Int J Clin Exp Med 2016;9(6):10018-10027
www.ijcem.com /ISSN:1940-5901/IJCEM0025309

Original Article

USP7 promotes tumorigenesis in breast cancer and enhances MRI detection

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Received January 31, 2016; Accepted May 1, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: Epithelial mesenchymal transition (EMT) is a complex process in cancer cell development, special in breast cancer development. It involved in cell growth, invasion, metastasis and so on. EMT is regulated by multiple mechanism, although there are numerous reports about how EMT occur, the detail mechanism still unknown. Here, we showed that the deubiquitinating enzyme, USP7 is high expression in breast cancer and positive associate with lymph node metastasis and a poor prognosis. We next performed the mass spectrometry and co-IP assays to detect USP7 can physically associated with Snail, a transcription factor participate EMT process. We also found USP7 can promote EMT through deubiquitinating Snail, thereby stabilize Snail, and increase cancer cell invasion and metastasis ability. Interesting, USP7 also enhances MRI detection. In brief, our experiments revealed a novel mechanism of USP7 in facilitating tumorigenesis and metastasis of breast cancer cells, suggesting that USP7 might be a potential therapeutic target for treating breast cancer.

Keywords: USP7, EMT, Snail, MRI

Introduction

Post-translational modification of proteins have been confirmed play an important role in cancer development. Such as methylation, acetylation, ubiquitination and so on. Several protein can be ubiquitinated, it serves as a crucial cellular regulatory event. Its function to control the activity and stability of proteins [1]. Ubiquitination is mostly regulated by E3 ubiquitin ligases which have specificity [2]. However, ubiquitination is counterbalance by deubiquitinating enzymes that reverse, the action of E3 ubiquitin ligases [3, 4]. But how these two opposing work control of biological processes and cellular signaling have not been fully resolved.

Ubiquitin-specific protease 7 (USP7) is a deubiquitinate enzyme that can cleave K48-linked and K63-linked ubiquitin chains [5-7]. USP7 also regulates the ubiquitination of many proteins, such as XPC protein which involved in genome nucleotide excision repair or UV-induced PCNA [8]. Furthermore, USP7 also regulate the MDM2-p53 pathway which impacts numerous cellular and pathophysiological processes, including immune responses, transcription, DNA repair and cancer [5, 8].

EMT, short for Epithelial mesenchymal transition, is a complex biological processwhich result inepithelial cells obtaining myofibroblastic features and have a mesenchymal phenotype [9]. Eventually, tumor have strong invasion and metastasis ability [10]. The distinct characterize of EMT is decreased expression levels of epithelial marker, such as E-cadherin, and increased expression levels of mesenchymal marker, such as N-cadherin. Transcription factor play a crucial role in EMT. Snail has been reported that promote EMT process. It is a zinc-finger transcription factor and associated with lymph node metastasis and a poor prognosis in Breast carcinoma patients.

Here we investigated the molecular mechanism of USP7 in breast carcinoma. We found USP7 not only high expression, but also associated with lymph node metastasis and a poor prognosis. Meanwhile, we demonstrated USP7 also promote EMT through stabilize Snail. Our results reveal a novel mechanism of USP7 pro-

mote EMT, and found USP7 as a potential biomarker for breast cancer. Interesting, USP7 also enhances MRI detection.

Materials and methods

Cell culture, reagents, and antibodies

Human breast cancer cells, MCF-7 and MDA-MB-231, and human normal breast cells, MCF-10A were purchased from ATCC (USA). Cells were culture at 37°C with 5% CO_2 in DMEM contained with 10% fetal bovine serum (FBS) and 2 U/mL penicillin G, 2 mg/mL streptomycin. Proteasome inhibitor, MG132 was obtained from Sigma-Aldrich (USA). Antibodies against human USP7 was purchased from Sigma-Aldrich (USA). EMT Antibody Kit was obtain from Cell Signaling Technology. Anti-β-Actin antibodies were purchased from Abcam (UK).

Immunopurification and mass spectrometry

Stably expressing FLAG-USP7 in MCF-7 cells, forty-eight hours after transfection, lysate MCF-7 cells and applied to an equilibrated FLAG column following the manufacturer's suggestions. Washed the column and eluted protein complex by FLAG peptides (Sigma). The eluted solution resolved on SDS-PAGE, using silver stained kit to stain gel, splice special band and subjected to LC-MS/MS sequencing.

Cell invasion assay

Using Matrigel coated transwtell chamber filters (Chemicon Incorporation). After transfected with shRNA, MDA-MB-231 cells were starved for 24 hr in DMEM, and placed. 3×10^5 of cells in 500 μl serum free media to the upper chamber of the transwell. The chamber was then transferred to a well containing 500 μl of DMEM containing 10% FBS (fetal bovine serum). Cells were incubated for 24 hr at 37°C. Used cotton swabs removed the cells in the top membrane, and stained with 0.5% crystal violetand counted the remaining cells.

