frequent relapses, and the absence of targeted receptor-based drug treatments [3]. This emphasizes the need for gene therapy, where we propose Quiescin Q6 sulfhydryl oxidase 2 (QSOX2) as a potential therapeutic target.

QSOX2 is a member of the QSOX gene family, and it plays a crucial role in catalyzing disulfide linkages within unfolded proteins [4]. This function occurs both intracellularly and extracellularly [5]. These disulfide bonds are essential for maintaining protein structural integrity and functionality, impacting various biological processes [6]. In specific contexts, the overexpression of enzymes involved in disulfide bond formation can lead to an excess of disulfide bonds, contributing to cancer cell survival and unfavorable outcomes. Notably, protein disulfide isomerase (PDI), another disulfide bond-forming enzyme [7], has been associated with aggressive behavior in different cancers, such as ovarian cancer and melanoma [8]. Excessive disulfide bond formation, facilitated by PDI, enhances protein stability, promoting cancer cell invasion and angiogenesis [9]. Similarly, QSOX2, as a disulfide bond-forming enzyme, may influence proteins related to cancer cell proliferation and migration, potentially contributing to malignant traits.

QSOX2 remains relatively unexplored, but it recently has been linked to cancer progression. E2F transcription factor 1 (E2F1) has been shown to influence QSOX2 during the cell cycle, a process critical to non-small cell lung cancer (NSCLC) cell proliferation. Additionally, QSOX2 has shown prospect as a biomarker for tracking tumor size and treatment progress in NSCLC [5]. Increased QSOX2 expression has been associated with aggressive cancer progression and unfavorable prognoses in colorectal cancer (CRC), suggesting its potential as a novel biotherapy target for CRC patients [10]. Despite these insights, the role of QSOX2 in BC remains a significant gap in understanding, and the molecular mechanisms driving BC progression are yet to be elucidated. Importantly, our study stands as a pioneering effort, aiming to fill this critical void by being the first investigation into the relationship between QSOX2 and breast cancer, holding the potential to unveil crucial aspects of the role of QSOX2 in BC and contribute to advancing our understanding of this complex disease.

In this study, we demonstrate the genetic upregulation of QSOX2 in BC through patient data analysis and *in -vitro* experiments using TNBC cell lines. Notably, TNBC cells exhibited pronounced QSOX2 overexpression, which promotes cell proliferation and migration. To gain comprehensive insight into the underlying mechanisms responsible for these observed phenomena, we hypothesized that integrins may be involved as downstream effectors of QSOX2.

Integrins are cell surface molecules known to impact cancer cell behavior, influencing survival, migration, and drug resistance [19]. These molecules have subunits, with disulfide bonds playing a crucial role in their function [20]. Enzymatic disulfide bond exchange is essential for proper integrin signaling and structure [21]. Disrupting these bonds can affect integrin function, including the expression of αIIbβ3 [21].

Of particular relevance is the subunit integrin β1 (ITGB1), which is associated with cancer metastasis [19]. Studies involving ITGB1 deletion in mouse models have demonstrated reduced cell proliferation, and previous research has linked ITGB1 to cancer cell activation, as indicated by the proliferation marker Ki-67 [22,23]. These findings support our hypothesis that elevated QSOX2 enhances integrin structure and function by promoting disulfide bond formation, thereby accelerating TNBC cell proliferation and migration.

Specifically, the targeted reduction of QSOX2 expression led to a notable decrease in ITGB1 levels within TNBC cells. Moreover, QSOX2 knockdown weakens the stability of ITGB1. This phenomenon is supported by reduced ITGB1 retention upon halting protein synthesis and decreased ITGB1 synthesis in the absence of proteasomal degradation. Consequently, genetically lowering QSOX2 levels in TNBC patients could reduce the function of ITGB1, thereby counteracting the aggressiveness of TNBC and potentially yielding improved cancer outcomes.

2. Material and methods

2.1. Cell culture

Human BC cell lines were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). Normal epithelial MCF-10A was cultured in MEBM Basal Medium (CC-3151, Lonza, Basel, Switezerland) and MEGM Supplements (CC4136, Lonza, Basel, Switezerland). SKBR-3 and T-47D were cultured in RPMI 1640 media with L-glutamine (SH30027.01, Hyclone, UT, USA). MCF-7, MDA-MB-231, and Hs578T cells were cultured in DMEM/High glucose with L-glutamine and sodium pyruvate (SH30243.01, Hyclone, UT, USA). All culture media were supplemented with 10% fetal bovine serum (1600044, Gibco, MA, USA) and 1% antibiotic–antimycotic (15240-062, Gibco, MA, USA). All cells were cultured in a humid incubator at 37 °C with 5% CO2, and their mycoplasma contamination was verified to be absent.

2.2. RNA isolation and quantitative RT-PCR

RNA isolation and quantitative RT-PCR were processed as described previously [11]. Briefly, reverse transcription with isolated RNA was performed by using CellScript™ cDNA Master Mix (CDS400, CellSafe, Gyeonggi-do, Republic of Korea) as per the manufacturer's instructions. All the specific primers used for PCR amplification are listed in Supplementary Table 1. The comparative ΔΔCt method was employed to assess the relative mRNA levels, with the GAPDH gene serving as an internal reference for normalization.

2.3. siRNA transfection

Negative control siRNA (Bioneer, Daejeon, Republic of Korea) and QSOX2 siRNA were utilized for knockdown experiments. The cited sequence of #1 QSOX2 siRNA is as follows: Sense: GCAGCCAUUACGUGGCUAUtt, AntiSense: aaAUAGCCACGUAAUGGCUGC [5]. The pre-designed and combined #2 QSOX2 siRNA was purchased from Bioneer (169714-1, 169714-2, 169714-3, Bioneer, Daejeon, Republic of Korea). Each siRNA was transfected using Lipofectamine RNAiMAX Transfection Reagent (13778030, Invitrogen, MA, USA) according to the manufacturer's instructions. Alternatively, siRNA transfection was performed using the NEPA21 Super Electroporator (Nepa Gene, Chiba, Japan) via cuvette electroporation, according to the manufacturer's instructions. The specific electroporation conditions for the poring pulse and transfer pulse are listed in Supplementary Table 2.

2.4. Protein extraction and western blot

Protein extraction and Western blot were carried out as described previously [12]. Briefly, for secretory protein collection, media supernatant was treated with the protease inhibitor cocktail. Anti-β-Actin (1:3000, A5441-.2 ML, Sigma-Aldrich, MO, USA), anti-QSOX2 (1:1000, ab121376, Abcam, Cambridge, UK), anti-ITGB1 (1:1000, #4706, Cell Signaling, MA, USA), anti-pAKT T308 (1:1000, #9275S, Cell Signaling, MA, USA), anti-pAKT S473 (1:1000, #9271S, Cell Signaling, MA, USA), and anti-total AKT1 (1:1000, #9272S, Cell Signaling, MA, USA) were used as primary antibodies. Goat anti-rabbit (A120-101P, Bethyl, TX, USA) and Goat anti-Mouse (Bethyl, A90-116P, TX, USA) were used as secondary antibodies.

