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Original Article

Williams syndrome transcription factor is a target of pro-oncogenic Ser158 phosphorylation mediated by Ras-MAPK pathway in human breast cancer

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Abstract: Background: Williams Syndrome transcription factor (WSTF) has been implicated in a variety of cellular events. However, the roles of WSTF in human tumorigenesis have not been elucidated. Methods: The molecular and cellular assays were used to identify whether WSTF in MCF-7 cells is a downstream target of Ras-mitogen-activated protein kinase (MAPK) signaling pathway. Results: Ser158 of WSTF was phosphorylated following activation of MAPK signal, suggesting that WSTF may be a downstream target of MAPK signaling pathway. Conclusions: $WSTF^{Ser158}$ ($WSTF^{S158}$) phosphorylation by Ras-MAPK contributes to tumorigenesis.

Keywords: WSTF, Ras, MAPK, phosphorylation, breast cancer

Introduction

The *Williams syndrome transcription factor* (*WSTF*) gene (also known as *BAZ1B*) is one of a number of genes within a heterozygous deletion that exists in individuals with Williams syndrome [1]. WSTF protein is a multifunctional atypical kinase, which has been implicated in a variety of cellular events, including chromatin assembly, RNA polymerase I and III gene regulation, vitamin D metabolism and DNA repair [2].

As a subunit of several ATP-dependent chromatin remodeling complexes, WSTF plays dual roles of transcriptional activation and inhibition [2]. In addition, WSTF could recognize and bind to histone by WSTF/Acf1/cbpq46 (WAC), bromodomain and plant homeodomain (PHD) domains, and specifically regulate post-translational modifications (PMTs) primarily on the flexible terminal tails of histones [2]. Histone modifications, including methylation, acetylation, phosphorylation and ubiquitination, are epigenetic markers regulating gene expression, either transcriptional activation or silencing [3]. Mutations in histone-modifying enzymes often cause human diseases, including cancer [3]. Moreover, WSFT plays a role in regulation metabolism of vitamin D, which insufficiency could have an etiological role in various cancers [1, 3]. These indicate that WSTF probably contribute to human tumorigenesis.

It is known that PTM is a powerful way to modify the behavior of proteins, and thereby cellular behavior. Mitogen-activated protein kinase (MAPK) is protein phosphokinase that communicates with other proteins in the Ras-MAPK pathway by adding phosphate groups to the serine, threonine, and tyrosine of protein, which acts as an "on" or "off" switch [4]. The Ras-MAPK pathway is aberrantly activated in many human cancers [5]. Drugs that reverse the "on" or "off" switch are being investigated as cancer treatments [4].

In this study, serine 158 (Ser158), a residue located close to the N terminus of WSTF, was identified as a phosphorylation site. Further-

more, we clarified that the Ser158 of WSTF is a phosphorylation target of Ras-MAPK signaling pathway in breast cancer. In particular, for the first time, we demonstrated that the MAPK-dependent phosphorylation of WSTF plays an important role in oncogenesis of breast cancer.

Materials and methods

Cells and patient samples

Human MCF-7 cells were obtained from the Cell Culture Center of Peking Union Medical College. The cells were maintained in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) and 1% Penicillin/Streptomycin (Solarbio, China) at 37°C with 5% CO_2.

The 30 pair adult patient samples of breast cancer tissue and matched adjacent normal tissues were provided by Alenabio Technology Co., Ltd., Xi'an (X777910-899).

Peptide competition assay (PCA)

The peptide competition experiment was performed with MCF-7 cells lysates. Transfer and immobilize the antigen on PVDF membrane and prepare two identical test samples for analysis by PCA. The polyclonal antibody for phosphorylated-158Ser WSTF (anti-P-WSTF^{S158}) was synthesized by Bohaitongda Bio-Tech Co., Ltd., Beijing. Using P-WSTF and non-P-WSTF peptides and a P-WSTF^{S158} antibody, where an antibody concentration of 1 μg/ml and a 200-fold molar excess of peptide are used in a total reaction volume of 2 ml. The P-WSTF^{S158} antibody was pre-incubated with P-WSTF or non-P-WSTF peptides for 2 h at 37°C with gentle rocking. Centrifuge the samples for 15 min at 4°C (10000-15000 rpm/min) to pellet any immune complexes. Carefully remove the supernatant. The pre-incubated antibody in each of the two samples is ready for use. Pipette the contents of each sample onto the two identical test samples for immunoblotting (i.e. Western Blot strips). Incubate each strip for 2 h at 37°C, washing with PBS 3× at room temperature (RT). Transfer each strip to a new solution containing a Goat Anti-Rabbit IgG secondary antibody (ZB-2301, Zhongshan Golden Bridge, China, dilution 1:5000). Incubate each strip for 40 minutes at 37°C, washing with PBS 3× at RT.

