Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

ANXA2 Regulates RANKL-Induced Osteoclast Differentiation Through STAT3 Signaling in Breast Cancer

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Background: Bone metastasis affects nearly 70% of patients with advanced breast cancer, significantly influencing patient survival. Osteoclasts play a crucial role in osteolysis and the proliferation of bone tumor cell metastasis. Although previous studies have established Anxa2 as a critical factor in the invasion and metastasis of breast cancer, its involvement in bone metastasis remains poorly understood.

Methods: The correlation between ANXA2 expression and survival was analyzed in breast cancer cohorts. Enrichment analysis was performed to explore ANXA2-associated signaling pathways. RAW264.7 cells were induced to differentiate into osteoclasts using conditioned media from breast cancer cells, and osteoclastogenesis was quantified using the TRAP assay. Breast cancer cell lines with either Anxa2 overexpression or knockdown were established to assess the impact on osteoclastogenesis. The mRNA and protein expression levels were analyzed by RT-PCR and Western blot. The role of STAT3 in regulating RANKL expression was evaluated using a dual luciferase reporter assay.

Results: ANXA2 was significantly upregulated in breast cancer patients and associated with poor survival. GO and KEGG analyses revealed that ANXA2 substantially modulated signaling pathways involved in bone metastasis. Furthermore, ANXA2 notably enhanced the differentiation of RAW264.7 cells into osteoclasts and upregulated genes associated with osteoclast differentiation. Additional investigation showed that ANXA2 markedly activated the STAT3 signaling pathway and increased RANKL expression. The dual luciferase reporter assay demonstrated that STAT3 directly bound to the −1804 region of the RANKL promoter, thereby regulating RANKL expression.

Conclusion: This study identifies ANXA2 as a key regulator of osteoclast differentiation through STAT3-mediated upregulation of RANKL, driving bone metastasis in breast cancer. These results highlight the potential of targeting the ANXA2/STAT3/RANKL axis as a therapeutic strategy to combat bone metastasis.

Keywords: ANXA2, breast cancer, osteoclast, RANKL, STAT3

Introduction

Breast cancer is the most common malignancy in women, with metastasis serving as the leading cause of mortality.[1] Around 65% of patients develop osteolytic bone metastases, resulting in severe bone-related complications that significantly reduce survival rates.[2,3] Therefore, effective interventions are essential to prevent and manage bone metastases, ultimately improving patient survival.

The crosstalk between breast cancer cells and the bone microenvironment, which enables bone-targeted metastasis, is a central topic in oncology research. Bone colonization is initiated by the formation of a pre-metastatic niche, with primary tumor cells influencing this niche through the secretion of soluble cytokines prior to the infiltration of circulating tumor cells into the bone marrow.[4,5] Breast cancer cells release factors such as parathyroid hormone-related peptide (PTHrP), IL-6, and IL-11, which stimulate osteoclast differentiation and activation, thus accelerating bone resorption.[6–8]

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In parallel, these cells secrete Receptor Activator of Nuclear Factor-κB Ligand (RANKL), which promotes osteoclast activity by directly binding to RANK receptors.[9] Activated osteoclasts, in turn, release growth factors and cytokines such as TGF-β, IGF, and VEGF, further driving tumor cell proliferation and metastasis.[10–13] This feedback loop represents a key mechanism in breast cancer bone metastasis. A comprehensive understanding of these molecular pathways is critical for the development of novel strategies and therapeutic targets to prevent and treat breast cancer.

The ANXA2 (Annexin A2) belongs to the family of membrane-associated proteins, interacting with various ligands to regulate cellular functions.[14–16] Recent studies highlight ANXA2's integral involvement in cancer progression, particularly by enhancing tumor cell proliferation, invasion, and angiogenesis.[14,17] Mechanistically, ANXA2 promotes proliferation and metastasis by inducing epithelial to mesenchymal transition via the EGF/EGFR signaling pathway.[18] Moreover, ANXA2 significantly elevates the expression of cyclin D1 and MMP2/9 at both the mRNA and protein levels, which are essential regulators of cell proliferation and invasion.[19] In addition, ANXA2 regulates both the cytoskeleton and extracellular matrix (ECM), two factors crucial for bone metastasis. ANXA2 boosts tumor cell motility and invasiveness by increasing F-actin expression and aggregation, alongside other cytoskeletal regulators, thus facilitating actin cytoskeleton remodeling.[20] Through plasminogen activation, ANXA2 generates plasmin, promoting ECM degradation and enabling cell migration and tumor invasion.[21,22] These findings suggest that ANXA2 not only contributes to the initial metastatic spread but also supports the sustained growth of bone metastases. However, the detailed molecular mechanisms underlying ANXA2's role in breast cancer bone metastasis remain to be completely defined.

This study examines the regulatory role of ANXA2 in RANKL expression, a key factor in osteoclast differentiation and breast cancer bone metastasis. The data reveal that ANXA2 modulates the STAT3 signaling pathway, thereby influencing RANKL expression and secretion. This, in turn, supports osteoclast differentiation, establishing a bone microenvironment conducive to the colonization and proliferation of breast cancer cells. These results suggest potential avenues for novel therapeutic strategies for breast cancer.

Materials and Methods

Cell Culture

MDA-MB-231, SK-BR-3, RAW264.7 cell were acquired from the American Type Culture Collection (ATCC). All cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), and incubated at 37°C in a humidified atmosphere with 5% carbon dioxide.

Data Acquisition

The breast cancer dataset from The Cancer Genome Atlas (TCGA) was accessed via the UCSC Xena browser (http://xena.ucsc.edu/). Raw counts and FPKM data from 1211 primary human breast cancer tissues were utilized for subsequent analysis. Our study utilized transcriptomic data obtained from the Gene Expression Omnibus (GEO) repository. We analyzed two distinct microarray datasets: GSE21653, comprising genetic profiles from 266 primary tumor cases, and GSE50567, containing expression data from 35 patients with primary tumors.

Bioinformatics Analysis

Differential expression genes (DEGs) in tissue samples categorized as $ANXA2_{low}$ and $ANXA2_{high}$ were assessed using the Edge R program. Differentially expressed genes (DEGs) were identified using a threshold of $|log_2FC| > 0.5$ and $p\text{-value} < 0.05$. To elucidate the biological significance of these DEGs, we conducted Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. Ethical approval for the project was granted by the Ethics Committee of Tianjin First Central Hospital.

