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IMP3 promotes TNBC stem cell property through miRNA-34a regulation

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Abstract. – OBJECTIVE: To explore the expression and function of insulin-like growth factor II (IGFII) mRNA binding protein (IMP3) in the Triple Negative Breast Cancer (TNBC).

MATERIALS AND METHODS: According to previously reported gene expression array, we found that IMP3 had significantly higher expression in the CD44+CD24-ESA+ cell cluster, tumor initiating cell or cancer stem cell (CSCs), compared to other tumor cells. Based on the GEO database (GEO accession No. GSE6883), we detected the mRNA levels of IMP 1,2 and 3 by quantitative polymerase chain reaction (q-PCR) in CD44+CD24-ESA+ cell cluster and other breast tumor cell clusters. Besides, we measured IMP3 expression in microsphere of breast cancer, which exerted more significant tumor stem cell properties. The effects of IMP3 on breast cancer cell stem cell properties were studied by RNA interference and overexpression approaches in vitro. Furthermore, we predicted and identified microRNA, which could target and regulate IMP3 from bioinformatics analysis, and verified the interaction by luciferase assays and rescue experiments.

RESULTS: Previously reported data showed that IMP3 expression was significantly upregulated in CD44+CD24-ESA+ cell cluster from breast cancer tissues. Besides, we found IMP3 had higher expression in mesenchymal cells rather than epithelial cells, which was also significantly elevated in SUM159 and T49D cell lines cultured as microsphere rather than adherent cells or differentiated cells. CD44+CD24-ESA+ cell cluster proportion was significantly decreased after silencing IMP3 in SUM1315, and its ability to develop into microsphere was significantly inhibited. By re-expressing IMP3 in SUM315, we restored the self-renewal capacity and tumorigenesis potential of SUM315. Through relative predicting website, we found several miRNAs which could regulate IMP3. miR-34a with highest score was chosen for further analysis. Mimicking miR-34a significantly downregulated IMP3 expression and inhibited its ability to develop into microsphere, while overexpressing IMP3 could rescue this process.

CONCLUSIONS: IMP3 plays a vital role in maintaining stem cell properties of breast cancer cells, which could be regulated by mir-34a.

Key Words:
Breast cancer, IMP3, Cancer stem cell, miR-34a.

Introduction

Breast cancer has become the highest incidence of female malignancy in China[1]. According to statistics from the International Agency for Research on Cancer in 2016, breast cancer accounts for 15% of neoplastic tumor, and is the primary cause of mortality in women under 45 years old[2]. More severely, the incidence and mortality of breast cancer have an upward tendency in China, seriously damaging the health and life of women[3]. So far, the molecular mechanism underlying breast carcinogenesis is not so clearly clarified, while the stem cell properties of some breast cancer cells make the treatment even more difficult[4,5]. Therefore, finding the key molecule and the regulatory pathway during the early breast carcinogenesis and development is of great importance in the research of breast cancer. It could also improve the prognosis and treatment of breast cancer, thereby reducing the mortality.

Triple-negative breast cancer (TNBC) is a subtype of breast cancer that lacks expression of the estrogen and progesterone receptor and does not overexpress human epidermal growth factor 2 receptor protein (HER2)[6-8]. IMP3 (insulin-like growth factor-II mRNA-binding protein 3) is a family member of the insulin-like growth factor-II mRNA-binding proteins (IGF2BPs/IMPs). IMP3 was first found in pancreatic cancer, while other studies revealed that IMP3 is highly expressed in several aggressive cancers, such as

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cervical cancer, lung cancer, breast cancer, colon cancer and so on. The high expression of IMP3 in cancer tissues indicates that it might act as an oncogene during tumorigenesis[9]. Further research revealed that IMP3 is a translational activator of IGFII mRNA, which could further promote the proliferation of leukemic cells.

Meanwhile, aberrantly overexpressed IMP3 has been proposed as poor prognostic biomarker in various cancers. Further investigations show that IMP3 functions as specific diagnostic marker in gastric and colon cancer. Therefore, identification of IMP3's function in cancer stem cell property could contribute to the tumor screening and early diagnosis, thus representing a potential therapeutic target for treatment in breast cancer.