Transfection of gene expression plasmids and siRNAs

The coding regions of human wild type (WT) *WSTF* and *H-Ras* were cloned from MCF-7 cDNA using PCR. The plasmids of pEGFP-N1-*WSTF*, pEGFP-N1-*WSTF*-Myc and pEGFP-N1-*H-Ras* were constructed, respectively. The corresponding mutant plasmids containing *WSTF*^{S158A}-HA, *H-Ras*$^{G12V/E37G}$, *H-Ras*$^{G12V/Y40C}$ and *H-Ras*$^{G12V/T35S}$ were constructed using the TaKaRa MutanBEST Kit (#D401).

The siRNAs against human *WSTF* (*WSTF*-si) *and c-Raf* (*c-Raf*-si), as well as the corresponding scramble siRNAs (control *WSTF*-si and control *c-Raf*-si), were all purchased from Shanghai GenePharma Company (GenePharma, China). The sequences were used as follows: 5'-GGAAGGAGAGAGAGUAUUATT-3' (*WSTF*-si), 5'-GUGCGAGGGGGUUGUAAUCTT-3' (control *WSTF*-si), 5'-GCACGCUUAGAUUGGAAUA-3' (*c-Raf*-si), 5'-AAGUCCAUGGUGACAGGAGAC-3' (control *c-Raf*-si).

MCF-7 cells were seeded in 6-well plates (2×10^5/well) and incubated overnight. The cells at 50% confluence were transfected with plasmid or siRNA using the Lipofectamine 2000 transfection reagent (Invitrogen, USA) and incubated. Forty-eight hours post-transfection, the cells were collected. Overexpression and silencing efficiency were tested by western blot and/or real time PCR. The experiments were repeated 3 times.

Immunoprecipitation (IP) assay

MCF-7 cells were transiently transfected with *WSTF*-Myc or *WSTF*^{S158A}-HA, and whole cell extracts were prepared 48 h after transfection. WSTF^{S158A} is a phosphorylation-deficient mutation of Ser158 to Ala in WSTF. The antibodies of anti-WSTF, anti-Myc and anti-HA (#2152, #2278 and #3724, Cell Signaling Technology, USA) were mixed with the protein G magnetic beads (#9006S, Cell Signaling Technology, USA), respectively. The cell extracts were incubated with magnetic beads-antibody complex at 4°C for 4 h. Magnetic beads-antibody-antigen complex was washed and the immunoprecipitated proteins were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membranes and probed using various antibodies.

Western blotting

Total cell lysates were obtained from MCF-7 cells RIPA buffer (Beyotime Institute of Biotechnology P0013B, Haimen, Jiangsu, China). Protein concentrations in the samples were determined by the BCA protein assay kit (Pierce, Rockford, IL USA). Cell lysate was loaded and run on a 10% SDS-PAGE, and the protein was transferred to a PVDF membrane (Millipore, Billerica, MA, USA) using the BioRad Semi-dry transfer system (BioRad, Hercules, CA, USA). The membrane was incubated with the primary antibody followed by the secondary alkaline phosphatase-conjugated the goat anti-rabbit (ZB-2301 dilution 1:5000, Zhongshan Golden Bridge, China) and goat anti-mouse antibodies (ZB-2305 dilution 1:5000, Zhongshan Golden Bridge, China). Primary antibodies were used as follows: anti-WSTF (Abcam, USA, ab51256), anti-P-extracellular signal-regulated kinase (anti-P-ERK1/2) (Abcam, USA, ab17942, 1:1000), anti-β-actin (#TA09, Zhongshan Golden Bridge, China), anti-Flag (#TA05, Zhongshan Golden Bridge, China), anti-Myc (Zhongshan Golden Bridge, China, #TA01), anti-AKT antibody (#9272, Cell Signaling Technology, USA), anti-P-Akt (Ser-473) (#4058, Cell Signaling Technology, USA), anti-pidermal growth factor (EGF) (E5036, Sigma, USA). Dilution was performed 1000-fold for all of the antibodies.