Construction of Plasmids, Lentivirus Packaging and Infection

The plasmids pCDH-Anxa2-GFP and pCDH-RANKL-GFP were synthesized via polymerase chain reaction (PCR) from breast cancer cell lines. The pLKO.1 vector containing Anxa2-targeting shRNA or RANKL-targeting shRNA was procured from Genechem Company (Shanghai, China). The Anxa2-targeting sequence was 5′-GGTCTGAATTCAAGAGAAA-3′,

and the RANKL-targeting sequence was 5′-AGAGGAAATCAGCATCGAGGT-3′. A sequence with equivalent GC content served as the negative control. Retroviral packaging was performed in 293T cells using Lipofectamine 2000 (Invitrogen) for vector transfection. Following collection of viral supernatants, breast cancer cells were transduced and subsequently selected with 1 µg/mL puromycin (Sigma) to establish stable cell lines.

Quantitative Real-Time PCR (qPCR)

TRIzol reagent was used to extract total RNA from cells. Using the reverse transcription kit, cDNA was created. SYBR Green Master Mix was used for qPCR using a QuantStudio 6 Flex machine. The values were adjusted to match the GAPDH mean expression. The PCR primers are showed as follows: DC-STAMP (forward: 5′-TACGTGGAGAGAAGCAAGGAA-3′, reverse: 5′-ACACTGAGACGTGGTTTAGGAAT-3′), V-ATPase-d2 (forward: 5′-CAGAGCTGTACTTCAATGTGGAC-3′, reverse: 5′-AGGTCTCACACTGCACTAGGT-3′), TRAP (forward: 5′-CACTCCCACCCTGAGATTTGT-3′, reverse: 5′-CATCGTCTGCACGGTTCTG-3′), CTSK (forward: 5′-GAAGAAGACTCACCAGAAGCAG-3′, reverse: 5′-TCCAGGTTATGGGCAGAGATT-3′), RANKL (forward: 5′-CAGCATCGCTCTGTTCCTGTA-3′, reverse: 5′-CTGCGTTTTCATGGAGTCTCA-3′) and GAPDH (forward: 5′-AACTTTGGCATTGTGGAAGG-3′, reverse: 5′-ACACATTGGGGGTAGGAACA-3′).

Western Blot Analysis

Cell lysis was performed in RIPA buffer. The extracted proteins were resolved by SDS-PAGE and subsequently transferred onto PVDF membranes. Membranes were blocked with 5% non-fat dry milk before overnight incubation at 4°C with primary antibodies specific to the target proteins. After thorough washing, HRP-conjugated secondary antibodies were applied. Signal detection was achieved using an enhanced chemiluminescence (ECL) system, followed by quantitative analysis of band intensities with ImageJ software.

TRAP Staining

RAW264.7 cells were cultured overnight in 96-well plates. Following this, the cells were exposed to SK-BR-3 or MDA-MB-231 conditioned media (CM) at a 4:1 ratio. The medium was refreshed every two days. After four days, cells were fixed, stained, and analyzed for TRAP activity under a microscope. Osteoclasts were characterized as TRAP-positive multinucleated cells with more than three nuclei.

RANKL ELISA

RANKL secreted in vitro was detected by ELISA kit. Undiluted supernatant was put into microwells coated with anti-RANKL. Samples were replaced with a biotinylated antibody solution after 90 minutes of incubation at 37°C, and then they were incubated for another 60 minutes at the same temperature. The avidin-biotin-peroxidase complex (ABC) solution was added to the plates after they had been well cleaned, and they were then incubated for 30 minutes at 37°C. After giving the wells another wash, the color-developing agent was added, and they were then incubated for 15 minutes at 37°C in the dark. Stop solution was added to halt the reaction, and absorbance was measured on a plate reader at 450 nm.

Dual-Luciferase Reporter Assay

pRL-TK and pGL3-RANKL promoter or vector control were transiently co-transfected into cells cultured in 24-well plates for 24 hours. The luciferase activity was measured using the dual-luciferase reporter assay system (Promega), and Renilla luciferase luminescence intensity was used for normalization.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0.2. OS and RFS for each subgroup were compared via the Log rank test, and survival probability was determined through the Kaplan-Meier method. All experiments were conducted independently in triplicate. Data were presented as mean ± standard deviation (SD).

Data Availability

The datasets generated and/or analyzed during the current study were available from the corresponding author upon reasonable request.

Results

ANXA2 is Highly Expressed in Breast Cancer Patients and Predicts Poor Survival

To examine the role of ANXA2 in breast cancer progression, expression levels in both tumor and adjacent normal breast tissues were analyzed using publicly available datasets. Data mining of the ANXA2 mRNA profile from the GEO dataset GSE 21653 revealed elevated ANXA2 expression in tumor samples compared to normal counterparts, a finding validated by an independent dataset, GSE 50567 (Figure 1A and B). This suggests a potential role of ANXA2 in facilitating malignant progression. Furthermore, Kaplan-Meier analysis was performed to stratify patients into low ($ANXA2_{low}$) and high ($ANXA2_{high}$) ANXA2 mRNA expression groups. Survival analyses revealed that patients with higher ANXA2 expression exhibited poorer outcomes, as indicated by overall survival (OS: HR, 1.27; 95% CI, 1.03–1.56; log-rank P, 0.026) and recurrence-free survival (RFS: HR, 1.19; 95% CI, 1.08–1.32; log-rank P, 7e-04) (Figure 1C and D). Taken together, these results suggest that ANXA2 overexpression in breast cancer is associated with a poor survival.