2.5. Protein stability analysis

MDA-MB-231 and Hs578T cells underwent treatment with 10 μg/ml of cycloheximide (CHX; Sigma-Aldrich, C4859, MO, US) for a duration ranging from 6 to 12 h, or with 20 μM of MG-132 (Sigma-Aldrich, M7449, MO, US) for a period spanning 6–20 h. Following the treatments, cell lysates were collected using previously described methods for subsequent Western blot analysis.

2.6. Cell proliferation analysis

Cell proliferation was assessed through manual cell counting using a hematocytometer. Only live cells, which were stained with a 0.4% trypan blue solution (Gibco, 15250-061, MA, US), were counted under a microscope. For Sulforhodamine B (SRB) assay, cells were first seeded in 96-well plates. After fixing the cells with 100% MeOH for 1 h, they were washed with water and stained with 0.4% SRB dye (Santa Cruz, 3520-42-1, TX, US) in 1% acetic acid solution for 30 min at room temperature. 5 min after 10 mM Tris base solution (pH 10.5) was added to the washed and dried cells, the amount of SRB was measured fluorometrically at 510 nm. Ki-67 assay

procedure is described in the next following category.

2.7. Flow cytometry analysis

To assess proliferation using the Ki-67 marker, cells were fixed with 70% ethanol at −20 °C for 2 h 48 h after transfection, followed by washing with a cell staining buffer (Biolegend, 420201, CA, US). Flow cytometry analysis was conducted subsequent to a 30-min incubation with APC-conjugated Ki-67 antibody (Invitrogen, 17-5699-42, MA, US) at room temperature. For cell cycle arrest analysis,

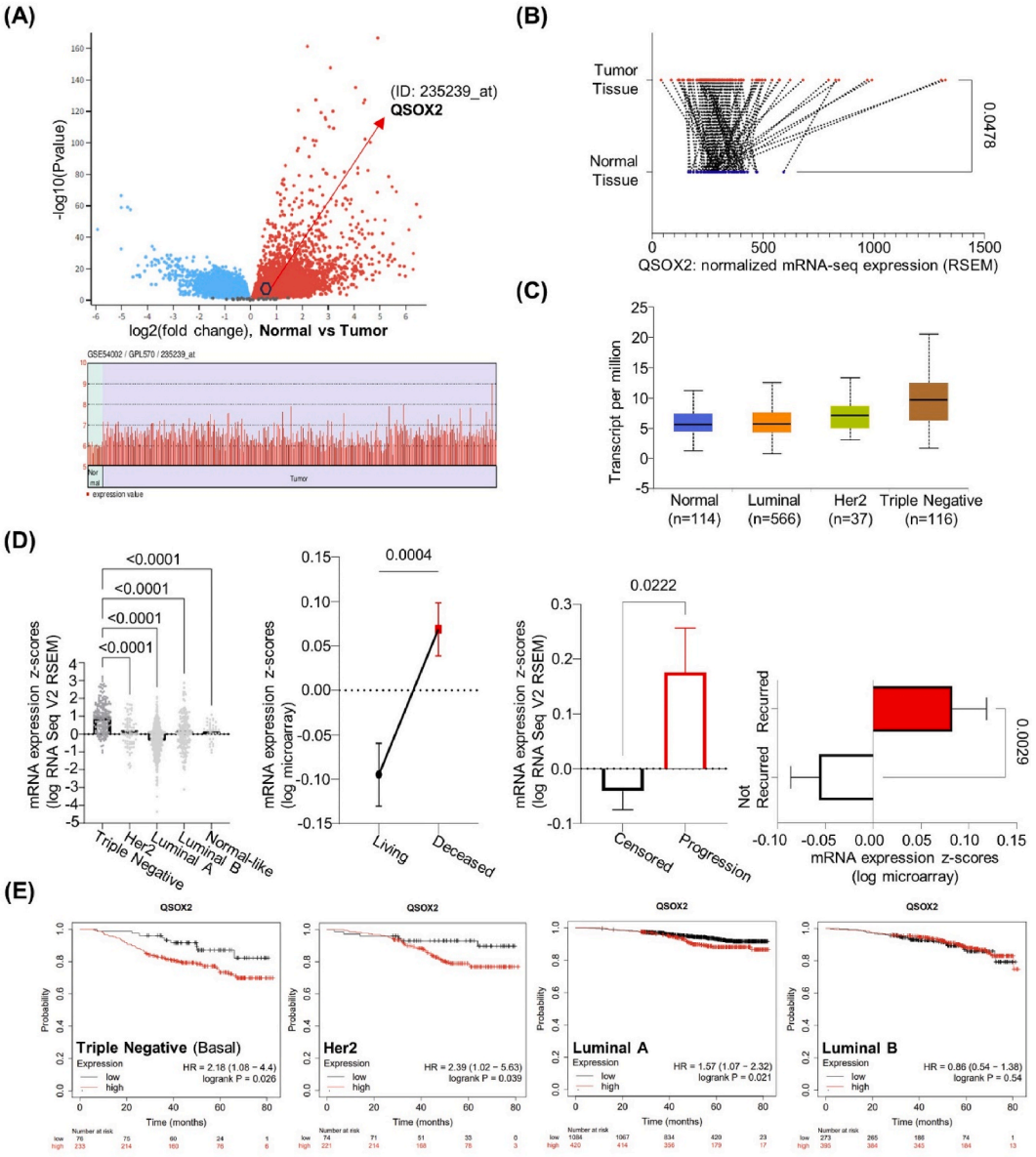

Fig. 1. QSOX2 expression patterns and implications in BC progression and prognosis. (A) QSOX2 (ID: 235239_at) RNA expression was compared in tumor and non-tumor tissue samples from clinical BC patients using a publicly available dataset (GSE54002) from Gene Expression Omnibus (GEO). (B) QSOX2 expression was analyzed via TCGA mRNA-seq analysis of paired BC patient samples (n = 96). (C) Using TCGA data via UALCAN, the specificity of QSOX2 expression was investigated across distinct BC subtypes. (Statistical significance; Normal-vs-Luminal: <1E-12; Normal-vs-TNBC: 8.476000E-03) (D) cBioPortal dataset was utilized for the following analyses. PanCancer Atlas (994 patients) from TCGA was used for QSOX2 mRNA expression comparison among BC subtypes and BC progression-free status inquiry. METABRIC (1904 patients) from Nature 2012 & Nature Commun 2016 was used for BC relapse-free status and overall survival status examination. All the values were depicted by mRNA expression z-scores relative to all samples. (E) A significant correlation between the high expression levels of the QSOX2 gene and shorter survival rate was verified by the Kaplan–Meier plot in each BC subtype. Statistical significance was assessed using Student's t-test, one-way, or two-way ANOVA, with a significance threshold established at $p < 0.05$.

whole cells were harvested and washed twice. The cells were fixed for more than 2 h with 70% ethanol added dropwise. The cell pellets were stained with a propidium iodide (PI) solution (Invitrogen, P3566, MA, US), and then RNase A (Thermo Fisher, EN0531, MA, US) was also added. Apoptosis analysis involved the use of the APC Annexin V Apoptosis Detection Kit with PI (Biolegend, 640932, CA, US). Briefly, cells were suspended in Annexin V binding buffer, followed by incubation with APC-Annexin V and PI solutions for 15 min at room temperature. Subsequently, Annexin V binding buffer was added, and flow cytometry analysis was performed using an FACS Aria II instrument (BD Biosciences, CA, US).