Western blotting

Extract total proteins from cell lysates and boil with SDS/PAGE loading buffer for 10 min. Then resolved using 10% SDS/PAGE gels and transferred onto PVDF membranes. For Western blot analysis, membranes first blocked with 5% dried skimmed milk at 37°C for 1 hr, then incubation with primary antibody at 4°C overnight or for 1 hr at room temperature followed by incubation with a secondary antibody. The blots were visualized using ECL reagent (Immunocruz, Santa Cruz biotechnology) on X-ray films according to the manufacturer's recommendation. β-actin antibodies was used as control.

GST pull-down assay

GST pull-down assaywas performed as previously described [11]. In short, use 50 μl of 50% glutathione-Sepharose 4B beads (Amersham Biosciences) to immobilized equal amounts of GST fusion proteins in 1 mL of GST pull-down binding buffer (10 mM Hepes, 3 mM $MgCl_2$, 100 mM KCl, pH 7.6, 5 mM EDTA, 5% glycerol, 3 mM $MgCl_2$, 0.5% CA630). Incubated for 90 min at 4°C with rotation, used GST pull-down binding buffer washed beads 4 times and resuspended adding 5 μl of in vitro transcribed/translated Snail for 2 h at 4°C with rotation. The beads were subsequently washed with ice-cold PBS. The beads then boiling in 30 μl of loading buffer to eluted bound proteins and resolved on SDS/PAGE.

qRT-PCR assay

Extracted total RNA from cells with the RNeasy Mini Kit (QIAGEN) following the manufacturer's protocol and then using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) to synthesis cDNA. The qRT-PCR was used to analyze resulting cDNA.

All qRT-PCR assay were repeated at least three times.

As follows are the primers of E-cadherin: 5'-GAAATCACATCCTACACTGCCC-3' and 5'-GTAGCAACTGGAGAACCATTGTC-3'; a-catenin-5'-TGTTACACAGGTTACAACCCT-3' and 5'-GATCATCTGCGAACTCTCCT-3'; N-cadherin-5'-TCAAAGCCTGGAACATATGTG-3' and 5'-GTTCAGGTAATCATAGTCCTGCT-3'; Fibronectin: 5'-CAATGTGAACGACACATTCCA-3' and 5'-ACCACTTGAGCTTGGATAGG-3'.

Immunohistochemistry (IHC)

Formalin-fixed and paraffin embedded (FFPE) breast tissue samples from patients with breast carcinoma. All the samples were