Protein expression levels in patient samples were examined by Western blot and quantified with ImageJ software. Protein expression levels in tumor samples were normalized to those of paired normal tissues ($P < 0.01$, Pearson's chi-square test).

Treatment of MCF-7 cells with activators and inhibitors of Ras-signaling pathway

The MCF-7 cells were serum-starved for 6 h in 0.1% FBS-containing DMEM medium followed by treatment with epidermal growth factor (EGF) for 60 min (15 ng/ml) or heregulin-β1 (HRG-β1) for 30 min, 60 min and 90 min (2 nM), respectively. Then, the cells were treated for 20 h with the following inhibitors respectively: the inhibitors of MAPK/ERK pathway 1049738-54-6 (Millipore, USA, 13 μM), PD184161 (ab143847, Abcam, USA, 10 μM) and U0126 (ab120241, Abcam, USA, 10 μM), the inhibitor of PI3-Kinase (PI3K)/Akt pathway Wortmannin (ab120148, Abcam, USA, 12 μM) and LY294002 (ab120243, Abcam, USA, 10 μM).

Confocal laser scanning microscopy

After fixing with 4% paraformaldehyde for 15 min, MCF-7 cells were treated by immunofluorescence dual-labeling method. In brief, rabbit anti-human WSTF antibody (ab51256, Abcam, USA) and mouse anti-human p44/42 MAP Kinase antibody (#9102, Cell Signaling Technology, USA) were diluted to 1:100 as primary antibody solutions; goat anti-rabbit Rhodamine-Labeled secondary antibody (Catalog No. 03-15-06, KPL, USA) and goat anti-mouse Fluorescein-Labeled secondary antibody (Catalog No. 02-18-06, KPL USA) were diluted to 1:500. Cells were blocked by 5% bovine serum albumin (BSA, Sigma, USA) 37°C for 30 min, incubated with primary antibody solutions at 37°C for 1 h, then cultured at 4°C overnight. After washing with PBS 3× at RT, cells were incubated with secondary antibody solutions (No. 02-18-06, 03-15-06, KPL USA) at 37°C for 1 h, and stained with DAPI (D1306, Life Technologies, USA, 1 ng/μl) for 5 min to counterstain the nucleus. After washing with PBS 3× at RT, the cells then observed under the confocal laser scanning microscope. The data were collected by a computer for digital imaging. The experiment was repeated 3 times [6].

Establishment of $WSTF^{S158A}$ stable MCF-7 cell line and in vivo tumorigenicity assay

The MCF-7 cell lines stable transfected with $WSTF^{S158A}$ plasmids were obtained from Bohaitongda Bio-Tech Co., Ltd., Beijing. The stable transfection was performed according to the manufacturer's instructions and reports [7-9].

The MCF-7 and $WSTF^{S158A}$ cells were injected (2×10^6 cells) orthotopically into the fourth mammary fat pad of nude mice (10 mice per group), respectively. The tumor volumes were measured twice weekly, and the data were tested statistically with a two-sided long-rank analysis [10]. Female BALB/C nude mice, 4-6 weeks old from Vital River Laboratory Technology Co. Ltd, Peking, China, were used in this study. Mice were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet. All animal studies were conducted with a North China University of Science

