Biochemical and Biophysical Research Communications xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Downregulation of DPF3 promotes the proliferation and motility of breast cancer cells through activating JAK2/STAT3 signaling

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ARTICLE INFO

Article history:
Received 4 April 2019
Accepted 25 April 2019
Available online xxx

Keywords:
DPF3
SWI/SNF complex
Migration
Invasion

ABSTRACT

It is reported that the genetic variation of DPF3 is a risk factor of breast cancer through large-scale association research. However, the expression, function and mechanism in breast cancer is unknown. We applied qPCR and western blotting to detect the levels of DPF3 in breast cancer tissues. MTT and Anchorage-independent growth ability assay were used to evaluate the effect of DPF3 on cell proliferation. Wound healing and transwell invasion assay were performed to detect the role of DPF3 on cell motility ability. Herein, we found that the mRNA and protein levels of DPF3 are both significantly downregulated in breast cancer tissues. And downregulation of DPF3 can promote the proliferation and motility of breast cancer cells. Further investigation illustrated that downregulation of DPF3 can activate the JAK2/STAT3 signaling. In conclusion, we found that the downregulation of DPF3 plays an indispensable function in the progression of breast cancer, and may be served as a novel therapeutic target to therapy breast cancer.

1. Introduction

Breast cancer still remains a public heath dilemma in the globe [1]. In 2018, it is predicted that there were approximate 2.1 million women newly diagnosed with breast cancer, accounting for almost 1 in 4 cancer cased among women. And breast cancer is also the leading cause related with cancer in over 100 countries [2]. Therefore, it is essential to explore the etiology and seek for the novel therapeutic therapy target of breast cancer.

The process that incipient cancer cells become tumorigenic and ultimately malignant needs multiple capabilities, of which, sustaining proliferation and activating invasion is the fundamental step [3,4]. Normal cells subtly balance production and release of the growth stimulant that instruct entry into cell cycle. Cancer cells, by breaking the homeostasis, become masters of their own destinies [5]. The greatest challenge to fight cancer is metastasis, and cancer cell migration and invasion is the fundamental ancient cellular behavior that helps cancer metastasis [6–8]. Therefore, we focused on cancer cell proliferation and motility and the mechanisms by which cancer cells obtain the capabilities for proliferation and motility.

In humans, it has been reported that the ATP-dependent chromatin remodeling factor, SWI/SNF complex, take an indispensable epigenetic role in substantial biology processes [9–11]. DPF3 is a cofactor that substoichiometrically interacts with SWI/SNF complex. The genetic variation of DPF3 is a risk factor of breast cancer and is associated with lymph node metastasis and tumor size through large-scale association research using specimens from women of European ancestry [12]. However, the expression, function and mechanism in breast cancer is unknown.

Herein, we found that the expression of DPF3 is significantly down-regulated in breast cancer tissues, and knockdown of DPF3 dramatically promotes the proliferation and motility of breast cancer cells. Besides, molecular experiments illustrated that the transcription of JAK2/START3 is significantly activated in DPF3-

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https://doi.org/10.1016/j.bbrc.2019.04.170
0006-291X/

silenced cells. The inhibitor of JAK2/STAT3, peficitinib, can significantly suppress the stimulating roles on proliferation and motility in DPF3-silenced cells. Our finding provided evidences that downregulation of DPF3 is closely associated with breast cancer and may be served as a novel therapeutic target for breast cancer.

2. Materials and methods

2.1. Cell culture and tissue specimens

Breast cancer cell MCF-7 was purchased from American Type Culture Collection (ATCC), and were cultured using Dulbecco's modified eagle medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS; HyClone). Fresh breast cancer tissues were collected form The First Affiliated Hospital of Sun Yat-sen University. Before using these patient tissues for research, Ethics approval from the Institutional Research Ethics Committee and patient informed consent were obtained.