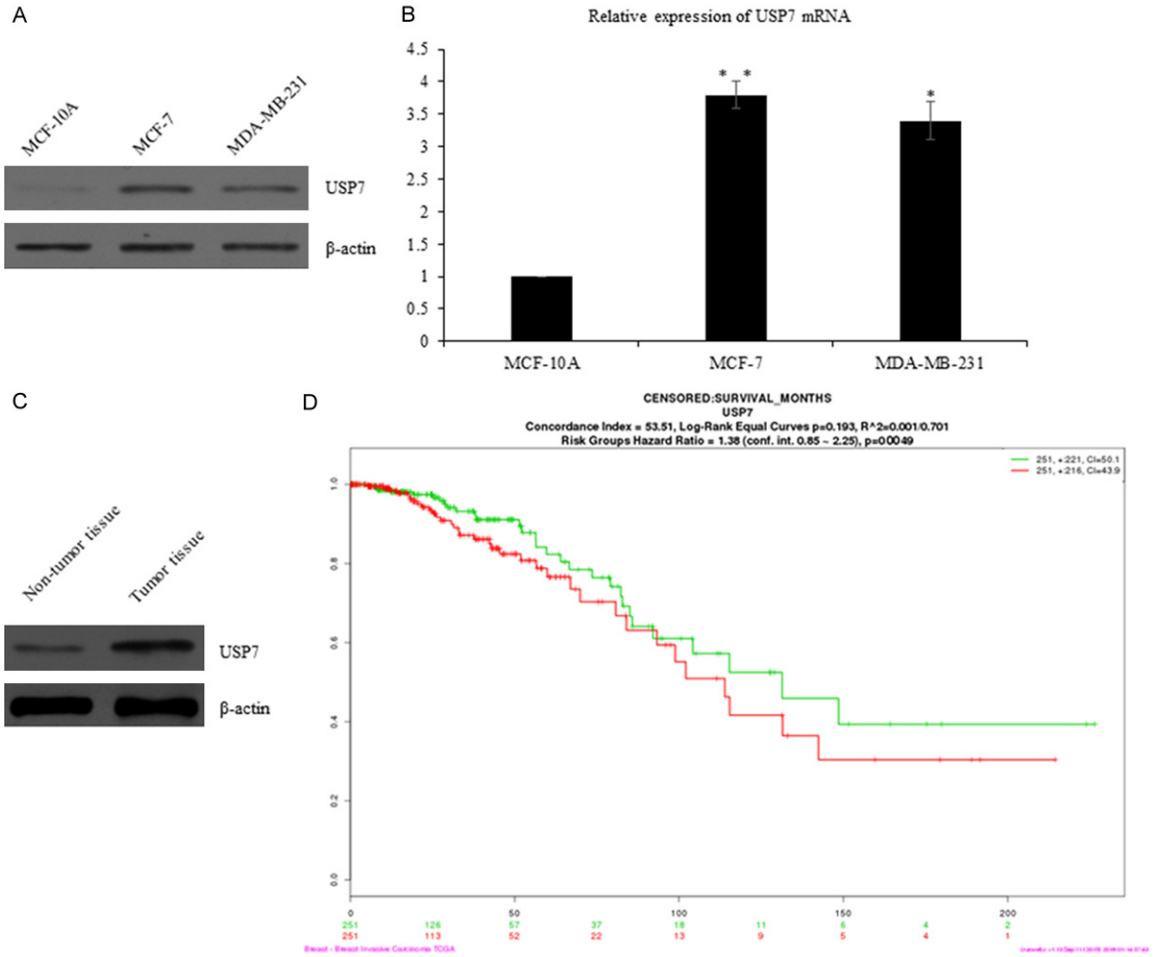

Figure 1. USP7 is High Expression in Breast Carcinoma and Positively Correlated with Poor Breast Carcinoma Prognosis. A. Western blot was used to detect the protein level of USP7 in human normal breast carcinoma cells MCF-10A and breast carcinoma cells MCF-7 or MDA-MB-231. B. Detected USP7 mRNA level in breast carcinoma cells by qRT-PCR. C. Measure the protein expression of USP7 between tumor tissue and non-tumor tissue from breast carcinoma patients by Western blot. D. The online tool analysis the relationship between USP7 and survival curves.

reviewed by three pathologists, and staged classified were according to the UICC-TNM classification. Breast specimens were collected between 2013 and 2015. 188 breast carcinoma tissues and adjacent normal tissues were enrolled for IHC. Then samples were de-paraffinized using xylene and then rehydrated through graded alcohols to water. Using 0.5% v/v hydrogen peroxide/methanol for 10 minutes to neutralized endogenous peroxidase. Wash slides in water twice. Antigen retrieval was carried out using 0.01 M citrate retrievalbuffer, and next blocked for 10 minutes using 10% normal goat serum in PBS. Sections were incubated with primary polyclonal antibodies anti-USP7 overnight at 4°C. At last, DAB was used to detection of bound antibody.

Statistical analysis

SPSS V.18.0 was used for statistical analysis. Results are reported as mean ± S.D. The chi-square test was used to examine. The various clinicopathological characteristics of USP7 expression between cancer and adjacent normal tissue were examined by chi-square test. The statistical significance was considered at a value of $P<0.05$.

Result

Usp7 is high expression in breast carcinoma and positively correlated with poor breast carcinoma prognosis

USP7 has been found high expression in different cancer and role a crucial function, to invest

Table 1. Clinicopathologic variables in 188 breast carcinoma patients

Variables	No. (n=188)	USP7 protein expression		*P* value
		Low (n=64)	High (n=124)	
Smoking				
<60	76	25	51	0.784
≥60	112	39	73	
Age				
<30	100	30	70	0.212
≥30	88	34	54	
Tumor size				
Small (≤3 cm)	87	50	37	0.001
Large (≥3 cm)	101	14	87	
Lymph node metastasis				
No	89	41	36	0.001
Yes	99	23	88	
Pathological grade				
I-II	82	52	30	0.001
III-IV	106	12	94	
Differentiation				
Well/moderate	84	24	60	0.155
Poor	104	40	64	

whether USP7 has a role in breast carcinoma, we used qRT-PCR and Western Blot to assess the mRNA and protein abundance of USP7 in human normal breast tissue cells MCF-10A and breast carcinoma cells MCF-7 or MDA-MB-231. As shown in **Figure 1A** and **1B**, not only the proteinlevels, but also the mRNA level of USP7 were significantly increased in carcinoma cells MCF-7 or MDA-MB-231 than normal breast tissue cells MCF-10A. To further explored USP7 expression level in breast carcinoma, than we used tumor tissues and adjacent normal tissues from 188 breast carcinoma patients. The results was similar with breast carcinoma cell, the protein level of USP7 was significantly higher in tumor tissues compared to the adjacent non-tumor tissues (**Figure 1C**).

Subsequently, we also performed IHC staining in our collect breast carcinoma patients' samples, the results revealed that USP7 protein was strongly up-regulated in breast carcinoma tissues compared to adjacent non-tumor tissues. Intriguing, we found USP7 expression also positively correlated with pathological grade, tumor size, andlymph node metastasis. However, smoking status, age and differentiation have no relationships with USP7 (**Table 1**).

Next, we used some bioinformatics website to analyze survival curve thatUSP7 high expression in breast carcinoma. As we assumed, high expression of USP7 would result in lower survival rate. (Hazard Ratio=1.38, P=0.0049, **Figure 1D**).

Knockdown USP7 inhibited breast carcinoma tumorigenesis in vitro

To investigated the cellular function of USP7 in breast carcinoma, we first knockdown USP7 by two different shRNA in MCF-7 cell, and used qRT-PCR to detect the mRNA levels of USP7, the results revealed USP7 were down-regulated almost 90% in MCF-7 cells when transfected USP7 shRNA, compared with control which MCF-7 cells transfected scramble shRNA (SCR). The USP7 shRNA#1 was used for further experiments (**Figure 2A**). The protein level of USP7 detected by Western Blot showed the same result (**Figure 2B**). Subsequently, monolayer colony formation assay was used to investigate the function of USP7 in breast carcinoma tumorigenesis. The data demonstrated that cells with an interference of USP7 effectively inhibited cell tumorigenesis, including foci formation frequency assay and cell growth rate assay in vitro assays (**Figure 2C** and **2D**). However, the opposite results can be seen in MCF-7 cells which over expressed FLAG-USP7 (data not shown). In brief, the abnormal expression of USP7 strongly influenced breast cancer tumorigenesis.

