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ORIGINAL PAPER

Long non-coding RNA Nkx2-2as/BTG2 axis attenuates breast cancer progression by targeting Wnt/β-catenin signaling

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Abstract

Breast cancer (BC) is the second leading cause of cancer-related deaths among women, primarily due to late-stage detection and therapy resistance. Therefore, identifying novel therapeutic targets is critical for improving BC outcomes. Long non-coding RNAs (lncRNAs) have recently emerged as promising candidates for cancer prognosis and treatment, owing to their ability to modulate oncogenic signaling pathways. Among them, the tumor-suppressive lncRNA Nkx2-2as has shown inhibitory effects in certain cancers; however, its role in BC remains poorly understood. To the best of our current knowledge, the relationship between Nkx2-2as and the Wnt/β-catenin signaling pathway in BC has not been previously characterized. To address this, we used computational tools including lncHUB2, RPISeq, GeneMANIA, TCGA and ENCORI to predict functional interactions of Nkx2-2as, which guided our focus toward its involvement in the Wnt/β-catenin signaling pathway, a key driver in BC progression. We hypothesized that Nkx2-2as may act as a pharmacologically actionable molecule in this context. To test this, MCF-7 breast cancer cells were transfected with either Nkx2-2as siRNA or an Nkx2-2as-pcDNA3.1 overexpression vector, individually and in combination. Overexpression of Nkx2-2as led to a significant reduction in proliferation (~85%), suppression of migration, and increased apoptosis. Conversely, silencing Nkx2-2as enhanced tumorigenic properties. Mechanistic analyses revealed that Nkx2-2as downregulates oncogenic targets such as β-catenin, TCF7 and MYC, while upregulating tumor suppressors AXIN2 and BTG2, the latter being a known inhibitor of β-catenin. Western blot analysis confirmed the transcriptional trends, showing decreased β-catenin and MYC and elevated BTG2 protein levels upon Nkx2-2as overexpression. These findings indicate that Nkx2-2as acts as a negative regulator of Wnt/β-catenin signaling through BTG2 activation, suggesting its potential role as a tumor suppressor and a candidate for RNA-based therapeutic strategies in BC. Targeting the Nkx2-2as/BTG2 axis may provide a conceptual framework for future studies aimed at developing RNA-based interventions to enhance chemosensitivity and overcome therapy resistance in BC.

Keywords Breast cancer · LncRNA · Nkx2-2as · Wnt/β-catenin signalling · BTG2 · RNA based therapy

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Introduction

Breast cancer (BC) is the second leading cause of cancer-related deaths among women worldwide. According to the National Cancer Registry Programme of India and GLOBOCAN 2020, the BC mortality rate in India (17.1 per 100,000 women) is notably higher than that in the UK (12.7 per 100,000 women). This high mortality rate is primarily due to late detection at locally advanced or metastatic stages. Therefore, it is critical to explore the genetic and epigenetic regulators that drive BC progression in order to identify reliable prognostic biomarkers and novel therapeutic targets. In this context, long non-coding RNAs (lncRNAs) have emerged as attractive candidates for next-generation RNA-based therapies due to their diverse mechanisms of action and ability to modulate key oncogenic pathways.

LncRNAs are a class of transcripts longer than 200 nucleotides with non-protein coding functions that represent an exciting frontier for RNA-targeting drug development. Although only 1–2% of the human genome encodes proteins (~ 20,000 genes), lncRNAs account for 60–70% of all transcribed RNAs, with over 95,000 identified in human [8]. Studies have revealed that lncRNAs can act as tumor suppressors or oncogenes by regulating chromatin structure, interacting with transcription factors, and sponging oncogenic microRNAs [14].

Recent findings have underscored the significance of non-coding RNA signaling network interactions in BC, which regulates tumor growth, stemness, and drug resistance between microRNAs and the Hippo-YAP/TAZ pathway, which shapes tumor growth and therapeutic response [22]. Furthermore, microRNAs such as miR-195, miR-34c, and miR-1246 have been identified as potential biomarkers for trastuzumab-resistant HER2-positive BC [20], while others function as oncogenic or tumor-suppressive regulators in inflammatory BC [19]. These emerging insights emphasize the intricate regulatory roles of ncRNAs in BC pathogenesis and reinforce the potential of lncRNA-based therapeutic interventions aimed at overcoming chemotherapy resistance [7].

The Wnt/β-catenin signaling pathway is abnormally activated in various cancers, including lung, colorectal, liver, and BC [27]. Canonical Wnt signaling is initiated by Wnt ligand binding to frizzled receptors and LRP5/6 co-receptors, leading to inhibition of the β-catenin destruction complex (composed of APC, AXIN, and GSK3β) [9]. This allows β-catenin accumulation in the cytoplasm and subsequent nuclear translocation, where it interacts with TCF/LEF transcription factors to activate oncogenic target genes, including cyclin D1, MYC, and survivin [23]. Aberrant activation of this pathway promotes uncontrolled cell proliferation, epithelial-mesenchymal transition, metastasis, and drug resistance [17, 18].

Several lncRNAs have been reported to modulate the Wnt/β-catenin signaling pathway across various cancer types. For instance, lncRNA OTUD6B-AS1 suppresses renal cell carcinoma by targeting Wnt/β-catenin signaling [24]. LncRNA LINC-PINT inhibits glioblastoma metastasis through Wnt/β-catenin suppression [31]. LncRNA DANCR which act as a negative regulator of β-catenin signalling to reduce odontoblast differentiation [4]. LncRNA CARMN blocks Wnt/β-catenin via miR-92a-3p/BTG2 regulation in cervical cancer [25]. In contrast, several lncRNAs such as HOTAIR, NEAT1, and CCAT2 have been shown to activate Wnt/β-catenin signaling, thereby promoting tumor proliferation, metastasis, and therapeutic resistance in different cancer types [10, 28, 30].

Despite growing evidence of lncRNAs regulating the Wnt/β-catenin signaling pathway in various cancers, the role of lncRNA Nkx2-2as in this context remains largely unexplored. Previous studies have demonstrated that Nkx2-2as exerts tumor-suppressive effects in medulloblastoma by sequestering miR-103 and miR-107, thereby upregulating BTG2 a well-established antagonist of Wnt signaling [29]. However, the functional understanding of this lncRNA remains limited. Since BTG2 inhibits β-catenin–dependent transcription of oncogenic Wnt target genes (e.g., *MYC*, *Cyclin D1*), its upregulation by Nkx2-2as could attenuate Wnt-driven proliferation, invasion, and chemoresistance in BC [2]. To bridge this gap, it is plausible that Nkx2-2as may regulate BTG2 expression by acting as a competing endogenous RNA (ceRNA) or by modulating transcriptional activity, thereby indirectly influencing β-catenin stability and downstream oncogene expression. Such regulation could serve as a molecular checkpoint linking epigenetic modulation and signaling pathway control in BC.

Given this regulatory relationship, we hypothesize that the Nkx2-2as/BTG2 axis may function as a key modulator of Wnt/β-catenin signaling in BC. However, to date, no studies have investigated the role of Nkx2-2as in modulating this pathway in any cancer type. Therefore, this study aims to elucidate the mechanistic link between Nkx2-2as, BTG2 and Wnt/β-catenin signaling in the regulation of BC progression. Furthermore, understanding this axis may provide a foundation for the future development of RNA-based therapeutic strategies targeting Wnt pathway dysregulation to overcome chemotherapy resistance in BC.

Materials and methods

In silico analysis of LncRNA Nkx2-2as

To investigate the functional characteristics of Nkx2-2as, a series of computational analyses were performed using publicly available bioinformatics tools and databases. The study focused on predicting secondary structure, subcellular localization, expression distribution across cell lines, and functional interactions between genes and transcription factors.

Secondary structure prediction

To investigate the thermodynamic stability and predicted folding pattern of Nkx2-2as, its canonical transcript was analyzed using RNA secondary structure modeling. Complementary DNA (cDNA) sequences for lncRNAs were downloaded from the GENCODE v41 (gencode.v41.long_noncoding_RNAs.gtf) (version 41, assessed march 2024) reference genome annotation (Homo_sapiens.GRCh38.ncrna.fa). The canonical transcript of Nkx2-2as was extracted, and its secondary structure was predicted using RNAfold v2.5.0 with default settings. RNAfold predicts minimum free energy (MFE) RNA structures based on thermodynamic stability. Transcripts lacking complete cDNA sequences or exceeding RNAfold input length limitations were excluded.

Subcellular localization prediction

To predict the subcellular compartment where Nkx2-2as is predominantly localized, localization analysis was performed using lncHUB2 (v2.0, accessed January 2024) [15], which integrates co-expression data from ARCHS4 and lncATLAS. Genes co-expressed with Nkx2-2as were ranked by Pearson correlation, and scaled scores were adjusted using CN-RCI (cytoplasmic/nuclear relative concentration index). Normalized localization scores (− 0.5 to 0.5) were computed, and ROC curves assessed prediction accuracy. To visualize expression across tissues, UMAP analysis was conducted in R v4.3.2 using 3,000 ARCHS4 samples after $\log_2$ transformation and quantile normalization. Each point represented one of 18,705 lncRNAs, with Nkx2-2as indicated by a black arrow.

Expression profiling across cell lines

To determine the tissue- and cell-type-specific expression pattern of Nkx2-2as, expression data were retrieved from lncHUB2 (v2.0), which integrates datasets from ARCHS4 and GENCODE v41. TPM (Transcripts Per Million) normalized expression values were extracted and compared to visualize the expression distribution of Nkx2-2as across different human tissues and cell lines.

Functional enrichment analysis

Functional enrichment analysis was performed to identify the potential biological functions and regulatory mechanisms associated with Nkx2-2as using the lncHUB2 platform [15]. The analysis was conducted to relate Nkx2-2as expression profiles to relevant pathways, diseases, and transcriptional regulators implicated in BC.

Enrichment analyses were carried out using datasets integrated within lncHUB2. The Enrichr library was used to compute the mean Pearson correlation coefficient (PCC) for each gene set by averaging the PCCs between Nkx2-2as and all genes in the respective set. Gene sets with higher mean PCC values were considered to have stronger co-expression relationships and were selected for further evaluation.

Disease enrichment was performed using DisGeNET to identify disorders potentially associated with Nkx2-2as dysregulation. ChEA (ChIP-X Enrichment Analysis) and ENCODE transcription factor binding datasets were utilized to predict putative transcriptional regulators of Nkx2-2as. The output data included ranked lists of transcription factors with corresponding mean PCC values, representing the correlation strength between their known target genes and NKX2-2AS expression [16]. Gene sets enriched in canonical signaling or metabolic pathways were mapped to KEGG categories. Results from DisGeNET, ChEA, and ENCODE analyses were compiled to infer possible biological and regulatory associations for Nkx2-2as in BC.

Molecular interaction possibility prediction using the RPISeq tool

To predict the potential interaction between LncRNA Nkx2-2as and the tumor suppressor gene BTG anti-proliferative factor 2 (BTG2), the RPISeq web server (http://pridb.gdcb.iastate.edu/RPISeq/) was used. RPISeq applies random fore st (RF) and support vector machine (SVM) classifiers, with interaction probabilities > 0.5 considered positive predictions. The nucleotide sequence of Nkx2-2as (NR_034170.1; Ensembl ID: ENSG00000258197) was retrieved from Ensembl, and the corresponding BTG2 protein sequence (UniProt ID: P78543) was obtained from UniProt. These IDs were used to ensure reproducibility and accuracy in data retrieval. RPISeq was selected due to its high predictive accuracy for RNA–protein interactions and validated performance across diverse datasets.

Analysis of gene-gene interaction networks

Gene-gene interaction networks were analyzed using the GeneMANIA plugin (v3.6.0; accessed June 2024) available at http://geneMANIA.org. GeneMANIA was chosen for its ability to integrate multi-dimensional omics data and prioritize gene interactions for hypothesis generation. This tool integrates data from protein–protein, protein–DNA, and genetic interactions, along with co-expression, co-localization, and pathway information [26]. Homo sapiens was selected as the target organism. The interactions between BTG2 and CTNNB1 (β-catenin) were examined to infer potential functional relationships.

To explore potential RNA–protein and RNA–RNA regulatory associations, ENCORI (starBase v3.0; accessed May 2024) was used (https://rnasysu.com/encori/). The interacti on between BTG2 and CTNNB1 was evaluated using high-throughput CLIP-seq datasets.

For reproducibility, lncHUB2 (v2.1; accessed May 2024) and GENCODE (GRCh38.p14) were used as reference databases for gene annotations. Transcripts with incomplete annotation, low confidence, or pseudogene overlap were excluded from the enrichment and interaction analyses.

Cell culture

The human BC cell line, MCF-7, was obtained from the National Centre for Cell Science (NCCS, Pune, India). Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (100 U/mL penicillin, 100 µg/mL streptomycin) at 37 °C in a humidified incubator containing 5% CO_2. Cells were used within passage numbers 5–20. When cultures reached ~ 80% confluence, cells were trypsinized with 0.25% trypsin–EDTA, centrifuged at 300 × g for 5 min, and collected for further assays.

