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Keywords
TNBC, miR-182-5p, macrophage polarization, Notch1

Abstract

Introduction
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Material and methods
After transfection with miR-182-5p mimics, inhibitors, and controls for 24 hours, exosomes were extracted by differential centrifugation from transfected MDA-MB-231. Macrophages co-cultured with these exosomes to illustrate the regulative effects of exo-miR-182-5p reprogram macrophage. Furthermore, breast cancer cells co-cultured with exo-miR-182-5p reprogrammed M2 macrophages to demonstrate the effects of reprogrammed M2 macrophages to influence breast cancer progression. After all, these findings were validated in cell derived xenograft (CDX) BALB/C nude-mice model.

Results
In this study, we demonstrate that exosome-derived miR-182-5p from TNBC cells reprograms M2 macrophage polarization through direct combined with Notch1, thereby enhancing breast cancer progression in vitro and in vivo. When co-cultured with exosomes from TNBC cells transfected with miR-182-5p mimics or inhibitors, macrophages showed altered Notch1/Hes1 pathway expression, leading to M2 polarization and subsequent changes in reactive oxygen species (ROS), inflammation, and other biochemical markers. Furthermore, breast cancer cells co-cultured with exosomes reprogrammed macrophages exhibited increased colony formation, migration, and invasion, as well as reduced apoptosis. These findings were validated in a BALB/C nude-mice model.

Conclusions
This study pioneers the elucidation of the feedback loop mechanism between breast cancer cells and macrophages mediated by the exosome-derived miR-182-5p/Notch1 pathway, highlighting its role in macrophage reprogramming. Although the therapeutic application of miR-182-5p inhibitors as anticancer agents remains in the early stages, targeting macrophage polarization represents a promising avenue for breast cancer therapy.

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INTRODUCTION

The tumor microenvironment (TME) consists of non-cancerous cells and components within a tumor, including stromal cells and immune cells. The critical roles of the TME in tumor progression and responses to therapy, particularly immunotherapy, are well-established [1, 2]. Notably, the infiltration of CD4+ T cells is recognized as a key factor in the efficacy of immunotherapy [3]. Recently, tumor-associated macrophages (TAMs), a significant component of the TME, have gained attention for their role in tumor progression and their potential as targets for clinical intervention [4]. Macrophages are categorized into M1 (classical) and M2 (alternative) phenotypes. M1 macrophages are associated with tumor- cytotoxic effects and antitumor immune responses, whereas M2 macrophages contribute to tissue repair and immunosuppression [5]. Dynamic shifts from M1 to M2 phenotypes in macrophages are observed in various inflammatory conditions and are linked to tumor-related tissue damage [6]. Numerous studies have demonstrated a correlation between the M2 polarization of tumor-associated macrophages (TAMs) and poorer prognosis in various human cancers [7]. Additionally, aberrant Notch pathway expression, identified in these malignancies, influences key cellular processes including proliferation, differentiation, apoptosis, survival [8], as well as functions critical for immune regulation such as macrophage activation [9-10].

MicroRNAs are small, 17-25-nucleotide, single-stranded, non-coding RNAs that primarily mediate post-transcriptional gene silencing by binding to the 3'-untranslated region (UTR) or the open reading frame (ORF) region of target mRNAs [11-12]. The involvement of miRNAs in various biological activities, including cell proliferation, differentiation, migration, as well as disease initiation and progression, particularly in tumor pathology, has been

extensively documented [13]. For example, miR-325 targets lipocalin 15 to inhibit proliferation, migration and invasion of breast cancer cells [14]. Similarly, miR-610 functions as a tumor suppressor by reducing cisplatin resistance in hepatocellular carcinoma through the targeted silencing of hepatoma-derived growth factor [15]. Moreover, the down-regulation of miR-182 expression is associated with progression and metastasis of osteosarcoma [16]. Overexpression of microRNA-182 has been documented to be associated with metastasis and poor patient prognosis in breast cancer patients [13]. Functional studies have demonstrated that up-regulation of miR-182 in cancer cells significantly contributes to accelerated tumor proliferation, migration, invasion, epithelial-mesenchymal transition, metastasis, stemness, and therapy resistance, through the suppression of multiple genes [17-21]. In breast cancer, miR-182 has been shown to regulate trastuzumab resistance and tamoxifen sensitivity [19-20]. Additionally, previous studies have demonstrated that breast cancer cells can induce miR-182 expression in macrophages, which in turn influences M2 polarization [15].

Exosomes, originating from the membranes of multivesicular bodies (MVB), have diameters ranging from 50 nm to 150 nm [22]. These vesicles are abundantly present in various body fluids and serve as vehicles for intracellular communication, transporting proteins, lipids, nucleic acids, and other substances [23]. Due to their ease of extraction and stability in biological environments, miRNA contents have emerged as preferred molecules in exosome for research [22]. Recent studies have demonstrated the diagnostic potential of exosomal miRNA across a range of cancers, including bladder, ovarian, hepatoblastoma, colorectal, and glioblastoma [24-25]. Specifically, the detection of certain mRNAs in exosomes not only aids in cancer diagnosis but also in evaluating the progression of the tumor microenvironment.

Exosomes facilitate critical communication between tumor and non-tumor cells within the microenvironment, playing vital roles at different stages of tumor development, such as immune regulation, microenvironment remodeling, angiogenesis, invasion, and distant metastasis [26-27]. For example, miR-126, delivered to the lungs by exosomes from MDA-MB-231 breast cancer cells, effectively inhibits the proliferation and migration of lung cancer cells, thereby suppressing tumor development [28].

While the contribution of miR-182-5p to the regulation of breast cancer cell malignancy and breast cancer cells induced miR-182-5p expression in macrophages influences M2 polarization is well-established, the precise mechanisms underlying how exosomal miR-182-5p from tumor cells influences macrophage polarization remain to be elucidated. This study aims to elucidate the mechanisms underlying the communication between cancer cells and macrophages during reprogramming, by employing exosomes from breast cancer cells with either overexpressed or inhibited miR-182-5p.

MATERIALS AND METHODS

Clinical mRNA expression analysis

The miR-182-5p binding site on Notch1 was analyzed with the following parameters "has-miR-182-5p" and "Notch1" at the "miRNA-Target" section on the Starbase Database (starbase.sysu.edu.cn).

Clinical miR-182-5p and Notch1 mRNA expression in normal and breast cancer patients were also analyzed on the Starbase Database. Under the "Pan-Cancer" section, searching "has-miR-182-5p" in "miRNA Differential Expression", or "Notch1" expression in "Gene Differential Expression", with the following parameter "Breast Invasive Carcinoma" in cancer

type.

Clinical Samples.

This study used two treatments naïve TNBC continuous cohorts: all treatments naïve pathogenic conformed female TNBC patients in Xinjiang Tumor Hospital from January 1, 2013 to October 1, 2015 and from January 1, 2022 to June 1, 2022 included in the screening. Exclude patients without complete pathological or clinical information, paraffin-embedded primary tumor specimens of 157 patients from January 1, 2013 to October 1, 2015 were used for immunohistochemical detection of Notch1, and frozen primary tumor and adjacent tissues of 30 patients from January 1, 2022 to June 1, 2022 were used for the analysis of biochemical markers and the expression of miR-182-5p. Samples were obtained with informed patient consent and approval by the Medical Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University (G-2019050).

Mouse Experiments.

The BALB/c nude mice were obtained from Hangzhou Medical College. For the fat-pad injection assays, 5×10^6 MDA-MB-231 cells were mixed with 100 μl of Matrigel and injected into the mammary fat pads on each side of 8-week-old female mice. After 7 days, miR-182-5p agomir, miR-182-5p antagomir, and the negative control oligonucleotides were injected into the tumors every 3 days for a total of 7 times. All animal studies were conducted according to the guidelines for the care and use of laboratory animals and were approved by the Medical Ethics Committee for Animal Experiments of the Affiliated Tumor Hospital of Xinjiang Medical University (IACUC-20190226-49).

Other procedures

All of the other procedures are established standard techniques and are described in the Supplementary Files.

Statistical Analyses

All statistical analyses were performed using SPSS 19.0 software. Values are expressed as means ± SEM. Pair-wise comparisons were made using one-way ANOVA. A probability value (p) < 0.05 was considered significant, with those < 0.01 or < 0.001 being even more so.