2.8. Cell migration analysis

For transwell assay, 2.5 x 10^5 cells in serum free media were seeded to the inner well of the chamber (Corning, 3422, NY, US), and 10% FBS supplemented media was placed in the outer well. After a certain time period, the chamber membrane was washed and fixed with 4% PFA (P2031, Biosesang, Gyeonggi-do, KR) for 2 min. Then, the cells were lysed with 100% MeOH for 10 min, and the cells were dyed with 0.2% crystal violet. In the wound healing assay, cells treated with mitomycin C (10 μg/ml, Merck, M4287, Darmstadt, DE) at a concentration of 1×10^6 cells per well were plated onto six-well plates and allowed to reach approximately 80% confluence. Then, artificial scratches were created in each well using sterile 1000-μl pipette tips. Cell migration distance was photographed at 0 and 48 h using a microscope.

2.9. Public data acquisition and analysis

The GEO dataset (GSE54002), obtained from the GEO database (http://www.ncbi.nlm.nih.gov/geo), was utilized to compare QSOX2 RNA levels between breast tumor and non-tumor tissues. These samples were acquired through laser capture microdissection (LCM). mRNA-seq data from The Cancer Genome Atlas (TCGA) project for BC were downloaded from FireBrowse (http://firebrowse.org). The RSEM-normalized expression data for QSOX2 consisted of 96 pairs, enabling the comparison of expression levels between tumor tissue and normal tissue from each patient. UALCAN (https://ualcan.path.uab.edu/index.html), an online publicly available database, was used to observe the mRNA expression of QSOX2 in major subtypes of breast invasive carcinoma. The association between QSOX2 mRNA expression and relapse-free status, progression-free status, overall survival status, and BC subtypes was analyzed using cBioPortal (https://www.cbioportal.org/). Survival analysis of BC patients was conducted using the Kaplan-Meier Plotter (http://kmplot.com/), a database created by integrating gene expression and clinical information from BC patients. The Cancer Cell Line Encyclopedia (CCLE) databank (https://sites.broadinstitute.org/ccle/) was employed to analyze the overall RNA expression of QSOX2 in various BC cell lines.

2.10. Statistical analysis

GraphPad Prism v9.0 software was employed for statistical analysis, and results were presented as the mean with the corresponding standard error of the mean (SEM) from three separate experiments or triplicate samples. Statistical significance was determined through Student's t-test, as well as one-way or two-way ANOVA, with a significance threshold set at $p < 0.05$.

3. Results

3.1. QSOX2 expression patterns and implications in BC progression and prognosis

Previous studies have indicated the elevated expression of QSOX2 in lung and colorectal cancers [5,10]; however, its expression and role in BC and other malignancies remain relatively unexplored. Consequently, our study aims to identify the function of QSOX2 in BC, addressing this gap in knowledge. Initially, we conducted a comparison of QSOX2 expression between tumor and non-tumor tissue samples obtained from clinical BC patients within a publicly available dataset (GSE54002). The analysis revealed a significantly higher level of total QSOX2 RNA expression in tumor tissues compared to non-tumor tissues (Fig. 1A). Moreover, in the TCGA mRNA-seq analysis comparing paired tumor and normal tissue samples obtained from each BC patient, the expression of QSOX2 was markedly elevated in tumor tissue (Fig. 1B). Subsequently, we considered whether QSOX2 expression exhibits specificity based on distinct molecular subtypes of BC. To investigate this aspect, we employed additional TCGA data via UALCAN. Upon analyzing mRNA levels, we observed that QSOX2 expression reached its peak in TNBC, surpassing levels in normal conditions as well as luminal and Her2-enriched BC (Fig. 1C). Likewise, analysis of the cBioPortal dataset for breast invasive carcinoma (TCGA, PanCancer Atlas) revealed that QSOX2 mRNA expression was the highest within the basal subtype, often known as TNBC, in contrast to other molecular subtypes of BC (Fig. 1D).

To explore the potential link between elevated QSOX2 expression and the prognoses of BC patients, we conducted an analysis utilizing TCGA dataset from PanCancer Atlas and METABRIC via the cBioPortal platform. Our findings revealed a positive correlation between QSOX2 expression and a higher rate of patient mortality (Fig. 1D). Moreover, patients with significantly higher QSOX2 expression levels exhibited an increased likelihood of experiencing cancer progression and recurrence (Fig. 1D). High QSOX2 expression was associated with worse survival rates not only in overall BC patients but also in those with TNBC, Her2-enriched, and Luminal A subtypes (Fig. 1E).

Prior to conducting *in -vitro* experiments, we initially assessed QSOX2 expression across a spectrum of 60 distinct human BC cell lines using CCLE data. Interestingly, among these cell lines, MDA-MB-231 and Hs578T, both of which are TNBC cell lines, exhibited the

highest levels of QSOX2 RNA expression (Fig. 2A). As a result, our attention became directed towards TNBC, allowing us to investigate the specific contribution of QSOX2 in driving cancer progression and the underlying mechanisms behind this phenomenon. To corroborate the expression profile observed in publicly available data for QSOX2 across human BC cell lines, we directly assessed the mRNA, protein, and secreted protein levels of QSOX2 in TNBC cell lines relative to a normal epithelial breast cell line. As anticipated, the expression of QSOX2 was significantly elevated in all components of the TNBC cell lines compared to the normal epithelial breast cells (Fig. 2B–D).

3.2. Impact of QSOX2 expression on TNBC cell proliferation and apoptosis resistance

To further investigate the role of QSOX2 within TNBC cells, we established an RNA interference experiment aimed at suppressing the expression of our target gene. The specific sequences of our siRNAs have been detailed previously. By employing two distinct siRNAs, we assessed the efficacy of our RNA interference in reducing mRNA, protein, and secreted protein levels of QSOX2. Both #1 and #2 siRNAs demonstrated significant knockdown of QSOX2 expression in comparison to the transfection with randomly scrambled (negative control) siRNA (Figs. S1A–S1C). Consequently, we employed these two siRNAs consistently throughout our study to meticulously scrutinize the impact on the phenotype of TNBC cells upon QSOX2 silencing.

