Overexpression of POU3F2 promotes radioresistance in triple-negative breast cancer via Akt pathway activation

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Research Article

Abstract

Purpose: POU3F2 is associated with malignant behaviors and poor prognosis in cancer. However, the function and mechanism of POU3F2 in breast cancer remains to be elucidated. The purpose of our study was to explore the role of POU3F2 in triple-negative breast cancer and radiotherapy.

Methods: POU3F2 expression was examined by RT-PCR and Western Blot. Proliferation of cancer cells was measured by MTT assay. Migration of cancer cells was determined by Transwell assay and wound healing assay. To determine which proteins interact with POU3F2, co-IP was preformed. Survival analysis was performed by online database resources GEPIA and The Kaplan–Meier plotter. DNA damage after radiation was examined by Comet Assay. Radiosensetivity was evaluated with Clonogenic survival assays. Tumor xenograft was established with MDA-BA-231 breast cancer cells in BALB/c nude mice to explore the effect of POU3F2 *in vivo.*

Results: We found that the expression of POU3F2 was significantly elevated in breast cancer cells, and higher POU3F2 expression was related to poor prognosis of patients with breast cancer. Functional assays revealed that POU3F2 promoted proliferation, migration, and invasion of triple-negative breast cancer (TNBC) cells in vitro and in vivo. In addition, knock-down of POU3F2 decreased the radioresistance of TNBC cells in vitro. Furthermore, POU3F2 could enhance the activation of the Akt pathway by interacting with ARNT2, thereby promoting proliferation and radioresistance in TNBC cells.

Conclusions: Our results provide the first evidence that high expression of POU3F2 promotes radioresistance in triple negative breast cancer via Akt pathway activation by interacting with ARNT2.

Introduction

Breast cancer is the most prevalent cancer of females around the world, with nearly 2.3 million new cases per year, and accounts for 11.7% of all cancer cases[1]. Triple-negative breast cancer (TNBC), one of the worst subtype accounting for 15%-25% of all breast cancer, lacks expression of estrogen receptor (ER), progresterone receptor (PR), and HER2 [2], therefore patients with TNBC does not respond to endocrine therapy or HER2-targeting therapies. Though immune checkpoint inhibitors have benefited TNBC patients, a lower response rate and inevitable immune checkpoint inhibitor (ICI)-related complications also limit the clinical applications. Currently, radiotherapy (RT) is still commonly utilized and plays a pivotal role in the treatment of TNBC. Adjuvant RT decreased the 10year recurrence rate of TNBC from 35–19%, resulting in significant improvement in 15-year survival rates for patients with TNBC[3]. However, compared with other breast cancer subtypes, TNBC is more resistant to RT, which often leads to disease relapse and metastatic dissemination after RT. Therefore, there is a pressing need to identify underlying molecular mechanisms to overcome radiotherapy resistance in TNBC and biomarkers for early recognizations patients who are likely to develop radioresistance.

POU Class 3 Homeobox 2 (POU3F2), also namely BRN2, is a member of POU3 family of transcription factors that bind to an octameric DNA sequence[4]. POU3F2 forms a transcriptional regulatory complex

by cooperating with itself (via homo-dimerization) and other proteins, including TATA binding protein (TBP), the transcriptional coactivator, p300, and Sox-10 in melanocytic regulation[5]. POU3F2 plays a significant role in cellular differentiation[6], could induce neuroendocrine-specific transcription factors and is associated with cell viability[7]. Recently, it has been reported that POU3F2 is involved in the pathogenesis and treatment resistance of small cell lung cancer[8] [9], prostate cancer[10, 11], melanoaoma[12], and glioma [13]. Cook and Sturm reported that POU3F2 expression upregulated in melanoma was related to increased tumorigenesis and cancer cell growth. Sakaeda et al. observed that POU3F2 was highly expressed in small lung cancer cells when compared with non-small cell lung cancer. However, whether POU3F2 mediates radioresistance in TNBC remains unknown.

In the present study, by examining the expression of POU3F2 in breast cancer cell lines and human tumor samples, and exploring the function of POU3F2 in the proliferation, migration and invasion, and radioresistance of TNBC cells. We demontrated that POU3F2 could interact with ANRT2 and activate Akt signaling, resulting in increased proliferation and radioresistance of TNBC cells.

Materials And Methods

Cell lines and culture

MDA-MB-231, BT549, BT474, MCF7, MDA-MB-453, and 76N-F2V ,MCF-10A were purchased from ATCC. MDA-MB-231 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Hyclone).MCF-10A, BT549, BT474, MCF7, MDA-MB-453, and 76N-F2V were grown in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum. All cells were grown at 37°C in a incubator at 5% CO_2.