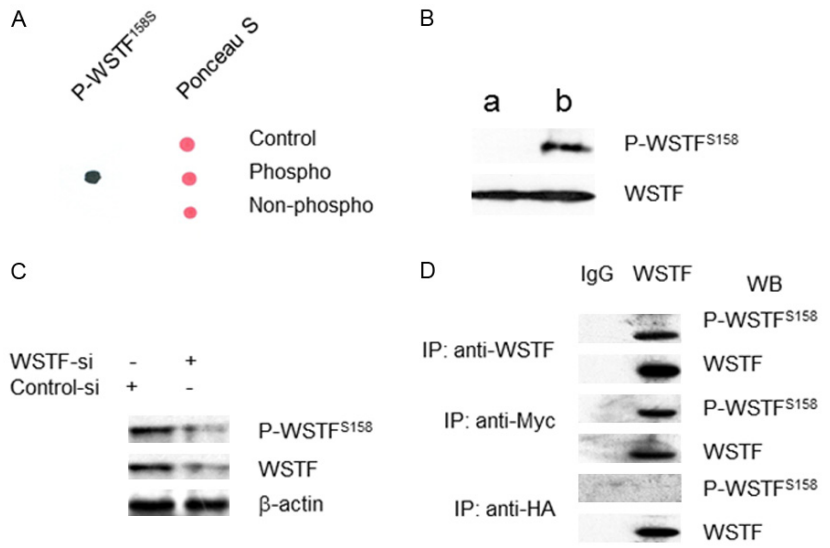

Figure 1. WSTF^{S158} is a phosphorylation target. A. Dot blot assay with WSTF peptides containing phosphorylated or non-phosphorylated Ser158. Equal amounts of unrelated (control), phospho- or non-phospho-peptides were immunoblotted with an anti-phospho-WSTF antibody. B. The peptide competition experiment was performed with MCF-7 cell lysates; the P-WSTF^{S158} antibody was pre-incubated with phospho-WSTF or non-phospho-WSTF peptides (a & b). C. Cell lysate from scramble or *WSTF* specific siRNA knockdown MCF-7 cells were probed 48 h after transfection. D. Endogenous WSTF were precipitated and tested with anti-WSTF or anti-P-WSTF^{S158} antibody (upper panel). Tagged WT or mutant WSTF were transfected and immunoprecipitated with antibodies against Myc or HA. Samples were immunoblotted with antibodies against WSTF and P-WSTF^{S158}.

and Technology Animal Care and Use Committee protocol specifically approved for this study and in accordance with the principals and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals. A value of $P < 0.05$ was considered as statistically significant.

Results

Detection of P-WSTF^{S158} in vitro and in vivo

To study if Ser158 of WSTF is a phosphorylation site, a polyclonal antibody against P-WSTF^{S158} was synthesized. The specific hybridization dot and band were detected in WSTF group by dot blot hybridization assay *in vitro* (**Figure 1A**) and peptide competition experiment in MCF-7 cell lysates, respectively (**Figure 1B**). In addition, the level of P-WSTF^{S158} was correspondingly decreased in WSTF-knockdown MCF-7 cells (**Figure 1C**). All these data confirmed the specificity of the P-WSTF^{S158} antibody.

Then, the immunoprecipitation assay was performed to verify if phosphorylation of WSTF^{S158} is a physical event in MCF-7 cells. As shown in **Figure 1D**, endogenous WSTF and phosphorylation status (P-WSTF^{S158}) were detected in WT MCF-7 cells (upper panel). In addition, the P-WSTF^{S158} band was detected in MCF-7 cells post-transfected with WT *WSTF*-Myc at 48 h (middle panel), but not in MCF-7 cells transfected with *WSTF*^{S158A}-HA (lower panel). It suggested that exogenous WT WSTF could be phosphorylated in MCF-7 cells. Therefore, WSTF could be phosphorylated at Ser158 site, and the phosphorylation of WSTF^{S158} is a physical event in MCF-7 cells.

Identification of Ras signaling pathway regulating phosphorylation of WSTF^{S158} in MCF-7 cells

It is known that RalGEF, PI3K and MAPK pathways could be preferentially activated by Ras mutations H-Ras$^{G12V/E37G}$, H-Ras$^{G12V/Y40C}$ and H-Ras$^{G12V/T35S}$, respectively [11]. Then, the MCF-7 cells were transfected with plasmids expressing Ras three kinds of mutations, respectively (**Figure 2A**). As presented in **Figure 2B**, 48 h later, the key proteins of the three kinds of pathways (P-ERK1/2, RalA-GTP and P-AKT) were upregulated in the different Ras mutation group, which suggested that the three kinds of pathway were all activated by Ras mutations. The level of P-WSTF^{S158} was correspondingly increased in the MAPK activation group, but no changes of P-WSTF^{S158} were detected in the cells with activated RalGEF or PI3K (**Figure 2B**). These results indicated that phosphorylation of WSTF^{S158} probably mediated by MAPK pathway.