Given the potential role of QSOX2 in driving TNBC progression and unfavorable patient outcomes, our initial investigation centered on understanding the impact of QSOX2 expression levels on TNBC cell phenotypes. At first, we evaluated TNBC cell proliferation. The SRB assay, which measures cellular protein content, provided insights into cell density [13]. QSOX2 suppression visibly hindered TNBC cell growth, corroborated by protein content measurements (Fig. 3A). Furthermore, Ki-67, a universally accepted marker, was employed to evaluate cellular proliferation [14]. Flow cytometry revealed significantly fewer Ki-67-stained proliferative TNBC cells after QSOX2 knockdown (Fig. 3B). Manual cell counting also indicated a substantial reduction in TNBC cell proliferation upon QSOX2 suppression (Fig. 3C). In summary, diminished QSOX2 expression in TNBC correlated with reduced TNBC cell division.

To decipher the mechanism underlying effect of QSOX2 knockdown on TNBC cell proliferation, we initially explored the cell cycle, considering the potential impact of G0/G1 phase arrest on cell expansion [15]. We observed a trend indicating reduced synthesis phase and an increase in G0/G1 phase in QSOX2-knockdown TNBC cells, although no significant differences were noted in TNBC cell cycle phases between the two conditions. (Fig. 3D). Consequently, we investigated the impact of QSOX2 knockdown on apoptosis in TNBC cells. Apoptosis, a process often disrupted in cancer cells, can offer a survival advantage [16]. We hypothesized that QSOX2 knockdown might reduce TNBC cell proliferation by promoting apoptosis. To assess apoptosis, we measured the mRNA levels of key genes, such as NOXA (also known as PMAIP1) and PUMA (also referred to as BBC3), which are recognized markers or inducers of apoptosis [17]. As expected, QSOX2 knockdown resulted in higher mRNA expression of these apoptosis markers in TNBC cells (Fig. 3E). Consistent with our previous results, inhibiting QSOX2 led to a noticeable increase in apoptotic cells, as demonstrated through Annexin

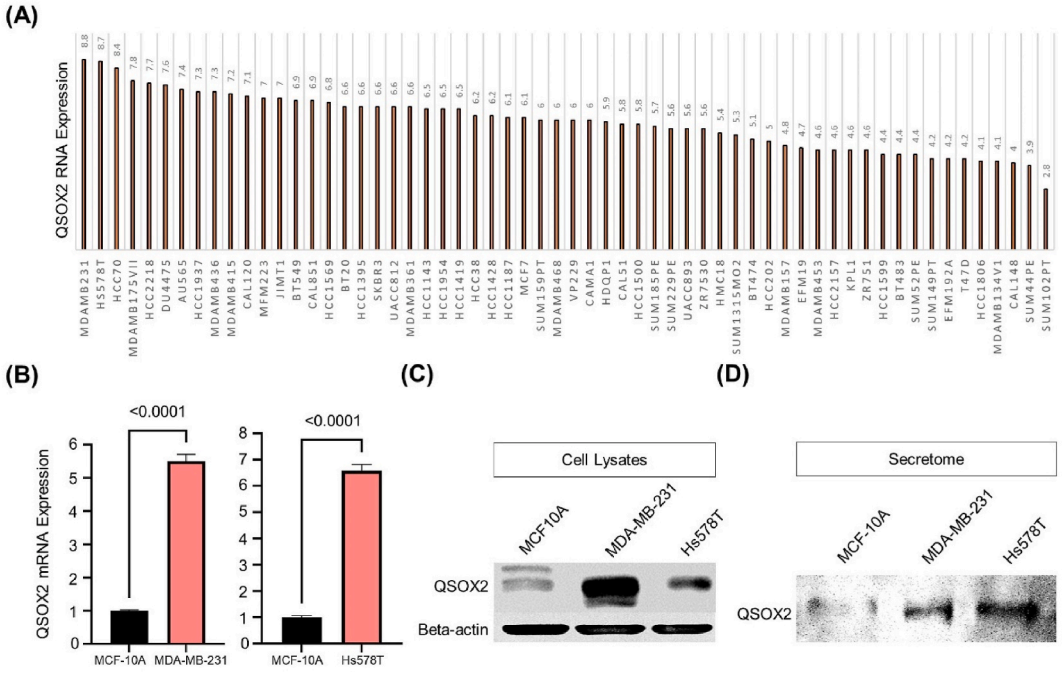

Fig. 2. QSOX2 is overexpressed in human TNBC cell lines. (A) QSOX2 RNA expression was evaluated in 60 diverse human BC cell lines using CCLE data. (B–D); (B) mRNA, (C) protein, and (D) secreted protein levels of QSOX2 were examined in TNBC cell lines compared to normal epithelial breast cells. Statistical significance was assessed using Student's t-test, one-way, or two-way ANOVA, with a significance threshold established at $p < 0.05$. Full non-adjusted images can be found in Supplementary Materials.