Quantitative real-time PCR analysis

Total RNA was isolated from all cancer cell lines using Trizol (Invitrogen), according to the manufacturer's protocol. Reverse transcription was performed using a PrimeScript® RT reagent kit (Takara). Subsequently, qPCR was carried out using SYBR®-Green I fluorescent dye (Takara). The qPCR primers were as follows:

POU3F2 forward: 5'-CGGCGGATCAAACTGGGATTT-3'

POU3F2 reverse: 5'-TTGCGCTGCGATCTTGTCTAT-3'

GAPDH forward: 5'-CTGTTGCTGTAGCCAAATTCGT-3'

GAPDH reverse: 5'-ACC CACTCCTCCACCTTTGAC-3'.

Western blot

Whole cell extracts were prepared using lysate buffer DS (Beyotime), and 1x protease inhibitors. Protein concentration was determined using a BCA protein assay (Cwbio). Immunoreactive bands on the

membranes were visualized using an ECL detection kit. Primary antibodies used in this study were as follows: anti-POU3F2 (Abcam), anti-GAPDH (CST), anti-FLAG (CST), anti-ANRT2 (CST), anti-pAkt (CST), anti-survivin (CST), anti-Bcl-2 (Abcam). Secondary antibodies (Cwbio).

Proliferation assay

Proliferation of cancer cells was measured by MTT assay. Briefly, cells were plated into 96-well plates (2×10^3 cells/well) in 200 μL of culture medium. After incubation for 24 hours, 48 hours, 72 hours, and 96 hours, the medium was removed, and 20 μl of MTT dye solution (Sigma-Aldrich) was added and incubated for 2 hours at 37°C. The cells were dissolved in DMSO, and the optical density (OD) was measured at 490 nm in a microplate spectrophotometer (Thermo Scientific).

Transwell assay and wound healing assay

For the transwell assay, cells (1×10^5 cells/μl) were resuspended in 200 μl of RPMI-1640 medium or DMEM and seeded in the upper chambers of transwell inserts (8 μm pore size, 24-well plates, Corning). The lower chambers were filled with 750 μl of RPMI-1640 medium or DMEM containing 10% FBS. After incubation, the migrated cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet at room temperature. The migrated cells were imaged with a microscope. For wound healing assay, cells (5×10^5 /well) were seeded into 6-well plates. When cells reached 100% confluence, sterile 200 μl pipette tips were used to scratch a straight line across the middle of the well. Detached cells were removed by PBS wash and the plates were incubated. A picture was taken to record the width at 0 hours, and after incubation for 12 hours the width of the scratch was measured and photographed.

Co-immunoprecipitation assays (CoIP)

Cells were harvested and washed with PBS three times. The clarified cell lysates were incubated for 16 h with the appropriate antibody at 4°C. A total of 30 μl of 50% slurry of protein A- and G-Sepharose (Amersham) was added. After incubation for 2 h at 4°C, the mixture was pelleted and washed several times with CoIP buffer. Each sample was analyzed by SDS-PAGE and immunoblotting with the appropriate antibodies.

Survival analysis

Survival analysis was performed using online database resources. GEPIA is an interactive online database that includes 9,736 tumors and 8,587 normal samples from TCGA and the GTEx projects, which analyses RNA sequencing expression. The Kaplan–Meier plotter webtool (http://kmplot.com/analysis/) was used to generate the survival plot.

Comet assay

Reagents and glue board were prepared according to the according to the manufacturer's protocol. Cells were harvested after 48 h in each condition and resuspended in 1% low-melting-point agarose (at 37°C). The agarose was allowed to gel at 4°C and the coverslip removed. Cells were lysed overnight in Alkaline Lysis (A1 lysis solution) solution supplemented with 1% triton. After lysis of the cells (typically 1 to 2

hours at 4°C) the slides were washed in distilled water to remove all salts and immersed in an electrophoresis solution.

Cells transfection and lentivirus transduction

Cells were seeded in 6-well plates and grown to 70–90% confluency at transfection. Opti-MEM (Gibco) was diluted according to the manufacturer's protocol. Then DNA was diluted into Opti-MEM medium and the DNA was added to the Lipofectamine3000 Reagent (Gibco). After incubation for 5 minutes, the DNA-liquid complexes were added to each well. Transfected cells were analyzed for gene expression two or three days later. siRNA was purchased from Invitrogen. For lentivirus packaging, transduction, and screening of stable cell lines, the whole procedure was conducted as previously detailed[14].

Clonogenic survival assays

Cells were seeded in plates, and were then exposed to radiation at the indicated doses (Varian2300EX). After incubation for 14 days, the cells were fixed and stained with 4% paraformaldehyde and 1% crystal violet. Colonies with more than 50 cells were counted by microscopy. Survival curves were generated using the multitarget single-hit model.

Animal study

Athymic nude mice (6-week-old female) were purchased from Guangdong Medical Laboratory Animal Center. MDA-MB-231 cells stably transduced with lentivirus or control were subcutaneously injected into the left mammary fat pads of 6-week-old female nude mice (n = 6 peer group). All mice were feeding in a standard SPF rearing center and experiments were approved by the Institutional Animal Care and Use Committee of Nanfang Hospital and obied the rules required by Guide for the Care and Use of Laboratory Animals.