2.2. Quantitative PCR (qPCR)

Total RNA extraction and qPCR were performed according to the methods described previously [13]. The primers are as follows: DPF3: forward, 5′- CCTCTCAGGAAGACCACGACAA-3′, reverse, 5′- CAGGTGAGTGTGAGCATAGTGG-3'; Cyclin D1, forward, 5′- TCTACACCGACAACTCCATCCG-3′, reverse, 5′- TCTGGCATTTTGGAGAGGAAGTG-3'; cyclin D3, forward, 5′- AGATCAAGCCGCACATGCGGAA-3′, reverse, 5′-ACGCAAGACAGGTAGCGATCCA-5'; cyclin E1, forward, 5′- TGTGTCCTGGATGTTGACTGCC-3′, reverse, 5′- CTCTATGTCGCACCACTGATACC-3'; CDK4, forward, 5′- CCATCAGCACAGTTCGTGAGGT-3′, reverse, 5′-TCAGTTCGGGATGTGGCACAGA-5'; C-Myc, forward, 5′- CCTGGTGCTCCATGAGGAGAC-3′, reverse, 5′- CAGACTCTGACCTTTTGCCAGG-3'; Survivin, forward, 5′- CCACTGAGAACGAGCCAGACTT-3′, reverse, 5′-GTATTACAGGCGTAAGCCACCG-3'; Bcl-xl, forward, 5′- GCCACTTACCTGAATGACCACC-3′, reverse, 5′- AACCAGCGGTTGAAGCGTTCCT-3'; Bcl-2, forward, 5′- ATCGCCCTGTGGATGACTGAGT-3′, reverse, 5′-GCCAGGAGAAATCAAACAGAGGC-3'.

2.3. Plasmids

For overexpression of DPF3, human full-length DPF3 gene was amplified by PCR and cloned into the pMSCV plasmid. To upregulate DPF3, two human shRNA sequence targeting DPF3 were cloned into pSuper-*retro*-puro plasmid. Stable cell overexpressing or downregulating DPF3 were produced using HEK293T cells through retroviral infection, and screened using 0.5 μg/ml puromycin for 10 days. The shRNA fragments are as following: Sh#1: 5′-CCGGAGTTACAACTCACGGCT-3'; Sh#2: 5′- CTGGCGCAAGAAGAGACGATT-3'.

2.4. Western blotting assay

Western blotting was carried out following the methods described previously [14]. The antibodies used in this study are as follows: *anti*-DPF3, *anti*-p-JAK1, *anti*-p-JAK2, *anti*-JAK3, *anti*-p-Tyk2, *anti*-p-STAT1, *anti*-p-STAT2, *anti*-p-STAT3, *anti*-p-STAT4, *anti*-p-STAT5, *anti*-p-STAT6, *anti*-GAPDH (Abcam).

2.5. MTT assay

Cells were cultured using 96-well plates. At the indicated time, cells were treated using 100 μL 0.5 mg/ml MTT reagent (Sigma) for 4 h. Later, the culture medium was removed, and 100 μL dimethyl sulphoxide (DMSO; Sigma) were added into each well. Finally, the absorbance was evaluated at 570 nm wavelength, and the value at 655 nm as the reference.

2.6. Anchorage-independent growth ability assay

The plates were coated using completed medium containing 1% agar (Sigma). The complete medium including 500 cells and 0.3% were plated on the top of the plates. 10 days later, the colonies larger than 0.1 mm were counted.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The activity of MMP2 and MMP9 in the culture supernatants was detected using ELISA kit (R&D Systems) according to the protocol.

2.8. Wound healing assay

The cells were cultured using 6-well plates. Until the cell confluence reached about 90%, we used a sterilized 10 μL pipette tip across the cell monolayer to create wound.

The debris of cells were removed by two washing using PBS. The cells were incubated for 24 h. Then, the wound healing was examined under Olympus camera.

2.9. Transwell invasion assay

The filter of the transwell plate (BD Biosciences) was coated using Matrigel. 1×10^4 cells were seeded into the transwell plate containing DMEM without FBS, and the transwell plate were put into the chamber containing the DMEM supplementing 0.5% FBS. 24 h later, the cells that did not traverse the filter were removed using cotton swabs, and the cells that traversed the filter were fixed using 4% paraformaldehyde and dyed using 1% crystal violet.

2.10. Statistical analysis

All statistical analysis was evaluated by the SPSS 22.0 software package. The statistical significance between groups was conducted by 2-tailed paired student's *t*-test. $P < 0.05$ was considered statistically significant, and all experiments repeated more than three times.

3. Results

3.1. DPF3 is significantly decreased in breast cancer tissues and closely associated with poor survival

To investigate the role of DPF3 in breast cancer, we firstly evaluated its expression level in the Cancer Genome Atlas (TCGA), which is the availably public dataset on cancer. The analysis showed that DPF3 is significantly decreased in breast cancer tissues compared with normal breast tissues (Fig. 1A). We also analyzed its level in paired tissues. As illustrated in Fig. 1B, the levels of DPF3 in the overwhelming majority of breast cancer tissues (T) is dramatically downregulated relative to that in corresponding adjacent normal tissues (ANT).