Then, the inhibitors for the MAPK and PI3K pathways were used. As shown in **Figure 2C**, the P-WSTF^{S158} level was decreased by MPAK pathway inhibitor U0126, but not by PI-3K path-

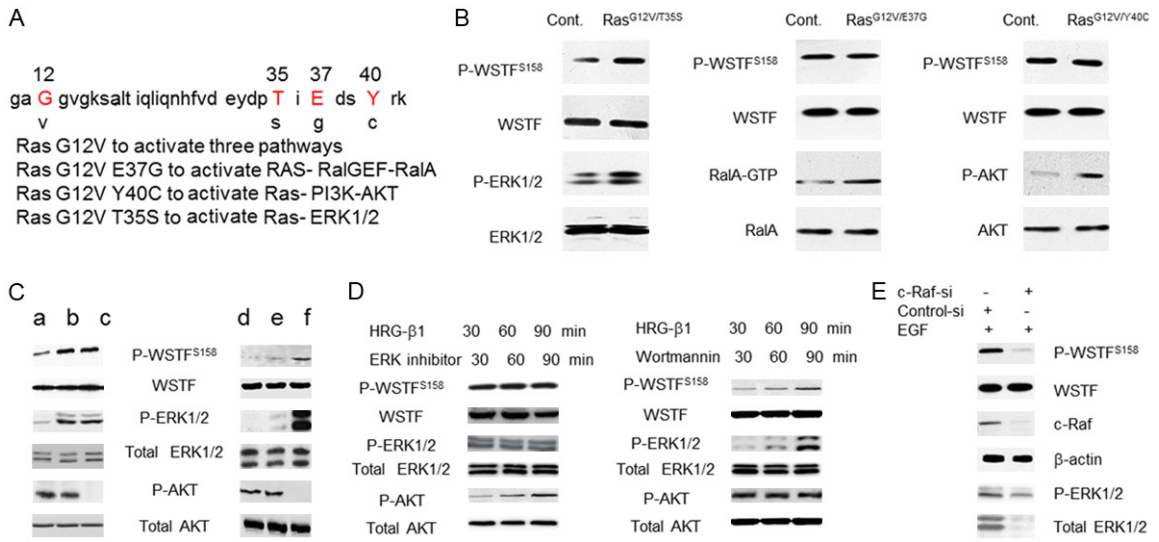

Figure 2. WSTF^{S158} is a target of MAPK pathway. A. The H-Ras$^{G12V/E37G}$, H-Ras$^{G12V/Y40C}$ and H-Ras$^{G12V/T35S}$ expression vectors were constructed to preferentially activate the RalGEF, PI3K or MAPK pathways, respectively. B. MCF-7 cells were transfected with empty-pEGFP-N1, pEGFP-H-Ras$^{G12V/E37G}$, pEGFP-H-Ras$^{G12V/Y40C}$ or pEGFP-H-Ras$^{G12V/T35S}$ plasmids. The P-WSTF^{S158} levels were then measured using western blotting. C. Left panel: The cells lysates of MCF-7 with or without ERK inhibitor (U0126, 10 μM) were analyzed by Western blotting with the P-WSTF ^{S158} antibody (1:1000) (a & b). The cells lysates of MCF-7 with the PI-3K inhibitor (LY294002, 10 μM) were used as control (c). The inhibitor was added into normal media 20 h before sample collection. The P-AKT, total AKT and P-ERK1/2 and total ERK1/2 were tested in order to confirm the potency and specificity of LY294002 and U0126 inhibitors. Right panel: MCF-7 cells cultured with normal media were treated with the ERK inhibitor (CAS NO. 1049738-54-6, 13 μM) (d), inhibitor of the MAPK and PI3-Kinase (PI3K)/Akt signaling pathways: PD184161 (10 μM) and Wortmannin (12 μM) (e & f) for 20 h followed by Western blotting. D. WT MCF-7 cells cultured in 0.1% FBS-containing DMEM media for 6 h followed by addition of 2 nM HRG-β1 in the absence or presence of ERK inhibitor (CAS NO. 1049738-54-6, 13 μM) or Wortmannin (12 μM). E. MCF-7 cells were cultured with EGF (15 ng/ml). Cell lysate from scramble or *c-Raf* specific siRNA knockdown MCF-7 cells were probed 48 h after transfection.