RESULTS

miR-182-5p and Notch1 Expression in TNBC: Analysis of Database and Clinical Samples

Previous studies have already established the oncogenic roles of miR-182, which promotes cancer invasion by targeting the Notch1/Hes1 pathway and reprogramming M2 polarization of macrophages in cancer cells and mice. Utilizing the Starbase Database (starbase.sysu.edu.cn), the 3'UTR of Notch1 was found to have a binding site for miR-182-5p (Fig.1A), indicating that has-miR-182-5p is predicted to interact directly with Notch1. To evaluate the relationship between miR-182-5p and Notch1 in breast cancer, the expression levels of miR-182-5p and Notch1 were analyzed using public databases and clinical breast cancer samples. A negative correlation in expression between miR-182-5p and Notch1 was demonstrated (Fig.1B-C).

To further explore the impact of Notch1 expression, Notch1 was detected by IHC in 157 FFPE TNBC samples from Xinjiang Tumor Hospital. Notch1 expression was not associated with age, menstrual period, tumor size, or Ki67 expression but was significantly related to BMI, lymphatic metastasis, histological grade, and tumor stage (Table 1). These results suggest that Notch1 expression is associated with metastasis but not with proliferation. Furthermore, overexpression of miR-182-5p was observed in 30 frozen TNBC samples compared to adjacent

non-tumorous tissue (Fig. 1D). The biochemical indicators of glycolysis, including glucose (GLU), lactate (LD), lactate dehydrogenase (LDH), hexokinase (HK), pyruvate kinase (PK), and adenosine triphosphate (ATP), were notably elevated in TNBC (Supplementary Fig.S1).

Construction of Exosomes from miR-182-5p Overexpression/Inhibition in MDA-MB-231 Cells

To evaluate the regulatory effects of miR-182-5p in reprogramming macrophages, constructs for miR-182-5p mimics, miR-182-5p inhibitors, and corresponding controls were developed. Initially, miR-182-5p levels were quantified by qPCR in human breast cancer cell line MDA-MB-231 and the normal human breast cell line MCF-10A. Our findings indicate that miR-182-5p was significantly overexpressed, and Notch1 expression was notably decreased in MDA-MB-231 cells (Fig.2A). Following the transfection with miR-182-5p mimics and inhibitors, the expression of miR-182-5p altered in MDA-MB-231 cells accordance with the plasmid (Fig.2B) and in exosomes derived from transfected MDA-MB-231 cells (Fig.2C).

miR-182-5p Regulates Notch1 by Combine Directly

To illustrate the mechanism of miR-182-5p regulating Notch1. Wild-type and mutation Notch1 luciferase plasmid were constructed. By using luciferase, miR-182-5p-mimics was proved to combine with wildtype-Notch1 directly, but not MT-Notch1 (Fig.2D).

Regulative Effects of Exosomal miR-182-5p on Macrophage Reprogramming

M0 macrophages were co-cultured with exosomes from MDA-MB-231 cells transfected with either miR-182-5p mimics or inhibitors. The expression changes of key genes in the Notch1/Hes1 pathway, including Notch1, Hes1, HK2, and PKM2, were evaluated at both RNA and protein levels. Additionally, macrophage biomarkers and biochemical indicators of

glycolysis were detected.

In summary, exosomes containing miR-182-5p mimics led to a reduction in the RNA (Fig.3A) and protein (Fig.3B) expression levels of Notch1 and Hes1. This was followed by an upregulation of HK2 and PKM2 expression (Fig.3A-B), which subsequently induced the polarization of macrophages towards the M2 phenotype (Fig.3C) and an increase in glycolysis indicators, including GLU, LD, LDH, HK, PK, and ATP (Supplementary Fig.S2). Conversely, exosomes with miR-182-5p inhibitors prompted an upregulation in Notch1 and Hes1 expression, which in turn restrained HK2 and PKM2 expression (Fig.3A-B). This induced polarization of macrophages towards the M1 phenotype (Fig.3C), and led to a decrease in GLU, LD, LDH, HK, PK, and ATP (Supplementary Fig.S2).

The Effects of Exosomal miR-182-5p-induced Macrophage Regulation on Cancer Progression

Our findings demonstrate that exosomal miR-182-5p from breast cancer cells can induce M2 polarization of macrophages through the regulation of the Notch1/Hes1 pathway, subsequently leading to variations in the biochemical indicators of glycolysis. However, the extent to which exosomal miR-182-5p-induced M2 polarization of macrophages and changes in the immune microenvironment affect breast cancer progression remains unclear.

To elucidate the impact of exosomal miR-182-5p-induced M2 polarization of macrophages on breast cancer progression, breast cancer cells (MDA-MB-231) were co-cultured with macrophages treated with exosomes. Co-culturing with macrophages treated with exo-miR-182-5p mimics significantly enhanced the colony formation (Fig.4A), migration/invasion (Fig.4B), and reduced apoptosis (Fig.4C) of MDA-MB-231 cells.

Conversely, co-culturing with macrophages treated with exo-miR-182-5p inhibitors significantly reduced colony formation (Fig.4A), migration/invasion (Fig.4B), and increased apoptosis (Fig.4C) of MDA-MB-231 cells.

The Effects of miR-182-5p Regulation on Breast Cancer Progression *In Vivo*

To investigate the impact of miR-182-5p on breast cancer progression in vivo, a cell-derived xenograft (CDX) model using BALB/c nude mice was established. MDA-MB-231 cells were transfected with miR-182-5p-agomir (to overexpress miR-182-5p), miR-182-5p-antagomir (to inhibit miR-182-5p), and corresponding controls. 24 hours post-transfection, 5×10^6 cells were injected into the armpit of BALB/c nude mice. The diameter of tumors was measured every 5 days until day 44, at which point the mice were euthanized for further analysis.

Tumor volume (Fig.5A) and weight (Fig.5B) significantly increased in the miR-182-5p-agomir treated mice, with proliferation confirmed by H&E staining (Fig.6A). M2 macrophage polarization was notably upregulated (Fig.5C). Conversely, tumor volume (Fig.5A), weight (Fig.5B), and proliferation (Fig.6A) significantly significant shrunk in the miR-182-5p-antagomir treated mice. M2 macrophage polarization was notably downregulated (Fig.5C).

Furthermore, the protein (Fig.6A-B) and RNA (Fig.6C) levels of Notch1, Hes1, HK2, and PKM2 changed in a manner consistent with the *in vitro* findings, leading to changes in biochemical indicators (Supplementary Fig.S3). There was one inconsistency: the protein levels of Notch1 did not show a clear trend, possibly due to ineffective staining by the Notch1 IHC antibody.

DISCUSSION

Notch family proteins, serving as transmembrane receptors, play a pivotal role in

determining cell fate by regulating differentiation, apoptosis, and proliferation. Aberrant expression of the Notch pathway, observed in various human cancers, influences proliferation, differentiation, apoptosis, and survival [8]. Specifically, elevated Notch expression has been correlated with poorer breast cancer outcomes [29]. The Notch family consists of four highly conserved transmembrane receptors, Notch1, Notch2, Notch3, and Notch4, exhibiting variable expression levels across different breast cancer genotypes [30]. Notch2 overexpression promotes tumor progression in estrogen receptor-positive luminal tumors [31], while Notch3 is implicated in regulating proliferation in HER2-negative breast cancer [32]. Additionally, Notch4 activation disrupts ductal and lobular growth, thereby inducing tumorigenesis [29]. Most studies focus on the Notch1 signaling pathway, which has been shown to govern migration and metastasis, and its inhibition reduces these processes [33]. A meta-analysis involving 4,463 patients across 17 studies found a negative correlation between increased Notch1 levels and recurrence-free survival, regardless of other prognostic factors or cancer type [34]. Notch1's critical role extends to resistance mechanisms against paclitaxel, suggesting its potential as a therapeutic target [35]. High Notch1 expression levels have been linked to reduced overall survival in breast cancer patients [36], particularly in HER2-positive breast cancer, where where Notch1 enhances stem cell survival, contributing to trastuzumab resistance [37]. Notch1's role in triple-negative breast cancer (TNBC) is particularly notable, with higher mRNA expression levels significantly associated with TNBC, but not with ER-negative samples [38]. Notch1 activation of the AKT pathway and promotion of EMT through MVP activation underscore its role in chemo-resistance in TNBC [39].