Identifying USP7 as a Snail-Interacting protein

To further investigated the cellular functions of USP7, we first expressed FLAG-USP7 in human breast carcinoma MCF-7 cells, then prepared cellular extracts and performed affinity purification and mass spectrometry. We identified several USP7-interacting proteins, which are involved in a variety of cellular functions including transcriptional regulation, cell apoptosis, proteindegradation (**Figure 1A**). Interestingly, we found eight matching peptides from EMT associated transcription factor, Snail, in the USP7 containing protein complex, this data

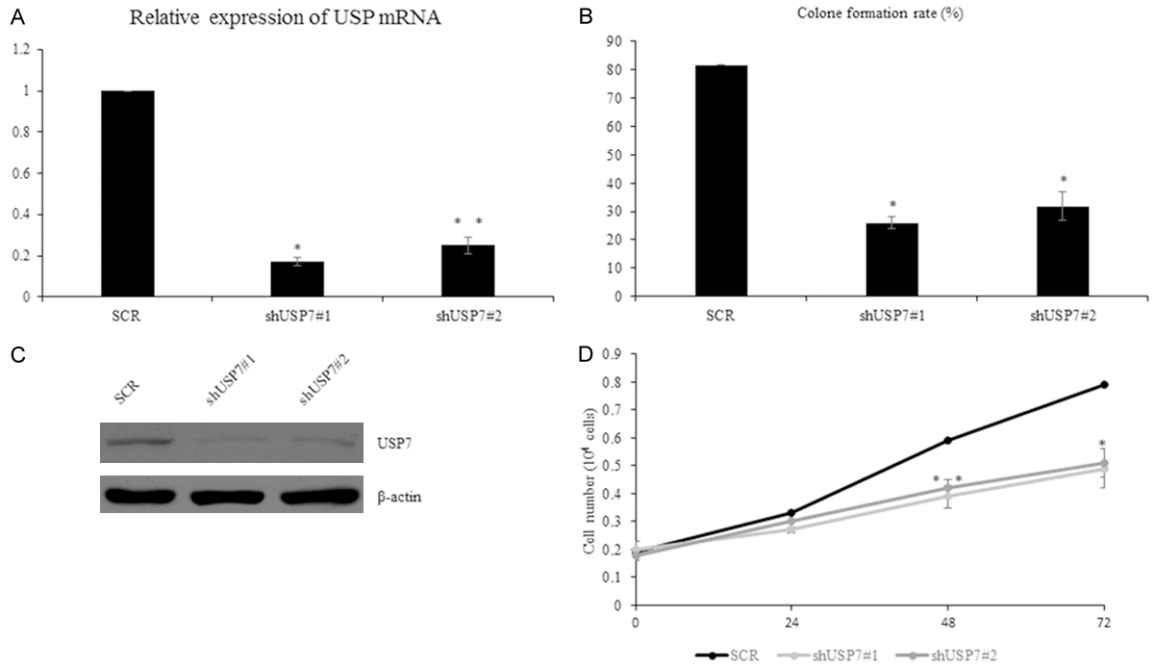

Figure 2. Knockdown USP7 Inhibited Breast Carcinoma Tumorigenesis in vitro. A. MCF-7 cells were transfected with SCR or shUSP7 and then performed qRT-PCR to verify the efficiency of shUSP7. B. Western blot was used to verify the efficiency of shUSP7. C. The silence of USP7suppress foci formation in monolayer culture. D. CCK-8 assay was used to draw the growth curves when MCF-7 cells were transfected with SCR or shUSP7.