Subsequently, we checked the expression of DPF3 in fresh paired breast cancer tissues (N) and corresponding adjacent normal tissues (ANT). DPF3 is markedly downregulated in breast cancer tissues (N) compared with matched adjacent normal tissues (ANT) both on mRNA and protein level (Fig. 1C and D).

Moreover, we explored DPF3 mRNA prognostic value through the Kaplan-Meier plotter database (www.kmplot.com) in patients with breast cancer. Breast cancer patients were divided into high expression and low expression using the median values of DPF3 mRNA. The analysis demonstrated that decreased DPF3 indicated worse overall survival (OS; Fig. 2A) and poor relapse free survival (RFS; Fig. 2B).

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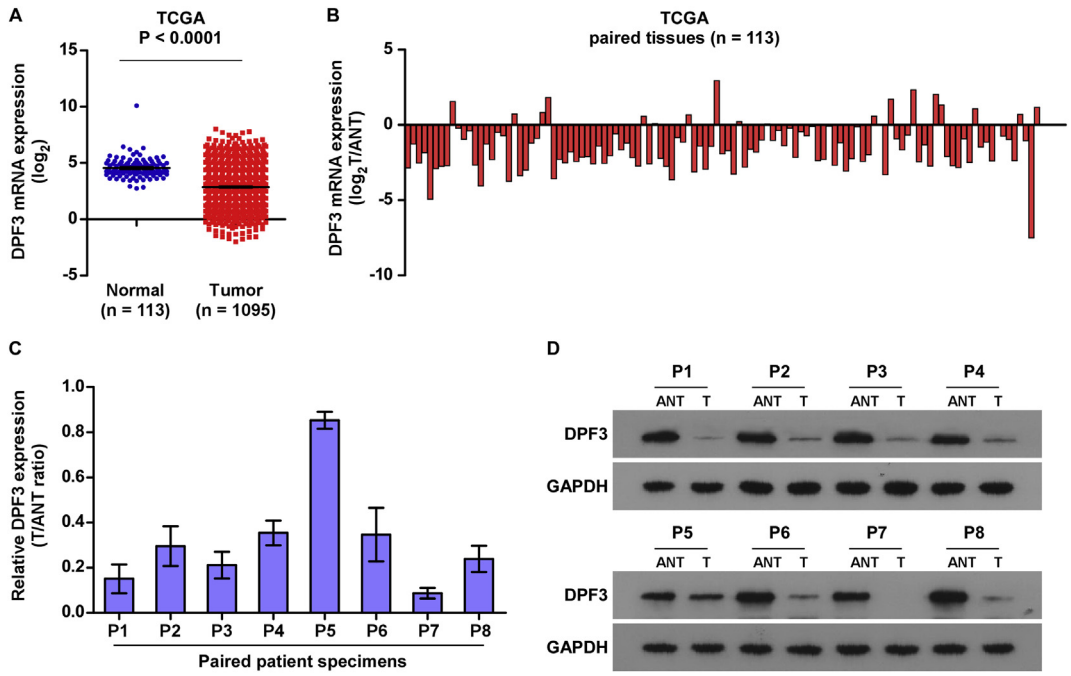

Fig. 1. DPF3 is significantly reduced in breast cancer tissues (A) The relative mRNA expression of DPF3 in normal breast tissues (Normal; n = 113) and breast cancer tissues (Tumor; n = 1095) by analyzing the publicly available dataset TCGA. (B) DPF3 mRNA expression are markedly reduced in breast cancer tissues (T) compared with that in matched adjacent normal tissues (ANT) using paired specimens by analyzing the publicly available dataset TCGA. (C) Relative DPF3 expression in paired fresh patient specimens by qPCR assay. (D) The protein expression of DPF3 in paired fresh patient specimens by western blotting assay.

Altogether, the levels of DPF3 is significantly decreased in breast cancer tissues and closely associated with poor relapse free survival and overall survival.