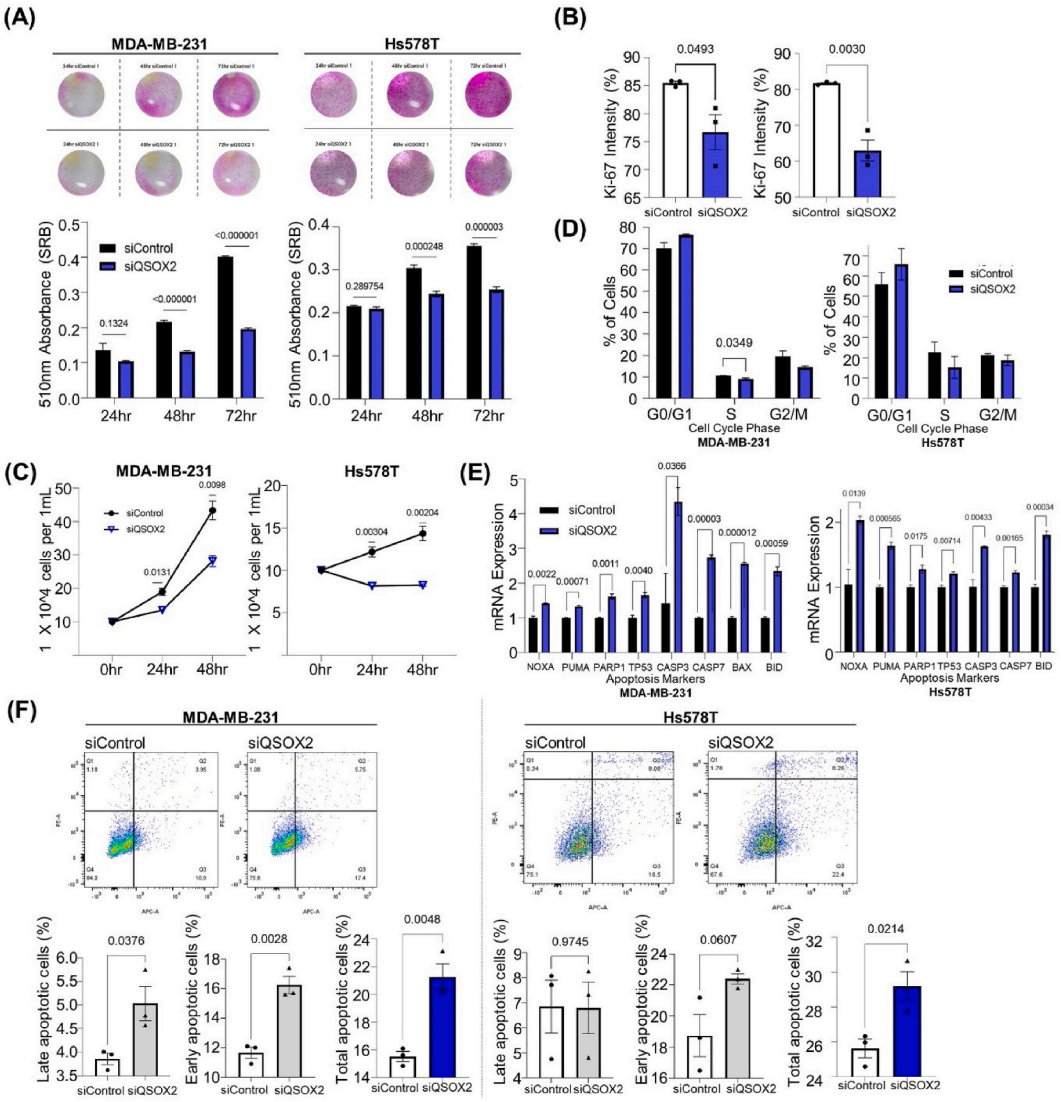

Fig. 3. QSOX2 promotes the proliferation of human TNBC cells. (A) TNBC cell proliferation was assessed using the SRB assay and revealed that QSOX2 suppression significantly inhibited TNBC cell growth, supported by protein content measurements.; The abbreviation "hr" indicates hours. (B) Ki-67 showed significantly fewer proliferative TNBC cells when evaluated using flow cytometry after QSOX2 knockdown. (C) Counting cells manually demonstrated a significant decrease in TNBC cell proliferation following QSOX2 suppression.; The abbreviation "hr" indicates hours. (D) No significant differences were observed among TNBC cell cycle phases regardless of QSOX2 levels. (E) In TNBC cells, the knockdown of QSOX2 resulted in increased mRNA expression of apoptosis markers. (F) Inhibition of QSOX2 resulted in a noticeable rise in apoptotic and necrotic cells, which was confirmed through Annexin V-PI staining followed by flow cytometry analysis. Statistical significance was assessed using Student's t-test, one-way, or two-way ANOVA, with a significance threshold established at $p < 0.05$.

V-PI staining followed by flow cytometry analysis (Fig. 3F). Altogether, our findings suggest that elevated QSOX2 expression in TNBC may contribute to apoptosis evasion, thereby promoting cancer cell survival and rapid proliferation.

3.3. *QSOX2 affects TNBC cell movement and EMT*

Additionally, to assess the alteration in migratory capacity, another hallmark of cancer cell aggressiveness, we investigated the impact of QSOX2 suppression on TNBC cell movement. According to the finding of wound-healing assay, the inhibition of QSOX2 led to a noticeable reduction in the number of migrating TNBC cells (Fig. 4A). Correspondingly, the *trans*-well assay demonstrated a decrease in the migration rate of TNBC cells upon QSOX2 knockdown (Fig. 4B). These results collectively confirm that the attenuation of QSOX2 expression also abates the migratory ability of TNBC cells.

Various factors play a role in governing the migration and invasion of cancer cells. For instance, conditions such as hypoxia and increased extracellular matrix (ECM) rigidity induce cancer cells to initiate a quest for more favorable surroundings [18]. In this study,

our focus centers on the role of EMT as a potential driving force behind the mobility of TNBC cells. Consequently, we examined the impact of QSOX2 knockdown on the EMT phenotype. Since the EMT process is set in motion by transcription factors like Snail and Zeb, often through multiple signaling pathways including the TGF-β pathway [18], we proceeded to analyze the mRNA expressions of these EMT-associated genes. Real-time qPCR revealed a downregulation of transcription levels in well-established mesenchymal marker genes in TNBC cells following QSOX2 knockdown (Fig. 4C). Conversely, the expression of epithelial marker genes exhibited a significant increase upon QSOX2 silencing (Fig. 4D). In summary, the observed results suggest that QSOX2 may play a role in promoting EMT in TNBC, thereby potentially facilitating cancer cell migration and invasion, which could ultimately contribute to an unfavorable patient prognosis.

3.4. The QSOX2-ITGB1 connection: examining integrin stability in TNBC

After investigating the effects of elevated QSOX2 expression in TNBC, our main goal was to understand the underlying molecular