Materials and Methods

Cell Culture and Transient Transfection

The breast-cell lines (SUM159, T47D and SUM315) were cultured in Dulbecco's modified eagle medium (DMEM) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). Cells were maintained in a humidified atmosphere at 37°C containing 95% air and 5% CO_2. For plasmids transfection, cells were digested by trypsin and seeded in 6-well plates. When the cells reached to 60% confluency, plasmids were transfected following the instructions of Fugene-6 kit. Silencing of IMP3 expression was performed utilizing shRNA. The lentiviral particles were thawed at room temperature, gently mixed in the medium and then added into the cell plates for overnight incubation. Primer pairs for the constructions of overexpression vector are as follows:

IMP3-forward 5'-GCCATAGAATTCATGAACAAACTGTATATCGGAAACC

IMP3-reverse 5'-ACAATAGCGGCCGCTTACTTCCGTCTTGACTGAGGT

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After quantification, reverse transcription reaction was performed using Prime Script RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. Properly diluted complementary Deoxyribose Nucleic Acid (cDNA) was used for the quantification of mRNA by Real-time polymerase chain reaction (PCR) using the ABI Prism 7500 FAST Detection System (Applied Biosystems, Foster City, CA, USA). The final volume of the reaction mixture contained the cDNA, deionized water, primers, and SYBR green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Relative expression of target genes was calculated after normalization by the $2^{\Delta\Delta Ct}$ method. The specific primers were shown in Table I.

Western Blot Analysis

For total protein isolation, cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The standard Western blot was performed as described be-

Table I. Primers used in this study.

Genes		Primers
IMP1	Forward	ATATGGGAGCTGAGATTCCG
	Reverse	GACAAACCAGGTGGACCTCT
IMP2	Forward	TTCTCAGGCCAGACAGATTG
	Reverse	TATGGTCAAGCCCTCCTTTC
IMP3	Forward	CCGCAGTTTGAGCAATCAGAA
	Reverse	CGAGAAAGCTGCTTGATGTGC
OCT4	Forward	GAGAAGGATGTGGTCCGAGT
	Reverse	GTGCATAGTCGCTGCTTGAT
NANOG	Forward	CCTATGCCTGTGATTTGTGG
	Reverse	GATCCATGGAGGAAGGAAGA
ALDH1A1	Forward	CACAGGTTTCGGCTTTGTAA
	Reverse	GCTCCAGCATCGAATTTGTA
ALDH1A2	Forward	ACGTCTGTCCCTCTCTGCTT
	Reverse	GTAAGGACCGTGGCTCAACT
ALDH1A3	Forward	CCCGTAACAGAACCAGTGTG
	Reverse	GATGAAGGGAAGGCAAATGT

low. Protein concentration in each cell lysate was quantified by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in nonfat milk, the membranes were incubated at 4°C overnight with primary antibodies. The blots were then washed in Tris-buffered saline and Tween-20 (TBST-20) buffer for 3 times and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2-3 h at room temperature. Protein bands were visualized with the electrochemiluminescence (ECL) (Beyotime, Shanghai, China) luminescence using Tanon Detection System.

Flow Cytometry Analysis

Cells were harvested with EDTA-free trypsin and cell supernatants were collected into the prepared flow cytometry tubes. After that, cell suspensions were added into corresponding tubes and centrifuged, washed in phosphate-buffered saline (PBS) twice and stained with 200 μL binding buffer containing Ca^{2+}. The cells were then incubated with corresponding antibodies in the dark, and analyzed using a FACS flow cytometer.

Statistical Analysis

Analyses were conducted by statistical analysis software statistical product and service solutions (SPSS) version 22.0 (Armonk, NY, USA). Pictures were edited with GraphPad Prism 6.0 (La Jolla, CA, USA). Comparisons were performed using Student's *t*-test between pairs of groups, and results were expressed as mean±SEM. x^2-test was used to examine the differences between the variables. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD). $p<0.05$ was considered to be statistically significant. *$p<0.05$, **$p<0.01$.