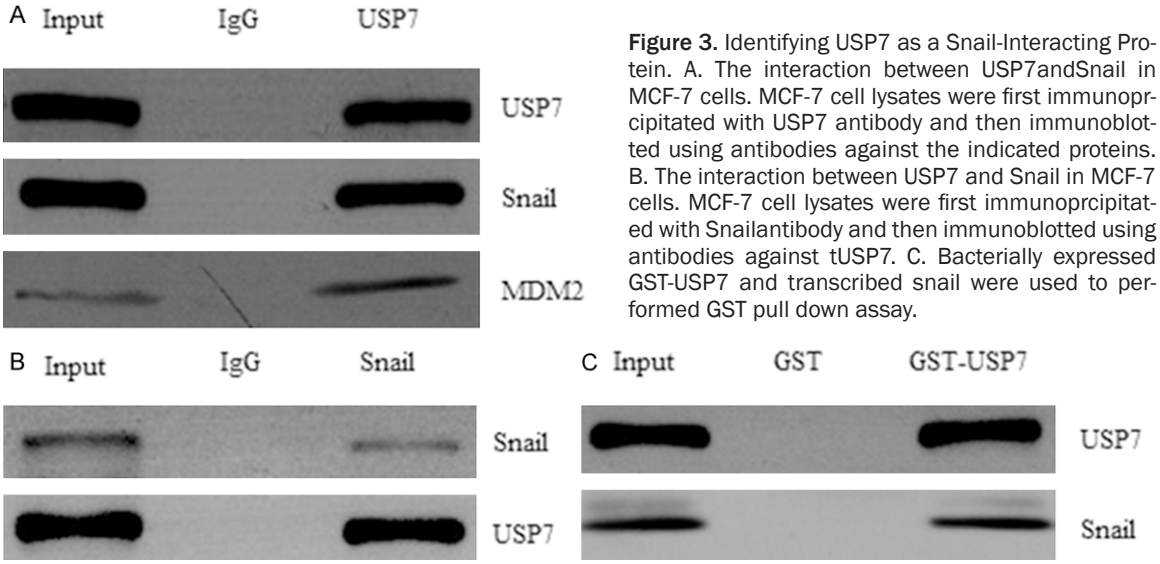

Figure 3. Identifying USP7 as a Snail-Interacting Protein. A. The interaction between USP7andSnail in MCF-7 cells. MCF-7 cell lysates were first immunoprcipitated with USP7 antibody and then immunoblotted using antibodies against the indicated proteins. B. The interaction between USP7 and Snail in MCF-7 cells. MCF-7 cell lysates were first immunoprcipitated with Snailantibody and then immunoblotted using antibodies against tUSP7. C. Bacterially expressed GST-USP7 and transcribed snail were used to performed GST pull down assay.

indicated that USP7 is associated with Snail in vivo. Next, we attempted to verify the interaction between USP7 and Snail were specific through co-immunoprecipitation (co-IP) followed by western blotting. Co-IP assays were performed in MCF-7 cell lysates with antibodies against USP7, and then immunoblotting with antibodies against Snail. The results showed that Snail was efficiently co-immunoprecipitated with USP7 (**Figure 3A**). Reciprocal immunoprecipitation with anti-Snail and immunoblotting with anti-USP7 also indicated that USP7 interacts with Snail in vivo (**Figure 3B**).

To investigated whether USP7 direct interact with Snail, we performed GST pull-down experi-

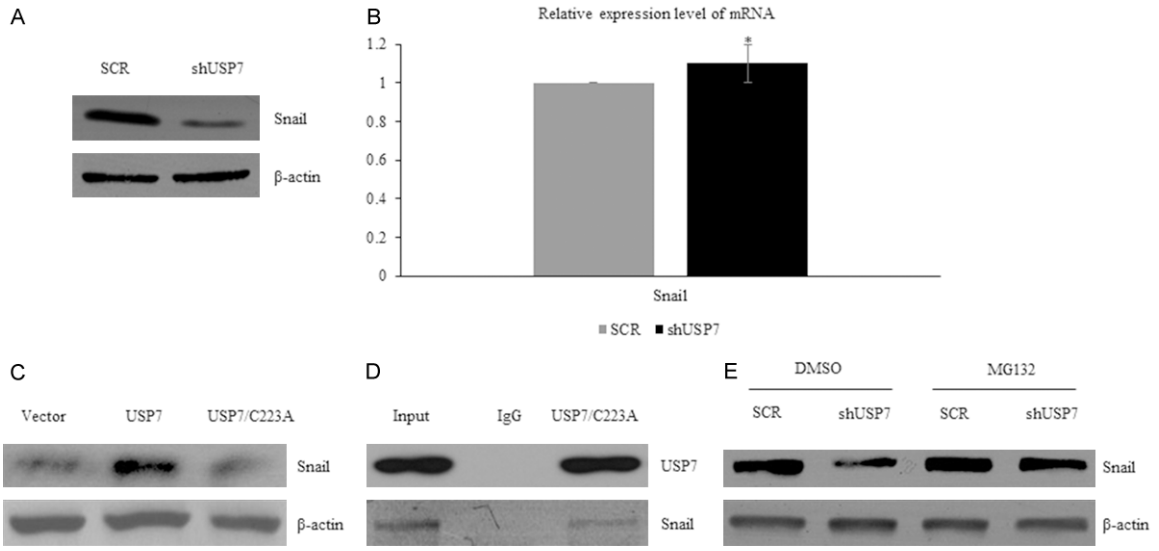

Figure 4. USP7 Suppresses Snail Ubiquitination and Stabilizes Snail. A. Knockdown USP7 in MCF-7 cells and follow by Western blot to detect Snail protein expression. B. qRT-PCR was used to measure Snail mRNA level when USP7 silence in MCF-7 cells. C. MCF-7 over express wide type USP7 and inactive USP7 (USP7/C223A) mutant to investigated whether Snail expression depend on USP7 deubiquitinase. Western blot was perform to detect the protein expression of Snail. D. MCF-7 cells over express inactive USP7 (USP7/C223A) mutant and performed co-IP assay, in order to investigate whether USP7/C223A also interact with Snail. E. Knockdown USP7 in MCF-7 cells and incubated with proteasome-specific inhibitor, MG132, used Western blot assay to measure Snail protein expression.