3.2. *Downregulation of DPF3 significantly promotes proliferation and motility of breast cancer cells*

Next, we investigated the biological function of DPF3 in breast cancer. Firstly, we established overexpressing or silencing DPF3 stable cell lines using breast cancer cell MCF-7 (Fig. 3A). Interestingly, we found that the mRNA expressions of cyclin D1, cyclin D3, cyclin E1 and CDK4 are significantly decreased in DPF3-upregulated cells, while dramatically increased in DPF3-downregulated cells (Fig. 4B), suggesting that DPF3 may be involved in proliferation of breast cancer cells. In accordance with that, MTT assay demonstrated that upregulation of DPF3 inhibits cell proliferation, but, inhibition of DPF3 exhibited the opposite outcome (Fig. 4C). Not only that, anchorage-independent growth assay showed that the colonies formed by DPF3-upregulated cells are significantly decreased both in number and size compared with that formed by control cells, while the colonies formed by DPF3-downregulated cells showed the opposite outcome (Fig. 4D).

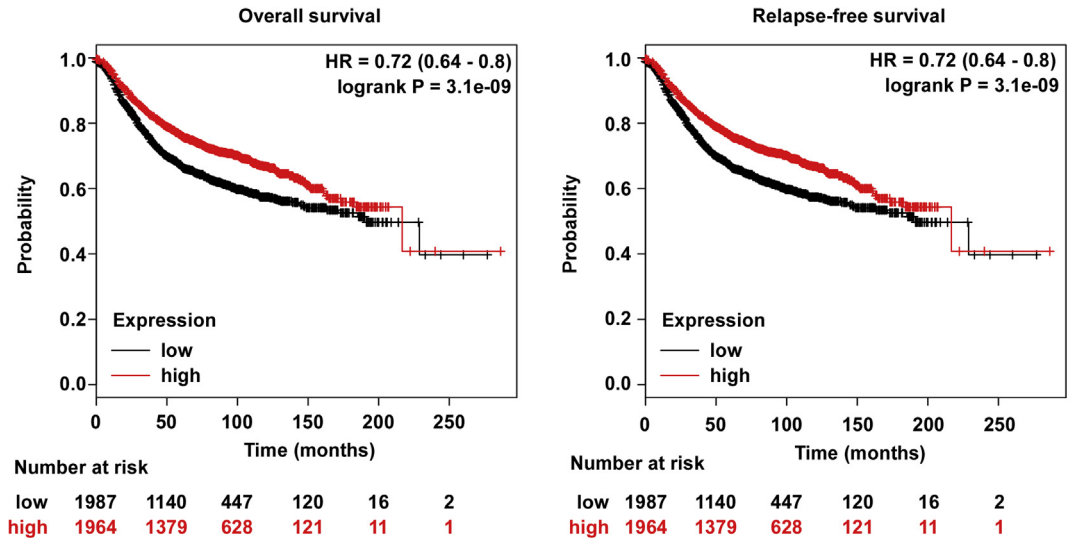

Fig. 2. Low expression of DPF3 correlated with poor survival of patients with breast cancer. (A) The overall survival of patients with breast cancer by analyzing the Kaplan-Meier plotter database. (B) The disease-free survival of patients with breast cancer by analyzing the Kaplan-Meier plotter database.