Results

IMP3 is Aberrantly Overexpressed in Triple-Negative Breast Cancer (TNBC) and is Relative to the CSC Property

According to previously reported gene expression array[10], we found that IMP3 expression was significantly higher in CD44+CD24-ESA+ cell cluster, the tumor initiating cells were isolated from breast tumor tissue in comparison with other breast tumor cell clusters (Figure 1A). We also accessed the expression level of IMP1 and IMP2, two other family members of the insulin-like growth factor-II mRNA-binding proteins. However, there were no differences in the expressions of IMP1 and IMP2 in these clusters. Meanwhile, we assessed IMP3 expression by immunoblot and qPCR in epithelial cells and mesenchymal cells. We found IMP3 expression was higher in mesenchymal cells compared with that in epithelial cells (Figure 1B). The breast cell lines SUM159 and T49D cells did not express IMP3 when cultured as adherent cells (Figure 1C). However, IMP3 expression was significantly up-regulated when cultured as microspheres. Moreover, collagenase-1-induced microsphere differentiation resulted in a significant decrease in IMP3 expression. In addition, we also observed that the expression of stem cell properties related genes in microspheres was significantly higher than that of adherent cells (Figure 1D). These data demonstrated that IMP3 expression is dysregulated in TNBC and relative to CSC properties.

Interfering IMP3 Expression Inhibits Stem Cell Properties of Breast Tumor Cells

After silencing IMP3 expression in SUM1315, we analyzed the CD44 +CD24-ESA + cell cluster by flow cytometry. Results showed that the percentage of CD44+CD24-ESA+cell cluster decreased significantly after IMP3 knockdown (Figure 2A and 2B). As self-renewal is an important feature of stem cells, we measured microsphere formation ability of SUM1315 cells passaged to P1, P2 and P3 (every 7 days). The results showed that with the increase of passages, microspheres formation of cells with low IMP3 expression was significantly reduced, relatively to the control group (Figure 2C). Next, we performed separate measurements of IMP3 expression levels and microsphere formation ability for the CD44 + CD24-ESA + cell cluster in SUM1315. Results indicated that after silencing IMP3 expression in the CD44 + CD24-ESA + cell cluster, the ability to form microspheres of CD44 + CD24-ESA + cell subpopulations at different cell concentrations decreased significantly (Figure 2D). I*n vitro* interference with IMP3 expression is effective in inhibiting the stem cell-like properties of breast tumor cells.

IMP3 Restoration Promotes Tumor Cell Self-Renewal and Tumorigenesis

Subsequently, we constructed IMP3 overexpression vector and performed rescue experiments.