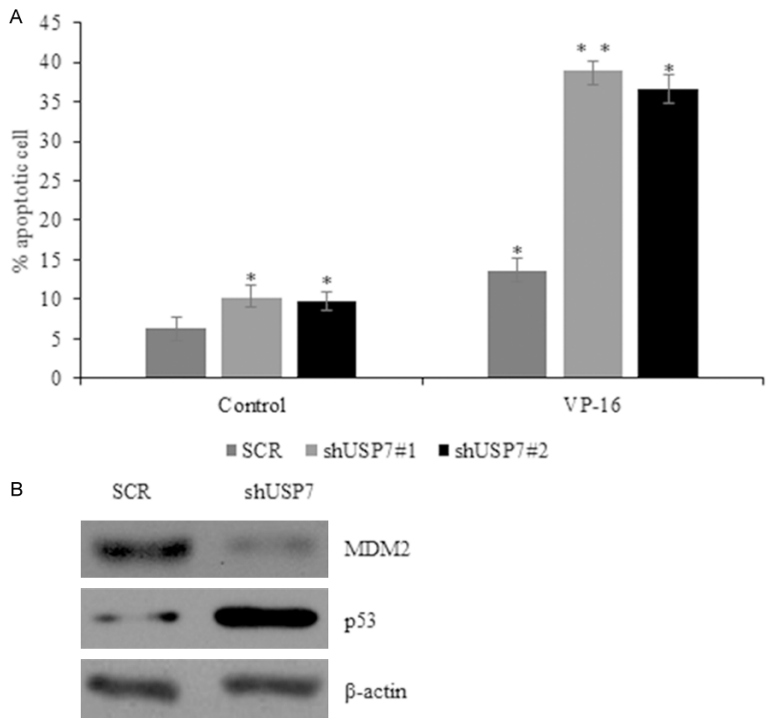

Figure 5. USP7 Suppresses Cell Apoptotic through p53-MDM2 Pathway. A. MCF-7 cells were transfected with SCR and shUSP7 and incubated with or without VP-16 for 24 hr, follow by Annexin V/PI staining and then performed flow cytometry assay to assess the apoptosis of MCF-7 cells. B. Western blot to detect the effect of USP7 in p53-MDM2 pathway.

ments, GST-USP7 was expressed in bacteria and subsequently incubated with vitro transcribed/translated FLAG-tagged Snail (**Figure 3C**). These experiments indicated that Snail could interact with USP7 directly in vitro. Collectively, these experiments support our observation that JMJD6 is physically associate with Snail in vivo.

USP7 suppresses snail ubiquitination and stabilizes snail

USP7 contains a carboxyl-terminal ubiquitin hydrolase domain that defines the C-19 class of peptidases. USP7 has already been reported that it can deubiquitinating MDM2 and SIRT1, so we assume USP7 may regulate Snail function via its deubiquitinase activity. We first knockdown USP7 in MCF-7