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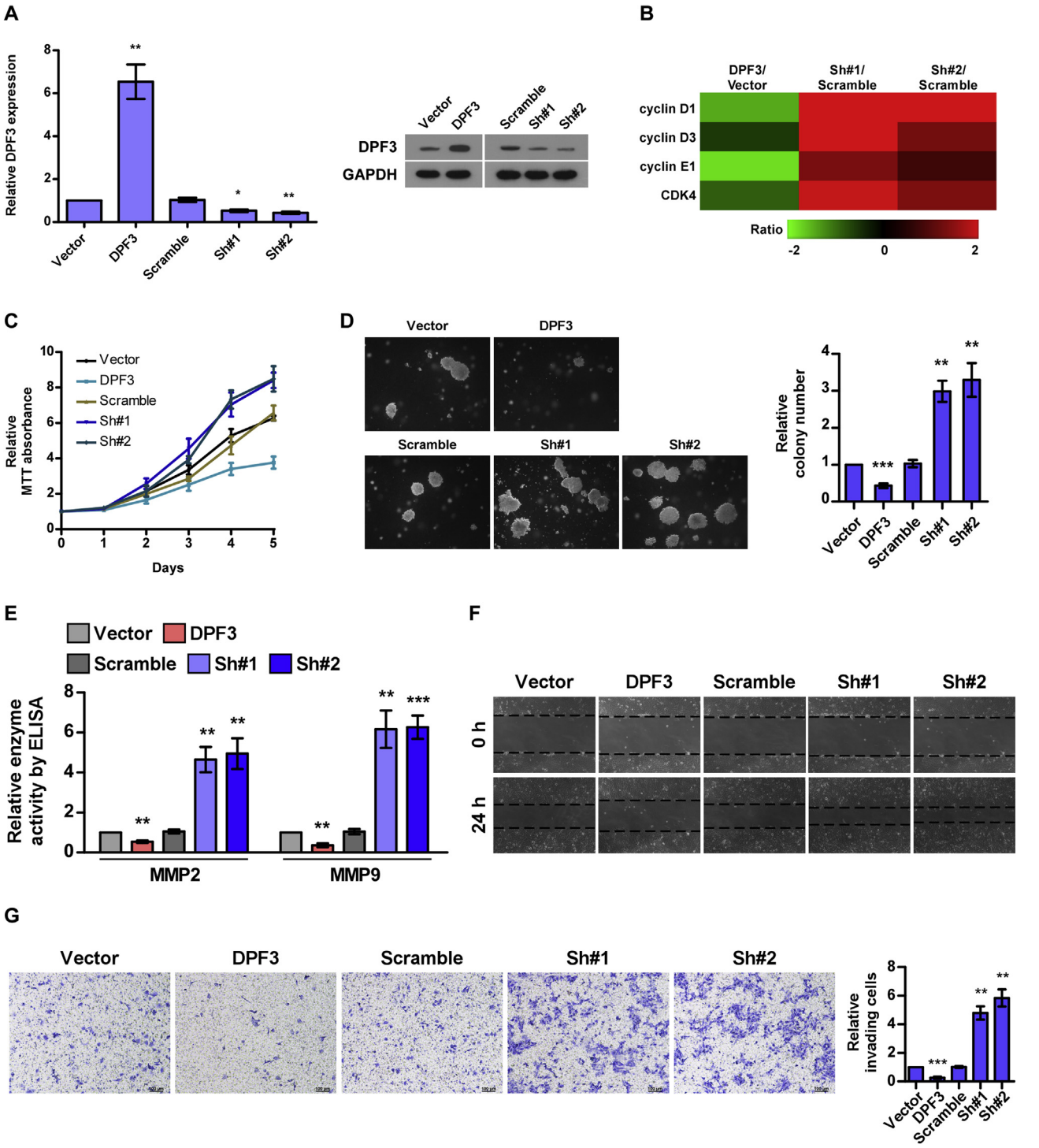

Fig. 3. Downregulation of DPF3 can promote the proliferation and motility of breast cancer cells. (A) The mRNA (left panel) and protein (right panel) expression of DPF3 in indicated stable cell lines. (B) Downregulation of DPF3 significantly elevates the mRNA expression of cyclin D1, cyclin D3, cyclin E1 and CDK4, but its overexpression induces the opposite outcome. (C) The capacity of different cells using MTT assay. (D) Representative images of colonies (left panel) and colony numbers (right panel) using anchorage-independent growth ability assay. (E) The enzyme activity of MMP2 and MMP9 in the culture supernatants was detected using ELISA. (F) Upregulation of DPF3 inhibits, while downregulation promotes the migration of breast cancer cells by wound healing assay. (G) Representative images of transwell (left panel) and invading cell numbers (right panel) using transwell invasion ability assay.*P < 0.05; **P < 0.001; ***P < 0.0001.

Moreover, the ELISA assay showed that the enzyme activity of matrix metalloproteinase-2 (MMP2) and −9 are both significantly inhibited in DPF3-upregulated cells, but markedly increased in DPF3-downregulated cells (Fig. 4E), suggesting that DPF3 may be involved in cell motility. Therefore, we examined the effect of DPF3 on cell motility using wound healing and transwell matrigel penetration assay. The wound healing assay showed that upregulation of DPF3 can inhibit, while downregulation significantly promotes the breast cancer migration (Fig. 4F). Similarly, transwell matrigel penetration assay demonstrated that the invasion ability is