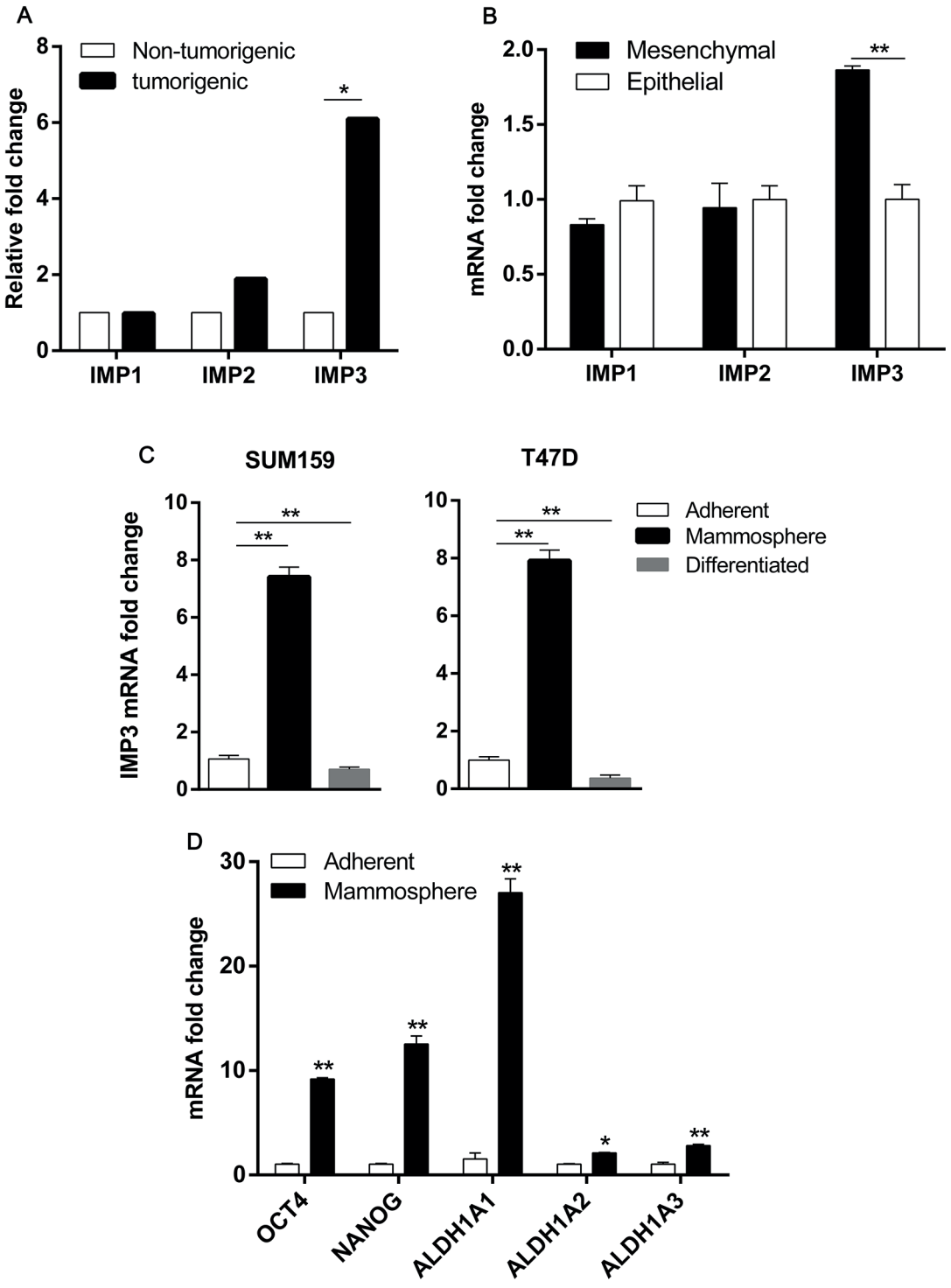

Figure 1. IMP3 was aberrantly overexpressed in TNBC. ***A,*** IMP3 expression was higher in CD44+CD24-ESA+ cell cluster in breast tumor than other cell clusters. ***B,*** IMP3 expression was higher in breast tumor mesenchymal cells compared with epithelial cells. ***C,*** The SUM159 and T49D cells did not express IMP3 when cultured as adherent cells and induced to differentiation by collagenase, but were significantly up-regulated when cultured as microspheres. ***D,*** Stem cell-like properties relative gene expressions were higher in breast tumor cell microspheres than those cultured as adherent cells.