Statistic

Data are expressed as mean ± standard deviation. Statistical comparisons were conducted using t-tests in SPSS software (version 26.0), followed by LSD test. A p-value of $+ < 0.05$ was considered to indicate a statistically significant difference.

Results

POU3F2 expression is upregulated in breast cancer and related to poor prognosis in breast cancer patients.

The expression of POU3F2 in human normal mammary epithelial cells, including MCF-10A and 76N-F2V, and five human breast cancer cell lines was assessed by qPCR and western blot. POU3F2 was upregulated at the mRNA (Fig. 1A) and protein levels (Fig. 1B) in breast cancer cell lines, particularly in the TNBC cell lines (BT549 and MDA-MB-231). In addition, we collected 30 breast cancer samples and 30 adjacent normal samples and evaluated the expression of POU3F2. We found that POU3F2 expression was increased more than ten-fold tumor tissue compared with normal adjacent breast tissue (Fig. 1C).

Consistent with the gene expression, the expression of POU3F2 protein levels were significantly higher in tumor samples than normal breast tissue (Fig. 1D). Given the high expression of POU3F2 in tumor samples, we speculated that POU3F2 might be prognostic factor of survival for breast cancer patients. We used KMplot [15] and GEPIA [16] to plot Kaplan-Meier survival curves, and found that high expression of POU3F2 was related to poor clinical outcome (p = 0.0028 and p = 0.016, respectively) (Fig. 1E and F). These data show that expression of POU3F2 was not only increased in breast cancer cell lines and breast tumor specimens, but that high expression of POU3F2 was associated with poor overall survival.

POU3F2 promote proliferation, migration, and invasion of TNBC cells

To investigate the biological functions of POU3F2 in TNBC cells, we transiently knocked down POU3F2 expression using siRNA (Fig.S1A and B), and overexpressed POU3F2 by transfecting cells with a POU3F2 overexpression plasmid (Fig.S1C and D). Knockdown of POU3F2 inhibited the proliferation of MDA-MB-231 and BT549 cells (Fig. 2A and B). POU3F2 overexpression enhanced proliferation in 76N-F2V and MCF-10A cells (Fig. 2C and D). To investigate the effect of short-term knockdown of POU3F2 on cell migration and invasion, we performed wound healing assays and transwell assays. In wound healing assays, we observed slower migration in MBA -MD-231 and BT495 cells with POU3F2 knockdown, when compared with control groups (Fig. 2E and F). Similarly in transwell assays, the number of POU3F2 knockdown MDA-MB-231 and BT495 cells in lower chamber were fewer than those of the control groups (Fig. 2G and H). These data demonstrated that POU3F2 facilitates the proliferation, migration, and invasion of TNBC cells. Next, we investigated whether overexpression of POU3F2 in MDA-MB-231 cells could enhance tumor growth in vivo. We subcutaneously inoculated nude mice with MDA-MB-231 cells infected with POU3F2-overexpressing lentivirus or control lentivirus. In line with our results in vitro, the POU3F2 overexpressing MDA-MB-231 tumors exhibited increased tumor growth, compared with the control group (Fig. 2I and J). There was also a significant increase in tumor weight in the POU3F2 overexpression groups (Fig. 2K). These results suggested that POU3F2 could promote proliferation, migration, and invasion in breast cancer cells and enhanced tumor growth in vivo.

POU3F2 enhances radioresistance of TNBC cells in virtro

TNBC often exhibits radioresistance. Transcription factors, such as STAT3[17]and NF-κB[18], are involved in cancer radioresistance[19]. Currently, the role of POU3F2 in radioresistance is unknown. To explore the effects of POU3F2 on radioresistance of MDA-MB-231 and BT549 cells, we irradiated cells with varying x-ray doses and performed colony forming assays. Compared with the vector control cells, MDA-MB-231 and BT549 cells with POU3F2 overexpression showed increased surviving fraction, indicating increased radioresistance (Fig. 3A and B). We performed a similar assay in which POU3F2 was knocked down in MDA-MB-231 and BT549 cells. POU3F2 knockdown reduced the surviving fraction, indicating a decrease in radioresistance (Fig. 3C and D). These results suggested that POU3F2 affects the sensitivity to radiotherapy in TNBC cells. Because ionizing radiation kills cancer cells mainly by means of damaging DNA[20], we examined weather short-term knockdown of POU3F2 in MDA-MB-231 and BT549 cells

affected DNA damage. Comet assays were performed to assess DNA damage in these cells, and we observed that POU3F2 knockdown in MDA-MB-231 and BT549 cells resulted in more DNA damage than the control groups (Fig. 3E and F). When DNA gets damaged and double-strand breaks occur, cells initiate repair mechanisms. H2AX phosphorylation is a classical marker of DNA double-stranded breaks[21]. To further confirm DNA damage at the protein level, we evaluated γ-H2AX expression when these cells were exposed to radiation. Two hours after irradiation, we found that γ-H2AX expression was increased in MDA-MB-231 and BT495 cells with POU3F2 knockdown, compar3F2 in vivo,we The results showed that ed to that of control groups (Fig. 3G and H). These results suggested that POU3F2 mediates radioresistance of TNBC cells.