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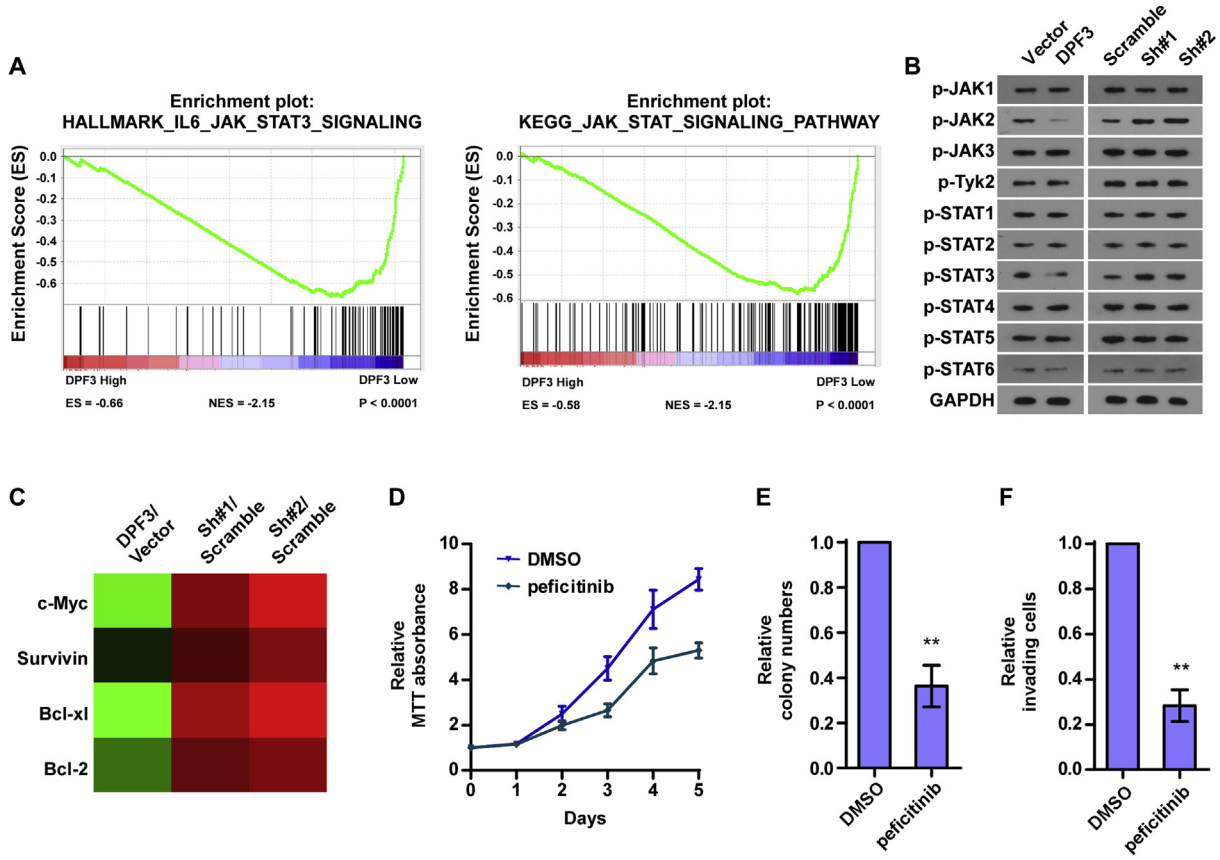

Fig. 4. Downregulation of DPF3 can activate the JAK2/STAT3 signaling. (A) GSEA illustrated that DPF3 may be involved in JAK/STAT signaling. (B) The phosphorylation levels of the main molecular, JAK1, JAK2, JAK3, Tyk2, STAT1, STAT2, STAT3, STAT4, STAT5 and STAT6, implicated in JAK/STAT signaling. Western blotting assay showed that downregulation of DPF3 activates the JAK2/STAT3 signaling. (C) The mRNA expression of JAK2/STAT3 signaling downstream genes. (D) MTT assay showed proliferation ability is significantly inhibited in cells treated with FLLL32, an inhibitor of JAK2/STAT3 signaling, compared with control using DPF3-silenced cells. (E) Wound healing assay showed migration ability is significantly inhibited in cells treated with FLLL32, an inhibitor of JAK2/STAT3 signaling, compared with control using DPF3-silenced cells. (F) Transwell invasion assay showed invasion ability is significantly inhibited in cells treated with FLLL32, an inhibitor of JAK2/STAT3 signaling, compared with control using DPF3-silenced cells. **P < 0.001.

significantly increased in DPF3-silenced cells, but suppressed in DPF3-upregulated cell (Fig. 4G).