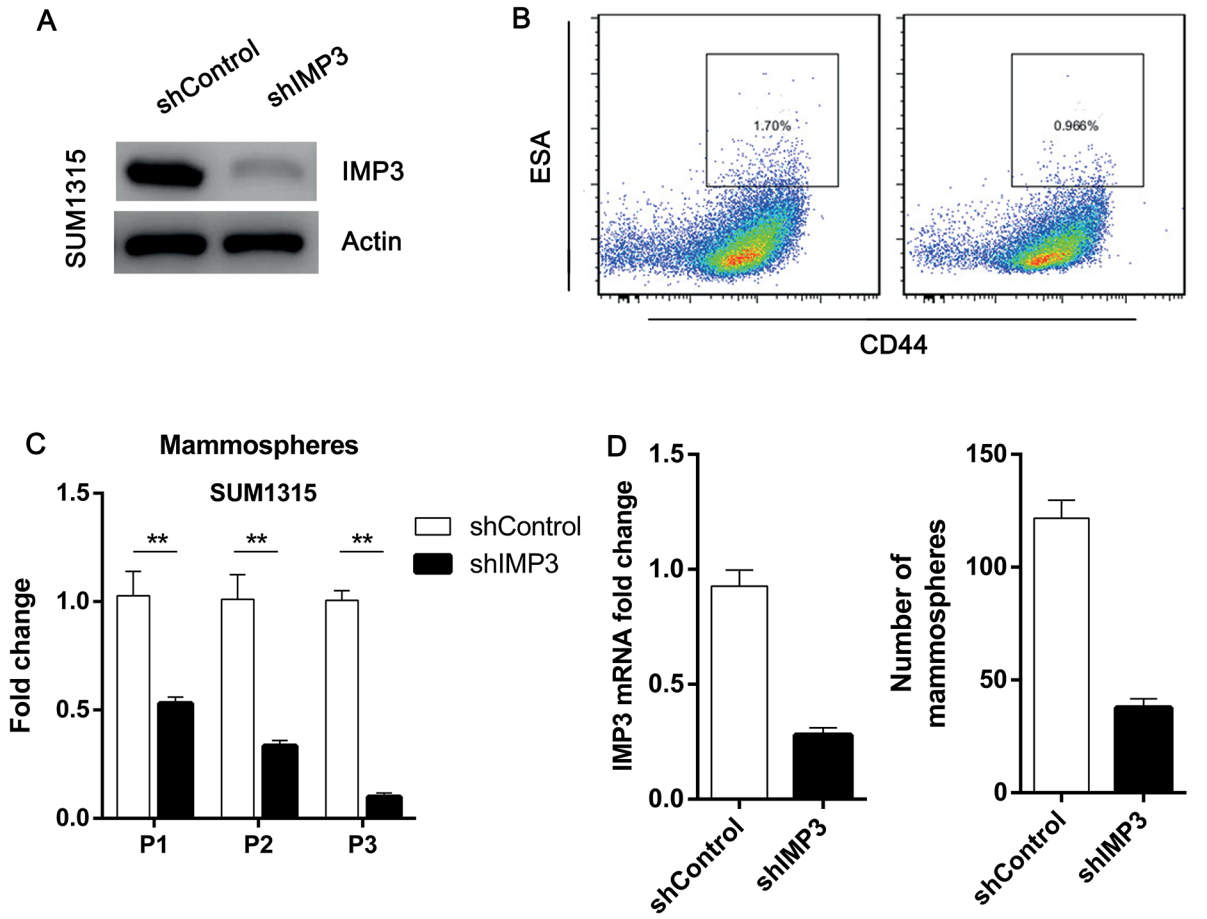

Figure 2. Interfering IMP3 expression inhibited stem cell properties of breast tumor cells. ***A,*** IMP3 expression was decreased in SUM1315 after IMP3 knockdown. ***B,*** Cd44+CD24-ESA+ cell cluster percentage significantly declined after IMP3 expression decreased. ***C,*** With the increase of passages (P1, P2, P3), microspheres formation of cells with low IMP3 expression was significantly reduced relative to the control group. ***D,*** After silencing IMP3 expression in the CD44 + CD24-ESA + cell cluster, the ability of forming microspheres of CD44 + CD24-ESA + cell subpopulations at different cell concentrations decreased significantly.

Flow cytometry results revealed that the percentage of CD44 + CD24-ESA + cell subpopulation was up-regulated after increasing IMP3 expression (Figure 3A and 3B). In addition, we examined the ability of tumor formation in mice as a measure of tumorigenicity. *In vivo* experiments in mice showed that the tumor-free survival of mice with low IMP3 expression was significantly higher than that of the control (Figure 3C). However, after up-regulating IMP3 expression, the tumor-free survival of mice was significantly reduced (Figure 3D).

IMP3 is a Vital Target for MiR-34a

To explore the possible mechanism of IMP3 overexpression in CD44 + CD24-ESA + cells, we used an online bioinformatics server (miRNA.org) to predict microRNAs that may target the regulation of IMP3. We found miR-34a scored highest in a series of microRNAs that might target IMP3 (score = -1.098) (Figure 4A). To test the role of miR-34a in regulating IMP3, we overexpressed miR-34a and found that IMP3 expression was significantly downregulated in the miR-34 overexpression group by immunoblot (Figure 4B). Luciferase assay further revealed that miR-34a negatively regulated wild type IMP3, but not with variant IMP3 (Figure 4C). In addition, the ability of miR-34a-overexpressed cells to form microspheres was significantly reduced. In summary, IMP3 was a potentially vital target for miR-34a (Figure 4D).

Re-Expression of IMP3 Rescue the Tumor Suppressor Effect Caused by miR-34

To further test whether IMP3 was the target for miR-34a, we conducted rescue experiments