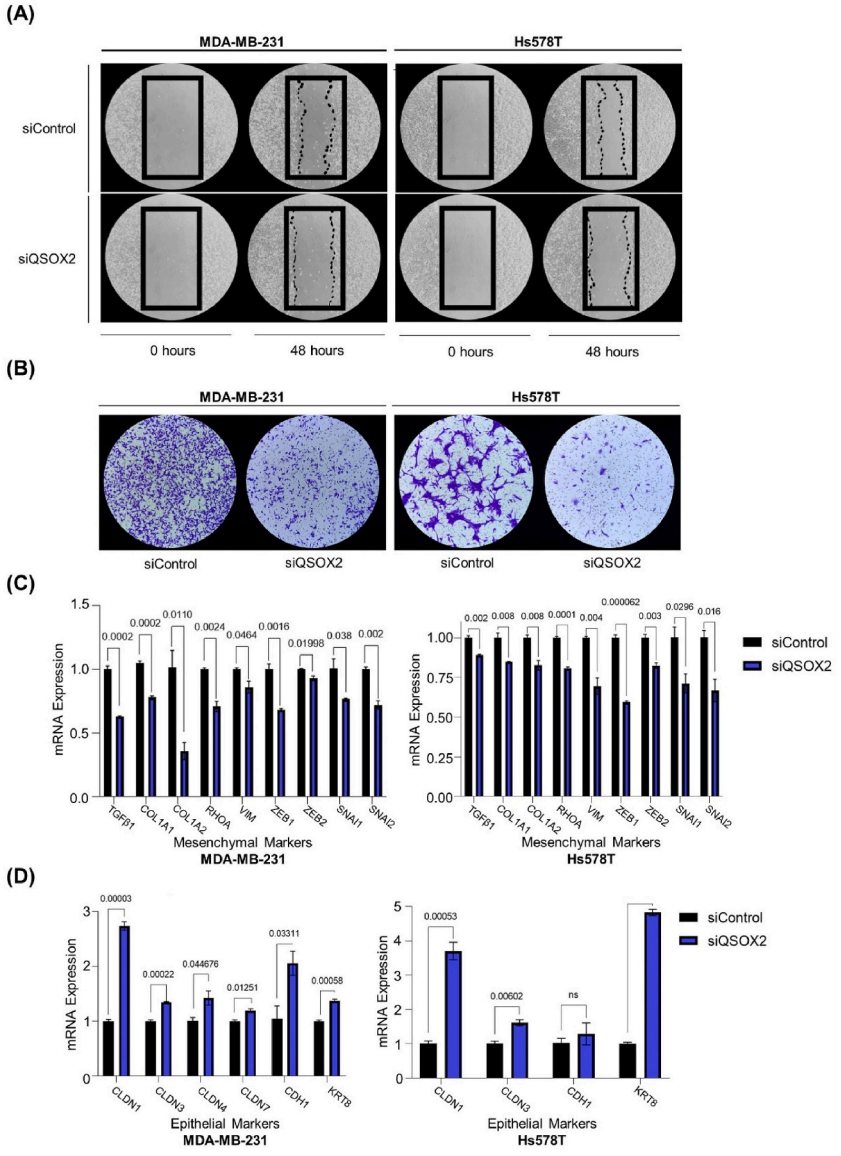

Fig. 4. QSOX2 facilitates the migration of human TNBC cells. (A) The results of the wound-healing assay indicated a significant decrease in the number of migrating TNBC cells due to QSOX2 inhibition. (B) The *trans*-well assay showed a reduction in the migration rate of TNBC cells following QSOX2 knockdown. (C) Real-time qPCR indicated a decrease in transcription levels of established mesenchymal marker genes in TNBC cells after QSOX2 knockdown. (D) The expression of genes associated with epithelial markers significantly rose following QSOX2 silencing. Statistical significance was assessed using Student's t-test, one-way, or two-way ANOVA, with a significance threshold established at $p < 0.05$.

mechanisms. We aimed to clarify how increased QSOX2 levels drive TNBC cell proliferation and migration while inhibiting apoptosis and promoting EMT. Our investigation led us to discover a potential link between QSOX2 and integrins.

To understand the influence of QSOX2 on integrins, we evaluated the mRNA levels of specific integrins associated with BC malignancy following QSOX2 knockdown. Notably, Integrin β3 (ITGB3) and Integrin β4 (ITGB4) expressions showed slight decreases in each cell line (Fig. 5A). However, the most substantial reduction was observed in ITGB1 expression, not only across both TNBC cell lines but also in both siRNA transfected conditions (Fig. 5A). These results were consistent at the protein level (Fig. 5B). As a result, ITGB1 was selected as the representative integrin affected by QSOX2 for subsequent investigations. Additionally, to implicitly confirm the diminished function of ITGB1 following QSOX2 suppression, we examined protein kinase B (AKT) signaling, which is one of the conventional integrin downstream signaling pathways [22]. As a result, a reduction in the levels of active or phosphorylated AKT was observed upon QSOX2 knockdown (Fig. 5C).

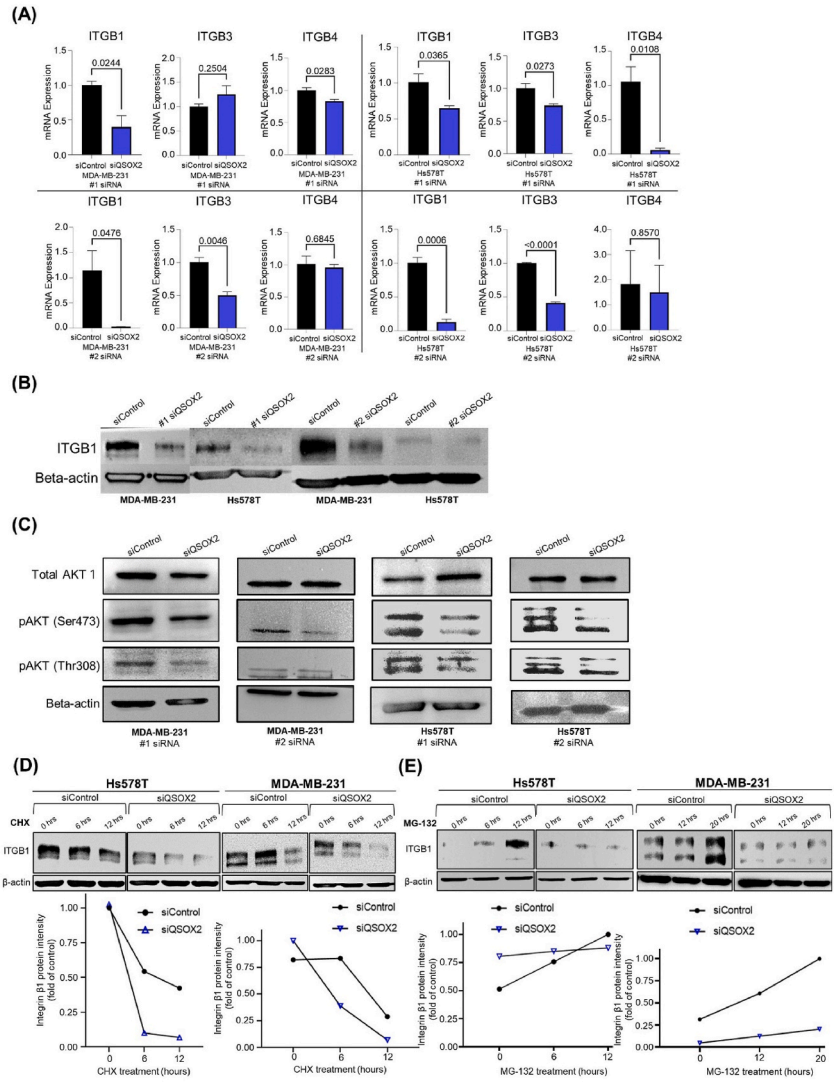

Fig. 5. Upregulated QSOX2 excessively stabilizes ITGB1 in human TNBC cells. (A) The mRNA levels of integrins related to BC malignancy were assessed 48hr after QSOX2 knockdown, noting slight decreases in Integrin β3 (ITGB3) and Integrin β4 (ITGB4) expressions, but the most significant reduction occurred in ITGB1 expression, observed consistently across both TNBC cell lines and both siRNA transfected conditions. (B) The total protein expression was reduced 48hr after siRNA-QSOX2 transfection in TNBC cells. (C) To implicitly confirm reduced ITGB1 function due to QSOX2 suppression, the AKT signaling pathway was investigated, revealing decreased levels of active AKT (phosphorylated) after QSOX2 knockdown. (D) After introducing QSOX2-suppressing siRNA, protein synthesis was halted with cycloheximide (CHX), showing that stable QSOX2 expression improved ITGB1 protein stability in both TNBC cell lines. (E) QSOX2 knockdown accelerated the degradation of ITGB1 protein, and this effect was further confirmed using MG-132, a proteasome inhibitor, which increased ITGB1 protein degradation when QSOX2 expression was suppressed. Statistical significance was assessed using Student's t-test, one-way, or two-way ANOVA, with a significance threshold established at $p < 0.05$. Full non-adjusted images can be found in Supplementary Materials.