Table 1 qRT-PCR primers for mRNAs

qRT-PCR Primers for mRNAs sequences 5' − 3' direction	
AXIN2 -F	TTCTGCAAAAGAGAGCTTCCA
AXIN2 -R	ATTGCATCCGTTGCATTCTC
TCF7(TCF1)-F	CAATCTGCTCATGCCCTACC
TCF7(TCF1)-R	CTTGCTTCTGGCTGATGTCC
β-catenin -F	GCTACTCAAGCTGATTTGATGGA
β-catenin -R	GGTAGTGGCACCAGAATGGATT
MYC -F	GGGCTTCTCAGAGGCTTGG
MYC -R	GTCCTTGCTCGGGTGTTGTA
β-actin -F	AAATCTGGCACCACACCTTC
β actin -R	AACGGCAGAAGAGAGAACCA
Nkx2-2as -F	GACAACTGGTGGCAGATTTCGCTT
Nkx2-2as -R	AGCCACAAAGAAAGGAGTTGGACC
BTG2 F	CTGGGCAGAGAGTGAAAAG
BTG2 R	CTTCCATCCTAACCCCAAT

Quantitative real-time polymerase chain reaction

Total RNA was isolated from MCF-7 cells using TRIzol reagent (HiMedia) following the manufacturer's protocol. Complementary DNA (cDNA) synthesis was performed using the PrimeScript RT Reagent Kit (Takara, Japan). qRT-PCR was carried out in a 10 µL reaction mixture containing 1 µL cDNA, 200 nM primers, and 5 µL SYBR Green Master Mix (Thermo Fisher Scientific) using a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). The thermal profile included 50 °C for 2 min (UDG activation), 95 °C for 2 min (polymerase activation), and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate, and the mean Ct value was used if the coefficient of variation was < 10%, as suggested by MIQE guidelines [3]. Gene expression was normalized to ACTB (β-ACTIN), and relative expression was calculated using the 2^−ΔΔCt method. Primer sequences are listed in Table 1.

Plasmid amplification, transfection, and knockdown assay

The Nkx2-2as-pcDNA3.1 plasmid (50 ng) was transformed into competent *E. coli* (C404010, Thermo Fisher Scientific) using standard heat-shock transformation (42 °C for 90 s). Transformants were selected on ampicillin (100 µg/mL) LB plates and plasmids extracted using the PureLink™ HiPure Midiprep Kit (K210014, Thermo Fisher Scientific) as per manufacturer's protocol.

For overexpression studies, MCF-7 cells were transfected with Nkx2-2as-pcDNA3.1 using Lipofectamine 3000 (Thermo Fisher Scientific). After 48 h, cells were selected using neomycin (G418; 0.5 µg/mL) until stable clones were established. Transfection efficiency was verified by measuring Nkx2-2as expression using qRT-PCR, with empty pcDNA3.1 vector serving as a control.

For knockdown experiments, siRNA targeting Nkx2-2as (Genscript, USA, catalogue no. SC1518, Sense: 5′-AAGG UUCAGAAGGAGAGGCAU-3′, Antisense: 5′-UGCCUC UCCUUCUGAACCUUU-3′) was transfected using Lipofectamine RNAiMAX reagent according to manufacturer instructions. The final siRNA concentration was 50 nM, and cells were incubated for 48 h before analysis.

For combination treatments, MCF-7 cells were first transfected with Nkx2-2as-pcDNA3.1 for 24 h, followed by siRNA transfection for an additional 24 h under identical conditions. All experiments were performed in triplicate biological replicates ($n = 3$).

Cell proliferation assay

Cell proliferation was measured using the MTT (3–4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide) assay. MCF-7 cells were seeded in 96-well plates (3×10^3 cells/well) and incubated for 0, 24, and 48 h. At each time point, 10 µL MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. The resulting formazan crystals were dissolved in 200 µL DMSO, and absorbance was measured at 570 nm with background correction at 630 nm using an ELISA microplate reader (BioTek Synergy HTX). Cell proliferation (%) was calculated as:

$$\text{Cell proliferation } (\%) = (\text{OD of test}/\text{OD of control}) \times 100$$

Scratch assay

The scratch wound-healing assay was conducted to determine the impact of Nkx2-2as knockdown and overexpression on the migratory ability of MCF-7 cells. Cells were seeded in 6-well plates, treated according to experimental conditions, and grown to confluence. A linear scratch was made using a 200 µL pipette tip, and cells were washed twice with PBS to remove debris. Images were captured at 0, 24, and 48 h post-scratch using an Olympus CKX53 inverted microscope. Wound closure was quantified using ImageJ (v1.54f) software. Three independent fields were analyzed per replicate ($n = 3$).

Apoptosis and nuclear morphology assays

DAPI staining

DAPI (4',6-diamidino-2-phenylindole) staining was used to observe nuclear morphology and detect apoptotic changes induced by Nkx2-2as knockdown or overexpression. Cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 1% Triton X-100, and stained with 1 µg/mL DAPI for 15 min in the dark. Fluorescence was observed under a Nikon Eclipse E800 microscope using excitation/emission wavelengths of 340/480–500 nm.

Acridine orange and ethidium bromide (AO/EtBr) double staining

Acridine orange/ethidium bromide (AO/EtBr) double staining was performed to visualize and distinguish viable, apoptotic, and necrotic cells following modulation of Nkx2-2as expression. Cells were fixed with 4% paraformaldehyde and stained with a 100 µg/mL AO–EtBr mixture for 15 min at room temperature. Stained cells were visualized under a Nikon Eclipse E800 fluorescence microscope. Viable, early apoptotic, and late apoptotic/necrotic cells were identified based on differential fluorescence emission.

Flow cytometry

Flow cytometry was used to quantify apoptotic cell populations and analyze changes in cell cycle distribution resulting from Nkx2-2as modulation. Apoptosis was quantified using Annexin V–FITC/PI staining. Cells were fixed in 70% ethanol overnight at 4 °C, treated with 1 mg/mL RNase A for 30 min at 37 °C, and stained with Annexin V–FITC and PI (50 µg/mL) in PBS containing 0.5% Tween-20. Analysis was performed on a BD Accuri C6 flow cytometer (Becton Dickinson, USA), recording 10,000 events per sample. Gating was adjusted using unstained and single-stained control cells.

Colony formation assay

The colony formation assay was performed to assess the long-term proliferative potential and clonogenic ability of MCF-7 cells after Nkx2-2as knockdown, overexpression, or combined treatment. Cells were seeded at 500 cells/well in 6-well plates and treated according to experimental groups. After 10–14 days, colonies were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet, and counted manually (≥50 cells/colony) using ImageJ. Experiments were performed in triplicate ($n = 3$).

Western blot analysis

MCF-7 cells were seeded in 6-well plates and divided into four groups: (1) control MCF-7 cells, (2) LncRNA Nkx2-2as knockdown, (3) LncRNA Nkx2-2as overexpression, and (4) LncRNA Nkx2-2as overexpression followed by knockdown. After 48 h post-transfection, cells were harvested and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors. The lysates were kept on ice for 30 min and centrifuged at 12,000 rpm for 15 min at 4 °C to remove cell debris. Protein concentrations were determined using the Lowry method with bovine serum albumin (BSA) as the standard. Equal amounts of protein (40 µg) were mixed with 4× SDS-PAGE loading buffer, boiled at 95 °C for 5 min, and separated by 10–12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membranes (Amersham, GE Healthcare Life Sciences, USA), which were blocked with 5% BSA in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated overnight at 4 °C with the following primary antibodies: Phospho-β-Catenin (Ser33/37/Thr41) Rabbit mAb (Cell Signaling Technology, #9561, 1:1000), MYC (D84C12)

Rabbit mAb (Cell Signaling Technology, 1:1000), BTG2 (1A5) Mouse mAb (Santa Cruz Biotechnology, sc-517187, 1:1000), and β-Actin (8H10D10) Mouse mAb (Cell Signaling Technology, 1:1000) used as an internal loading control. After washing with TBST, membranes were incubated with appropriate HRP-conjugated secondary antibodies (anti-rabbit IgG, 1:10,000; anti-mouse IgG, 1:10,000) for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) substrate and detected with a gel documentation system (Image Quant LAS 500, GE Healthcare Life Sciences, USA). Band intensities were quantified using ImageJ software for densitometric analysis.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM) from at least three independent experiments. Comparisons between two groups were performed using the Student's t-test, while multiple group comparisons used one-way ANOVA followed by Tukey's post-hoc test. Statistical analyses and graph generation were performed using GraphPad Prism v8.0.2 (263) (GraphPad Software, USA). A p-value < 0.05 was considered statistically significant.

Results

This study sought to explore the functional interaction between the lncRNA Nkx2-2as and the Wnt/β-catenin signaling pathway in BC progression. The results revealed that lncRNA Nkx2-2as overexpression significantly inhibited MCF-7 cell growth, migration, through Wnt/β-catenin signaling suppression. The following results presents the key findings.

Structural, localization, and expression analysis of Nkx2-2as

Comprehensive computational analyses were conducted to characterize the structural configuration, subcellular localization, and expression profile of lncRNA Nkx2-2as, providing an overview of its molecular attributes relevant to cancer biology. The following results present the key findings. The predicted secondary structure of the canonical Nkx2-2as transcript, generated using RNAfold, exhibited a thermodynamically stable conformation with a minimum free energy (MFE) of −860.90 kcal/mol, indicating potential RNA motifs that may contribute to RNA–protein interactions or regulatory functions (Fig. 1A). Subcellular localization analysis using lncHUB2 showed a CN-RCI score of −1.25, consistent with a nuclear-enriched distribution of Nkx2-2as in MCF-7 cells (Fig. 1B). Expression profiling across diverse cell lines, based on transcriptomic data from ARCHS4 and GENCODE, revealed a notable reduction in Nkx2-2as expression in multiple cancer cell lines, including MCF-7, relative to non-cancerous tissues (Fig. 1C). This reduced expression pattern was further visualized through UMAP clustering of 3,000 samples, which displayed a distinct expression landscape consistent with downregulation of Nkx2-2as in cancer cells (Fig. 1D).

Pathway and Functional enrichment analysis of Nkx2-2as

Functional enrichment identified pathways and regulators associated with Nkx2-2as activity. KEGG pathway analysis revealed significant enrichment ($p < 0.05$, FDR < 0.1) in metabolic and cellular processes, including neomycin, kanamycin, and gentamicin biosynthesis (fold enrichment = 4.1, $p = 3.2 \times 10^{-4}$), starch and sucrose metabolism (fold enrichment = 3.6, $p = 4.5 \times 10^{-3}$), galactose metabolism, synaptic vesicle cycle, mitophagy, autophagy, and ferroptosis (Fig. 2A–B). These pathways are enriched in processes related to metabolic regulation, vesicle trafficking, and programmed cell death.

DisGeNET disease enrichment (adjusted $p < 0.05$) showed associations with lymphomas, leukemias, spastic syndromes, autoimmune diseases, and neurodevelopmental disorders, indicating enrichment in immune-related and oncogenic disease categories (Fig. 2A–B).

ChEA and ENCODE transcription factor enrichment analyses predicted regulation of Nkx2-2as by multiple transcription factors, including ER (MCF-7), SOX4, EZH2, BATF, CBX3, and VDR. Several of these transcription factors exhibited moderate-to-strong correlations with Nkx2-2as expression (mean Pearson $r > 0.45$), supporting their potential regulatory association (Fig. 2C). Together, these enrichment patterns highlight the association of Nkx2-2as with metabolic and cell death–related pathways and its putative regulation by key transcriptional and epigenetic factors.

LncRNA Nkx2-2as is downregulated in MCF-7 cells

The basal expression levels of key genes, including lncRNA Nkx2-2as, BTG2, and β-catenin, were analyzed in untreated MCF-7 cells using RT-PCR. The Ct values were normalized to the reference gene β-actin (average Ct = 21.2) to calculate ΔCt values using the formula: ΔCt (target gene) = Ct (target gene) – Ct (β-actin). To facilitate biological interpretation, the relative expression level of each gene compared to the reference was determined using the 2^(-ΔCt) method (Fig. 3A). The obtained 2^(-ΔCt) values are presented in Table 2.