POU3F2 promotes proliferation and radioresistance by interacting with Aryl hydrocarbon receptor nuclear translocator 2

Previous studies have suggested that protein-protein interaction are important in mediating responses to radiotherapy[22].We speculated that POU3F2 might interact with other proteins to mediate radioresistance in TNBC. We screened several proteins that could interact with POU3F2 by immunoprecipitation (IP) and mass spectrometry analysis. To further determine which proteins interact with POUF2, we conducted co-IP and found that POU3F2 binds to aryl hydrocarbon receptor nuclear translocator 2 (ARNT2, Fig. 4A). ARNT2 has been correlated with tumor progression, and has been reported to act as an anti-tumor gene[23, 24]. To determine the role of ARNT2 role in TNBC cells, pAKT, survivin, and BCL-2 were evaluated by western blot. Overexpression of ARNT2 inhibited Akt phosphorylation and the activation of signaling downstream of Akt (Fig. 4B). In addition, we observed Akt and its down stream signaling could be activated by POU3F2 overexpression (Fig. 4C). To explore whether POU3F2 could mediate radioresistance by binding to ARNT2, we conducted the corresponding rescue experiments. Overexpression of ARNT2 reduced the increased proliferation capability (Fig. 4D and E) and increased surviving fraction (Fig. 4F and G) in TNBC cells with POU3F2 overexpression. These results suggest that POU3F2 mediates radioresistance through the AKT pathway by interacting with ARNT2.

Discussion

TNBC is the most aggressive subtype of breast cancer. Compared with non-TNBC tumors, TNBC often presents with larger tumor size, increased tumor grade, and higher proliferation index when diagnosed[25]. Despite the initial response of TNBC to treatment, about 30% patients will develop tumor relapse and 20% of patients will succumb to TNBC metastasis within five years[26]. It is urgent to illuminate underlying molecular mechanism mediating radioresistance and identify potential targets for TNBC to improve radiosensitivity. In this study, we found that the expression of POU3F2 was closely related to progression of TNBC, and it could be used as a biomarker of radioresistance and a potential target to develop novel therapeutic strategies for TNBCs.

POU3F2 is a reprogramming transcription factor that regulates cellular differentiation, contributes to tuomr progression and induces stem-like cancer cells in different kinds of tumors, such as glioblastoma, melanoma and small cell lung cancer. Here, our results showed that POU3F2 not only promoted proliferation, migration, and invasion, but also mediated radioresistance in TNBC. Previous studyies had demonstrated that POU3F2 formed a transcriptional regulatory complex by cooperating with itself and other proteins, including TATA binding protein (TBP), the transcriptional coactivator, p300, and Sox-10 in melanocytic regulation[5]. By co-IP experiment, we found that POU3F2 could bind to ARNT2 protein that has been reported to act as an anti-tumor gene [23, 24]. ARNT 2 is a transcription factor related to adaptive responses against cellular stress from xenobiotic substances[27]. The expression level of ARNT2 mRNA was positively correlated with favorable prognosis of breast cancer, and the presence of ARNT2 was significantly correlated with the smaller tumor size and improved 5-year survival rate after breast cancer diagnosis[28]. In the present study, our results provide the evidence that low expression of ARNT2 due to POU3F2-was associated with radioresistance in TNBC.

The Akt pathway contributes to many cellular functions in cancer, including survival, apoptosis, and proliferation[29]. It can be activated by irradiation and involved in mediating radioresistance in many tumor types, including glioma[30], prostate cancer[31], and lung cancer[32]. Growing evidence revealed that the Akt serving as key factor played a key role in prostate cancer progression and radiation resistance by activating pathway proteins or through mutations in the pathway[33]. Blocking PI3K/Akt signaling by inhibiting EGFR could improve radiosensitivity and affect tumor growth through a variety of mechanisms, including inhibition of neovascularization through VEGF[34]. It has been reported that ARNT2 inhibits cancer cell proliferation via negatively regulating the Akt pathway[35]. In this research, our data showed that POU3F2 overexpression could increase the viability of TNBC cell lines by activating the Akt pathway upon irradiation. Meanwhile, ARNT2 overexpression in these cells reduced Akt activation. Thus, we speculated that POU3F2 might prevent the functions of ARNT2 by interacting directly with the ARNT2 protein, and later inhibiting the activation of Akt pathway. However, the specific binding sites of POU3F2 and ARNT2 are not defined, and additional investigation is needed to identify the specific binding sites.