Several studies have reported regulatory mechanisms between miRNA and Notch1 in

cancer cells. For instance, hsa-miR-599 downregulation in breast cancer deactivates the BRD4/Jagged1/Notch1 axis, thus inhibiting cancer progression [40]. MiR-34a suppresses breast cancer cell proliferation and invasion, potentially through targeting Notch1 [41]. Overexpression of OIP5 inhibits hsa-miR-139-5p, leading to the de-repression of NOTCH1, a core oncogene in breast cancer progression [42]. EVs carrying miR-887-3p target BTBD7 and activate the Notch1/Hes1 signaling pathway, promoting drug resistance in breast cancer cells [43]. Previous research has established the importance of miRNAs in breast cancer through the Notch1 pathway. However, few studies have explored miRNA/Notch1 regulation of macrophage polarization. In breast cancer, one study investigated miRNA affecting macrophage polarization via the microRNA-146a-5p/NOTCH1 Axis [44]. Nonetheless, the precise role and mechanism of miRNA in regulating Notch1 to reprogram M2 macrophage polarization remain unclear.

In this study, we demonstrate that exosomal miR-182-5p reprograms macrophage polarization from M1 to M2 by reducing Notch1 expression, thereby enhancing breast cancer progression both *in vitro* and *in vivo*. Through bioinformatics analysis, we identified a direct interaction between miRNA-182-5p and the Notch1 3'UTR (Fig.1A), confirmed by a luciferase assay (Fig.2D). Clinical analysis showed a correlation between miR-182-5p expression and Notch1 expression in human tumor tissues (Fig.1B-D). After transfection with miR-182-5p mimics, inhibitors, and controls for 24 hours (Fig.2B), exosomes were extracted from transfected breast cancer cells (MDA-MB-231) and quantified for miR-182-5p expression by RT-qPCR (Fig.2C). Macrophages co-cultured with these exosomes exhibited altered Notch1/Hes1 pathway expression (Fig.3A-B), resulting in changes in polarization (Fig.3C), and

modifications of biochemical indicators (Supplementary Fig.S2). Furthermore, breast cancer cells co-cultured with reprogrammed M2 macrophages showed increased colony formation (Fig.4A), migration, and invasion (Fig.4B), as well as reduced apoptosis (Fig.4C). These findings were validated in a BALB/c nude-mice model *in vivo* (Fig.5-6). This study is the first to elucidate the breast cancer-macrophage feedback loop mechanism via the exosome-miR-182-5p/Notch1 pathway for macrophage reprogramming. Based on our finding, exosomal miR-182-5p inhibitors could use as anti-tumor drugs for TNBC by regulating macrophage polarization.

Exosomal miRNAs have been found to play a positive role in antitumor immunity. Exosomes derived from breast cancer deliver miR-130 and miR-33, which downregulate TGF-β and IL-10, ultimately inducing the polarization of M1 macrophages [45]. Human HPV+ HNSCC-derived exosomes have been shown to transport miR-9 to macrophages, inducing M1 macrophage polarization and increasing tumor radiosensitivity [46]. Moreover, miR-1827 delivered by exosomes from human umbilical cord mesenchymal stem cells (MSCs) has been shown to inhibit macrophage M2 polarization and prevent colorectal liver metastasis [47]. Furthermore, exosome represent an ideal delivery system, offering many advantages including low immunogenicity, high stability, and targeted delivery capability [26]. Numerus studies have demonstrated that exosomes hold promise as a reliable vector for gene therapy in cancer treatment. For instance, exosomes loaded with glucose-regulated protein 78 siRNA have been shown to overcome tumor cell resistance to sorafenib in hepatocellular carcinoma [48]. Similarly, exosome-mediated siRNA delivery has been effective in reducing S100A4 expression, significantly inhibiting postoperative metastasis in triple-negative breast cancer

[49]. In summary, employing an exosomal miR-182 inhibitor could represent a viable therapeutic strategy for TNBC.

Even while using exosomal miR-182-5p inhibitors as anti-tumor drugs is still far from clinical application, drugs that regulate macrophage polarization may hold promise for breast cancer treatment. Such as the CSF1R signaling is vital for the differentiation and survival of the mononuclear phagocyte system, particularly macrophages [50], making CSF1R signaling in TAMs a compelling therapeutic target. Regorafenib, an antiangiogenesis inhibitor, has been shown to reduce tumor-associated macrophages and potently inhibit CSF1R, with proven efficacy in advanced colorectal cancer (CRC), gastrointestinal stromal tumors (GIST), and hepatocellular carcinoma (HCC) [51]. Several small molecules like Pimicotinib (ABSK021），ARRY-382, PLX7486, BLZ945, and JNJ40346527, and monoclonal antibodies (mAbs) include emactuzumab, AMG820, IMC-CS4, cabiralizumab, MCS110, and PD-0360324, targeting CSF1R or CSF1 has emerged as a promising anti-tumor strategy.

CONCLUSION

Taken together, our study highlights the role of exosomal miR-182-5p from breast cancer cells in reprogramming M2 macrophage polarization by targeting Notch1, leading to accelerated tumor progression through regenerative feedback. Our findings suggest that inhibiting miR-182-5p in TAMs offers a potential strategy for targeting these cells, emphasizing the effectiveness of miR-182-5p-inhibiting RNA interference (RNAi) therapy. Although RNAi therapy remains a long-term goal in cancer treatment, drugs targeting macrophage polarization, such as CSF1R inhibitors, may offer alternative and immediate therapeutic options for breast cancer patients with miR-182-5p overexpression. Further prospective and interventional

clinical studies are necessary to validate miR-182-5p's potential as both a prognostic marker and a therapeutic target in cancer.

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Figure Legends

Graphical abstract：The positive feedback loop mechanism between breast cancer cells and macrophages mediated by the exosome-derived miR-182-5p/Notch1 pathway. The M2 macrophage polarization induced by exosomal miR-182-5p from TNBC cancer cells, then the exosomes induced M2 macrophage polarization accelerated tumor progression.

Figure 1. MiR-182-5p and Notch1 expression in clinical samples. (A) 3'UTR of Notch1 had binding site of miR-182-5p in Starbase Database(starbase.sysu.edu.cn). **(B)** miR-182-5p was overexpression and **(C)** Notch1 was low-expression in breast cancer compare to normal tissue in Starbase Database. **(D)** miR-182-5p was overexpression in 30 TNBC samples. Numeric data were expressed as the mean ± SEM. ***$p < 0.001$.

Figure 2. Constructed exosomes from miR-182-5p overexpression/inhibition TNBC cell-line. (A) miR-182-5p was overexpression and Notch1 was low-expression in MDA-MB-231 cell. The cell **(B)** and exosome **(C)** miR-182-5p expression changed significantly according to miR-182-5p-mimics/inhibitor transfected to MDA-MB-231 cell. **(D)** miR-182-5p was proved to combine with Notch1 directly by Luciferase Reporter Assays. *miR-182-5p-mimics compare to Mimics-NC. $^{\#}$miR-182-5p-inhibitor compare to Inhibitor-NC. Numeric data were expressed as the mean ± SEM. */$^{\#}p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 3. The effect of exosomes from miR-182-5p overexpression/inhibition TNBC cells on macrophages polarization. The RNA **(A)** and proteins **(B)** of Notch1/Hes1 pathway

expression in macrophages were significant modified with TNBC exosomes contain miR-182-5p mimics/inhibitors. **(C)** The macrophage M2 polarization transferred by TNBC exosomes induction contain miR-182-5p mimics/inhibitors. *exo-miR-182-5p-mimics compare to exo-mimics-NC. #exo-miR-182-5p-inhibitor compare to exo-inhibitor-NC. Numeric data were expressed as the mean ± SEM. */#$p < 0.05$; **/##$p < 0.01$; ***/###$p < 0.001$.