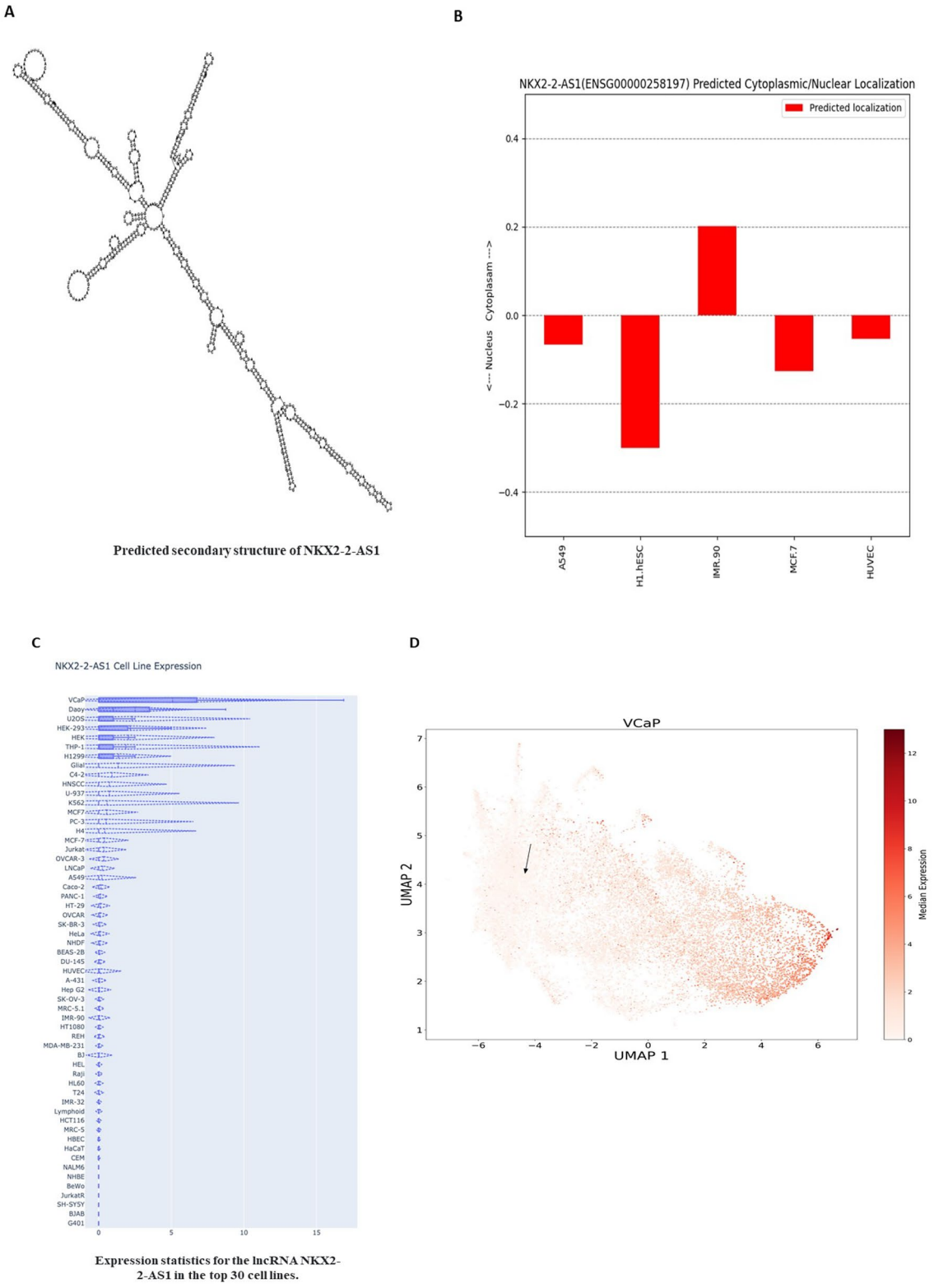


Fig. 1 Structural. localization, and expression analysis of lncRNA Nkx2-2as. (**A**) RNAfold prediction of Nkx2-2as shows a stable secondary structure with minimum free energy, suggesting functional RNA motifs. (**B**) lncHUB2 subcellular localization analysis indicates nuclear enrichment of Nkx2-2as in cell lines. (**C**) Transcriptomic profiling from ARCHS4 and GENCODE shows reduced Nkx2-2as expression in cancer cell lines, including MCF-7. (**D**) UMAP clustering of 3,000 samples highlights distinct Nkx2-2as expression patterns, (indicated by black arrow), consistent with a tumor-suppressive role

Fig. 2 (**A**, **B**) KEGG and DisGeNET enrichment analyses showing pathways and disease categories associated with Nkx2-2as, including metabolic and cellular processes, (adjusted $p < 0.05$, FDR < 0.1). (**C**) ChEA and ENCODE transcription factor enrichment identifying ER, (MCF-7), SOX4, EZH2, BATF, VDR, and CBX3 as predicted upstream regulators. Mean Pearson correlation coefficients, ($r > 0.45$) between these transcription factor target genes and Nkx2-2as expression indicate consistent co-expression patterns

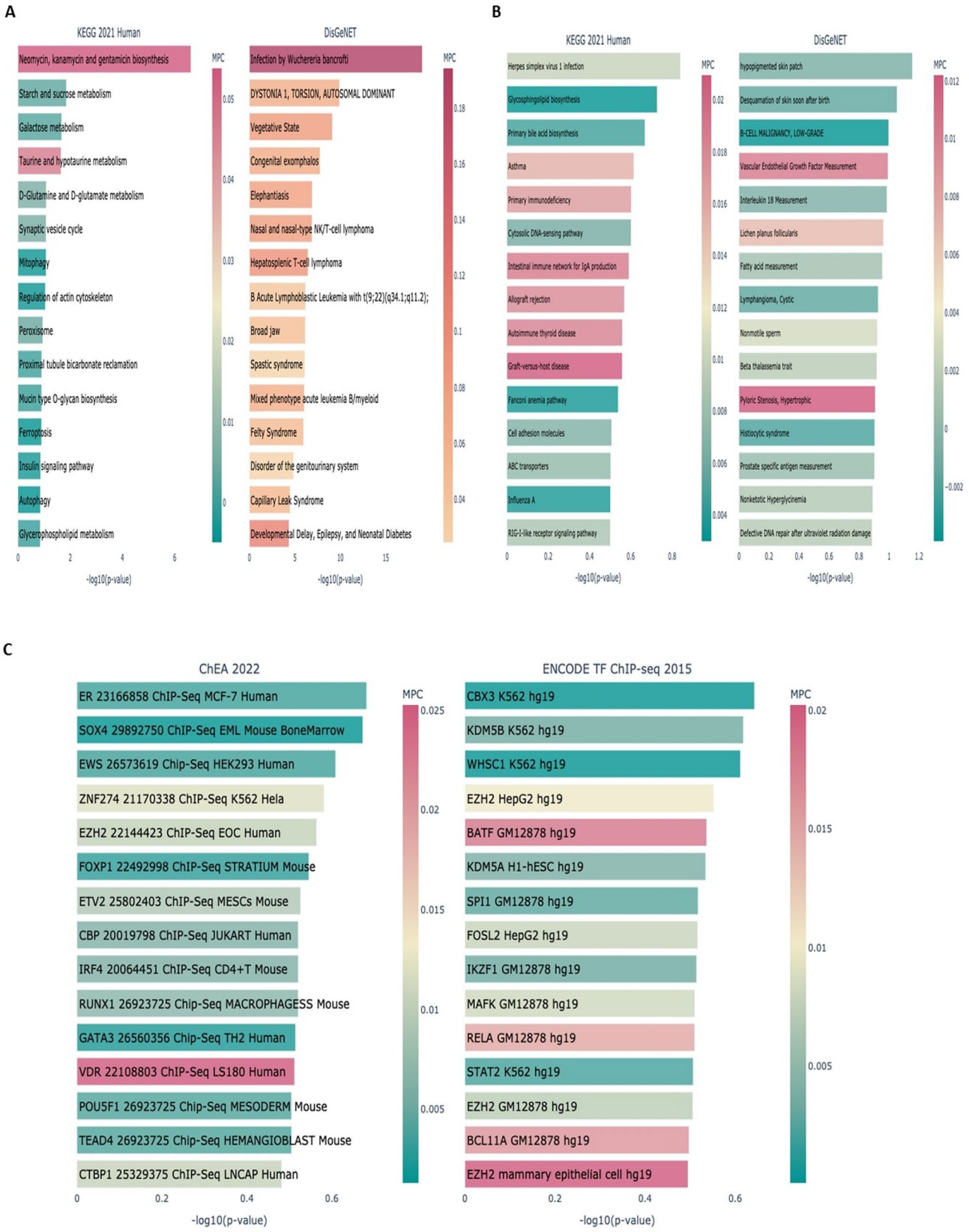


Fig. 2 (continued)

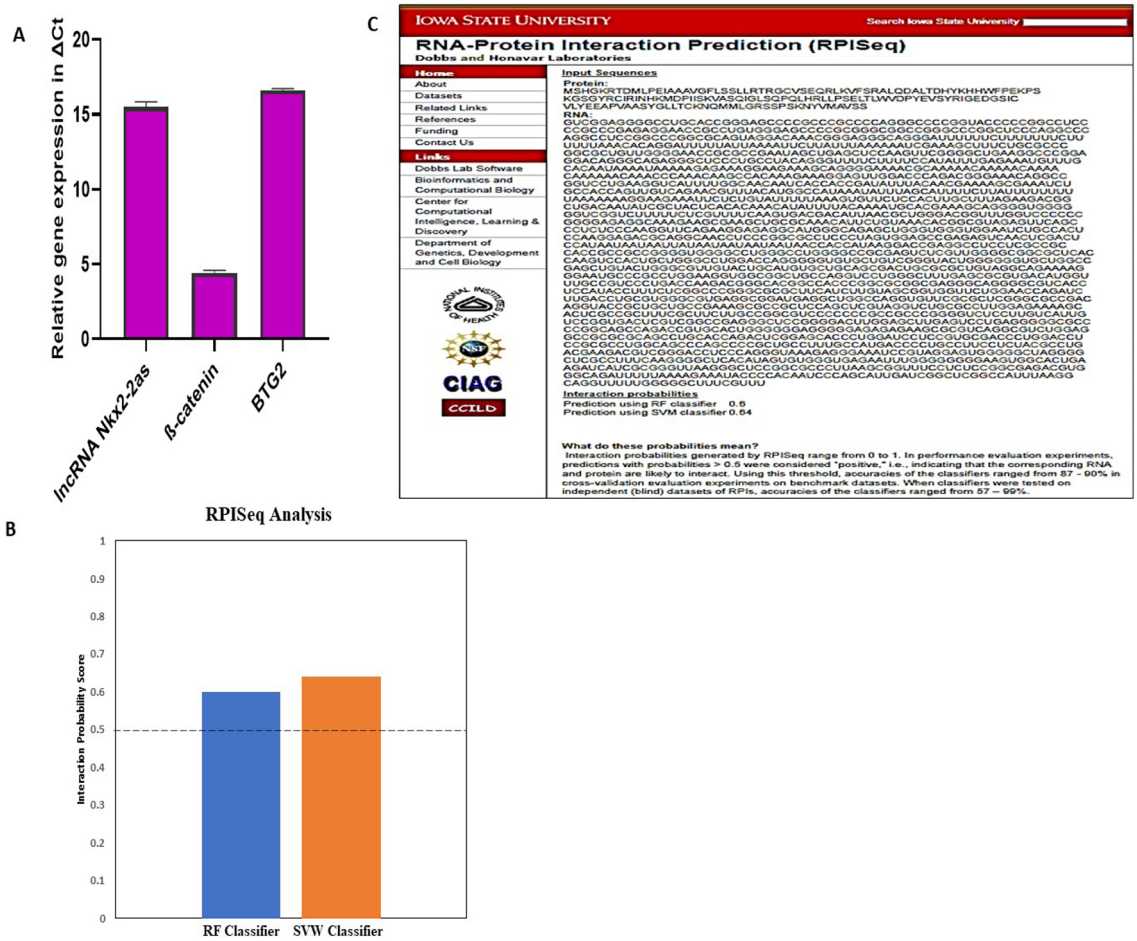


Fig. 3 Expression and interaction analysis of Nkx2-2as, BTG2, and β-catenin. (**A**) RT-PCR ΔCt analysis showed the relative expression levels of lncRNA Nkx2-2as, BTG2, and β-catenin in MCF-7 cells, with β-actin as the internal control. (**B**, **C**) RPI-Seq analysis predicted a strong likelihood of interaction between Nkx2-2as and BTG2, with RF and SVM classifier scores of 0.6 and 0.64, respectively. (**D**) Gene network analysis identified BTG2 as a potential modulator of β-catenin via Wnt pathway components including LEF1, TCF7L2, GSK3B, and APC. (**E**) ENCORI-based correlation analysis revealed an inverse expression relationship between BTG2 and β-catenin (CTNNB1) in BC samples, suggesting BTG2's role in suppressing Wnt/β-catenin signaling

Analysis of the relative expression levels (2^(-ΔCt)) revealed distinct expression patterns among the genes. β-catenin displayed the highest expression level, while lncRNA Nkx2-2as and BTG2 showed markedly low abundance in MCF-7 cells. The elevated expression of β-catenin suggests a constitutively active Wnt/β-catenin signaling pathway in this cell line. This expression profile is consistent with external transcriptomic data from ARCHS4 and GENCODE, which also indicate low expression levels of Nkx2-2as in MCF-7 cells.

RPI-Seq-based prediction of Nkx2-2as–BTG2 interaction

RNA–protein interactions (RPIs) play crucial roles in various cellular processes, including transcriptional and post-transcriptional regulation. The RPI-Seq analysis of lncRNA Nkx2-2as predicted a strong interaction potential with BTG2, as indicated by both the Random Forest (RF) and Support Vector Machine (SVM) classifiers. In RPI-Seq, scores above 0.5 are considered *positive predictions*, suggesting a likely RNA–protein interaction. The RF and SVM classifiers yielded scores of 0.60 and 0.64, respectively, indicating a positive likelihood of interaction between Nkx2-2as and BTG2. The reported range of 87–90% represents the overall classifier accuracy of the RPI-Seq model, reflecting the reliability of the prediction rather than a probability or statistical significance. Thus, RPI-Seq predicted a positive likelihood of interaction between Nkx2-2as and BTG2, supporting their potential functional association (Fig. 3B and C).