In conclusion, POU3F2 was upregulated in TNBC cell lines, and higher expression of POU3F2 was prognostic of poor outcome in patients with TNBC. Mechanistically, POU3F2 could enhance the activation of the Akt pathway by interacting ARNT2, thereby promoting proliferation and radioresistance in TNBC cells. Our data provides novel insight into the radioresistance of TNBC, and suggest that POU3F2 may be considered as a novel biomarker for prognosis of patients with TNBC. Targeting POU3F2 could also be potential strategy to overcome radioresistance to improve survival outcomes for patients with TNBC.

Declarations

FUNDING INFORMATION

DISCLOSURE

The authors declare no conflict of interest.

ETHICS STATEMENT

All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanfang Hospital and obied the rules required by Guide for the Care and Use of Laboratory Animals.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A

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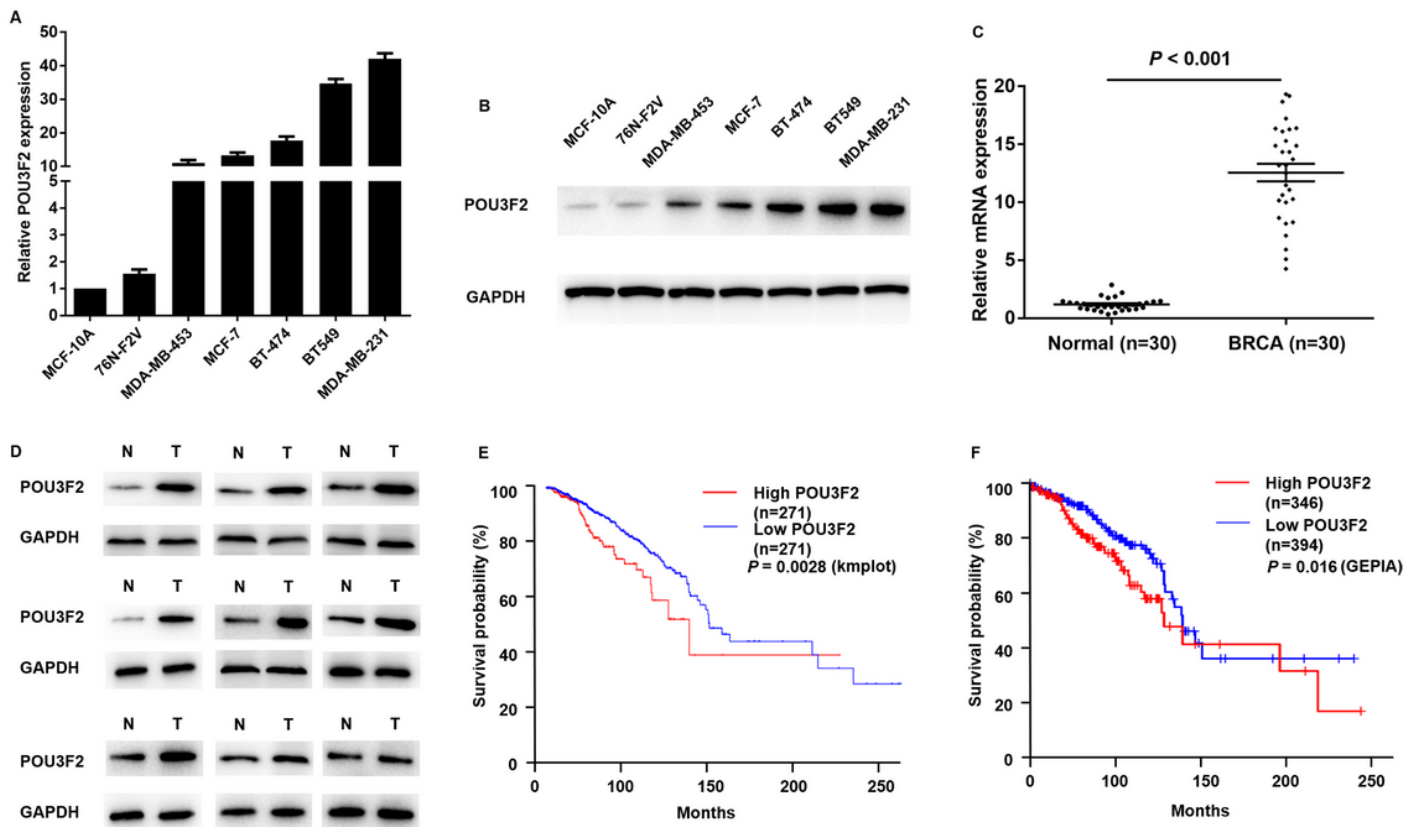
Figures