Figure 4. The influence of exosomal miR-182-5p induced macrophages M2 polarization to breast cancer progression. The survival **(A)**, migration/invasion **(B)**, and apoptosis **(C)** of MDA-MB-231 were altered notably when co-cultured with exosomal miR-182-5p induced macrophages. *M0-exo-miR-182-5p-mimics compare to M0-exo-mimics-NC. #M0-exo-miR-182-5p-inhibitor compare to M0-exo-inhibitor-NC. Numeric data were expressed as the mean ± SEM. ***/###$p < 0.001$.

Figure 5. The phonotype shifted by miR-182-5p overexpression/inhibition *In vivo*. The tumor volume **(A)**, tumor weight **(B)**, macrophages polarization **(C)** were shifted vitally in miR-182-5p agomir/antagomir nude-mice model. *miR-182-5p-agomir compare to agomir-NC. #miR-182-5p-antagomir compare to antagomir-NC. Numeric data were expressed as the mean ± SEM. */#$p < 0.05$; **$p < 0.01$; ***/###$p < 0.001$.

Figure 6. The mechanism switched by miR-182-5p overexpression/inhibition *In vivo*. The proliferation by H&E **(A)**, as well as protein **(B)** and RNA **(C)** of Notch/Hes1 pathway were re-programmed outstandingly by miR-182-5p agomir/antagomir in nude-mice model. *miR-

182-5p-agomir compare to agomir-NC. #miR-182-5p-antagomir compare to antagomir-NC. Numeric data were expressed as the mean ± SEM. */#p < 0.05; ##p < 0.01; ***p < 0.001.

Supplementary Figure 1. The biochemical indicators transformation of glycolysis in clinical samples. The biochemical indicators of glycolysis, including glucose (GLU), lactate (LD), lactate dehydrogenase (LDH), hexokinase (HK), pyruvate kinase (PK), and adenosine triphosphate (ATP), were notably elevated in 30 TNBC patients compared to adjacent normal tissue. Numeric data were expressed as the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

Supplementary Figure 2. The biochemical indicators transformation in macrophages. The biochemical indicators of glycolysis in macrophages were significant increased according to exosomal miR-182-5p-mimics, and decreased according to exosomal miR-182-5p-inhibitor. *exo-miR-182-5p-mimics compare to exo-mimics-NC. #exo-miR-182-5p-inhibitor compare to exo-inhibitor-NC. Numeric data were expressed as the mean ± SEM. #p < 0.05; **/##p < 0.01; ***p < 0.001.

Supplementary Figure 3. The biochemical indicators transformation in nude-mice graft model. The biochemical indicators of glycolysis in nude-mice graft model were notably increased according to miR-182-5p-agmir, and decreased according to miR-182-5p-antagomir. *miR-182-5p-agomir compare to agomir-NC. #miR-182-5p-antagomir compare to antagomir-NC. Numeric data were expressed as the mean ± SEM. #p < 0.05; **/##p < 0.01; ***/###p < 0.001.

SUPPLEMENTARY MATERIALS

Cell Culture and Macrophage Differentiation.

Human breast cancer cell line MDA-MB-231, human breast epithelial cell line MCF-10A, human monocytic leukemia cell line THP-1, and human kidney epithelial cell line 293T were obtained from the Cell Bank of the Chinese Academy of Sciences. Lipofectamine 3000 (13778-030, Thermo Fisher) was used to transfect the miR-182-5p-mimics, miR-182-5p-inhibitor，or oligonucleotide inhibitor into MDA-MB-231. Follow-up experiments were generally carried out around 24h after the transfection.

For macrophage differentiation, THP1 cells were stimulated with 100 ng/mL PMA for 48 hours to obtain M0 macrophages. Then M0 macrophages were co-cultured with breast cancer cell-derived EVs.

EV Purification.

Breast cancer cell-derived EVs were isolated by differential centrifugation. Briefly, 8 ml of cell supernatant from transfected MDA-MB-231 cells was collected and filtered through a 0.22-μm filter. An equal volume of XBP was added, and the mixture was mixed five times. The mixture was then transferred to an exoEasy tube and centrifuged twice at 500 g for 3 minutes. Subsequently, 10 ml of buffer XWP was added, followed by centrifugation at 2,810 g for 7 minutes to remove the residual buffer. Next, 600 μl of XE was added to the membrane and incubated for 3 minutes, then centrifuged at 500 g for 5 minutes to collect the eluate in a new collection tube. Finally, the eluate was re-added to the rotating column membrane, incubated for 3 minutes, and centrifuged at 2,810 g for 8 minutes to collect the exosomes in a new centrifuge tube.

Cell Proliferation, Migration, and Invasion.

Cell proliferation was analyzed using the CCK-8 assay. Briefly, a total of 5×10^3 MDA-MB-231 cells were seeded in 96-well Costar plates and co-cultured with 5×10^3 M0 cells containing different exo-miRNAs for 24 hours. The plates were incubated for 1 hour after adding 10 μl of CCK-8 solution. The absorbance was measured at 450 nm using a microplate reader.

Cell migration and invasion assays were performed using Matrigel® Basement Membrane Matrix (354234, Corning). After starved 12 h, a total of 1×10^6 transfected MDA-MB-231 cells were seeded in the upper chambers in serum-free DMEM, while the lower chambers were loaded with DMEM containing 10% FBS. After 48 hours, non-migrating cells on the upper chambers were removed with a cotton swab, and cells that invaded through the Matrigel layer to the underside of the membrane were stained and counted. Cell migration assays were performed similarly, but without Matrigel.

Constructs and Reagents.

The miR-182-5p-mimics, miR-182-5p-inhibitor, miR-182-5p-agomir, miR-182-5p-antagomir, and the negative control oligonucleotides were purchased from GenePharma.

For western blotting and flow cytometric analyses, the following antibodies were used: β-actin (100166-MM10, Sinobiological), Notch1 (ab52627, Abcam), HES1 (BM4488, Boster), Hexokinase 2 (HK2, A01389, Boster), PKM2 (4053s, CST), PE Anti-Human CD86 Antibody (E-AB-F1012D, Elabscience), APC Anti-Human CD206 Antibody (E-AB-F1161E, Elabscience), FITC Anti-Mouse F4/80 Antibody (E-AB-F0995C, Elabscience), PE Anti-Mouse CD86 Antibody (E-AB-F0994D, Elabscience), APC Anti-Mouse CD206 Antibody (E-AB-

F1135E, Elabscience).

For IHC analyses, the following antibodies were used: Notch1 (20687-1-AP, Proteintech), HES1 (BM4488, Boster), HK2 (22029-1-AP, Proteintech), PKM2 (15822-1-AP, Proteintech).

Enzyme-linked immunoassay (ELISA) kits were purchased from Nanjing Jiancheng and used to analyze the levels of PK (A076-1-1), GLU (A154-1-1), LD (A019-2-1), LDH (A020-2), ATP (A095-1-1), HK (A077-3), and ROS (E004-1-1). Human TNF-α (EK182) and Human IL-10 (EK110/2) were purchased from Liankebio.

microRNA and mRNA Detection.

Total RNAs were extracted using TRIzol reagent (Invitrogen, 15596018), and mature microRNAs were reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (4366597, Invitrogen). Subsequently, the TaqMan MicroRNA Assay Kit (#000597, ABI) was used for qPCR detection. The data were normalized to U6 expression.

Supplementary Table.S1 qPCR primers for miRNA/mRNA.

Primer	5' to 3'
hsa-miR-182-5p F	agtttggcaatggtagaactc
hsa-miR-182-5p R	gtccagttttttttttttttagtgtg
Notch1 F1	GAGGCGTGGCAGACTATGC
Notch1 R1	CTTGTACTCCGTCAGCGTGA
Hes1 F1	TCAACACGACACCGGATAAAC
Hes1 R1	GCCGCGAGCTATCTTTCTTCA
HK2 F2	TGCCACCAGACTAAACTAGACG
HK2 R2	CCCGTGCCCACAATGAGAC
PKM2 F1	ATGTCGAAGCCCCATAGTGAA
PKM2 R1	TGGGTGGTGAATCAATGTCCA
hsa GAPDH_F	GGAGCGAGATCCCTCCAAAAT
hsa GAPDH_R	GGCTGTTGTCATACTTCTCATGG
mouse GAPDH F1	AGGTCGGTGTGAACGGATTTG
mouse GAPDH R1	TGTAGACCATGTAGTTGAGGTCA

Luciferase Reporter Assays.