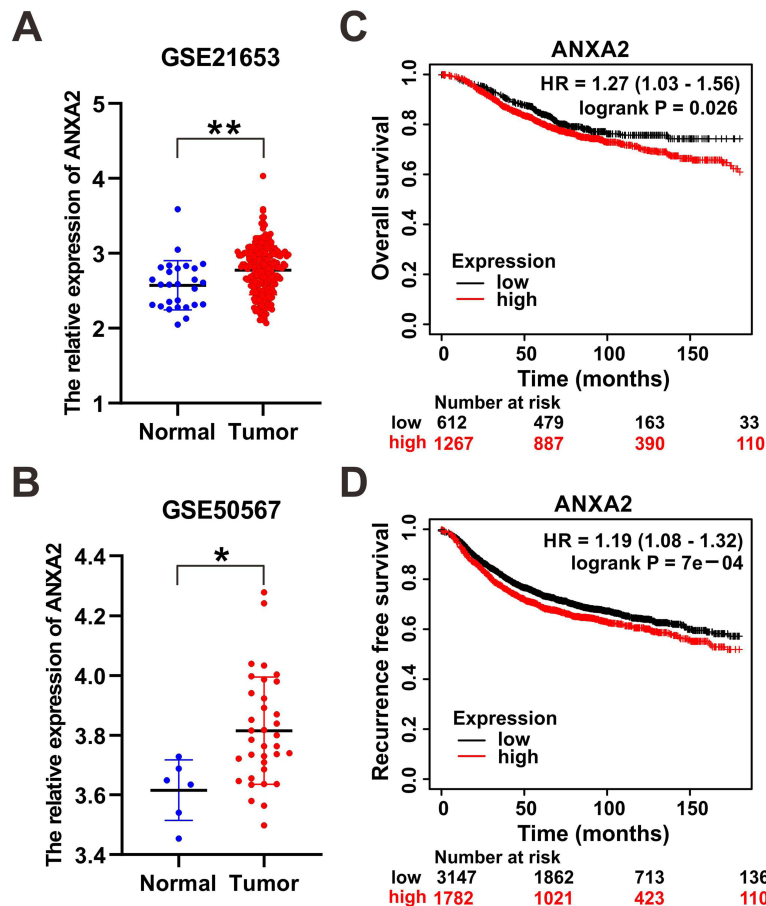

Figure 1 Elevated ANXA2 expression correlates with poor survival in breast cancer. (**A** and **B**) In two independent cohorts (GSE21653, GSE50567), ANXA2 expression was significantly higher in breast cancer tissues compared to adjacent normal tissues. Data are presented as mean ± standard deviation. (**C** and **D**) A Kaplan-Meier analysis of overall survival (OS) and relapse-free survival (RFS) based on ANXA2 mRNA levels was performed using the KM-plotter breast cancer database (http://kmplot.com/analysis). The optimal cutoff value was selected automatically. Data are shown as mean ± SD. *$P < 0.05$; **$P < 0.01$.

Gene Expression Difference Analysis and Functional Enrichment of TCGA Breast Cancer Data Based on ANXA2 Expression

To explore the molecular mechanisms underlying ANXA2's role in breast cancer development, the breast cancer data from the TCGA database were stratified into high and low ANXA2 expression groups, followed by gene expression differential analysis. This analysis revealed 1630 significantly differentially expressed genes, with 1073 genes downregulated and 557 genes upregulated (Figure 2A and B).

Functional enrichment analyses using GO and KEGG were then performed on these DEGs to elucidate the biological functions associated with ANXA2. Tumor cells are known to remodel the ECM, a crucial step in bone metastasis, which plays a significant role in tumor dissemination.[4,23–26] Notably, GO_BP analysis indicated that DEGs were predominantly involved in extracellular structure organization and extracellular matrix organization processes (Figure 2C). GO_CC analysis further identified the DEGs were primarily distributed in extracellular matrix and external encapsulating structure (Figure 2D). According to the GO_MF analysis, the DEGs were also closely linked to the extracellular matrix structural constituent (Figure 2E). KEGG pathway analysis highlighted the ECM-receptor interaction and cytokine-cytokine receptor interaction as the primary enriched pathways (Figure 2F). These data suggest that ANXA2 may play a crucial role in bone metastasis, thereby contributing to the progression.

ANXA2 Promotes Osteoclastogenesis in Breast Cancer

A stable MDA-MB-231 breast cancer cell line with reduced ANXA2 expression was established to assess the role of ANXA2 in osteoclastogenesis during breast tumor bone metastasis (Figure 3A). RAW264.7 cells were induced to differentiate into osteoclasts using conditioned media from MDA-MB-231 cells, and osteoclast production was quantified via the TRAP assay. ANXA2 knockdown significantly inhibited the differentiation of RAW264.7 cells into osteoclasts in MDA-MB-231-conditioned medium (Figure 3B). Additionally, the expression of osteoclast differentiation and activation-associated genes, including DC-STAMP, V-ATPase-d2, CTSK, and TRAP, was markedly reduced in the ANXA2 knockdown group compared to controls (Figure 3C). Conversely, a stable SK-BR-3 cell line overexpressing exogenous ANXA2 was constructed (Figure 3D). Culturing RAW264.7 cells in SK-BR-3-conditioned medium led to a substantial increase in mature osteoclast formation (Figure 3E), accompanied by significant upregulation of osteoclast differentiation and activation markers (Figure 3F). These results suggest that ANXA2 promotes osteoclast differentiation in breast cancer.

ANXA2 Affects Osteoclastogenesis by Regulating the Expression of RANKL in Breast Cancer

RANKL is a central regulator of osteoclast differentiation and activation. Binding of tumor-derived RANKL to the RANK receptor on osteoclast precursor cells triggers a cascade of downstream signaling events, involving tumor necrosis factor receptor-related factors, which activate key pathways to promote osteoclast differentiation and subsequent bone resorption. Bioinformatics analysis of TCGA breast cancer data revealed a positive correlation between ANXA2 expression and RANKL (Figure 4A). Further investigation in MDA-MB-231 cells demonstrated that ANXA2 knockdown significantly reduced both mRNA and protein levels of RANKL compared to the control group (Figure 4B and C). ELISA assays confirmed that ANXA2 knockdown led to a notable decrease in RANKL protein secretion (Figure 4D). Conversely, SK-BR-3 cells with ANXA2 overexpression exhibited markedly elevated RANKL expression and secretion (Figure 4E–G). Stable knockdown of RANKL in MDA-MB-231 cells via lentiviral-mediated shRNA mirrored the effects observed in ANXA2 knockdown cells, with a significant reduction in the number of mature osteoclasts differentiated from RAW264.7 cells (Figure 4H), as well as downregulation of DC-STAMP, V-ATPase-d2, CTSK, and TRAP expression (Figure 4I). These results were reversed upon overexpression of RANKL in SK-BR-3 cells (Figure 4J and K), suggesting that ANXA2 influences osteoclastogenesis by modulating RANKL expression.