Altogether, our results clarified that downregulation of DPF3 can significantly promote the capabilities for proliferation and invasion of breast cancer cells.

3.3. Downregulation of DPF3 hyperactivates the JAK2/STAT3 signaling

We further explored the underlying mechanisms of DPF3-mediated function. Gene set enrichment analysis (GSEA) illustrated that activated JAK/STAT gene sets are in the patients with low levels of DPF3 (Fig. 4A), suggesting that DPF3 may be involved in JAK/STAT signaling. Then, we detected the phosphorylation levels of the main molecular implicated in JAK/STAT signaling. As shown in Fig. 4B, only p-JAK2 and p-STAT3 have obvious changes among different cell lines, that is, overexpression of DPF3 significantly reduces the phosphorylation levels of JAK2 and STAT3, while its downregulation showed the opposite trend. The above mentioned results showed that downregulation of DPF3 can activate the JAK2/STAT3 signaling. We also examined the downstream genes expression of JAK2/STAT3 signaling. As demonstrated in Fig. 4C, the qPCR assay showed that *c*-Myc, Survival, Bcl-xl and Bcl-2 is significantly decreased in DPF3-upregulated cells, while substantially increased in DPF3-silenced cells. Finally, we used 5 μM FLLL32 (Selleck), a JK2/STAT3 inhibitor, to treat DPF3-silenced cells. MTT assay showed proliferation ability is significantly inhibited in cells treated with FLLL32 compared with control (Fig. 4D). Likewise, migration and invasion ability are also markedly suppressed in cells treated with FLLL32 compared with control (Fig. 4E and F).

Altogether, downregulation of DPF3 hyper-activates the JAK2/STAT3 signaling, and JAK2/STAT3 signaling is essential for down-regulation of DPF3 to take function in breast cancer progression.

4. Discussion

In the present study, we firstly investigated the expression and function of DPF3 on breast cancer progression. Our results showed that DPF3 levels are significantly reduced in breast cancer tissues. And cell function experiments clarified that downregulation of DPF3 markedly promotes the capabilities for proliferation and motility of breast cancer cells. Finally, the molecular experiments revealed that downregulation of DPF3 can activate the JAK2/STAT3 signaling.

The studies on DPF3 in cancers are poor. Hiramatsu et al. found that DPF3 takes an indispensable role in stemness maintenance of glioma initiating cells [11]. Theodorou et al. clarified that STAT5 can bind to the promoter of DPF3 to promote the progression of chronic lymphocytic leukemia [15]. In our study, we found that DPF3 is significantly decreased in breast cancer tissues. And down-regulation of DPF3 can activate the JAK2/STAT3 signaling. But, the detailed molecule mechanisms that down-regulation of DPF3

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activate this pathway is unclear. It has been reported that STAT5 can bind with DPF3 [16]. The STAT protein family structurally share five same domains. Therefore, we assume that STAT3 maybe bind with the promoter of DPF3 and also can interact with DPF3, which need to be checked in the future.

The STAT proteins take crucial function in multiple processed, including cell growth and differentiation, development, apoptosis, immune responses and inflammation [17,18]. The disorder of STAT signaling is involved in substantial human diseases, including cancer, autoimmune diseases [19,20]. Therefore, it is essential to exploit noteworthy therapeutic candidate to inhibit the hyperactivity of STAT signaling. We provided evidence that upregulation of DPF3 may be an effective method. But, more evidence needs to offer.

In conclusion, our study clarified that DPF3 takes an important in proliferation and motility of breast cancer cells, and may be served as a novel therapeutic target to therapy breast cancer.

Conflicts of interest

The authors declare no competing interests of this study.

Funding

This work was supported by Natural Science Foundation of China (No. 81602701); Natural Science Foundation of Guangdong Province (Nos. 2017A030313547, 2018A030313176); Science and Technology Projects Foundation of Guangdong Province (Nos. 2015A070710006, 2016A020215053, 2016A040403049, 2017A010105029); Science and Technology Projects Foundation of Guangzhou City (Nos. 201507020037, 201607010260); Scientific Research Projects of Guangdong Traditional Chinese Medicine Bureau (No. 20171056).

Acknowledgment

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.04.170.

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