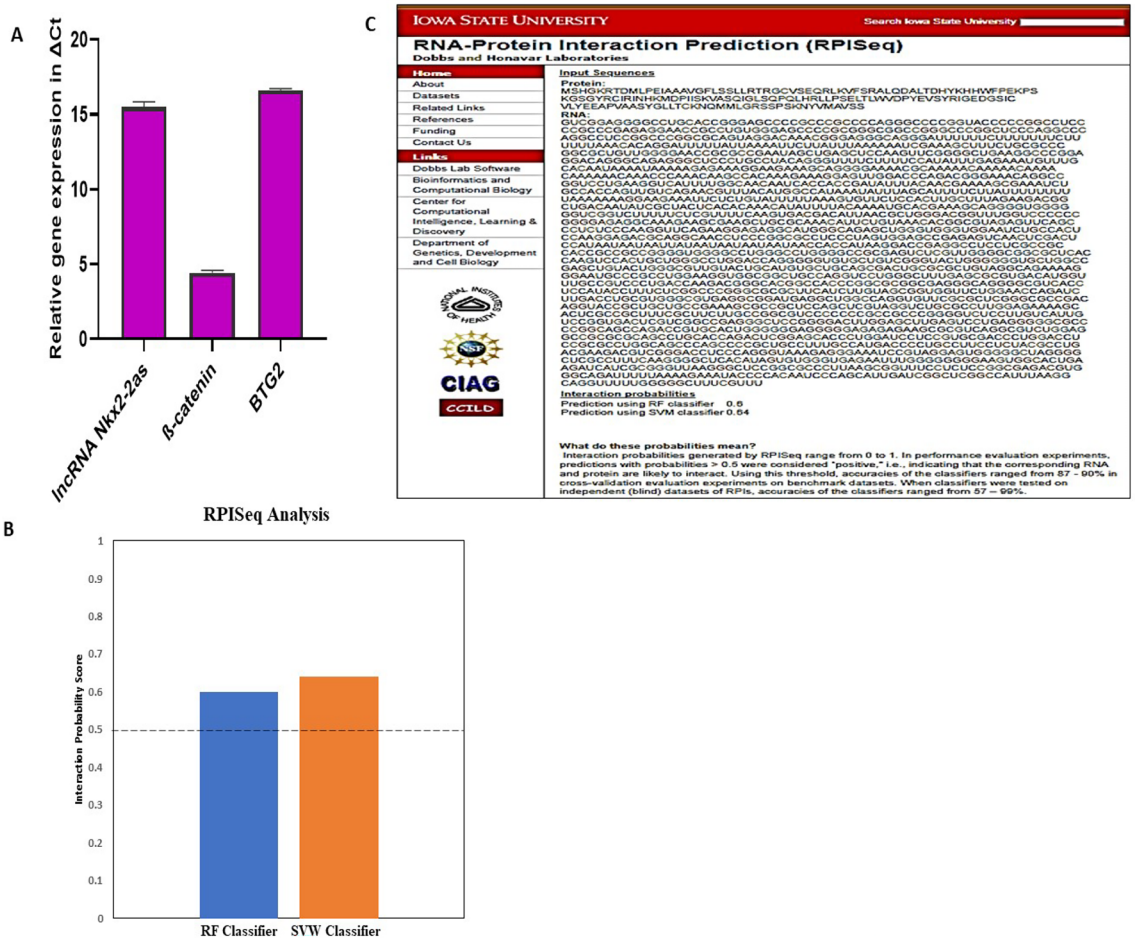


Fig. 3 (continued)

BTG2 inversely correlates with β-catenin expression in MCF-7 cells

The gene network analysis revealed multiple interaction links between BTG2 and β-catenin (CTNNB1) involving key components of the Wnt/β-catenin signaling pathway, including LEF1, TCF7L2, APC, CDH1, and GSK3B (Fig. 3D). These network associations indicate that BTG2 is connected to Wnt/β-catenin pathway elements implicated in cell proliferation and adhesion. Additional network nodes such as BTG1, TOB1, PRMT1, CHD8, HOXB9, and SALL1 were also linked to BTG2, reflecting its potential involvement in broader transcriptional and cell regulatory processes.

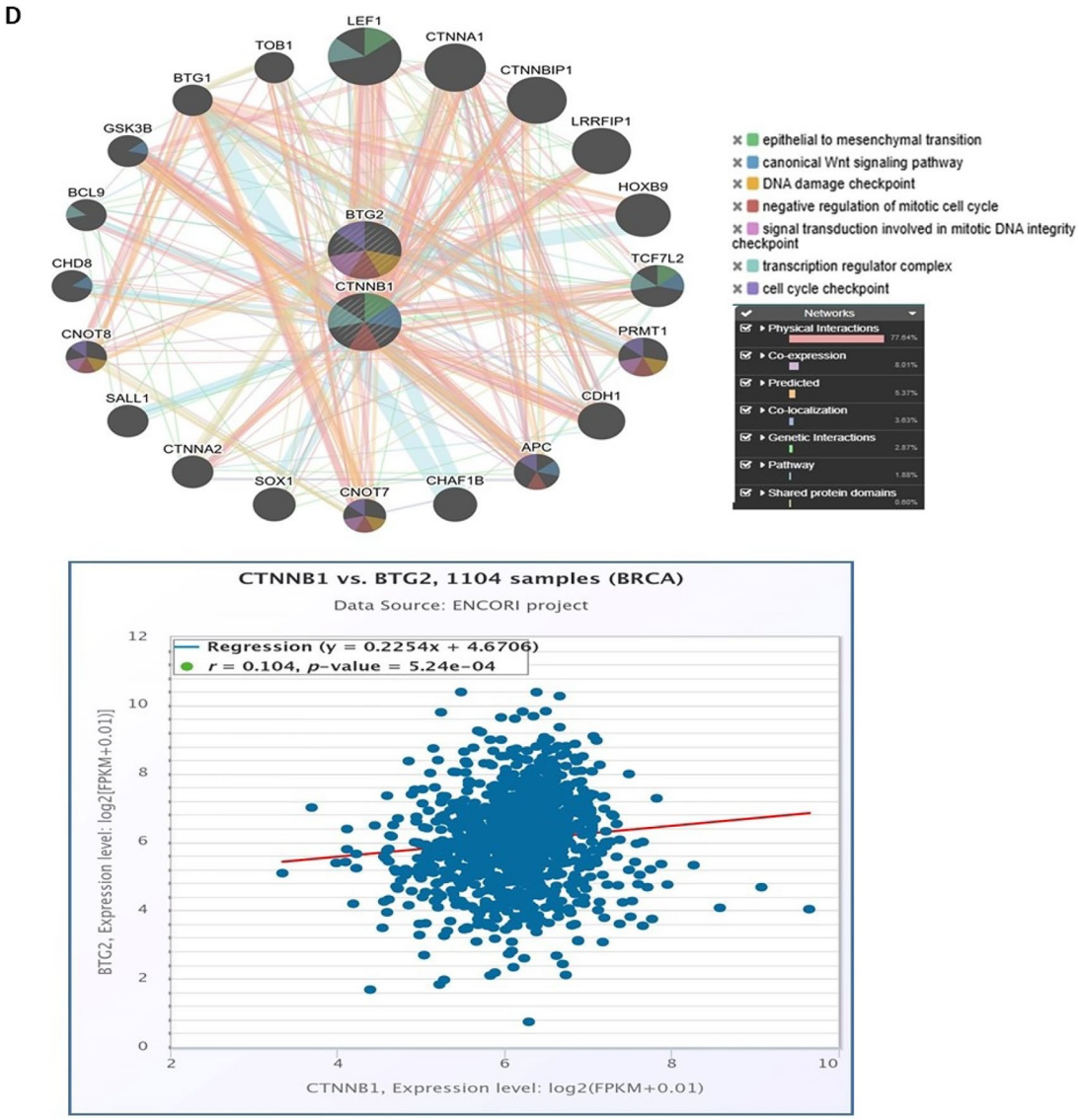
ENCORI-based correlation analysis of BC samples demonstrated an inverse relationship between BTG2 and β-catenin (CTNNB1) expression ($r = -0.104$, $p = 5.24 \times 10^{-4}$) (Fig. 3E). Higher BTG2 expression levels were associated with lower CTNNB1 expression, suggesting a possible negative regulatory connection.

Nkx2-2as modulation alters proliferation, colony formation, and migration in MCF-7 cells

MTT assay

To assess the influence of Nkx2-2as on BC cell proliferation, MCF-7 cells were transfected with the Nkx2-2as-pcDNA3.1 overexpression vector, siRNA for knockdown, and subjected to sequential treatment (overexpression followed by silencing). The MTT assay, initiated at 70% confluency, revealed a marked reduction in cell viability upon Nkx2-2as overexpression. After 48 h, cell viability decreased to 15.0 ± 0.42% ($p < 0.001$) compared to 95.2 ± 0.2% in the untreated control (Fig. 4A and B).

Morphological assessment indicated that overexpressing cells exhibited rounding and apoptotic-like features consistent with reduced proliferation. In contrast, siRNA-mediated knockdown of Nkx2-2as resulted in a slight increase in viability (86.6 ± 0.33%, $p > 0.05$), comparable to control cells, suggesting that suppression of Nkx2-2as alone exerts minimal impact under baseline conditions. In the sequential



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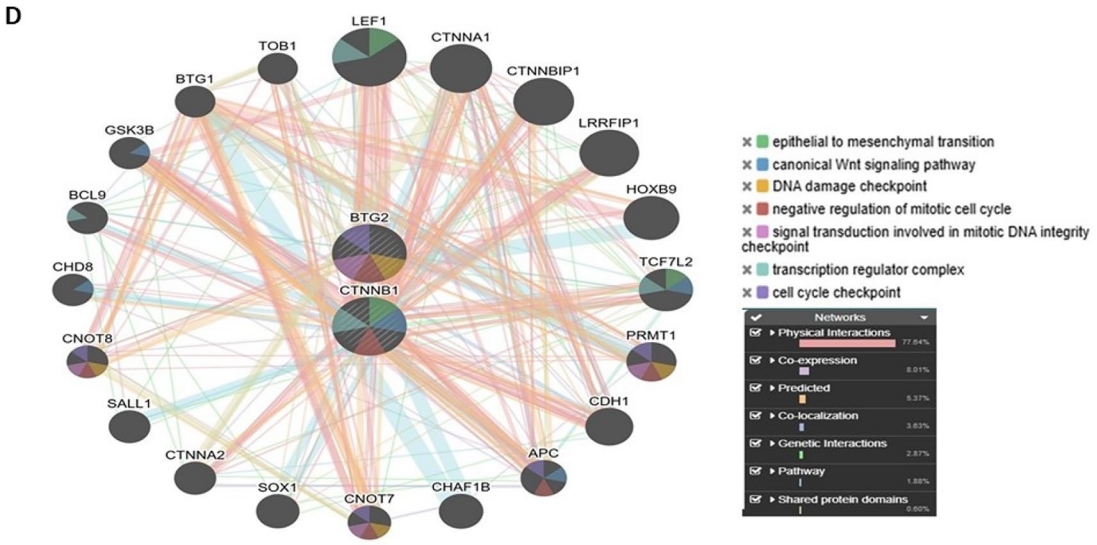
treatment group, initial overexpression decreased viability to 50.0±0.22% at 24 h and 35.0±0.05% at 48 h, whereas subsequent siRNA-mediated knockdown partially restored viability, indicating a reversal of overexpression-associated cytotoxic effects. Morphological observation supported this trend, showing visible stabilization of cell structure following knockdown (Fig. 4A and B).

Colony formation assay

To determine the long-term proliferative potential, colony formation assays were performed following Nkx2-2as modulation. Overexpression of Nkx2-2as markedly reduced both colony number and size compared with the untreated control ($p<0.01$), indicating decreased clonogenic potential (Fig. 4C). Colonies appeared sparse and less compact, consistent with diminished proliferation. Conversely, Nkx2-2as knockdown enhanced colony formation, reflected by increased colony density and size ($p<0.05$). In the sequentially treated group, colony formation was partially restored, suggesting that Nkx2-2as expression levels dynamically regulate the clonogenic capacity of MCF-7 cells.

Migration assay

Cell migration ability was assessed using the scratch assay. Nkx2-2as overexpression significantly impaired wound closure, with the scratch width increasing from 80 μm to 110 μm at 24 h and 180 μm at 48 h ($p < 0.01$), indicating reduced migratory capacity (Fig. 4D). Morphological evaluation qualitatively showed a shift toward rounded, less motile cell phenotypes. In contrast, Nkx2-2as knockdown



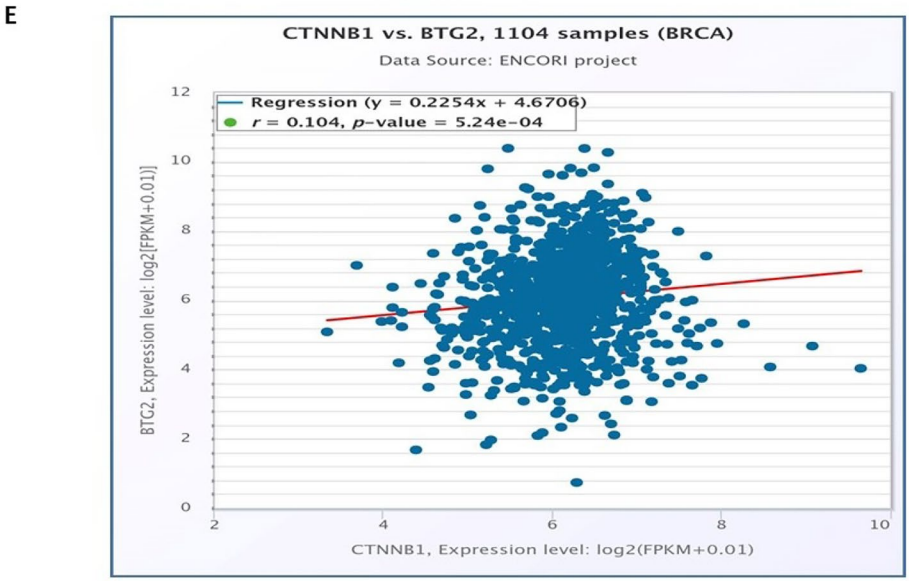


Fig. 3 (continued)

Table 2 Relative gene expression of LncRNA Nkx2-2as, BTG2, and β-catenin in control MCF-7 cells

Genes	(ΔCt) (Mean ± SD)	Relative expression ($2^{-\Delta Ct}$ (Mean ± SD))
lncRNA Nkx2-2as	15.84 ± 0.15	(1.70 ± 0.18) × 10^{-5}
β-catenin	4.28 ± 0.08	0.0515 ± 0.0029
BTG2	16.66 ± 0.05	(9.58 ± 0.33) × 10^{-7}

Data are shown as mean ± SD from at least three independent experiments. Statistical significance ($p < 0.01$) for differences in Ct values between genes was determined by one-way ANOVA with a post-hoc test

promoted wound closure, with the initial 80 µm scratch closing completely by 48 h, similar to control cells ($p < 0.05$). In the sequential treatment, siRNA-mediated silencing following overexpression mitigated the migration inhibition, with the scratch area remaining largely constant, indicating a partial restoration of migration potential.