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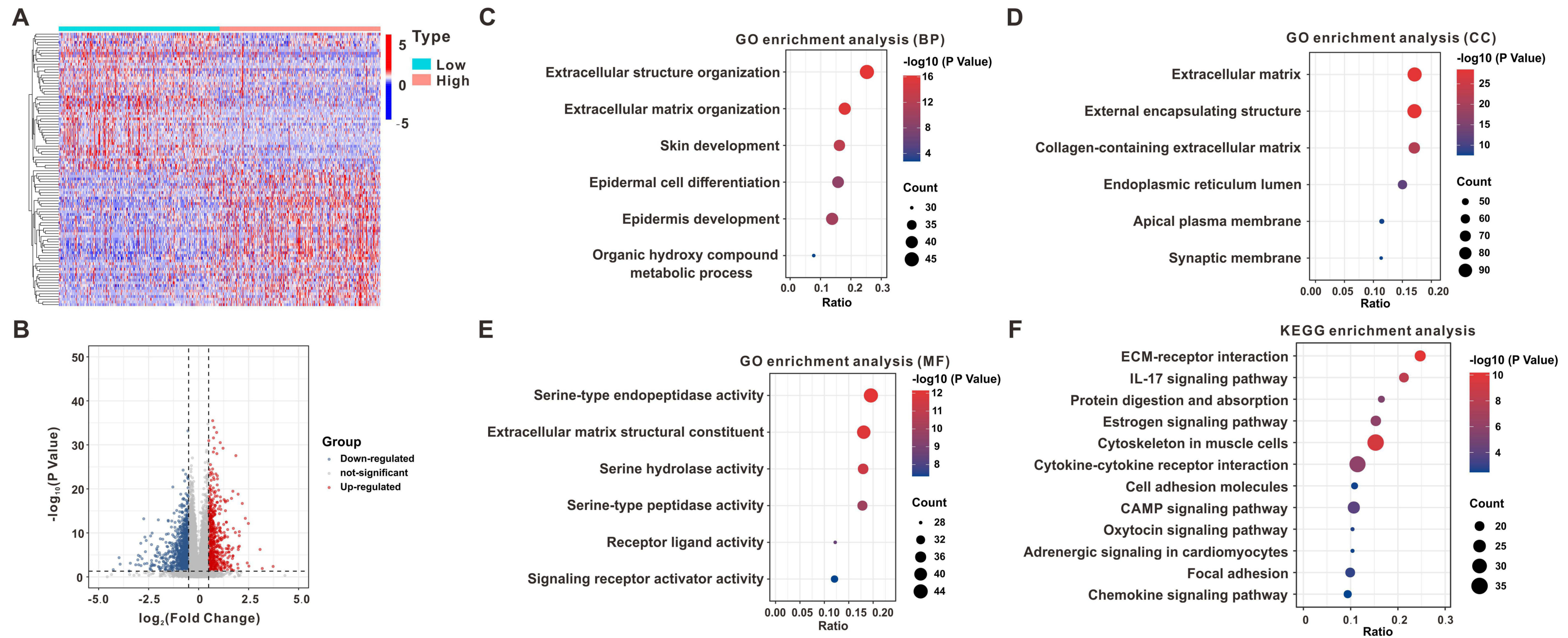

Figure 2 Gene expression profiling and functional enrichment analysis of TCGA breast cancer data based on ANXA2 expression. (**A** and **B**) A heatmap and volcano plot display differentially expressed genes between the $ANXA2_{low}$ and $ANXA2_{high}$ groups in the TCGA breast cancer dataset. (**C-F**) Bubble diagram of the GO enrichment analyses and KEGG enrichment analyses of the differentially expressed genes between the $ANXA2_{low}$ and $ANXA2_{high}$ groups in the TCGA breast cancer dataset. Bubble size indicates the number of DEGs enriched; bubble color indicates p-values.

Abbreviations: GO, Gene ontology; BP, biological process; CC, cellular components, MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