Next, we investigated the mechanism responsible for suppressing ITGB1 induction following QSOX2 knockdown. As mentioned previously, disulfide bonds play a vital role in maintaining the structural integrity of integrins, thereby enhancing protein stability through proper folding. Hence, an anticipated role of QSOX2 is to bolster the structural integrity of integrins by facilitating the formation of disulfide bonds. To test this hypothesis, experiments were designed to investigate the stability of the ITGB1 protein in TNBC cells.

Initially, following the introduction of siRNA to suppress QSOX2, the synthesis of all subsequent proteins within the cells was halted using CHX, a widely used laboratory reagent that impedes protein synthesis by inhibiting eukaryotic translation [24]. Results from both TNBC cell lines clearly showed that the ITGB1 protein was notably retained under consistent QSOX2 expression conditions (Fig. 5D). In contrast, QSOX2 knockdown accelerated ITGB1 protein degradation. For another experiment assessing ITGB1 protein stability, we used MG-132, a proteasome inhibitor that blocks ubiquitin-mediated proteolysis [19]. As expected, suppressing QSOX2 expression increased the degradation rate of the ITGB1 protein (Fig. 5E). On the contrary, when QSOX2 expression was sustained, the accumulation rate of ITGB1 protein was significant.

In summary, these experiments consistently demonstrate that the abnormal upregulation of QSOX2 in TNBC cells enhances the stability of the ITGB1 protein, as illustrated in Fig. 6A. This sustained stability contributes to the function of ITGB1, allowing cancer cells to drive rapid proliferation by evading apoptosis and promoting EMT-associated aggressive migration. While our in vitro experiments provide valuable insights into these molecular processes, we acknowledge the inherent limitations of not fully replicating the complex tumor environments present in patients. Therefore, the observed phenotypes may be part of a more intricate interplay within the in vivo context. Nonetheless, based on these confirmed phenotypes and attributes, targeting the QSOX2 gene for silencing in TNBC patients holds the potential to improve prognoses.

4. Discussion

TNBC often leads to recurrent instances and metastasis, resulting in a less favorable prognosis compared to other BC types [25]. Annually, TNBC affects approximately 42,000 women and is associated with a 37% mortality rate within 5 years, yielding a median survival of only 9 months post-recurrence [26]. Distant metastasis in TNBC primarily occurs in the lung (40%), followed by the brain (30%), liver (20%), and bone (10%) [3]. The absence of ER, PR, and HER2 receptors renders TNBC unresponsive to conventional hormone-based BC treatments. Therefore, the discovery of easily detectable universal genetic biomarkers for diagnosing TNBC occurrence or the risk of metastasis, as well as their modulation, holds promise for improving patient prognoses.

Yadav et al. highlighted TNBC identification through notable biomarker expression, including EGFR, VEGF, and Ki-67 [27] However, these biomarkers are broadly expressed in various cancers, limiting their effectiveness as treatment targets. To address the unique characteristics of TNBC, a focused approach is necessary. In contrast, our study explores the influence of QSOX2 on TNBC, revealing its specific expression in TNBC patients and cell lines. This research is the first to underscore the significance of QSOX2 in TNBC tumor progression and its underlying mechanisms.

The QSOX gene family, consisting of QSOX1 and QSOX2, plays a central role in introducing disulfide bonds to unfolded and reduced proteins through molecular oxygen reduction [4]. Essentially, the mammalian QSOX family influences the folding and stability of matrix proteins by facilitating intermolecular disulfide bridge exchange within target substrates [28]. While QSOX1 has been extensively studied in the context of cancer and its potential role in tumor cell migration and invasion at the tumor-stroma boundary has been explored in lung, pancreas, and prostate cancers [29], the same level of investigation has not been extended to QSOX2. Despite being a paralog of QSOX1, QSOX2 remains relatively unexplored due to its lower expression in most human tissues. Notably, QSOX1 and QSOX2 share only 40% similarity in primary amino acid composition and 68% resemblance in functional domains [30], indicating potential distinct roles for QSOX2 in molecular biochemistry that warrant further exploration. This study aims to elucidate the features and roles of QSOX2, particularly in the context of cancer and other biological contexts.

Prior studies have examined QSOX2 mostly in non-cancer scenarios. Maharaj et al. linked it to human growth regulation, associated with postnatal growth delay and mild immune issues [31]. Wang et al. explored the relationship between testosterone and QSOX2, demonstrating the constructive role of testosterone in QSOX2 biogenesis [32]. In cancer, only two studies have reported the molecular effects of QSOX2. One suggested QSOX2 as an E2F1-regulated gene, potentially serving as a serum biomarker for monitoring tumor

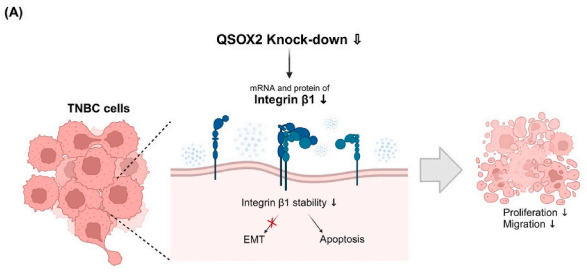

Fig. 6. Graphical Summary. (A) The elevated QSOX2 levels in TNBC cells promote cancer cell proliferation and migration by stabilizing the ITGB1 protein, suggesting that targeting the QSOX2 gene could be a promising therapeutic strategy for improving TNBC patient outcomes. The figure was created with BioRender.com.

progression and survival prediction in advanced NSCLC [5]. Another associated elevated QSOX2 expression with poor prognosis and tumor advancement in CRC [10]. Our study uniquely delves into the implications of QSOX2 in TNBC and its underlying mechanisms.