For Notch1 3'UTR assays, 293T cells were co-transfected with either the miR-182-5p-mimics or a negative control, along with the psiCHECK-2 vector containing the Notch1 3'UTR or its mutation at the end of the Renilla luciferase coding sequence. Lysates were collected 72 hours after transfection. Renilla luciferase activities were normalized to firefly luciferase activities to assess 3'UTR activities.

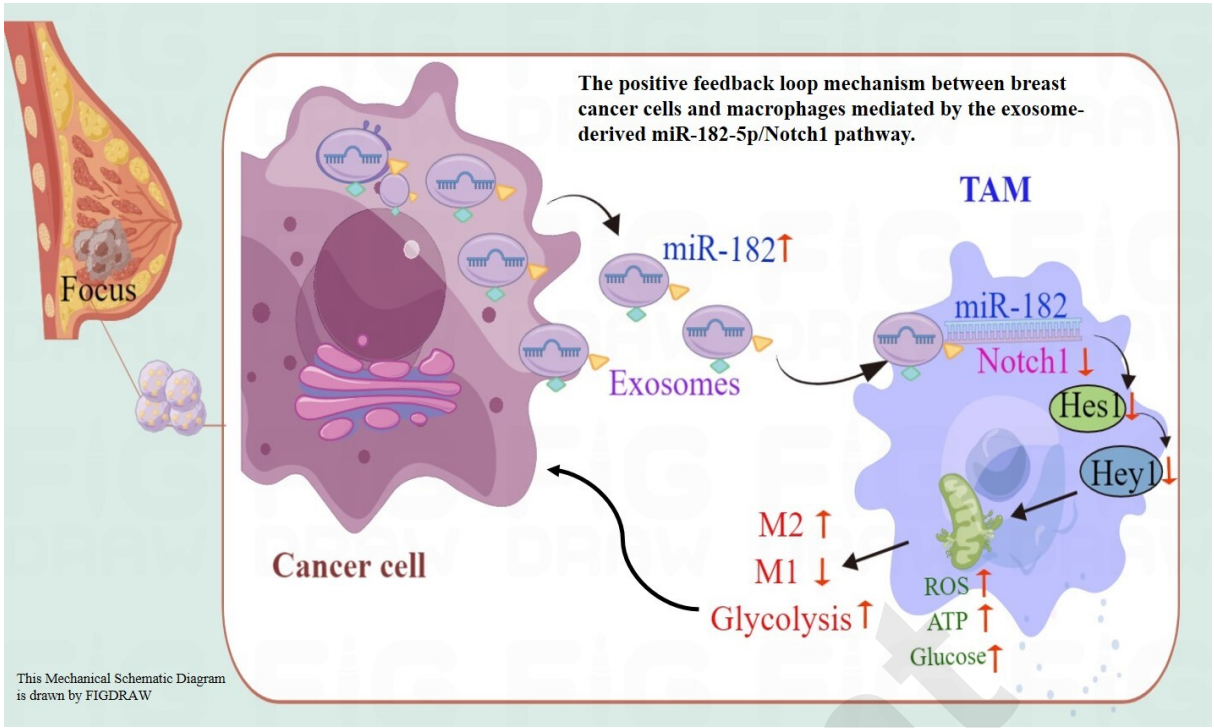
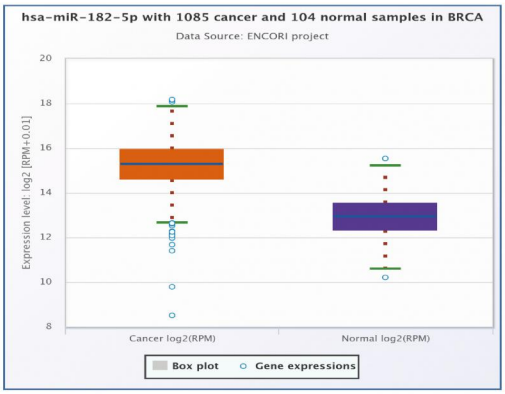

The positive feedback loop mechanism between breast cancer cells and macrophages mediated by the exosome-derived miR-182-5p/Notch1 pathway.
Focus
Cancer cell
miR-182↑
Exosomes
TAM
miR-182
Notch1↓
Hes1↓
Hey1↓
M2↑
M1↓
Glycolysis↑
ROS↑
ATP↑
Glucose↑
This Mechanical Schematic Diagram is drawn by FIGDRAW

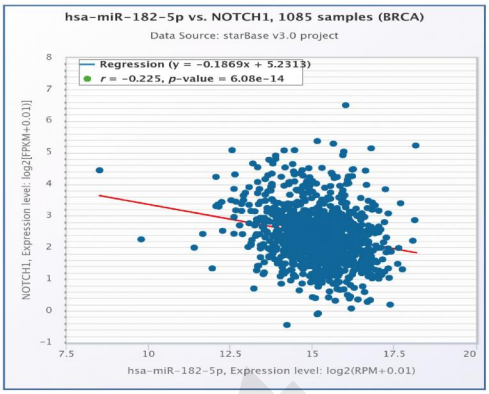
Table 1. The relationship between Notch1 expression and clinical information of patients.

	NOTCH1+	NOTCH1-	χ^2	*P value*
Age			0.0000	1.0000
≤50	46	51		
＞50	28	32		
Menstrual Period			0.0000	1.0000
MP	45	46		
Menopause	33	33		
BMI			6.2359	0.0442
18.5-23.9	22	40		
24-27	28	35		
>28	20	12		
Tumor Size			1.9133	0.3842
≤2cm	34	29		
2-5cm	41	46		
≥5cm	2	5		
Lymphatic Metastasis			11.6903	0.0006
N0	35	15		
N+	42	65		
Histological Grade			8.0658	0.0045
II	40	22		
III	38	57		
Ki-67			3.3809	0.0660
≤30	16	7		
＞30	62	72		
Tumor Stage			12.8251	0.0016
I	24	7		
II	39	54		
III	16	14		

A

B





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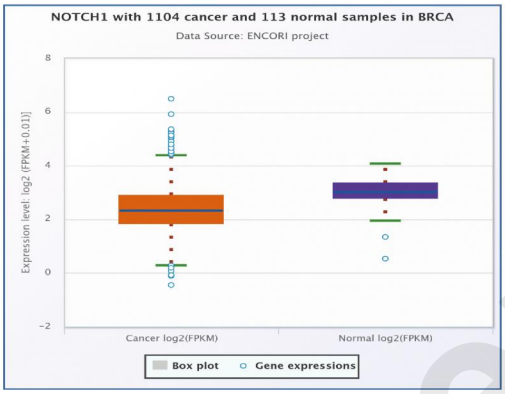