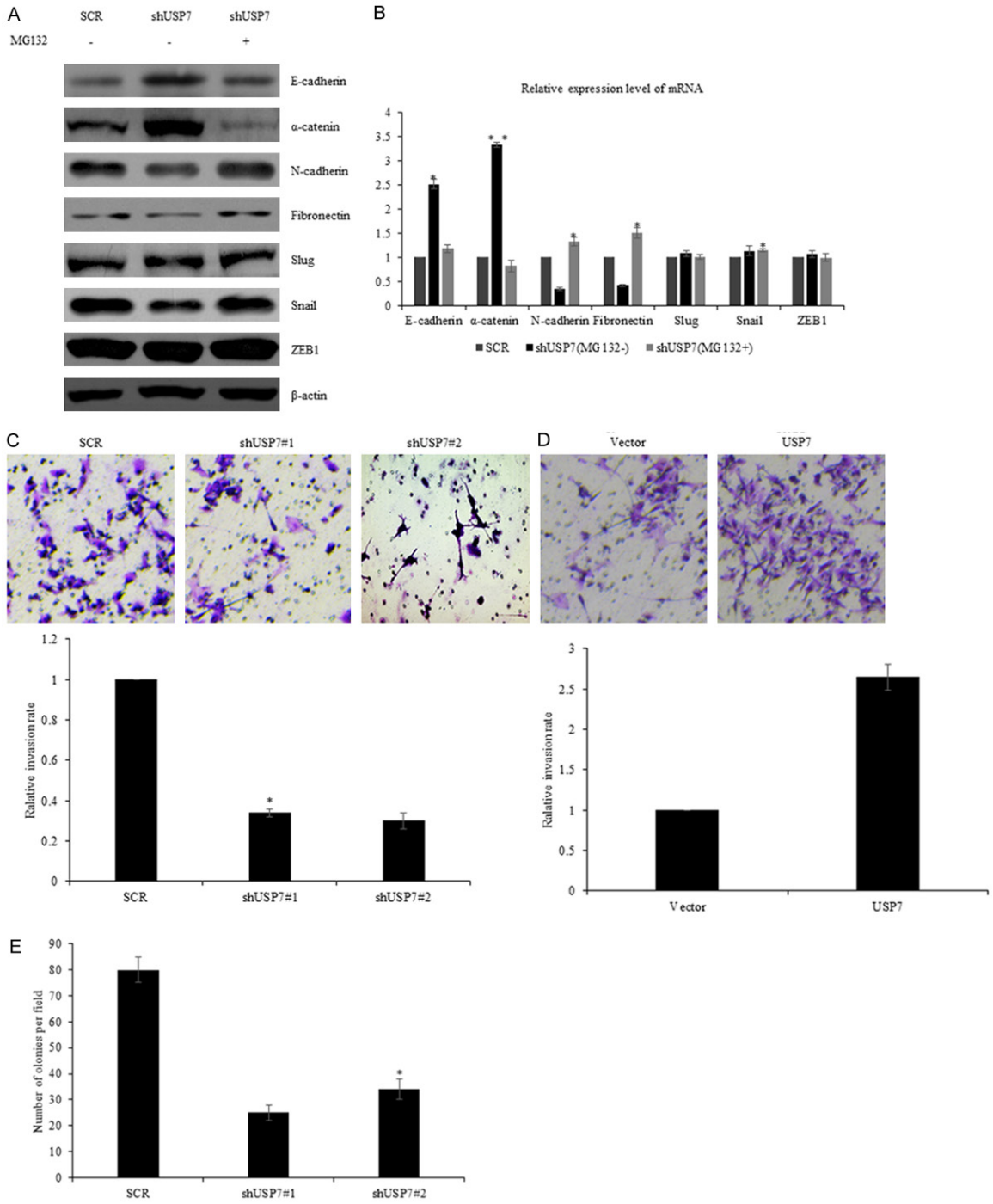

Figure 6. USP7 Regulates EMT through Stabilize Snail and Promote Breast cancer invasion. A. Silence USP7 in MCF-7 cells and challenge without or with MG132, subsequently performed Western blot to detect protein levels of EMT markersand transcript factors. B. Knockdown USP7 in MCF-7 cells and incubate without or with MG132, and follow by qRT-PCR to measure mRNA levels of EMT markers and transcript factors. C. Silence USP7 in MDA-MB-231 cells and culture in transwell chamber for 24 hr. Three independent. All experiments were carried out at least three independent experiments and representative photos were shown. D. Over express USP7 in MDA-MB-231 cells and culture in transwell chamber for 24 hr for in vitro invasion assay. Representative photos were shown.

cell. While knockdown USP7, the protein level of Snail was dramatically reduce (**Figure 4A**), but the mRNA level was not change (**Figure 4B**). That indicated USP7 may influence Snail

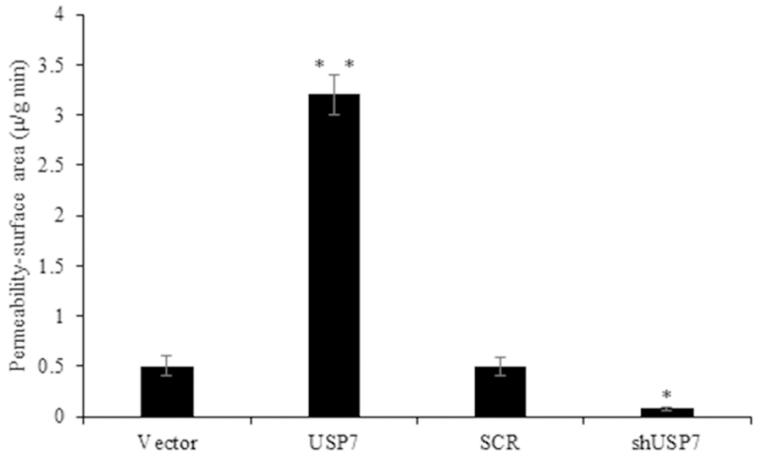

Figure 7. USP7 Enhances MRI Phenotyping detection. MCF-7 vector and MCF-7 USP7 overexpression tumor-bearing mice, or MCF-7 SCR and MCF-7 shUSP7 tumor-bearing mice were used to compare functional MRI maps of the permeability-surface area product and representative analyzed were shown.

through post transcription modification. Next we over expressed wide-type USP7 (wt-USP7) and catalytically inactive USP7 (USP7/C223A) mutant in MCF-7 cell, Snail only increased in wt-USP7, not change in inactive USP7 (USP7/C223A) mutant (**Figure 4C**). Furthermore, to investigated whether USP7/C223A has no effect on Snail because USP7/C223A did not interact with Snail. We performed co-IP assay, as shown in **Figure 4D**, USP7/C223A also interact with snail. Based on above experiments, we demonstrated that USP7 deubiquitinating snail, it is possible that USP7 can stabilize snail. In fact,we also found MG132, a proteasome-specific inhibitor, success to rescued Snail protein from degradation in USP7 knockdown cells (**Figure 4E**), indicating that polyubiquitination induces snail degradation through a proteasomal pathway.

Together with our finding that Snail interacts with USP7, these results indicate that USP7 function as deubiquitinase specifically for Snail and appear to regulate Snail protein stability.