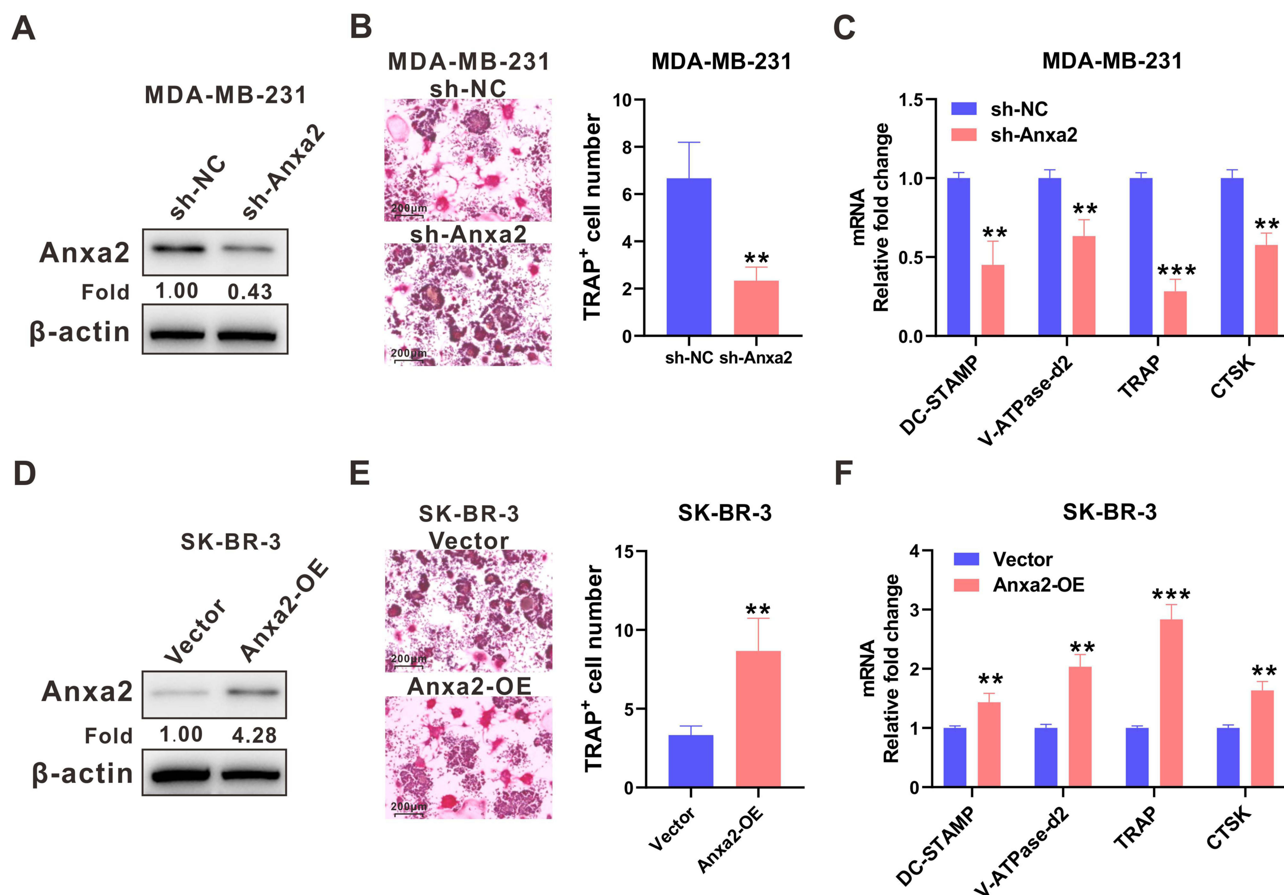

Figure 3 ANXA2 promotes osteoclastogenesis in breast cancer. (**A**) ANXA2 expression was assessed in sh-NC and sh-ANXA2 MDA-MB-231 cells. Quantitative analysis of signal intensity was performed using ImageJ software. The corresponding numerical results are presented below the figure. (**B**) Representative images and quantitative analysis of multinucleated TRAP+ cells were analyzed in RAW264.7 cells cultured with MDA-MB-231 conditioned medium for 4 days. n=3. (**C**) The mRNA expression levels of osteoclast-related genes were analyzed in RAW264.7 cells cultured with MDA-MB-231 conditioned medium for 4 days. (**D**) ANXA2 expression was measured in vector and ANXA2 overexpressing SK-BR-3 cells. Quantitative analysis of signal intensity was performed using ImageJ software. The corresponding numerical results are presented below the figure. (**E**) Representative images and quantitative analysis of multinucleated TRAP+ cells were analyzed in RAW264.7 cells cultured with SK-BR-3 conditioned medium for 4 days. n=3. (**F**) The mRNA expression levels of osteoclast-related genes were observed in RAW264.7 cells cultured with SK-BR-3 conditioned medium for 4 days. Data are presented as the mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$.

ANXA2 Regulates the Expression of RANKL in Breast Cancer Through the STAT3 Signaling Pathway, Thereby Affecting the Osteoclastogenesis

Previous studies have established that the activated STAT3 signaling pathway contributes to the elevated expression of LPS-induced RANKL in mouse osteoblast-like cells.[27] To explore the role of ANXA2 in this process, the effects of ANXA2 expression on the STAT3 pathway were examined in breast cancer cell lines MDA-MB-231 and SK-BR-3. Western blot analysis revealed a significant inhibition of the STAT3 signaling pathway following ANXA2 knockdown (Figure 5A and B). Knockdown of STAT3 similarly reduced RANKL expression, mirroring the effects observed with ANXA2 depletion (Figure 5C). In contrast, overexpression of ANXA2 in SK-BR-3 cells resulted in notable activation of the STAT3 pathway (Figure 5D and E), while STAT3 knockdown suppressed the increase in RANKL expression associated with ANXA2 overexpression (Figure 5F). These results suggest that ANXA2 regulates RANKL expression in breast cancer via the STAT3 signaling pathway, thereby influencing osteoclastogenesis.

STAT3 Transcriptionally Regulates RANKL Expression

Bioinformatics analysis identified four potential STAT3 binding sites within 2 kb of the RANKL promoter region (Figure 6A). ChIP assays confirmed that STAT3 binds to the RANKL promoter, specifically at the 2015/1762 site, while binding at the 1356/1002, 762/513, and 225/26 sites was minimal (Figure 6B). A dual luciferase reporter assay further