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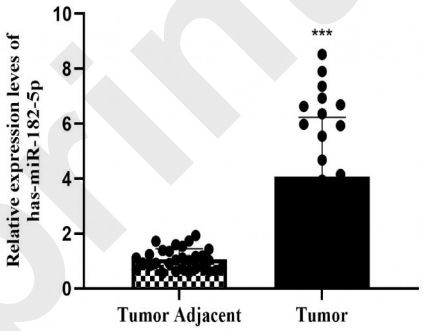
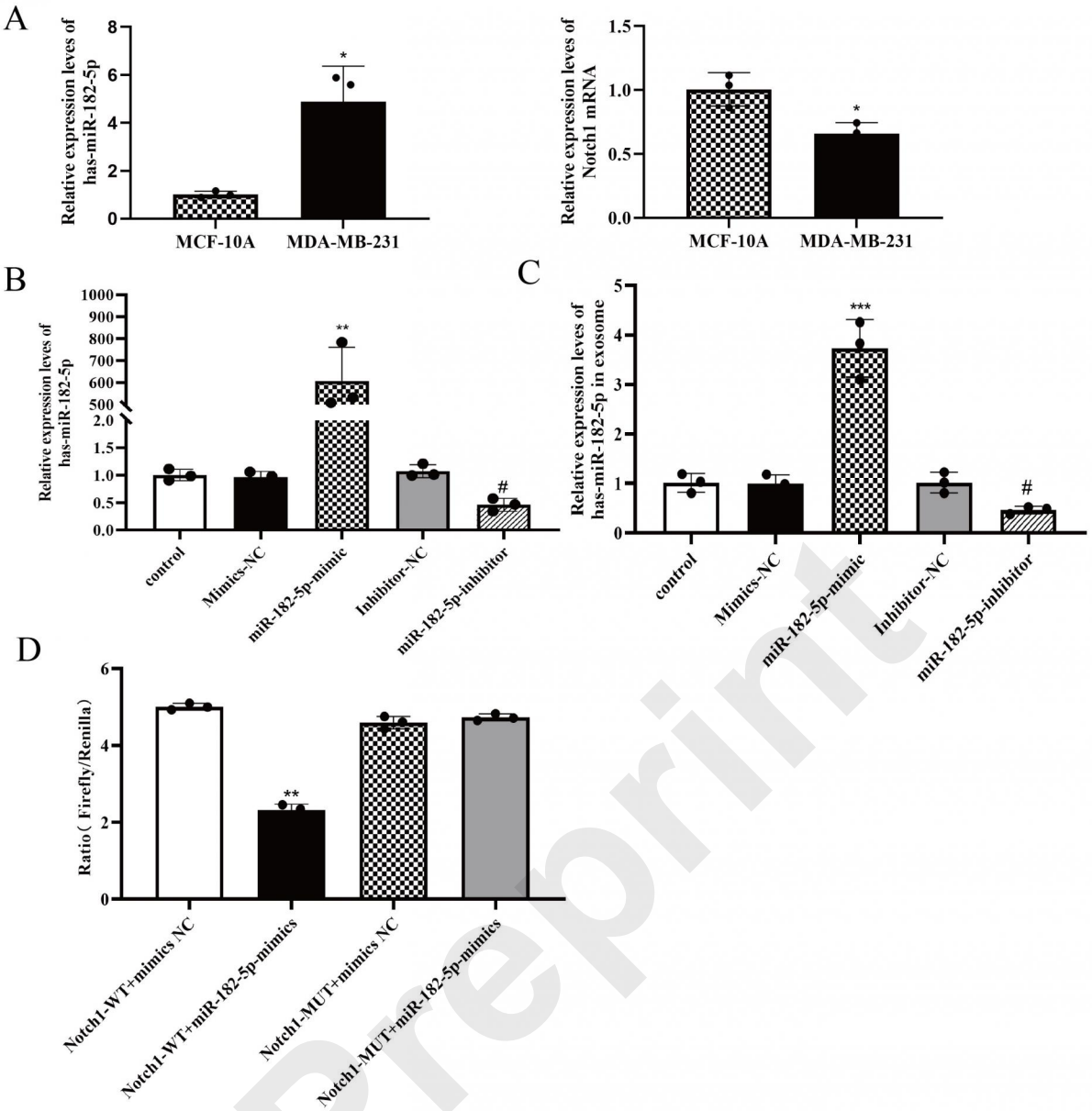
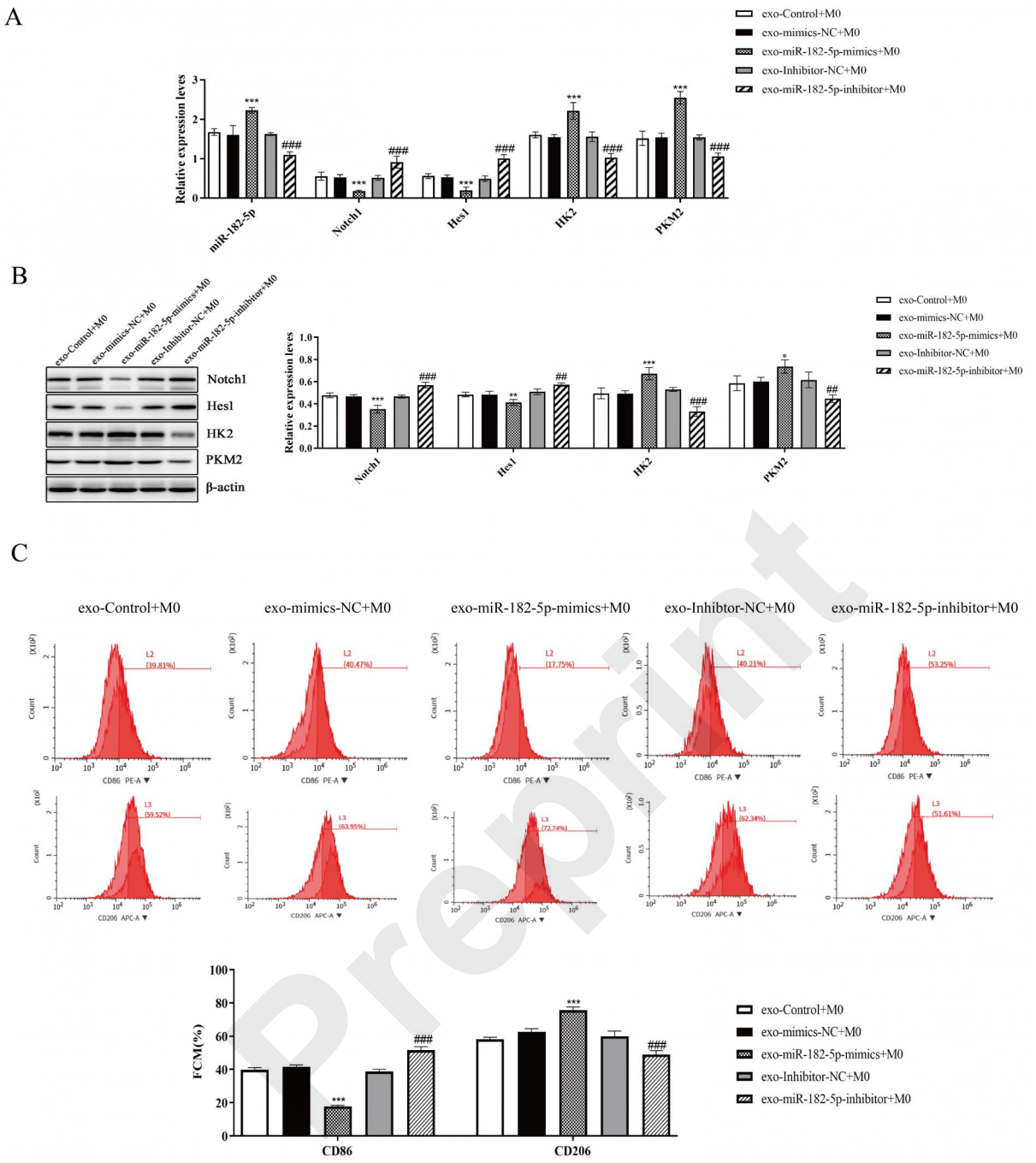