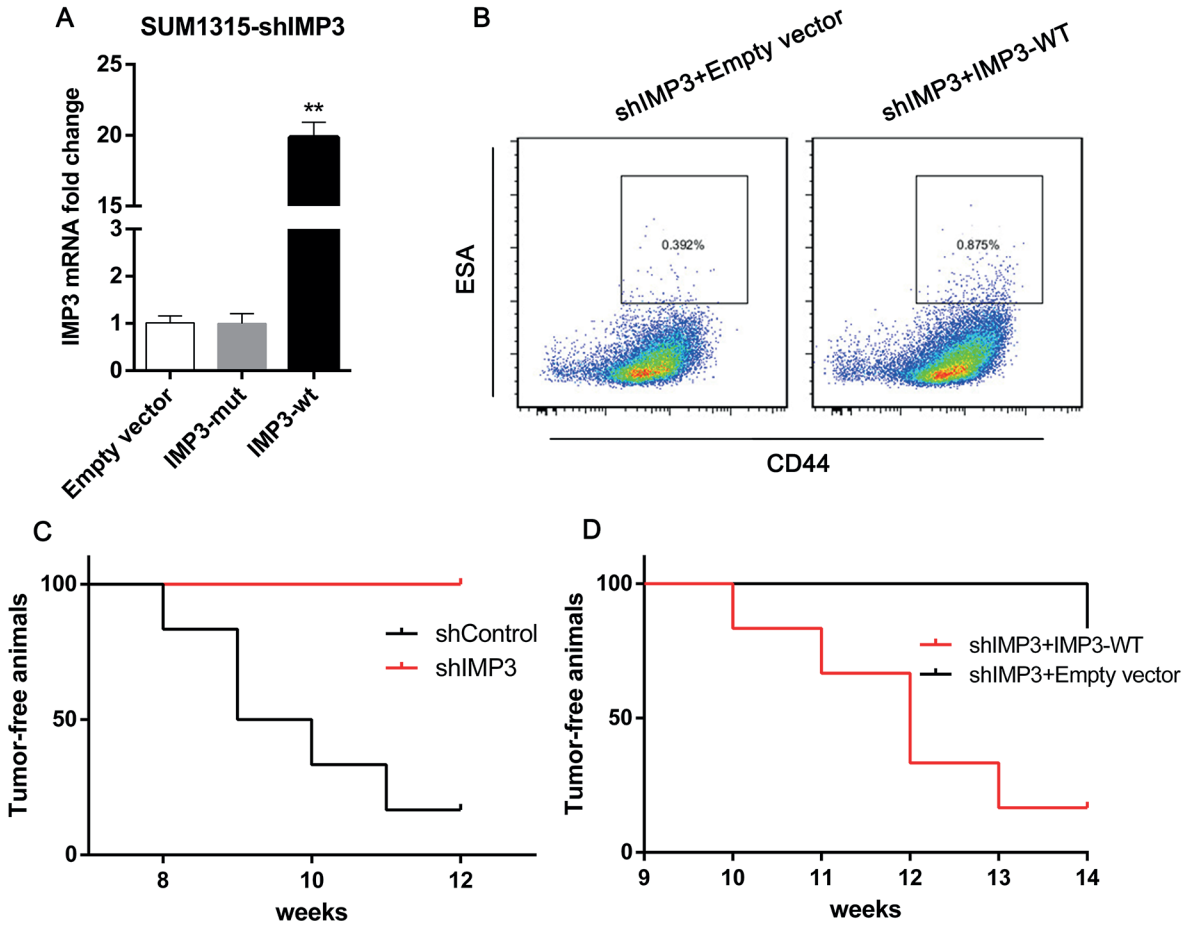

Figure 3. IMP3 restoration promoted tumor cell self-renewal and tumorigenesis. ***A,*** IMP3 was effectively overexpressed in SUM1315 cells transfected with IMP3 shrna**.** ***B,*** Cd44+CD24-ESA+ cell cluster percentage increased after IMP3 re-expression. ***C,*** Mice with low IMP3 expression had higher tumor-free survival than control groups. ***D,*** After up-regulating IMP3 expression, the tumor-free survival of mice was significantly reduced.

in miR-34a-overexpressing SUM1315 cells. We detected the protein level of IMP3 in SUM1315 cells transfected with IMP3 overexpression plasmids by Western blot, and determined the number of microspheres formed in each group (Figure 5 A and B). The results indicated that the ability of miR-34a-overexpressing SUM1315 cells to form microspheres was rescued after IMP3 was re-expressed.