Figure 1

Figure 1

POU3F2 is upregulated in breast cancer. (A) Real time PCR analysis of POU3F2 expression in breast cancer cell lines. Data represents the mean ± SD three replicates. (B) Western blot analysis of POU3F2 expressiom in breast cancer cell lines. (C)POU3F2 expression levels in para-tumor mammary tissues (n = 30) and tumor tissues (n = 30) assessed by RT-PCR. (D) Western blot analysis of POU3F2 in tumor and non-tumor tissue. (E-F) Kaplan−Meier survival analysis of overall survival of patients with high (n=271) and low (n=271) expression of POU3F2 by KMplot (E) and GEPIA (F).
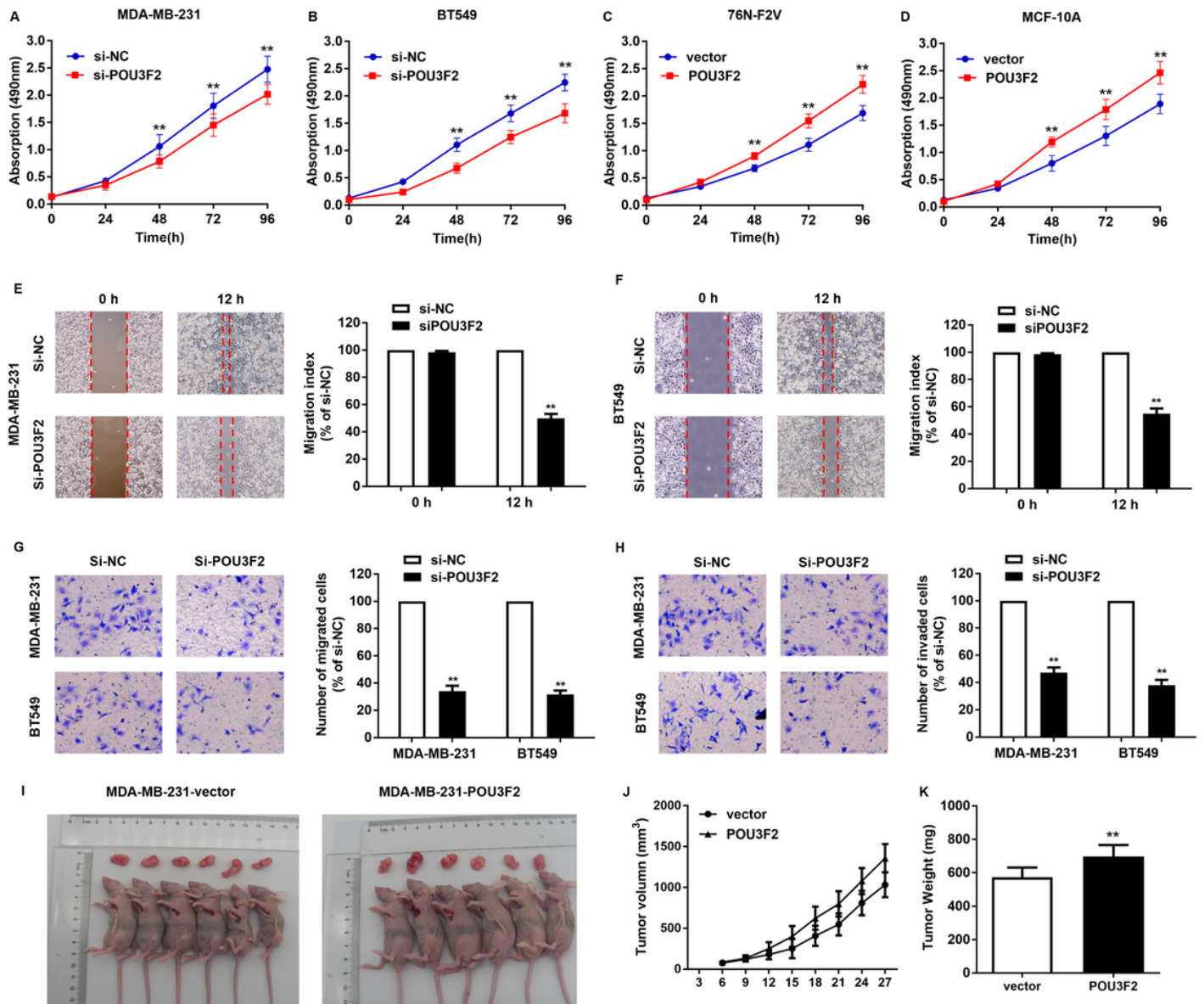
Figure 2

Figure 2

POU3F2 promotes cell proliferation and migration in vivo and in vitro.