Collectively, these findings indicate that Nkx2-2as modulation influences BC cell proliferation, clonogenicity, and migration, where overexpression suppresses and silencing reverses these cellular processes.

Nkx2-2as overexpression promotes apoptotic cell death in MCF-7 cells

DAPI staining

To examine nuclear morphology changes associated with Nkx2-2as modulation, MCF-7 cells were subjected to DAPI staining following overexpression, knockdown, and sequential treatments. Cells overexpressing Nkx2-2as

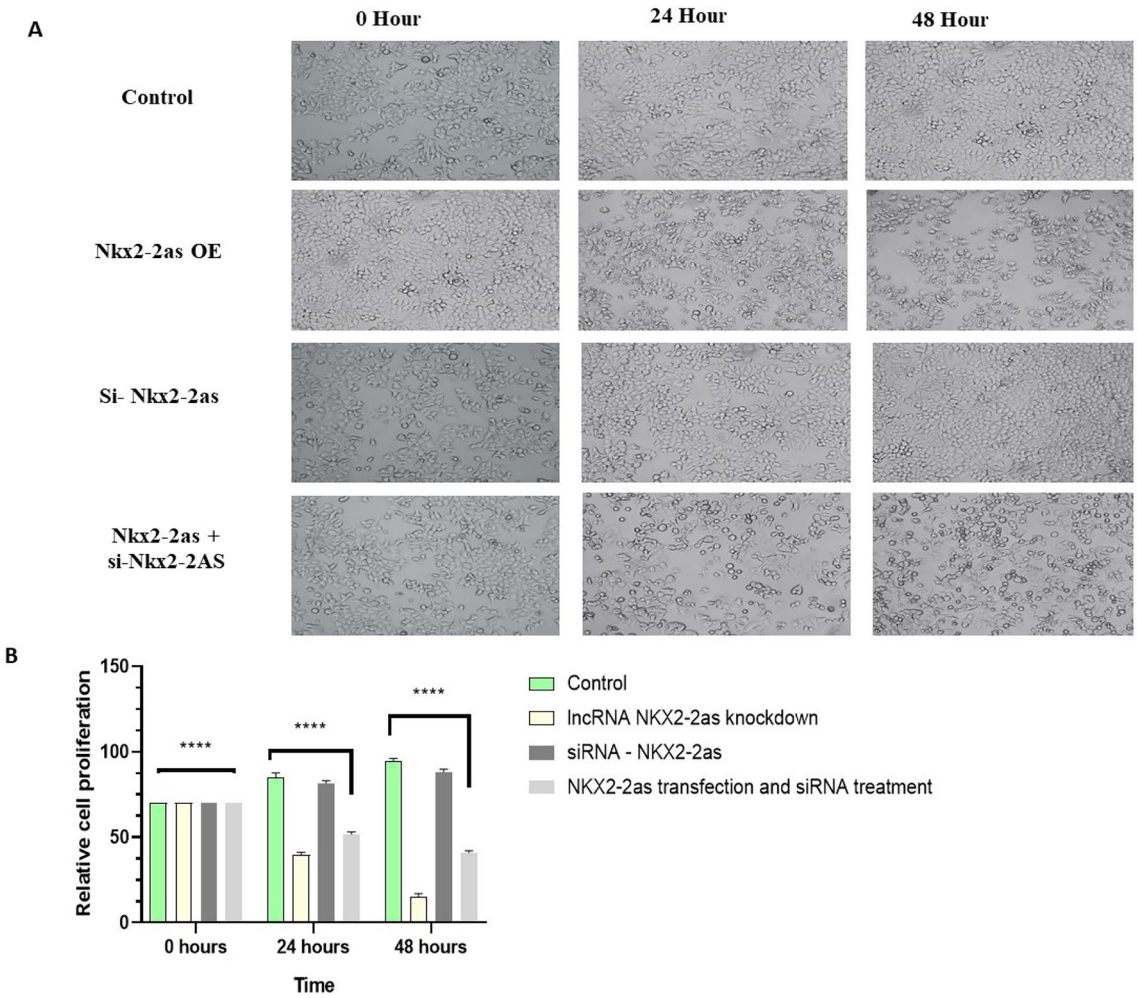
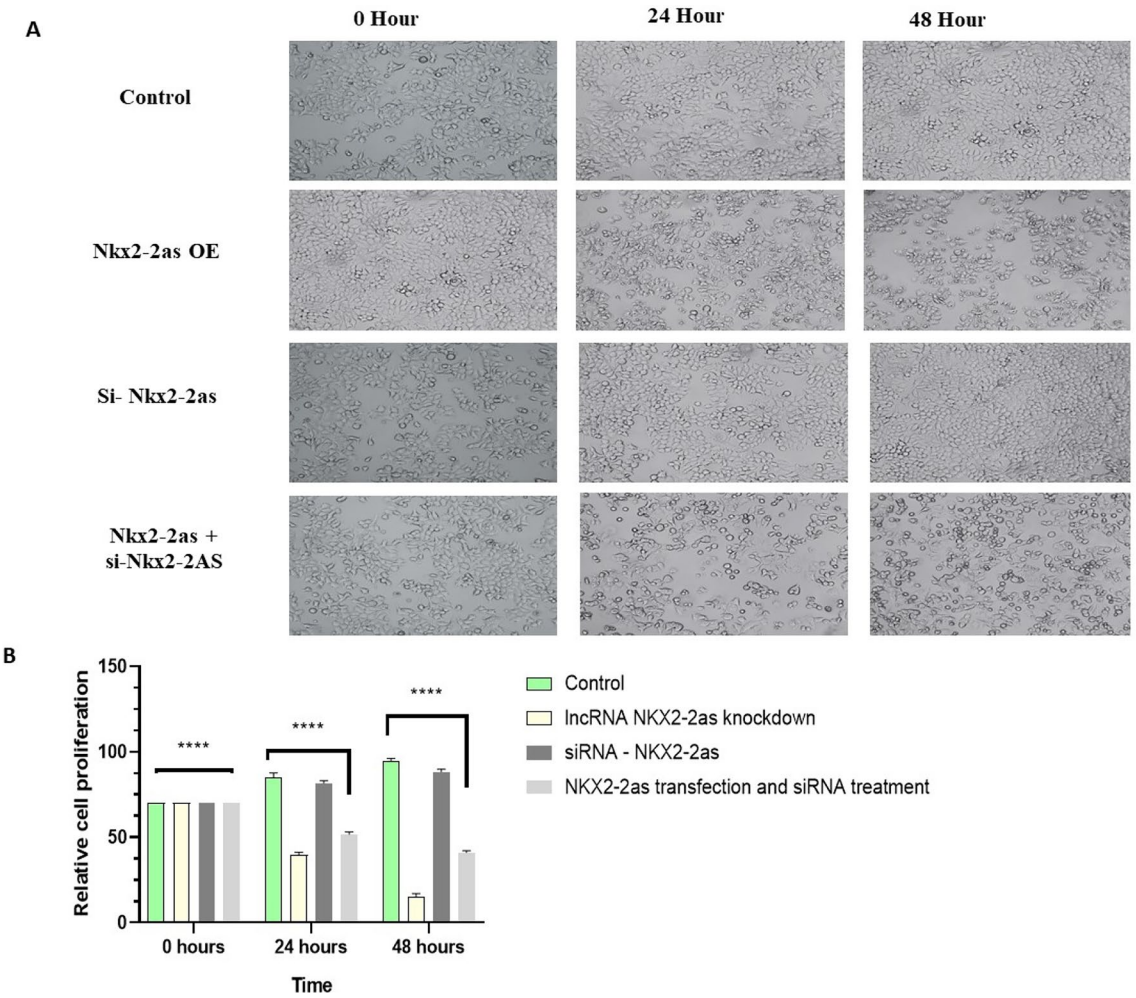


Fig. 4 (**A**, **B**) MTT assay showing reduced viability upon NKX2-2as overexpression, ($p<0.001$) and partial recovery following siRNA-mediated knockdown. (**C**) Colony formation assay demonstrating suppressed colony number and size in overexpressing cells, ($p<0.01$), enhanced clonogenic potential upon knockdown, ($p<0.05$), and partial restoration after sequential treatment. (**D**) Scratch assay showing impaired migration in NKX2-2as-overexpressing cells, (gap widened from 80 µm to 180 µm), while knockdown facilitated wound closure. Combined treatment reversed the inhibitory effect on migration. Data are presented as mean ± SD; significance determined by Student's t-test; $p<0.05$ considered statistically significant

displayed distinct nuclear alterations, including chromatin condensation, nuclear shrinkage, and increased fluorescence intensity, consistent with apoptotic morphology. Several cells showed nuclear fragmentation (orange arrows) and membrane blebbing (white arrows) (Fig. 5A). In contrast, siRNA-mediated knockdown of Nkx2-2as produced nuclei with normal morphology and uniform fluorescence comparable to control cells, suggesting minimal apoptotic change. In the sequential treatment group (overexpression followed by knockdown), a mixed population was observed—some cells retained apoptotic features such as chromatin fragmentation (orange arrows) and membrane blebbing (yellow arrows), while others displayed near-normal nuclear structure (Fig. 5A). Morphological assessment thus indicated that silencing Nkx2-2as partially reversed the apoptotic features observed upon its overexpression.

AO/EtBr dual staining

Acridine orange/ethidium bromide (AO/EtBr) staining was used to distinguish live, early apoptotic, and late apoptotic or necrotic cells (Fig. 5B). In the Nkx2-2as-overexpressing group, most cells exhibited orange-red fluorescence (white arrows), corresponding to late apoptotic or necrotic features, whereas control cells displayed uniform bright green fluorescence typical of viable cells. In siRNA-Nkx2-2as-transfected cells, fluorescence remained predominantly green with a few yellow-orange cells, suggesting a low proportion of early apoptotic cells. The combined treatment group showed a heterogeneous cell population, with both green (viable) and orange-red (apoptotic/necrotic) cells visible, indicating a partial recovery of viability after Nkx2-2as



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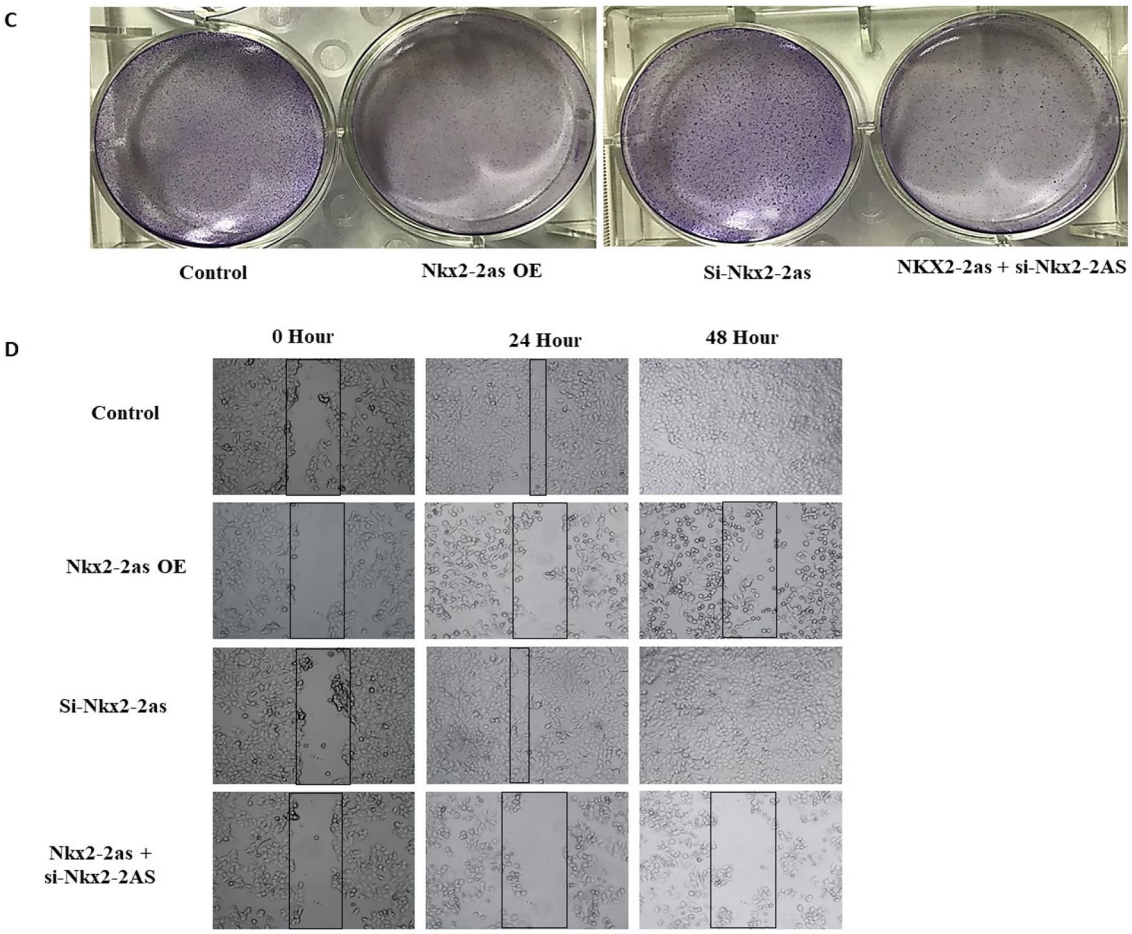
silencing. These fluorescence patterns qualitatively supported the morphological findings from DAPI staining.