Discussion

According to statistics, the incidence of breast cancer in China is about 169,000 each year, making it the second most common malignant tumor in women. The mortality rate of breast cancer in China is about 45,000, which has been becoming one of the most treats for women health[11]. Despite treatment in the early stage of disease, about 30% of patients will eventually undergo relapse and tumor metastasis. For patients diagnosed with metastasis, routine treatment could initially control the development of disease, while most tumor conditions will eventually deteriorate over time[12,13]. To reduce mortality and develop new therapeutic strategies, it is of great significance to fully grasp the molecular biology of breast cancer cells. Researches show that there exists a small amount of stem cell-like cells in tumor cell population. These cells possess the ability of self-renewal and specific differentiation and play a decisive role in maintaining tumor growth and angiogenesis, thus promoting tumor invasion and metastasis. These cells are generally considered to have stem cell-like pro-

perties, namely cancer stem cells (CSCs)[14,15]. The presence of breast cancer stem cells could enhance the proliferation, invasion and metastasis of breast cancer. Therefore, how to eradicate stem cell-like breast tumor cells has become an important topic for researchers.

IMP3, IMP1 and IMP2, are members of insulin-like growth factor II (IGF-1) mRNA-binding protein family. IGF-overexpression is associated with the pathogenesis of multiple organ tumors[16]. Therefore, it is considered that IMP family members are involved in carcinogenesis by stabilizing IGF II mRNA. IMP3 is expressed in developing epithelial tissue, muscle and placenta during human and mouse embryogenesis, but its expression level is extremely low in adult tissues. Samanta et al[17] measured IMP3 expression of 138 cases of breast cancer patients by immunohistochemistry and found that IMP3 expression was correlated with the histological grade, necrosis and determination of TNBC of breast cancer. The high expression of IMP3 in high-grade breast cancer and TNBC suggested that IMP3 may serve as a new marker of TNBC.

Our results indicate that the high expression of IMP3 was closely related to the occurrence of triple negative breast cancer and stem cell properties of breast tumor cells. The expression of IMP3 was higher in breast tumor cells with more significant stem cell properties, and knockdown of IMP3 in SUM1315 cell line significantly decreased the self-renewal and microsphere formation ability of breast tumor cells. In previous researches, the tumor-promoting activity of IMP3 has been focused on its mRNA activity. However, little is known about the mechanism underlying IMP3 binding to microRNAs during tumorigenesis[18-22]. In our work, miR-34a was predicted to target IMP3 by bioinformatics analysis. It is worth noting that miR-34a was found to have some inhibitory effect on prostate cancer, pancreatic cancer stem cells and gastric cancer stem cells, which is consistent with related studies of breast cancer. In