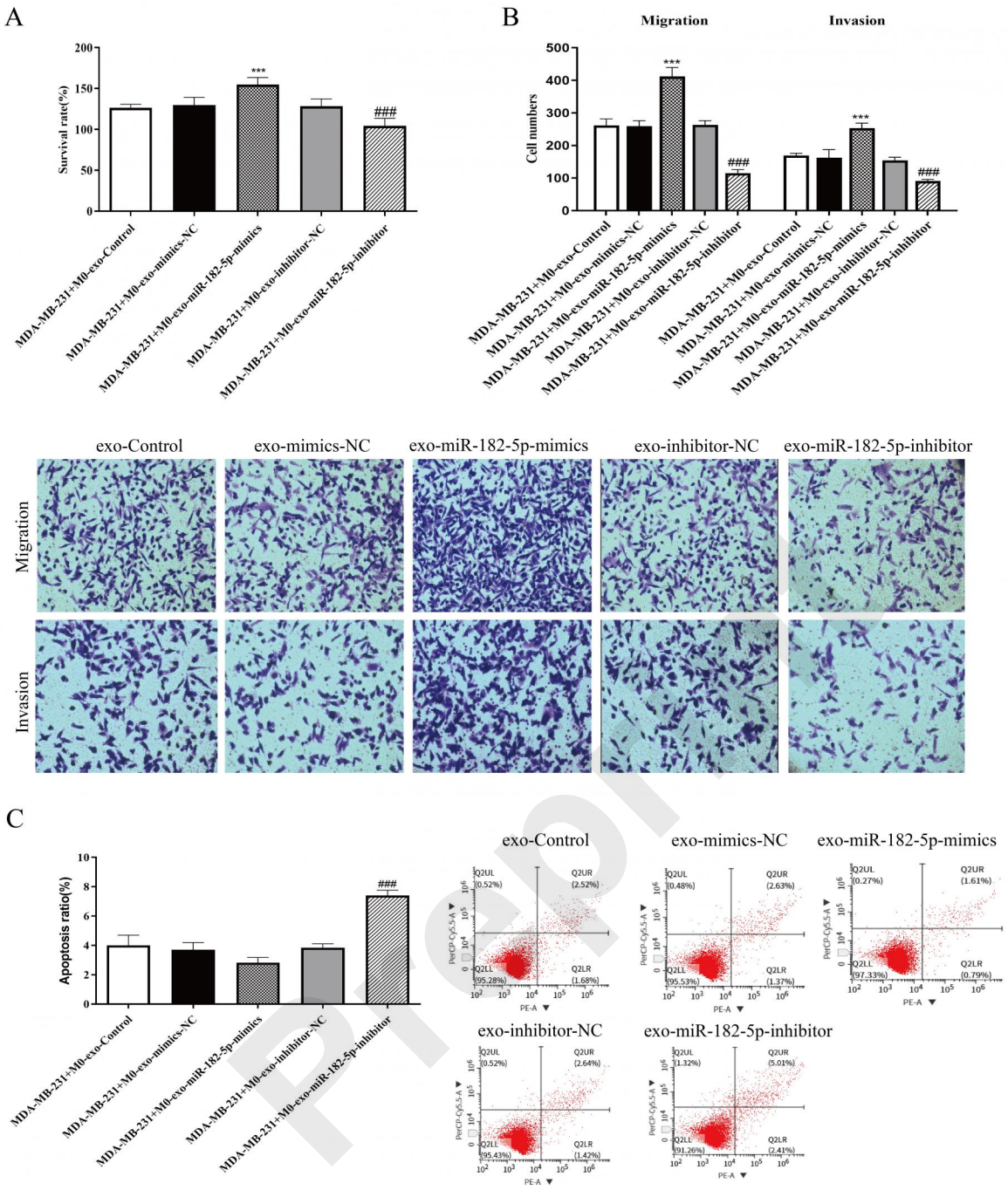
MiR-182-5p and Notch1 expression in clinical samples.



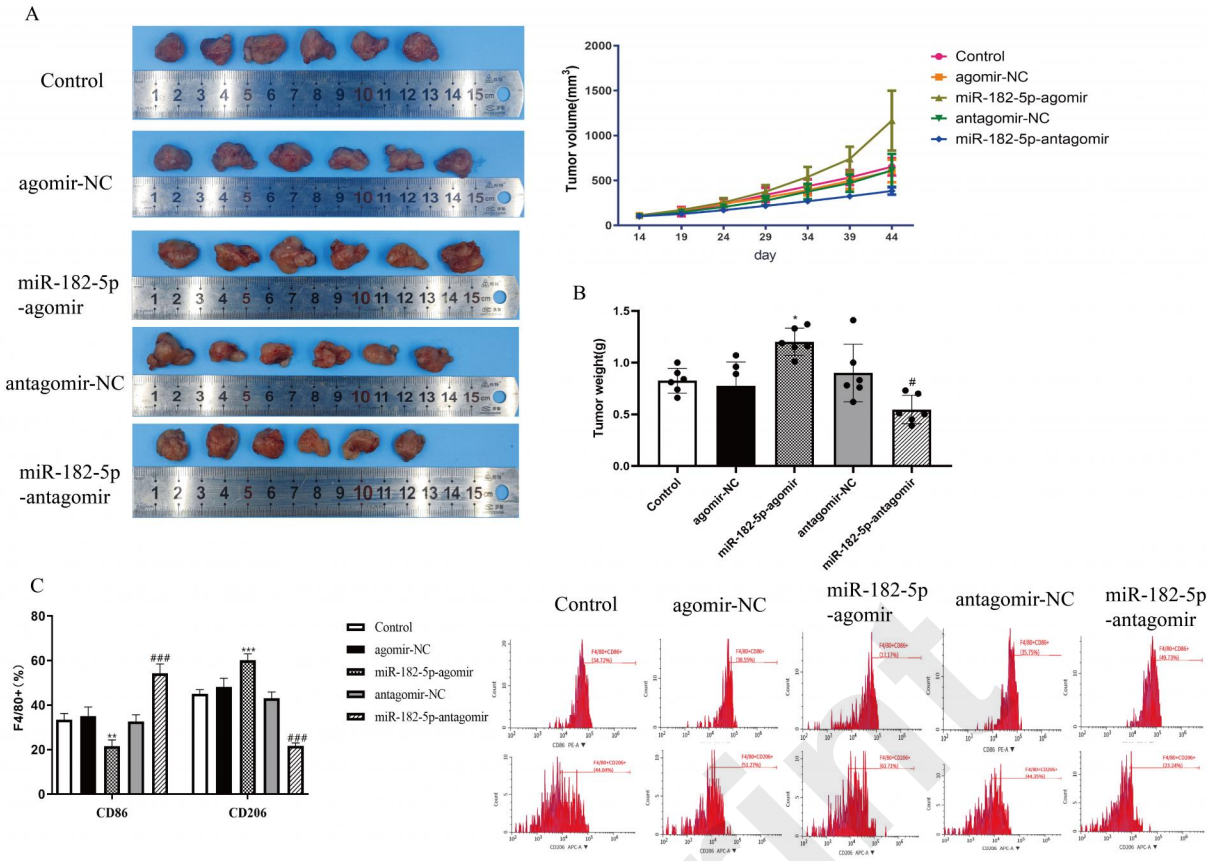
Constructed exosome from miR-182-5p overexpression/inhibition TNBC cell-line.



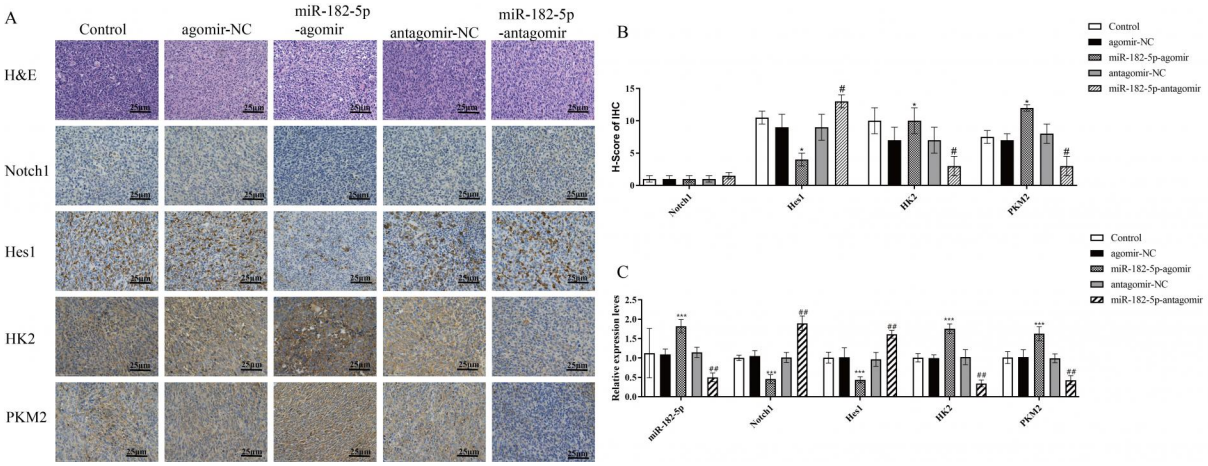
The effect of exosomes from miR-182-5p overexpression/inhibition TNBC cells on macrophages polarization.