USP7 suppresses cell apoptotic through p53-MDM2 pathway

There are several studies demonstrate USP7 regulate the apoptosis process in different carcinoma, such as Neuroblastoma [12], non-small cell lung cancer (NSCLC) [13]. To investigated the effect of USP7 expression on apoptosis in breast cancer cells, MCF-7 cells treated with SCR or USP7 shRNAs were incubation with or without VP-16 and used Annexin V/PI double staining to label apoptotic cells. Flow cytometry revealed that the cells which knockdown of USP7 resulted in an increased number of apoptotic cells than control cells (**Figure 5A**). Previous research has confirmed that USP7 stabilize the oncogene MDM2, thereby regulate the p53, and eventually inhibit cell apoptosis. The p53 is regulated by USP7 through the oncogene MDM2 [14-17]. But whether USP7 still regulate apoptosis decent on this manner in breast carcinoma was still unknown. Next, we explored whether the p53 and MDM2 expression change in USP7 knockdown. The results showed that MDM2 expression was dramatically decreased in MCF-7 cell which knockdown USP7 compared to control cells. However, p53 expression was significantly increased when USP7 expression was knocked down (**Figure 5B**). The results indicated that USP7 also mediated apoptosis by regulating the p53-MDM2 pathway breast cancer.

USP7 regulates EMT through stabilize snail and promote breast cancer invasion

Snail is a key transcription factor that regulate EMT process, our above experiment demonstrate that USP7 can interact and stabilize Snail, so we suppose USP7 maybe influence EMT through stabilize snail. To verify our hypothesis, we knockdown USP7 and detect EMT marker. Interestingly, no matter mRNA and protein level, USP7 knockdown increased the expression of E-cadherin and α-catenin, which as epithelial marker, however, mesenchymal marker, N-cadherin and fibronectin, were decreased (**Figure 6A** and **6B**). If used MG132 in USP7 knockdown cell, the epithelial marker and mesenchymal marker change would dramatically reverse. But other EMT associated transcription factors, such as ZEB1 or Slug did not found regulate by USP7 (**Figure 6A** and **6B**).

A great number of cancer occur EMT, special breast cancer. EMT influence a lot of cell pro-

gram, such as invasion. Besides, our previous analysis revealed that USP7 was positively correlated with lymph note metastasis, so USP7 maybe take part in carcinoma cell invasion. To certify our hypothesis, transwell assays were performed, we knockdown USP7 in the highly invasive human breast cancer cell line MDA-MB-231, the results demonstrated that the cell which knockdown USP7 invade number significantly reduce than control, it indicated that the invasion ability of MDA-MB-231 cells was dramatically weaken when USP7 was depletion (**Figure 6C**). Reciprocally, the number of invaded cell through matrigel when over expression USP7 in MDA-MB-231 cells was twice as much as control (**Figure 6D**). Together, USP7 can promote the invasion ability of MDA-MB-231 cells. We alsoinvestigated the function of USP7 in anchorage-independent cell growth in the MCF-7 cells. As shown in **Figure 6E**, we found USP7 depletion obviouslyinhibit the colony formation in soft agar. To sum up, USP7 take part in multiple cancer develop process, special in regulate cell invasion, and also increased cancer cells anchorage-independent grow ability.

USP7 enhances MRI phenotyping detection

We also performed MRI assay, the results indicated that USP7 up regulation resulted in MCF-7 get more angiogenic phenotype, and the permeability-surface area product was dramatically higher than the vector, however when USP7 was deletion, the permeability-surface area product was almost can not to detected (**Figure 7**).

Discussion

Numerous researchers have found that USP7 play a critical role in cell death and proliferation, and participate several crucial biological signaling pathways in tumorigenesis [18-22]. But the roles of USP7 in breast carcinoma is still unknown. Here, our research demonstrated that breast carcinoma express high levels of USP7. Furthermore, many clinical character, such as lymph node metastasis, tumor stage and tumor size were positive associated with high USP7 expression. Kaplan-Meier analysis also indicated that the breast carcinoma patients with high USP7 expression had dramatic worse survival than those with low USP7 expression. Subsequently, we utilized shRNA to knockdown USP7 expression, we found cell growth rate and foci formation in vitro were all effectively inhibited. Together, we showed that high expression of USP7 will promote breast carcinoma progression.

USP7 has been reported to regulate the p53-MDM2 pathway in several cancer [14, 17], but how about in breast cancer still unknown. Our results revealed that p53 was dramatic increased, however, MDM2 was significantly decreased in USP7 knockdown. MCF-7 cells compared with control cells. And Flow cytometry assay also demonstrated USP7 suppress cell apoptotic. Above data suggested USP7 also negatively regulate p53 levels through MDM2 in breast cancer, eventually suppress cell apoptotic.

We found that USP7 knockdown significantly influence EMT, the epithelial marker, E-cadherin was up regulate, but the mesenchymal marker, Vimentin and N-cadherin were down regulate. Furthermore, the cell invasiveness ability decrease was found after the USP7 deletion. These results indicated that USP7 may take part in EMT, and promote the invasion ability of cancer cells. Meanwhile, USP7 enhanced MRI phenotyping detection. Overall, we have demonstrated that USP7 over express in breast carcinoma, and high expression of USP7 play a crucial role in tumor invasion and metastasis in breast carcinoma. Our results reveal a novel mechanism of USP7 promote EMT, and found USP7 as a potential biomarker and potential therapeutic targetfor breast cancer.

Disclosure of conflict of interest

None.

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