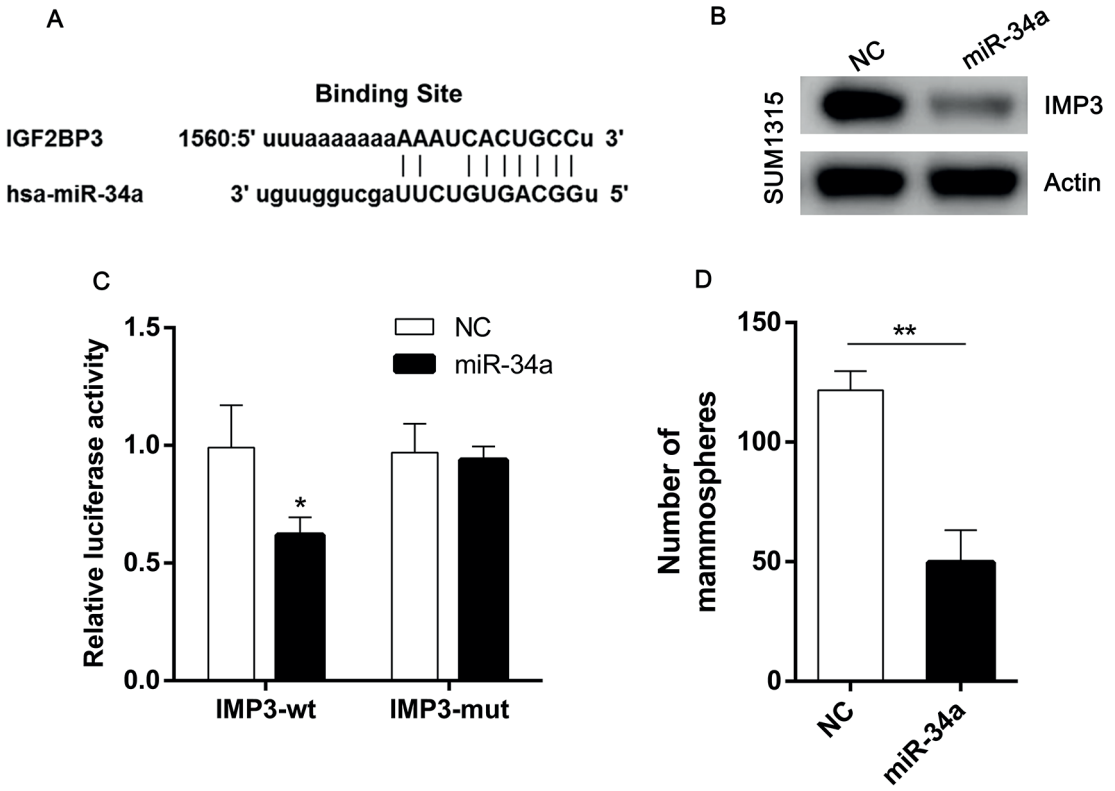

Figure 4. IMP3 acted as a target for MiR-34a. ***A,*** Predicted-binding site of IMP3 and microRNA. ***B,*** IMP3 expression decreased after miR-34a overexpression. ***C,*** Luciferase assay showed that miR-34a was negatively correlated with the expression of IMP3. ***D,*** The ability of the miR-34a overexpression group to form microspheres was significantly reduced compared to the control group.

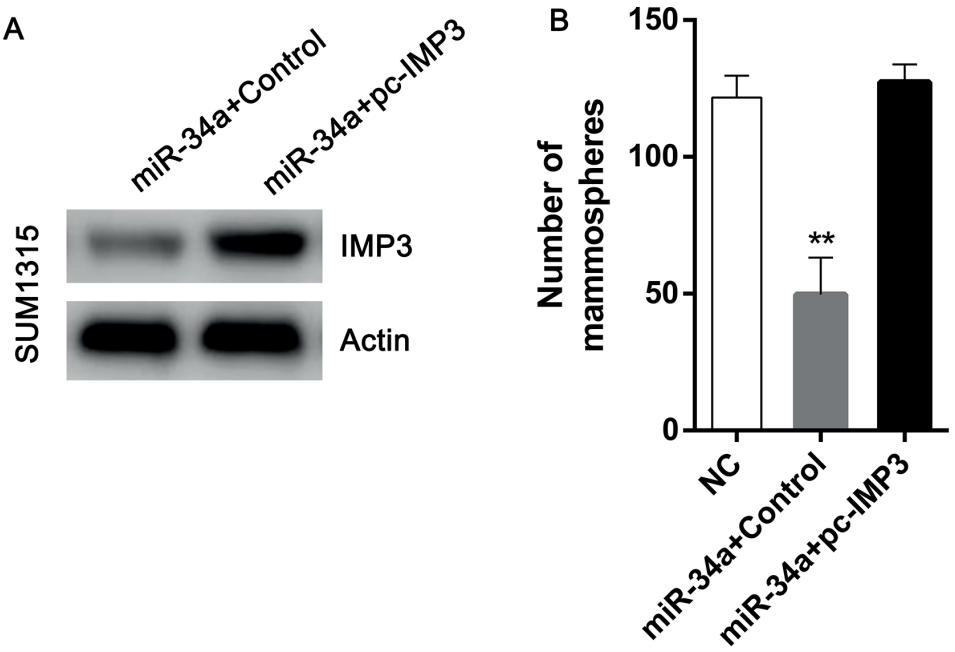

Figure 5. Re-expression of IMP3 rescued the tumor suppressor effect caused by miR-34. ***A,*** IMP3 expression in SUM1315 after transfected with IMP3 plasmid. SUM1315 was overexpressed with miR-34a in advance. ***B,*** The ability of SUM1315 to form microspheres was significantly rescued after IMP3 expression.

the miR-34a overexpression group, IMP3 expression was significantly reduced and the ability of SUM1315 microsphere formation also decreased, while IMP3 re-expression could rescue the phenotype. This indicates that miR-34a does reduce the expression level of IMP3 protein, further affecting its tumorigenicity.

In summary, we systematically studied the new mechanisms by which IMP3 promoted the maintenance of stem cell-like properties in breast tumor cells. These findings suggest that IMP3 may be an ideal target for breast cancer treatment in the future.

Conclusions

Our investigation suggested that IMP3 maintained the stem cell properties of breast cancer and was regulated by miR-34a.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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