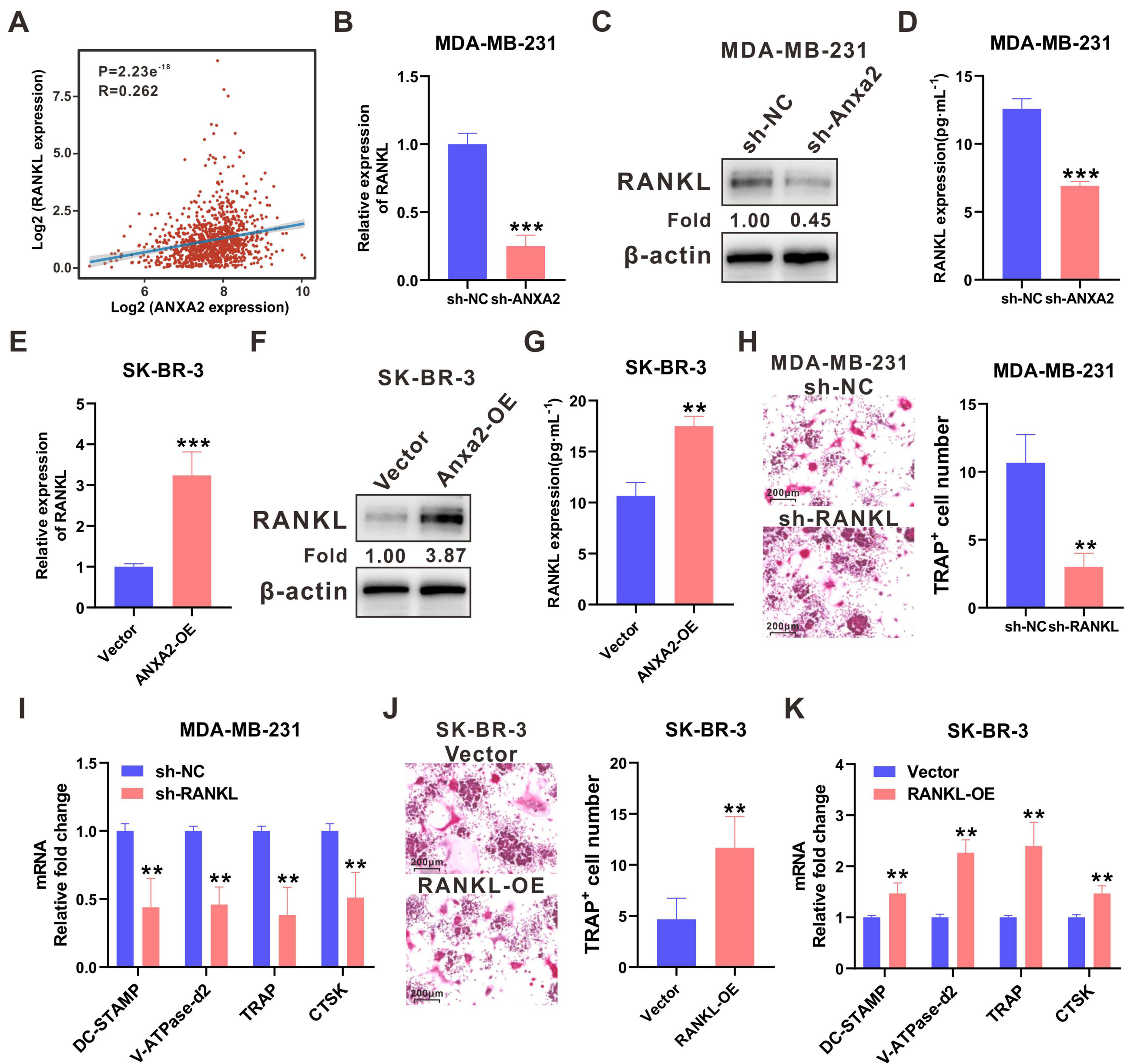

Figure 4 ANXA2 affects osteoclastogenesis by regulating the expression of RANKL. (**A**) Using the web tool GEPIA (gepia.cancer-pku.cn), a positive correlation between ANXA2 and RANKL expression was identified in TCGA breast cancer data. (**B** and **C**) Relative mRNA and protein expression of RANKL was measured in MDA-MB-231 cells after ANXA2 knockdown. Quantitative analysis of signal intensity was performed using ImageJ software. The corresponding numerical results are presented below the figure. (**D**) RANKL secretion was assessed by ELISA in sh-NC and sh-ANXA2 MDA-MB-231 cells. (**E** and **F**) Relative mRNA and protein expression of RANKL was measured in SK-BR-3 cells after ANXA2 over-expression. Quantitative analysis of signal intensity was performed using ImageJ software. The corresponding numerical results are presented below the figure. (**G**) RANKL secretion was assessed by ELISA in vector and RANKL overexpressing SK-BR-3 cells. (**H**) Representative images and quantitative analysis of multinucleated TRAP+ cells were analyzed in RAW264.7 cells cultured with sh-NC and sh-RANKL MDA-MB-231 conditioned medium for 4 days. n=3. (**I**) The mRNA expression levels of osteoclast-related genes were analyzed in RAW264.7 cells cultured with sh-NC and sh-RANKL MDA-MB-231 conditioned medium for 4 days. (**J**) Representative images and quantitative analysis of multinucleated TRAP+ cells were analyzed in RAW264.7 cells cultured with vector and RANKL overexpressing SK-BR-3 conditioned medium for 4 days. n=3. (**K**) The mRNA expression levels of osteoclast-related genes were analyzed in RAW264.7 cells cultured with vector and RANKL overexpressing SK-BR-3 conditioned medium for 4 days. Data are presented as the mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$.

elucidated the effect of STAT3 on RANKL promoter activity in the MDA-MB-231 cells. Cells transfected with the wild-type STAT3 binding motif at the −1804/+32 site exhibited higher promoter activity, whereas cells transfected with the −1253/+32 binding motif or the mutant −1804/+32 motif displayed significantly reduced activity (Figure 6C). Additionally, ANXA2 knockdown in the MDA-MB-231 cells diminished RANKL promoter activity, while ANXA2 overexpression in the SK-BR-3 cells enhanced it (Figure 6D). These results demonstrate that STAT3 may contribute to RANKL upregulation through direct promoter binding.

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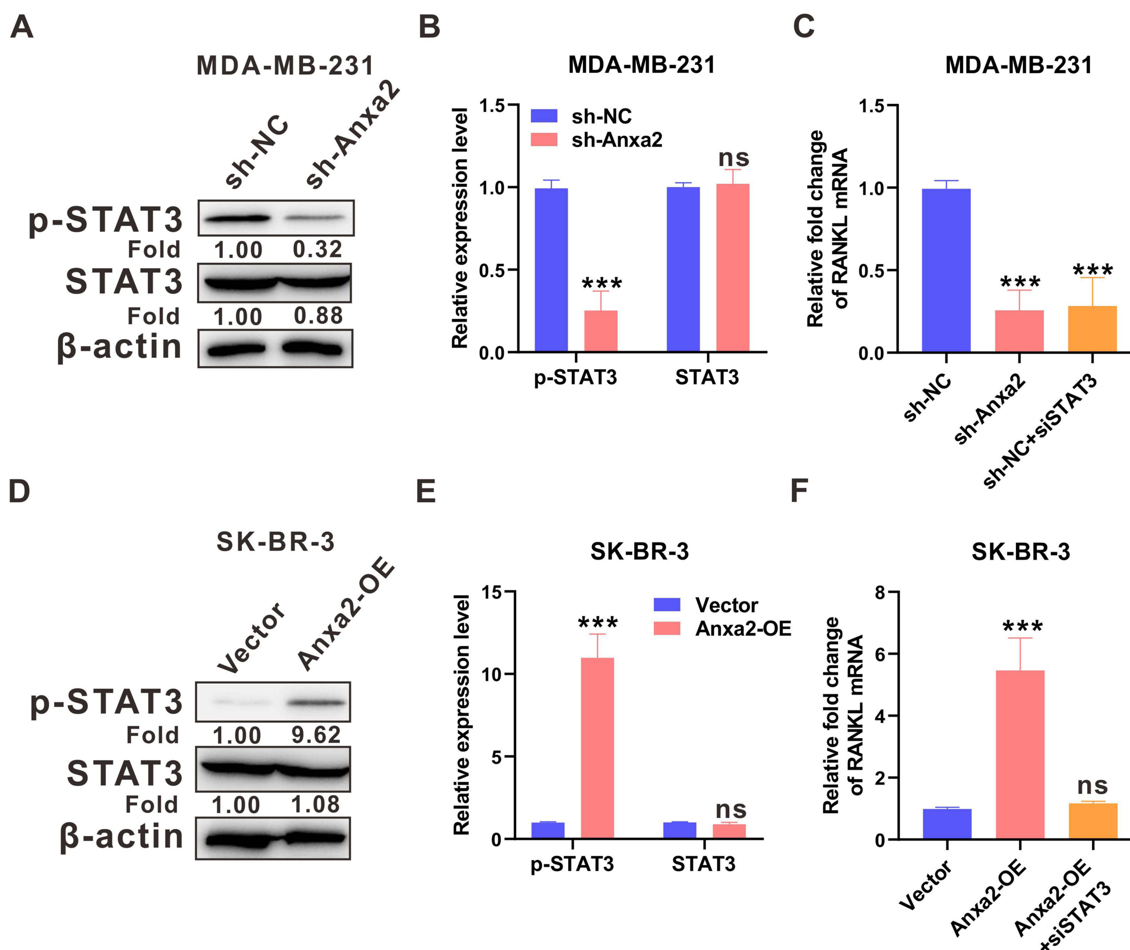

Figure 5 ANXA2 regulates the expression of RANKL through the STAT3 signaling pathway. (**A** and **B**) Protein lysates from sh-NC and sh-ANXA2 MDA-MB-231 cells were analyzed for phosphorylated STAT3 by Western blot. Quantitative analysis of signal intensity was performed using ImageJ software. The corresponding numerical results are presented below the figure. (**C**) Relative mRNA levels of RANKL in MDA-MB-231 cells following ANXA2 or STAT3 knockdown. (**D** and **E**) Protein lysates from vector and ANXA2-overexpressing SK-BR-3 cells were analyzed for phosphorylated STAT3 by Western blot. Quantitative analysis of signal intensity was performed using ImageJ software. The corresponding numerical results are presented below the figure. (**F**) Relative mRNA levels of RANKL in SK-BR-3 cells following ANXA2 overexpression or STAT3 knockdown. Data are presented as the mean ± SD. ***$P < 0.001$; ns, no significance.