Flow cytometry (Annexin V-FITC/PI)

Quantitative assessment of apoptosis was performed using flow cytometry with Annexin V-FITC/PI staining (Fig. 5C). Overexpression of Nkx2-2as resulted in a marked increase in late apoptotic cells (50.3% ± 1.2, $p < 0.001$) and a reduction in viable cells (18.2% ± 0.5) relative to control cells (99.7% ± 0.1). Early apoptotic (13.7% ± 0.8) and necrotic (17.8% ± 0.6) populations were also elevated. In contrast, Nkx2-2as knockdown yielded a moderate proportion of early apoptotic cells (32.0% ± 1.0) with 67.4% ± 0.7 viability, indicating limited apoptotic induction ($p > 0.05$). In the sequential treatment group, early apoptosis was observed in 72.6% ± 1.5 of cells, whereas late apoptotic and necrotic fractions decreased to 1.5% ± 0.1 and 0.04% ± 0.01, respectively ($p < 0.01$), with an increase in viable cells (25.9% ± 0.6). These data suggest that siRNA-mediated silencing following overexpression partially mitigated late-stage apoptotic effects. Statistical analyses were performed using triplicate samples ($n = 3$), and data are expressed as mean ± SD.

Nkx2-2as suppresses Wnt/β-catenin signaling via BTG2 activation

To explore the regulatory role of lncRNA Nkx2-2as in BC, we investigated its impact on the Wnt/β-catenin signaling pathway and cell proliferation in MCF-7 cells through both overexpression and knockdown strategies. Overexpression of lncRNA Nkx2-2as resulted in a dramatic 150-fold increase in transcript levels (Fig. 6A; $p < 0.001$), confirming successful transfection. This was accompanied by a significant reduction in β-catenin (CTNNB1) expression (0.40-fold vs. control; $p < 0.01$), indicating attenuation of Wnt/β-catenin signaling activity. Correspondingly, transcript levels of downstream effectors TCF7 and *MYC* were also decreased ($p < 0.05$), suggesting a general suppression



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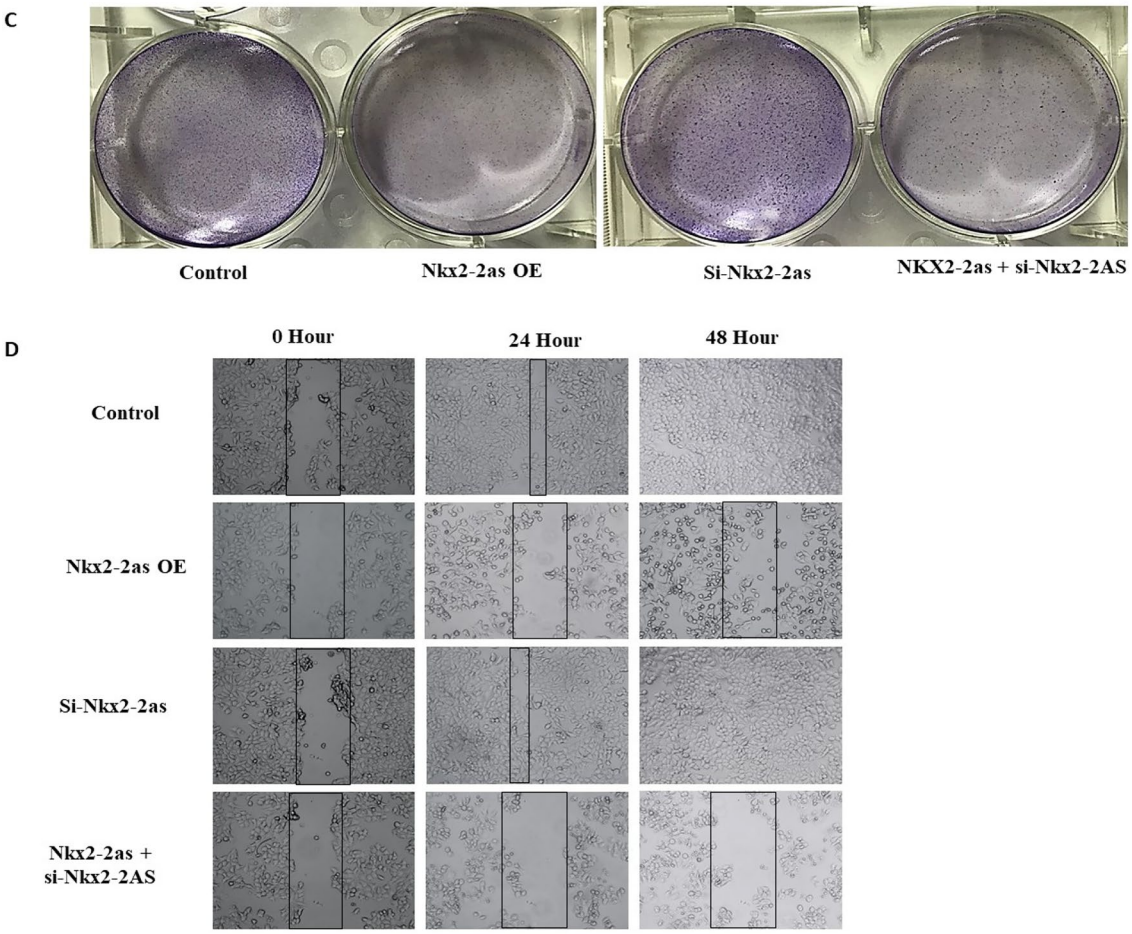
of pathway activation. Conversely, genes associated with β-catenin degradation and cell cycle control, including BTG2 and AXIN2, were upregulated by 8.5-fold and 4.5-fold, respectively ($p<0.01$; Fig. 6B).

Knockdown of lncRNA Nkx2-2as using siRNA achieved a 96% reduction in its expression (0.04-fold vs. control; $p<0.001$) and resulted in a mild elevation in β-catenin levels (1.79-fold; $p<0.05$), together with increased expression of TCF7 and *MYC* (Fig. 6C). This pattern suggests a reactivation of Wnt/β-catenin signaling under reduced Nkx2-2as expression. In the sequential overexpression–knockdown (rescue) group, Nkx2-2as expression decreased to 1.28-fold (vs. 150-fold in overexpression), with partial restoration of β-catenin and its target gene levels and reduced expression of BTG2 and AXIN2 (Fig. 6D).

Western blot analysis was performed to validate the transcriptional changes of β-catenin, MYC, and BTG2 at the protein level following modulation of lncRNA Nkx2-2as expression in MCF-7 cells. Consistent with the qRT-PCR results, overexpression of lncRNA Nkx2-2as markedly reduced the protein levels of β-catenin and MYC, indicating suppression of Wnt/β-catenin signaling activity. In contrast, BTG2 protein expression was strongly upregulated compared with control cells, confirming its positive regulation by Nkx2-2as (Fig. 7A).

Conversely, siRNA-mediated knockdown of lncRNA Nkx2-2as led to an increase in β-catenin and MYC protein levels, accompanied by a decrease in BTG2 expression (Fig. 7A), suggesting reactivation of Wnt signaling upon Nkx2-2as silencing. In the combined overexpression–knockdown (rescue) group, β-catenin and MYC levels were partially restored toward control values, while BTG2 expression declined relative to the overexpression condition (Fig. 7A).

Densitometric quantification normalized to β-actin revealed a consistent pattern with the mRNA data, β-catenin expression decreased from 1.00 (control) to 0.19 in the overexpression group and increased to 1.15 upon knockdown. MYC levels followed a similar trend, declining to 0.18 after overexpression and rising to 0.63 after knockdown. BTG2 protein levels showed an inverse relationship, increasing



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significantly to ~6.9-fold with Nkx2-2as overexpression and reducing to ~2.1-fold following knockdown (Fig. 7B).

These findings corroborate the transcriptional data, demonstrating that lncRNA Nkx2-2as exerts a negative regulatory effect on β-catenin and MYC while enhancing BTG2 expression, thereby indicating its role in suppressing the Wnt/β-catenin signaling pathway in BC cells.

Discussion

Long non-coding RNAs have emerged as pivotal regulators of oncogenic and tumor-suppressive signaling networks in BC, primarily through their influence on transcriptional, post-transcriptional, and signaling pathways. In this study, we explored the role of lncRNA Nkx2-2AS in modulating the Wnt/β-catenin signaling cascade and related cellular processes in MCF-7 breast cancer cells. The findings collectively suggest that Nkx2-2as may function as a tumor-suppressive lncRNA, influencing proliferation, apoptosis, and migration through transcriptional regulation of β-catenin-associated genes and the tumor suppressor BTG2.

Computational and experimental data support the hypothesis that Nkx2-2as is downregulated in BC and may exert its effects through nuclear regulatory mechanisms. In silico analyses predicted its nuclear localization and possible interaction with transcription factors such as ER, SOX4, EZH2, BATF, and the vitamin D receptor transcription factors known to govern hormone response and chromatin remodeling [13]. The observed positive correlation between Nkx2-2as and BTG2, and negative correlation with β-catenin (CTNNB1), suggests a regulatory axis where Nkx2-2as expression is associated with enhanced BTG2 activity and reduced Wnt/β-catenin signaling.

Mechanistically, Nkx2-2as overexpression was associated with downregulation of β-catenin, TCF7, and MYC, and concurrent upregulation of BTG2 and AXIN2, two well-characterized negative regulators of the Wnt/β-catenin pathway. BTG2, in particular, is known to suppress proliferation and promote differentiation in MCF-7 cells by interfering with β-catenin-mediated transcription [1]. This is consistent