(A-D) Proliferation of MDA-MB-231 and BT549 cells with knock-down of POU3F2 was assayed by MTT. (D-F) Wound healing assays were performed to examine the effect of POU3F2 on cell migration; ** $p < 0.01$. (G-H) Transwell assay. (I) Representative images of tumors from each group mice (n = 6), on the 21st day after inoculation when the tumor volume in the vector group reached approximately 500 mm^3. (J) Tumor volume growth curves in each group (n = 6). (E) Tumor weight was determined after euthanasia; ** $p < 0.01$.
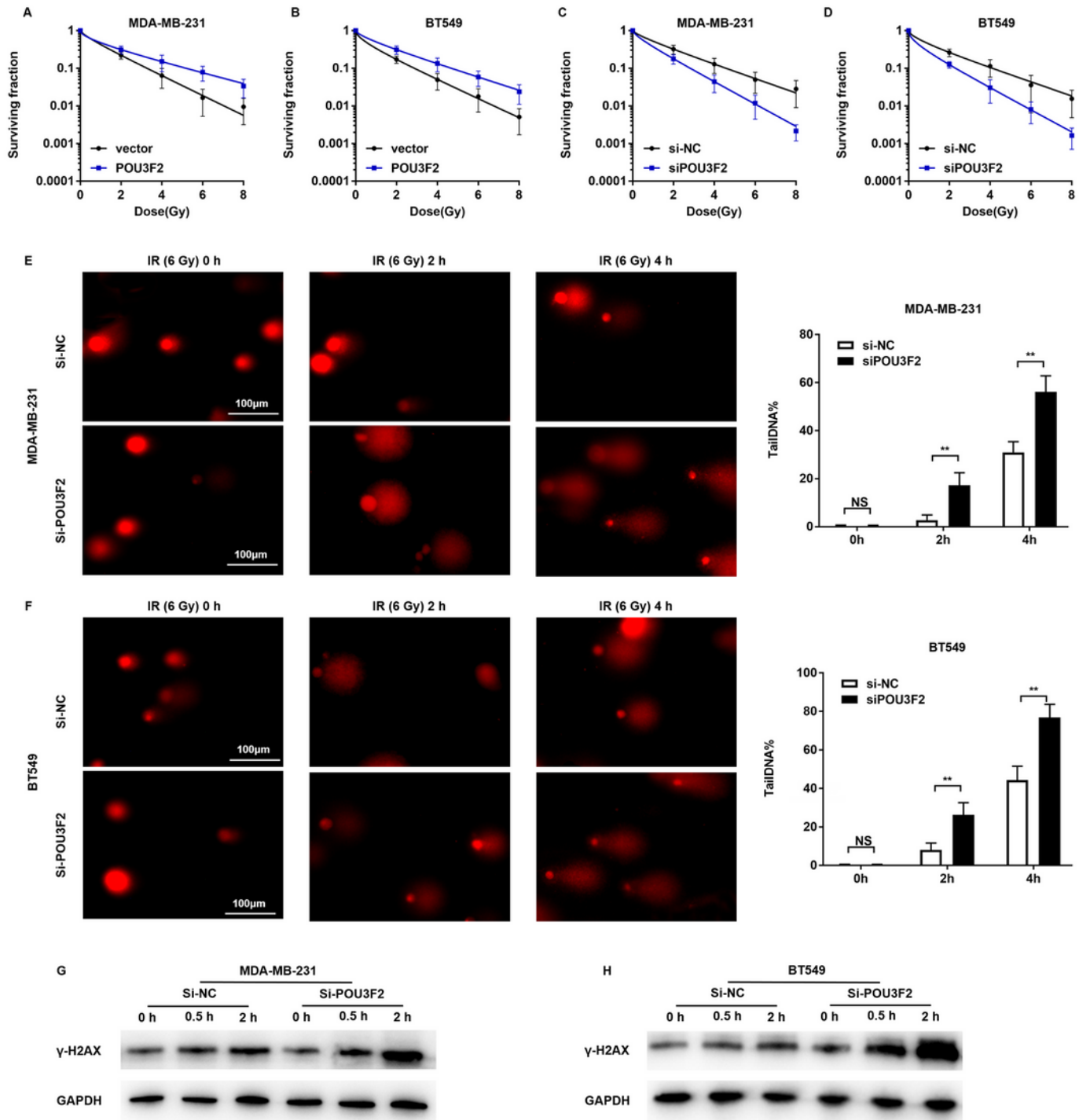
Figure 3