Discussion

Breast cancer predominantly affects women, with little reduction in the mortality rate due to metastatic disease despite advancements in the 5-year survival rate.[1] This presents a significant threat to women's health. As patients age, survival rates decline, with a median survival time of only 19 months. Bone metastases account for up to 65% of metastatic breast cancer cases.[28] Therefore, identifying novel therapeutic targets and improving the survival of breast cancer necessitate an in-depth understanding of the underlying regulatory mechanisms driving bone metastasis.

This study indicates that high expression of ANXA2 in breast cancer patients correlates with poor survival. Differential gene and pathway analyses identified key signaling pathways associated with bone metastasis. Additionally, ANXA2 activates the STAT3 signaling pathway, which regulates RANKL promoter activity. This, in turn, modulates RANKL transcription and expression, promoting osteoclast differentiation. Consequently, this creates a bone microenvironment that contributes to the initiation and progression of bone metastasis.

Research has established that ANXA2 is aberrantly expressed in various malignant tumors, with its role in tumor growth being context-dependent, either promoting or inhibiting progression depending on the cancer type.[29,30] In addition, ANXA2 acts as a mediator between tumor and host cells in bone tissue, promoting prostate cancer's specific metastasis to bone, while our previous studies have identified a strong association between ANXA2 and the invasion and metastasis of breast cancer.[18,19,31,32] However, limited research exists on the exact mechanisms through which ANXA2

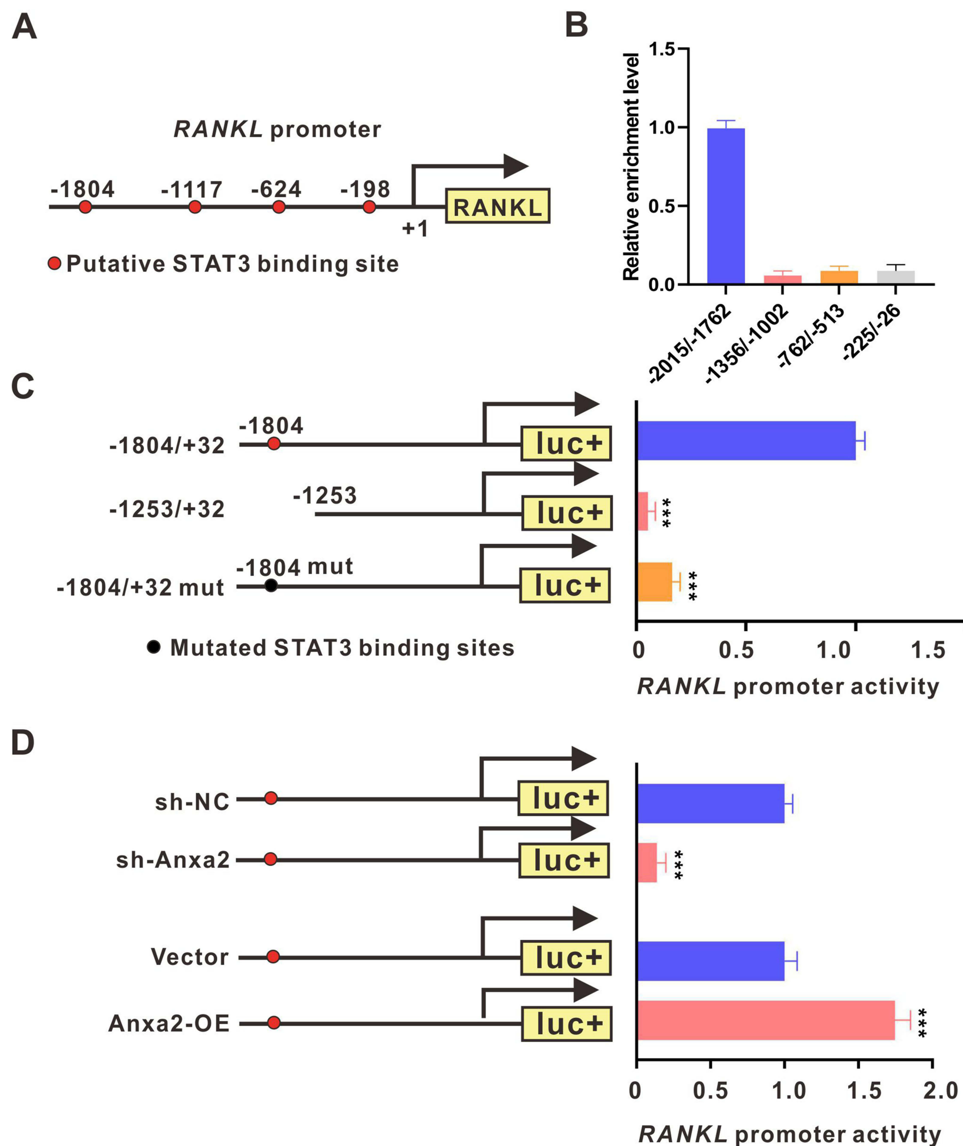

Figure 6 STAT3 transcriptionally regulates RANKL expression. (**A**) Schematic depicting potential STAT3-binding sites in the RANKL promoter. (**B**) STAT3 recruitment to the RANKL promoter in RANKL-His-overexpressing MDA-MB-231 cells were assessed via ChIP with an anti-His tag antibody. (**C**) Dual-luciferase reporter assay in MDA-MB-231 to evaluate RANKL promoter activity in the presence and absence of the wild-type STAT3-binding region, as well as with a mutant STAT3-binding motif. (**D**) RANKL promoter activities were compared by comparing: control and ANXA2-knockdown groups in MDA-MB-231 cells; vector and ANXA2-overexpression groups in SK-BR-3 cells. Data are presented as the mean ± SD. ***P < 0.001.

contributes to bone metastasis in breast cancer. In the current study, GEO bioinformatics data and online database analysis were utilized to confirm the aberrant expression of ANXA2 in breast cancer, with higher ANXA2 levels correlating with poorer patient survival. Further analysis of breast cancer data from the TCGA database categorized samples based on high and low ANXA2 expression, followed by subsequent bioinformatic analysis. GO and KEGG analyses revealed significant enrichment of the extracellular matrix-related pathway. It is well established that tumor cells remodel the ECM, a key process that facilitates bone metastasis, a critical stage in tumor spread.[4,23–26] This indicates that ANXA2 may promote the initiation and progression of breast cancer through its influence on the bone metastasis process.