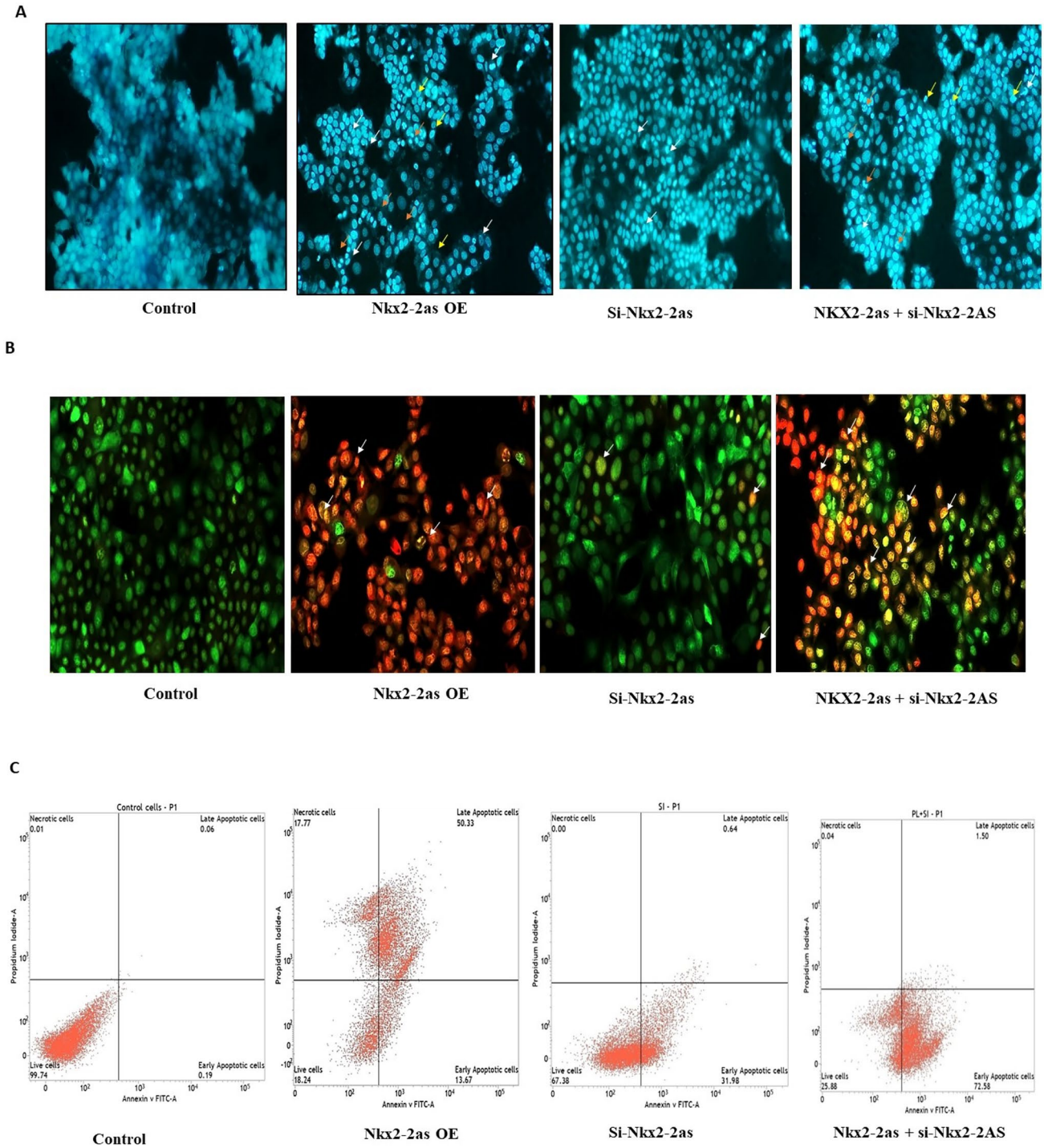
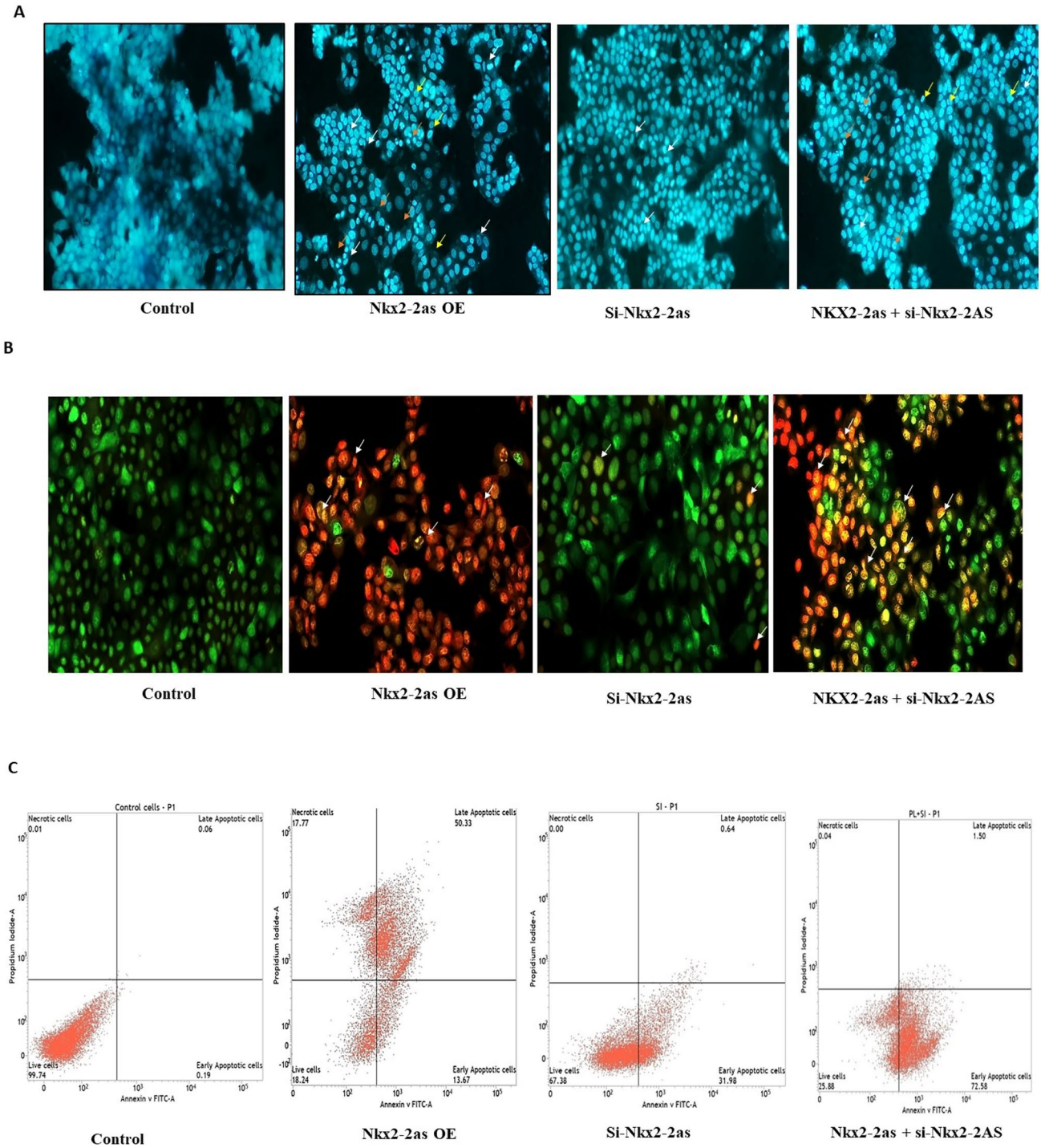


Fig. 5 (**A**) DAPI staining showing chromatin condensation, nuclear fragmentation (orange arrows), and membrane blebbing (white arrows) in NKX2-2as-overexpressing cells. Knockdown preserved normal nuclear morphology, while sequential knockdown after overexpression led to partial restoration (yellow arrows). (**B**) AO/EtBr dual staining revealed increased orange-red fluorescence (late apoptotic/necrotic cells, white arrows) in the overexpression group. Knockdown maintained green fluorescence (viable cells), and combined treatment showed mixed fluorescence indicative of partial recovery. (**C**) Annexin V-FITC/PI flow cytometry showed elevated late apoptotic fraction in NKX2-2as-overexpressing cells (50.3% ± 1.2, $p < 0.001$), minimal apoptosis in knockdown cells, and partial rescue in sequentially treated cells. Data represent mean ± SD from three independent experiments ($n = 3$). Statistical significance determined by Student's t-test; $p < 0.05$ considered significant



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with findings in other malignancies; for instance, BTG2 has been shown to function as a tumor suppressor in skin cancer by directly downregulating β-catenin, cyclin D1, and MYC protein levels, thereby inhibiting proliferation and invasion [6]. The Nkx2-2as–BTG2–β-catenin relationship observed here appears to be correlative and transcriptionally mediated, rather than through direct RNA binding or miRNA sponging, as no evidence of miRNA sequestration or sequence complementarity was detected in this study. However, the possibility of indirect regulation through chromatin-associated interactions or transcriptional modulation cannot be excluded and warrants further exploration.

These observations align with the broader context of ncRNA-mediated tumor suppression. For instance,

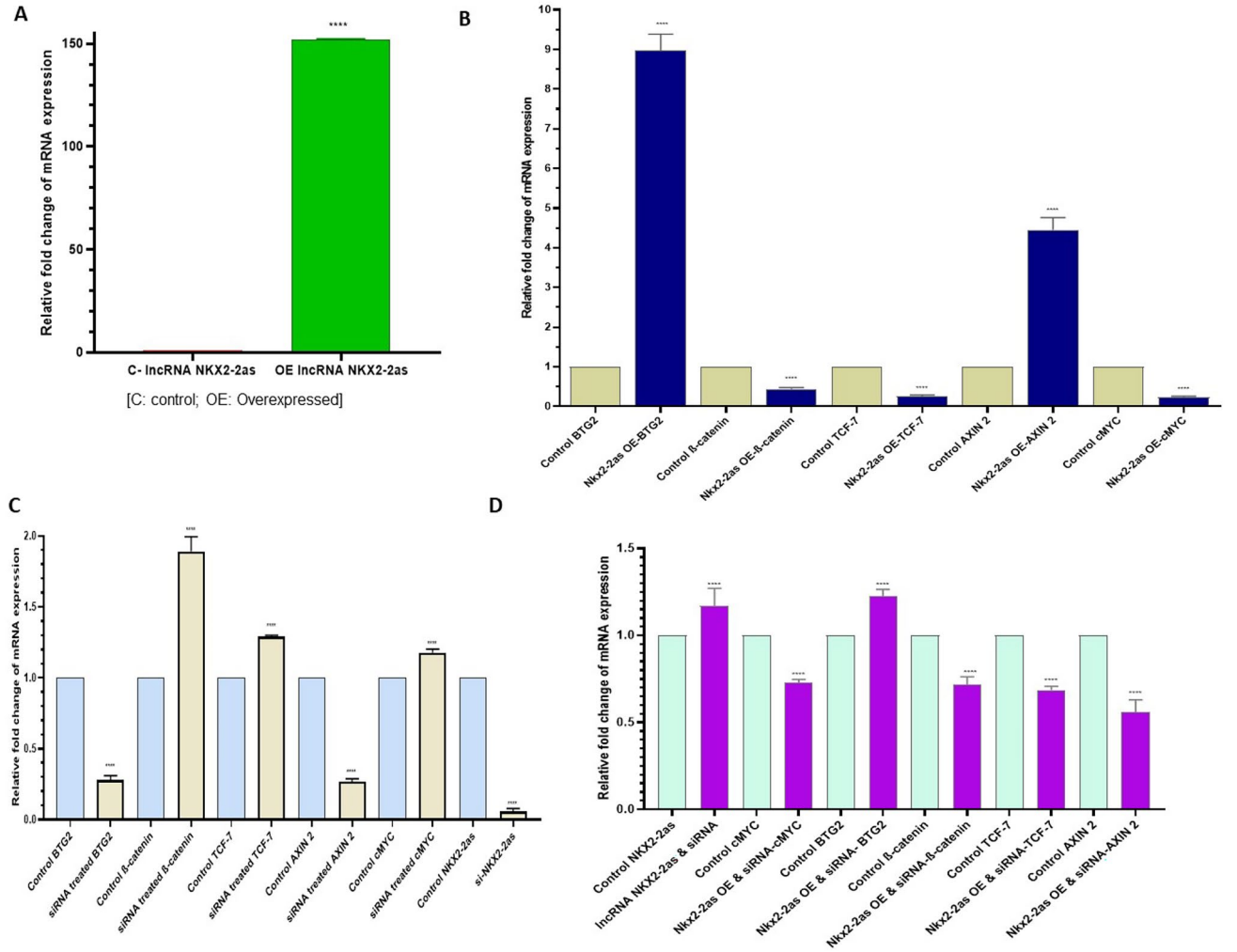
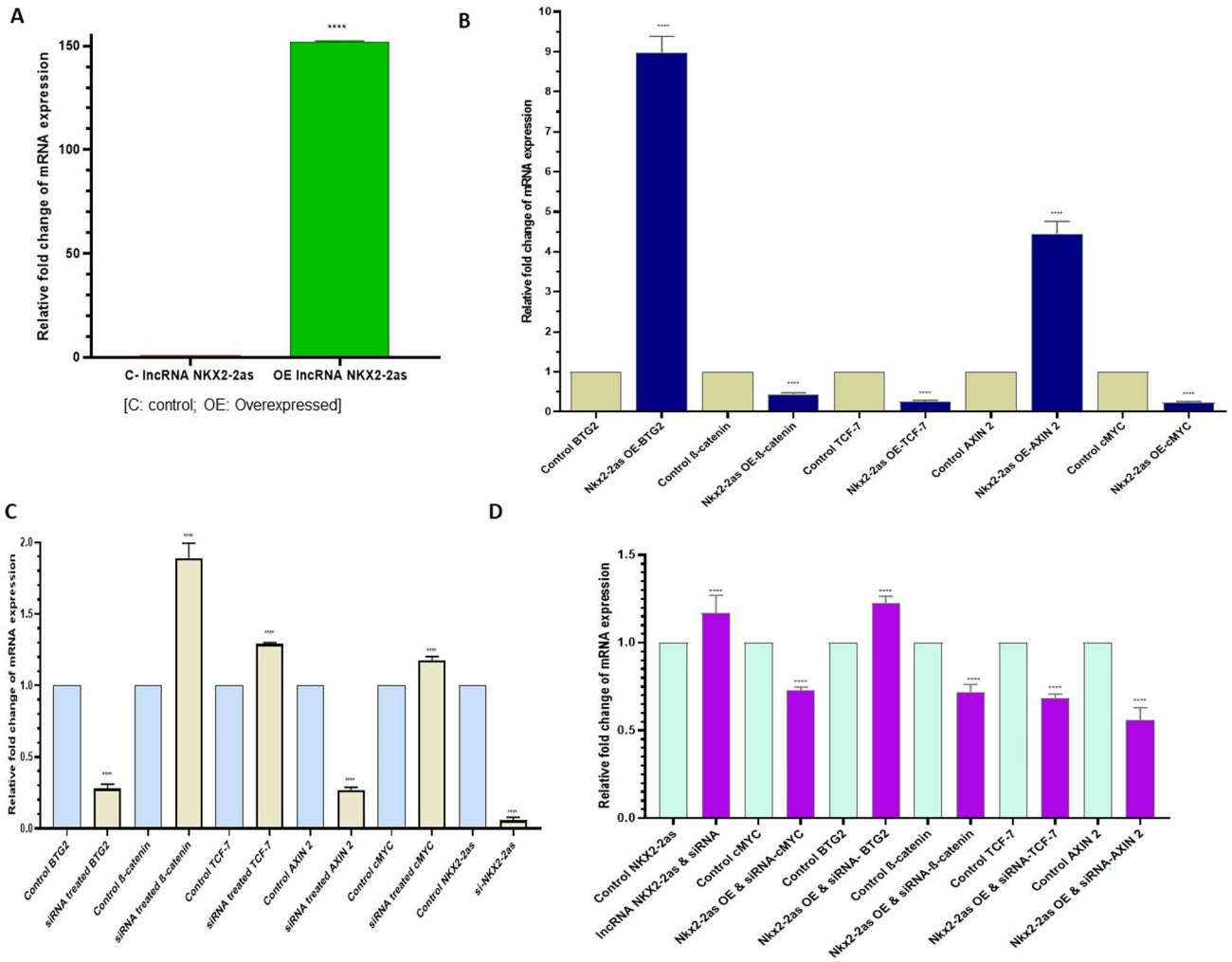