To comprehensively interpret the observed effects of QSOX2 on TNBC cells, integrins were highlighted as primary downstream targets. Integrins are critical in influencing cancer cell behavior, impacting survival, differentiation, migration, invasion, and drug resistance [19]. Notably, ITGB1 significantly contributes to cancer metastasis, especially in invasive BC, indicating aggressive tumor profiles when associated with fibronectin [33]. Silencing ITGB1 inhibits migration, invasion, and store-operated calcium influx in TNBC cells, making it a promising biomarker candidate for predicting BC cell behavior and TNBC patient survival [34]. Our study recognized the crucial role of ITGB1 in cancer progression and showed that heightened QSOX2 expression enhances ITGB1 stability, amplifying downstream signaling, promoting rapid proliferation, and facilitating aggressive migration via EMT.

Our study found that QSOX2 directly modulates ITGB1, although an alternative hypothesis suggests intermediary involvement in the observed ITGB1 decline after QSOX2 knockdown. The challenge of fully explaining the transcriptional reduction of ITGB1 within this study prompted this proposition. Mechanistically, we propose that QSOX2 enhances integrin structural integrity by facilitating disulfide bond formation, bolstering stability and downstream signaling pathways, vital for cancer cell processes. Aberrant QSOX2 upregulation in TNBC drives malignancy by enhancing cancer cell proliferation and invasiveness. Understanding the role of QSOX2 can inform targeted therapy for TNBC and other BC subtypes, improving patient outcomes and providing valuable treatment insights.

In line with our findings, similar phenotypes and mechanisms observed in other protein studies can inform and advance research and therapeutic development. For example, CD24 knockdown in MCF-7 BC cells resulted in reduced proliferation and influenced integrin β1 binding, leading to decreased proliferation and EMT [35]. Another study highlighted the role of acyl transferase DHHC3 in the palmitoylation of integrin α6β4, affecting cellular functions and Src signaling, with implications for α6β4 stability in cancer processes [36]. GPNMB, highly expressed in TNBC, interacted with α5β1 integrin, affecting integrin recycling, stability, and tumor progression [37]. Incorporating these findings into our investigation of integrin stability offers potential for enhancing therapeutic strategies aimed at TNBC patients, with the goal of inhibiting tumor advancement and enhancing patient outlooks.

The strength of our findings could have been enhanced by incorporating *in -vivo* experiments that closely mimic human patient scenarios. Nevertheless, existing research has provided insights into the impact of QSOX2 on tumor progression through *in -vivo* models. Jiang et al. conducted a xenograft tumorigenesis study to explore the influence of QSOX2 on CRC cell proliferation [10]. Their investigation revealed that xenograft tumors originating from shControl cells exhibited significantly greater weight and size compared to those arising from shQSOX2 cells [10]. Furthermore, the outcomes of immunohistochemistry staining indicated that stable knockdown of QSOX2 correlated with reduced Ki-67 staining intensity in the tumor sections [10]. These findings align with our data, particularly the decrease in Ki-67 observed in TNBC cells following QSOX2 knockdown, suggesting similar results in comparable *in -vivo* models.

While our study emphasizes the elevated expression of QSOX2 in TNBC, particularly focusing on the MDA-MB-231 and Hs578T cell lines, it is crucial to address the concern raised about the generalizability of our conclusions to all TNBC subtypes. Recognizing potential variability in QSOX2 expression and function across different TNBC subtypes is essential for a comprehensive understanding of its role in breast cancer. To overcome this limitation, future studies should extend their investigations to include a broader range of TNBC cell lines representing diverse molecular subtypes. This approach will provide a more complex perspective on the involvement of QSOX2 in TNBC progression, considering the inherent heterogeneity within this subtype. Broader validation across various TNBC subtypes will enhance the robustness and applicability of our findings, contributing to a more comprehensive understanding of the role of QSOX2 in breast cancer pathogenesis.

Moreover, we recognize that our study has limitations, including the reliance on robust public datasets such as TCGA and METABRIC for a comprehensive analysis of QSOX2 expression in breast cancer. While these datasets offer valuable resources for large-scale analyses, it is crucial to acknowledge limitations related to variations in patient demographics, treatment histories, and other clinical factors. The observed patient heterogeneity in these datasets may influence outcomes and should be considered when interpreting our results. Future studies could enhance the depth of our understanding by incorporating more diverse datasets, including longitudinal patient data and treatment response information, to provide a detailed perspective on the role of QSOX2 in breast cancer progression. Additionally, conducting in vitro experiments and validating our findings in independent patient cohorts would further strengthen the robustness of our research.

Also, in translating our findings to clinical applications, targeting QSOX2 in breast cancer therapy presents challenges and opportunities. Developing effective drug delivery methods for precise QSOX2 targeting is a notable challenge, requiring careful consideration of nanoparticle-based systems. These systems could enhance specificity but necessitate preclinical testing for safety and efficacy. Addressing potential off-target effects of QSOX2 inhibition is crucial, demanding systematic investigations into normal tissues' responses. The feasibility of QSOX2-targeted treatments may depend on the genetic diversity within breast cancer, requiring an individualized approach for each patient. Rigorous preclinical studies are essential to assess safety, efficacy, and specificity. Combinatorial approaches, like pairing QSOX2 inhibitors with existing therapies, could offer synergistic benefits, enhancing treatment outcomes.

In summary, our study addresses the rising global burden of TNBC. We've discovered a new role for QSOX2 in TNBC, shedding light on a mechanism that significantly influences cancer behavior. Our research not only provides insights into the complex pathways driving TNBC but also highlights QSOX2 as a potential treatment target, particularly its impact on integrin stability like ITGB1. This contributes to the broader understanding of using integrins for therapy. Our work reinforces the significance of personalized medicine, where genetic markers like QSOX2 can improve TNBC prognosis and treatment outcomes. Future studies can further explore the clinical applications of QSOX2 as a predictive marker and treatment target, ultimately benefiting TNBC patients and advancing cancer biology.

5. Conclusion

In summary, our study investigated the role of QSOX2 in BC progression and prognosis, particularly in TNBC. Elevated QSOX2 expression was associated with higher mortality rates and increased cancer progression and recurrence across various BC subtypes. Functional experiments demonstrated the significance of QSOX2 in TNBC, affecting cell proliferation, apoptosis resistance, and migration. Mechanistically, QSOX2 appeared to drive EMT in TNBC cells, potentially enhancing their invasiveness. Additionally, we uncovered a link between QSOX2 and ITGB1 stability, a critical regulator of cancer cell behavior. Targeting QSOX2 in TNBC and other BC subtypes may disrupt ITGB1 stability, offering potential for improved patient outcomes. Our research provides valuable insights into BC progression and future therapeutic development in breast cancer treatment.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Data availability statement

All data generated or analyzed in this study are included in this published article and its supplementary information files. Generated data are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

A-In Kim: Writing – original draft, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Ji Hoon Oh:** Data curation. **Je-Yoel Cho:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author used CHAT-GPT in order to verify the grammatical accuracy of the English text. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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