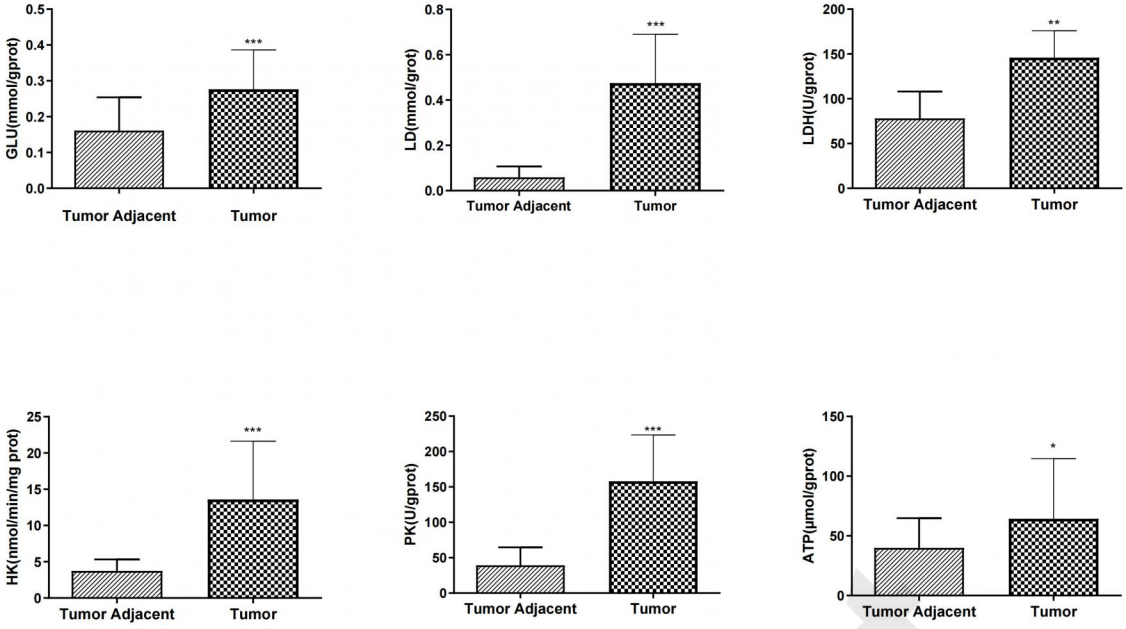
The influence of exosomal miR-182-5p induced macrophages M2 polarization to breast cancer progression.



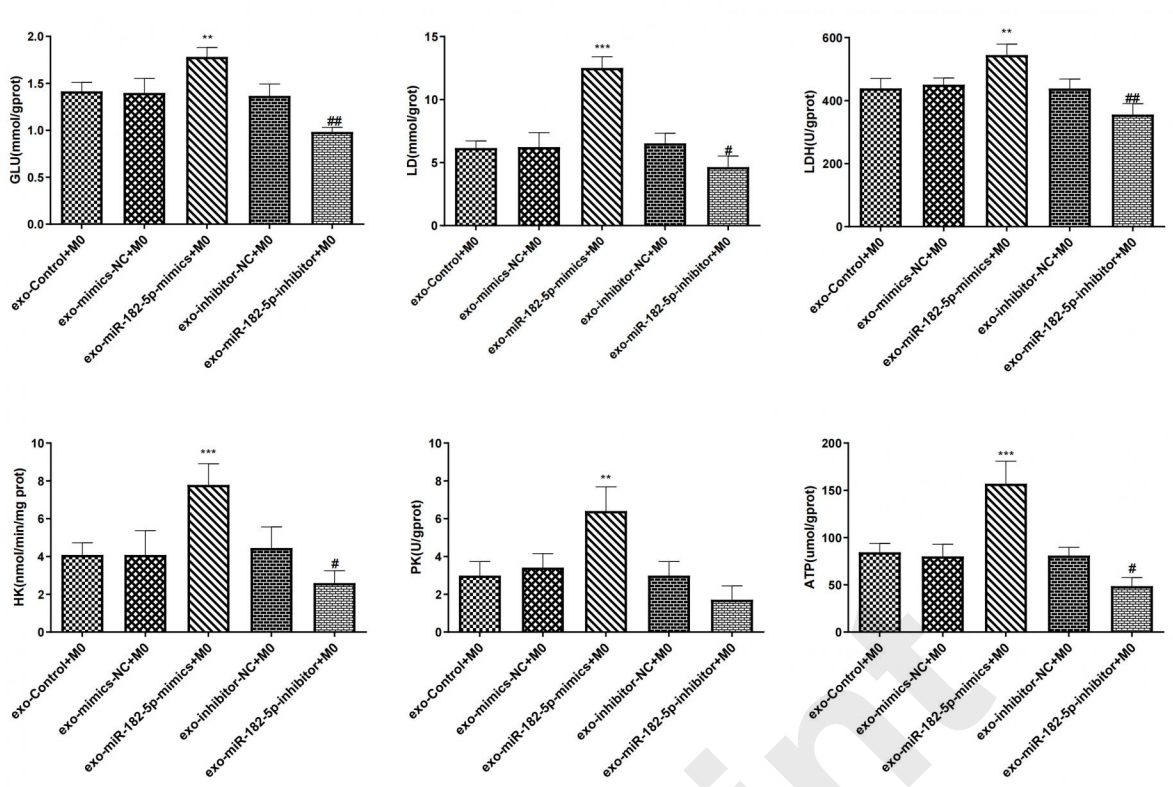
The phonotype shifted by miR-182-5p overexpression/inhibition In vivo.



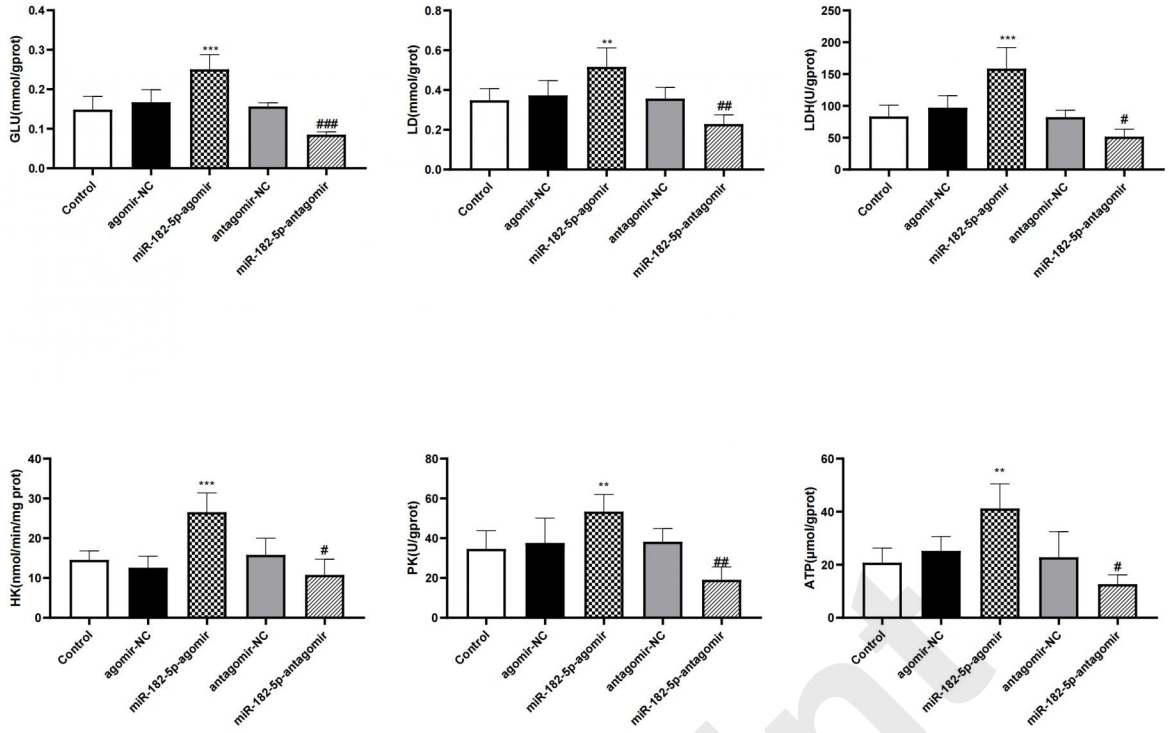
The mechanism switched by miR-182-5p overexpression/inhibition In vivo.



The biochemical indicators transformation of glycolysis in clinical samples.



The biochemical indicators transformation in macrophages.



The biochemical indicators transformation in nude-mice graft model.