Fig. 6 (**A**) Overexpression of Nkx2-2as resulted in an approximately 150-fold increase in transcript levels, ($p<0.001$, $n=3$), accompanied by a significant reduction in β-catenin, (CTNNB1), TCF7, and cMYC expression, ($p<0.05$, $n=3$), consistent with decreased Wnt/β-catenin pathway activity. (**B**) Under the same condition, BTG2 and AXIN2 transcripts were strongly upregulated, (8.5-fold and 4.5-fold, respectively; $p<0.01$, $n=3$), suggesting increased β-catenin turnover–associated gene expression. (**C**) siRNA-mediated knockdown of Nkx2-2as led to a 96% reduction in its expression, ($p<0.001$, $n=3$) and a moderate increase in β-catenin, (1.79-fold), TCF7, and MYC expression, ($p<0.05$, $n=3$), indicating partial reactivation of Wnt signaling. (**D**) In rescue experiments, (overexpression followed by knockdown), partial restoration of β-catenin signaling components and downregulation of BTG2 and AXIN2 expression were observed, ($p<0.05$, $n=3$), reflecting intermediate pathway activity between overexpression and knockdown conditions. Data are presented as mean ± SD from three independent biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test, ($p<0.05$ considered significant)

miR-491 has been shown to inhibit oncogenic signaling and induce apoptosis across multiple cancer types [21], while miR-98 modulates drug resistance and cell cycle progression through similar transcriptional networks [11]. Recent studies on lncRNAs further reinforce this paradigm in BC. For example, lncRNA GAS5, frequently downregulated in breast tumors, functions as a tumor suppressor by sponging miR-221-3p to upregulate DKK2, thereby inhibiting Wnt/β-catenin signaling, promoting apoptosis, and enhancing sensitivity to chemotherapy such as doxorubicin [5]. Similarly, lncRNA TPTEP1 suppresses triple-negative breast cancer progression by acting as a miR-1343-3p sponge to activate the SIRT3/FOXO3a axis, which represses Wnt/β-catenin activity and impedes proliferation, migration, invasion, and metabolic reprogramming [12]. Additionally, lncRNA CARMN exerts tumor-suppressive effects in BC by regulating epithelial-mesenchymal transition and metastasis, with evidence of crosstalk involving the miR-92a-3p/BTG2 axis in modulating Wnt/β-catenin-related pathways in related malignancies [25]. By analogy, Nkx2-2as may act as a higher-order regulator, fine-tuning transcriptional programs that converge on Wnt/β-catenin signaling. Integrating these insights, our data suggest that Nkx2-2as exerts tumor-suppressive effects through transcriptional coordination of



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BTG2 and β-catenin pathway genes, ultimately influencing proliferation and apoptotic balance in BC cells. Together, the transcript and protein expression data establish that lncRNA Nkx2-2as suppresses the Wnt/β-catenin signaling pathway through BTG2-mediated regulation in BC cells. The coordinated decrease in β-catenin and MYC levels, coupled with enhanced BTG2 expression, indicates that Nkx2-2as acts as a critical upstream modulator of this tumor-suppressive cascade. These findings not only strengthen the mechanistic understanding of the Nkx2-2as–BTG2–β-catenin axis but also highlight its potential as a target for future RNA-based therapeutic interventions in BC.

While these findings provide new mechanistic insights, they are based on in vitro models and require further validation in in vivo systems and clinical cohorts. Although we attempted to assess the clinical relevance of Nkx2-2as expression using the TCGA breast cancer dataset, this lncRNA is not well annotated in TCGA, and expression or survival correlation data are currently unavailable. Therefore, a prognostic analysis could not be conducted. To overcome this limitation, we utilized lncHUB and ENCORI (StarBase) databases, which contain curated and predictive information on Nkx2-2as expression, interacting partners, and co-expression patterns. These complementary in silico analyses provided supportive evidence of its potential role in regulating BTG2 and β-catenin signaling in BC. Future studies integrating RNA immunoprecipitation, chromatin interaction mapping, and dual-luciferase assays could help clarify whether the regulatory interactions between Nkx2-2as, BTG2, and β-catenin are direct or mediated by transcriptional intermediates.

Conclusion

This study demonstrates that lncRNA Nkx2-2as plays a critical role in suppressing BC progression by modulating the Wnt/β-catenin signaling pathway and apoptosis through

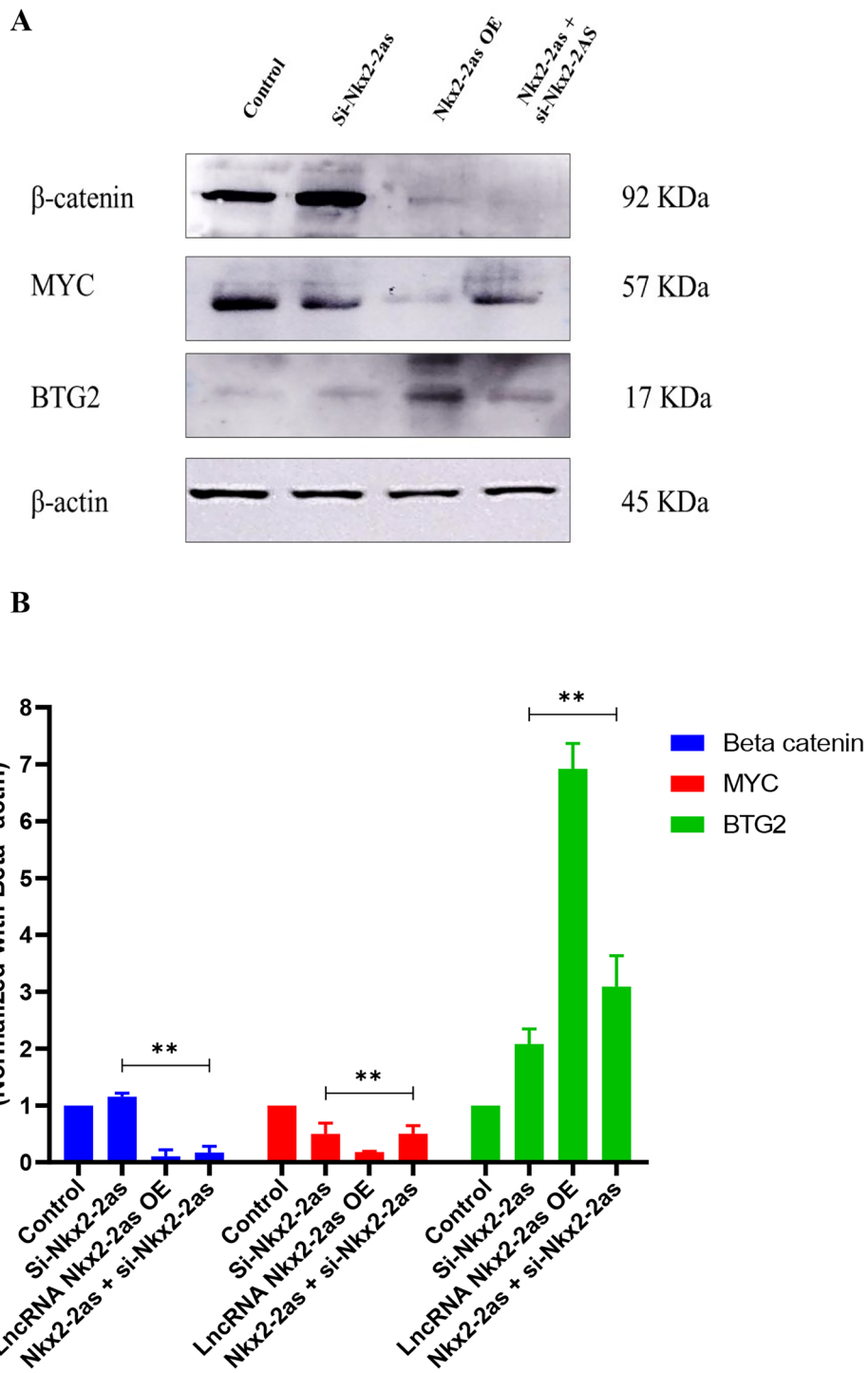
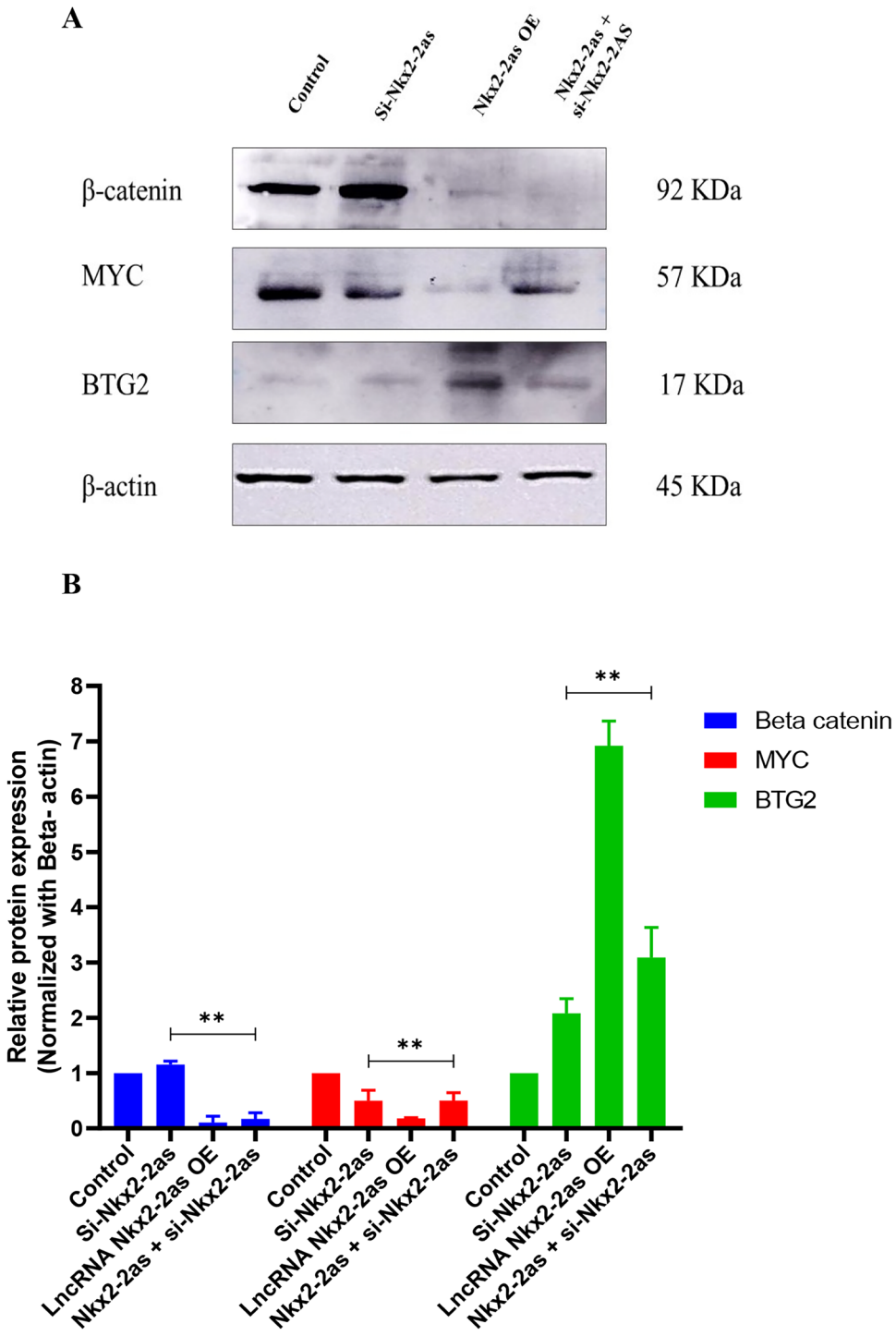


Fig. 7 **(A)** Representative blots showing changes in β-catenin, MYC, and BTG2 protein levels after Nkx2-2as knockdown, overexpression, and rescue treatments. β-actin served as a loading control. **(B)** Densitometric quantification normalized to β-actin. Overexpression of Nkx2-2as reduced β-catenin and MYC while increasing BTG2 levels, whereas knockdown showed the opposite trend, confirming suppression of Wnt/β-catenin signaling by Nkx2-2as. Data represent mean ± SD ($p < 0.05$, $p < 0.01$)

RNA-mediated mechanisms. Our findings highlight the therapeutic potential of targeting lncRNA Nkx2-2as, as its overexpression significantly inhibits MCF-7 cell proliferation and migration while inducing apoptosis. These effects were achieved through the downregulation of key Wnt/β-catenin components (β-catenin, TCF-7, and MYC) and the upregulation of the tumor suppressor BTG2, reinforcing the concept of RNA-based intervention in cancer signaling pathways. Notably, the knockdown of Nkx2-2as reversed these anti-tumorigenic effects, further emphasizing its role as a regulatory lncRNA with therapeutic relevance. The lncRNA Nkx2-2as/BTG2 axis thus represents a novel regulatory network that could be exploited for RNA-targeting drug development in BC. In summary, our study provides both transcriptional and protein-level evidence that lncRNA Nkx2-2as functions as a suppressor of Wnt/β-catenin



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signaling in BC via BTG2-mediated regulation. Overexpression of Nkx2-2as inhibited β-catenin and MYC while upregulating BTG2, a pattern consistently observed in both qRT-PCR and Western blot analyses. These findings reinforce the mechanistic role of Nkx2-2as in attenuating oncogenic signaling and emphasize its potential translational relevance as a prognostic biomarker and therapeutic target in BC.

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Author contributions Anjali K. Ravi: Writing – original draft, review & editing, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. Saradhadevi Muthukrishnan: Validation, Formal analysis, Data curation, Conceptualization. Gayathiri Gunasangkaran, Marie Arockianathan Pushpam, Vijaya Anand Arumugam, Kunnathur Murugesan Sakthivel: Formal analysis.

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Data availability The data supporting this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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