A dynamic equilibrium in bone metabolism is maintained under physiological conditions through osteoblast-mediated bone formation and osteoclast-mediated bone resorption.[33–35] This balance is disrupted when tumor cells invade bone tissue, primarily via the secretion of cytokines such as RANKL, PTH-rP, and IL-6, which favor bone resorption.[36,37] Osteoclast formation, the sole process responsible for bone resorption, is induced by the binding of RANKL to its receptor RANK,

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thereby activating the PI3K/AKT pathway and modulating the expression of associated genes.[38–40] This study demonstrated that ANXA2 knockdown substantially reduced the number of mature osteoclasts differentiated from RAW264.7 cells cultured in MDA-MB-231 conditioned medium. Moreover, the expression of genes associated with osteoclast differentiation and activation, including DC-STAMP, V-ATPase-d2, CTSK, and TRAP, was significantly reduced. This downregulation was reversed when cells were cultured in SK-BR-3 conditioned media overexpressing ANXA2. These results align with prior research in prostate cancer that the expression of ANXA2 could drive the tumor cells to the bone niche, altering bone homeostasis.[31,32] Moreover, Zhou et al demonstrated that elevated plasma ANXA2 levels show a significant negative correlation with hip bone mineral density, while in vitro evidence confirms ANXA2-mediated suppression of osteoblast proliferation.[41] These findings suggest that ANXA2 may promote bone metastasis through dual mechanisms: enhancing osteoclast differentiation while inhibiting osteoblast proliferation. However, the underlying molecular mechanisms of ANXA2-induced osteoblast suppression remain incompletely characterized and require further investigation.

Additionally, the impact of ANXA2 signaling on RANKL expression in breast cancer cells was also examined. Previous research has shown that in mouse osteoblast-like cells, the upregulation of LPS-induced RANKL is mediated by enhanced STAT3 signaling.[27] In the present study, ANXA2 overexpression strongly activated the STAT3 pathway, and the increase in RANKL expression induced by high ANXA2 levels was markedly attenuated following STAT3 knock-down, consistent with prior findings. Earlier investigations have demonstrated that Anxa2 can directly interact with STAT3 upon EGF stimulation, leading to STAT3 phosphorylation at tyrosine 705 and translocation to the nucleus, where it acts as a transcription factor.[18] Based on these findings, we hypothesize that Anxa2 directly binds STAT3, thereby enhancing its transcriptional activity and promoting RANKL upregulation. Furthermore, it was observed that that STAT3 may contribute to RANKL upregulation through direct promoter binding. However, we cannot exclude the involvement of STAT3-mediated co-factor recruitment, chromatin accessibility, histone modifications, or indirect transcriptional effects, as these mechanisms were not experimentally validated here. Future studies should assess STAT3's interplay with epigenetic regulators to fully delineate its role in RANKL expression.

In conclusion, ANXA2 is significantly overexpressed in breast cancer patients and correlates with poor survival. By activating the STAT3 signaling pathway, ANXA2 regulates the transcription and expression of RANKL, promoting osteoclast differentiation (Figure 7). However, the therapeutic potential of targeting the ANXA2-STAT3-RANKL axis remains to be explored. Future in vivo studies employing STAT3 inhibitors or ANXA2-blocking agents in bone metastasis animal models are necessary to assess their efficacy and potential clinical applicability. Addressing these research gaps will provide deeper insights into the mechanistic and therapeutic roles of ANXA2 in breast cancer bone metastasis.

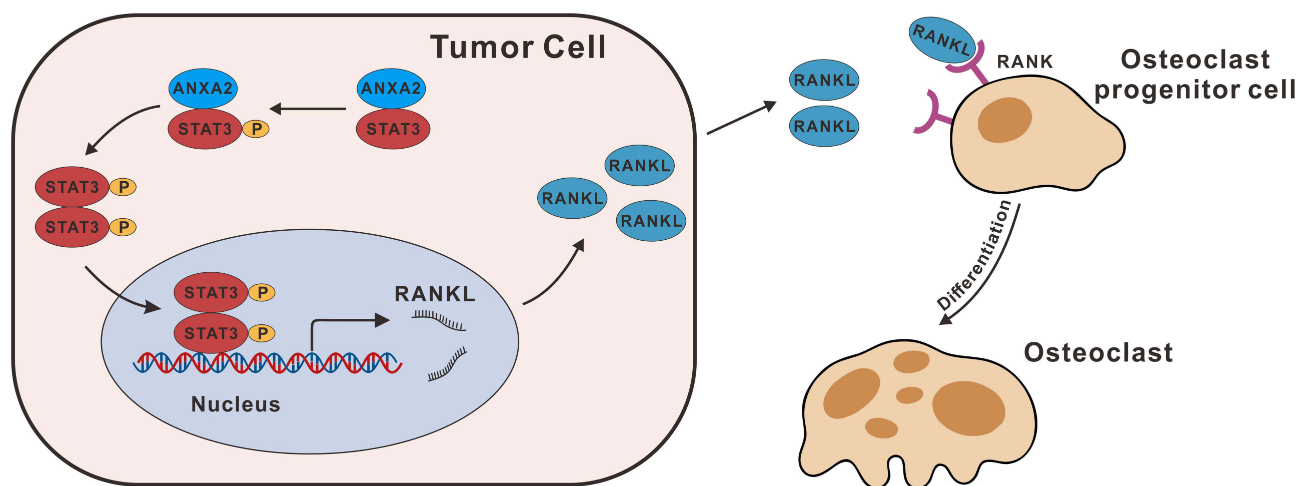

Figure 7 A schematic illustrating the direct interaction between ANXA2 and STAT3, leading to the activation of the STAT3 signaling pathway, which subsequently modulates RANKL transcription and expression, thereby promoting osteoclast differentiation.

Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Tianjin First Central Hospital.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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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