Figure 3

POU3F2 is related to radioresistance in TNBC in vitro. (A-B) Clonogenic survival assays of MB-MDA-231 and BT cells transfected with vector or POU3F2 overexpression plasmid. (C-D). Clonogenic survival assays of MB-MDA-231 and BT459 cells transfected with non-targeting control or POU3F2 siRNA. (E-F) DNA damage was analyzed by Comet assay; representative images. (E) MDA-MB-231 cells were transfected with non-targeting control or POU3F2 siRNA. (F) BT-549 cells were transfected with non-

targeting control or POU3F2 siRNA; ** p < 0.01). (G-H) Western blot analysis of γH2AX in MB-MDA-231 (G) and BT549 (H) with transient knockdown of POU3F2, followed by 6 Gy irradiation (IR) at indicated time points.
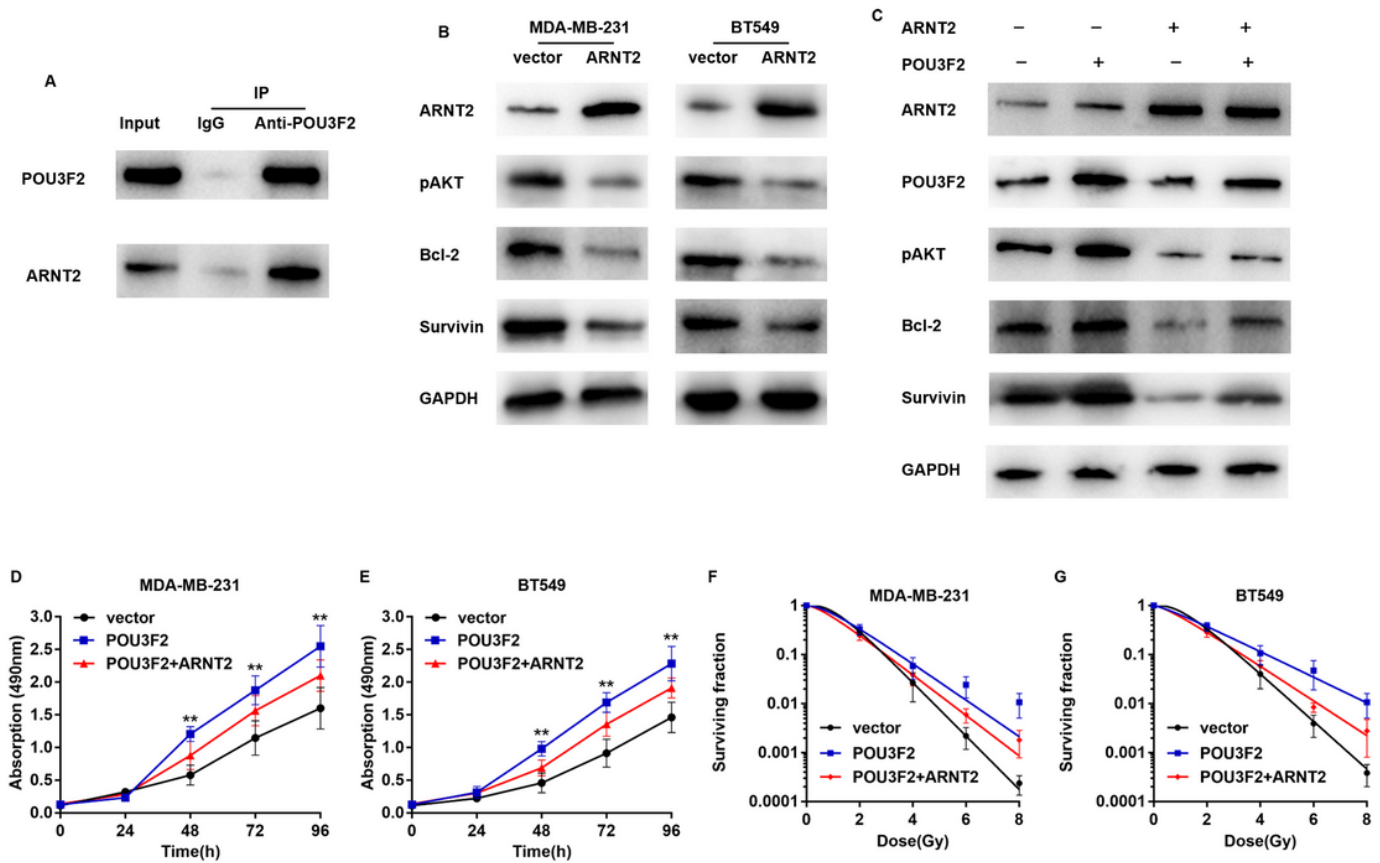

Figure 4

Figure 4

Underlying mechanism of *POU3F2* in radioresistance of TNBC. (A) Western blot analysis of POU3F2 interaction with *ARNT2*. (B) Western blot analysis of pAKT, BCL2, survin, and GAPDH in ANRT2 knockdown cells and control cells. (C) Western blot analysis of ARNT2, POU3F2, pAKT, BCL2, and survin in cells with overexpression of ARNT2 and/or overexpression of POU3F2. (D, E) MTT assays of (D) MDA-MB-231 cells and (E) BT549 cells with overexpression of POU3F2 +/- overexpression of ARNT2 (F-G) Clonogenic survival assay of (F) MDA-MB-231 cells and (G) BT549 cells with overexpression of POU3F2 +/- overexpression of ARNT2; ** p < 0.01.

Supplementary Files

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- Supplementalmaterials.doc