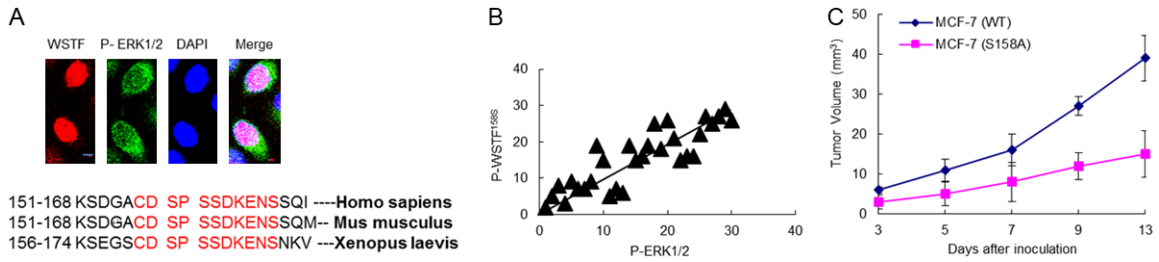

Figure 3. P-WSTF^{S158} is positively correlated with activity of MAPK pathway and contributes to tumorigenesis of breast cancer. A. (Upper panel) Confocal microscopy image demonstrating co-localization of WSTF and phosphorylated ERK1/2 protein in wild MCF-7. Bar represents 20 μm. (Lower panel) Comparison of the sequence of WSTF demonstrates that Ser158 is conserved in metazoans (mammals and frogs). B. Comparison of P-WSTF^{S158} levels and Ras-MAPK signaling activities in tumors and their adjacent normal tissues. Protein expression was examined by immunoblotting of 30 pairs of breast tissue samples and quantified with ImageJ software. Protein expression levels in tumor samples were normalized to those of paired normal tissues ($P < 0.01$, Pearson's chi-square test). C. *In vivo* tumor growth analysis was performed as described in the Material and Methods. The data points are mean ± SEM. A P value $P < 0.05$ was considered as statistically significant.

way inhibitor LY294002 (**Figure 2C**, left panel). The down-regulated P-WSTF^{S158} was obtained with other two MPAK inhibitors 1049738-54-6 and PD184161, but not the PI-3K inhibitor Wortmannin (**Figure 2C**, right panel). Moreover, under the treatment with HRG-β1, an activator of MAPK and PI3K pathway, the P-WSTF^{S158} level was maintained in MCF-7 cells simultaneously treated with MAPK pathway inhibitor (**Figure 2D**, left panel), while that was still

increased in MCF-7 cell simultaneously treated with PI3K pathway inhibitor Wortmannin (**Figure 2D**, right panel).

To further prove that P-WSTF^{S158} is a downstream target of Ras-MAPK pathway, c-Raf, which could be activated by Ras [12], was knocked down by specific siRNA. As shown in **Figure 2E**, the P-WSTF^{S158} was decreased in c-Raf-knockdown MCF-7 cells, even in the presence of EGF stimulation, an activator of Ras pathway [13-15].

All of these data indicated that WSTF^{S158} is a downstream target of MAPK pathway, but not RalGEF or PI3K-AKT pathway in MCF-7 cells.

Analysis the relationship between P-WSTF and MAPK pathway

As shown in **Figure 3A**, the co-localization of WSTF and P-ERK1/2 was detected in MEC-7 nuclei by confocal microscopy image. Further inspection of the sequence of WSTF showed Ser158 is conserved in metazoans (**Figure 3A**, lower panel). Given the data shown in **Figure 2**, we proposed that Ser158 probably is a direct or indirect downstream target of P-ERK1/2, a key protein in MAPK pathway. To discuss the relationship of P-WSTF and MAPK pathway in clinical specimen of breast cancer, protein expression levels of P-WSTF and P-ERK1/2 were examined by immunoblotting in 30 pairs of human breast cancer and matched adjacent normal tissues. Pearson's statistical analysis showed a positive correlation of the level of P-WSTF^{S158} and the activity of MAPK pathway (**Figure 3B**).

Demonstration of the function of WSTF^{S158} phosphorylation on tumorigenesis

Then, the MCF-7 cells stable transfected with WSTF^{S158A} was generated and *in vivo* tumorigenicity assay was performed. As presented in **Figure 3C**, the transplanted tumor sizes were attenuated in the nude mice transplanted with WSTF^{S158A} MCF-7 cells compared with those in the nude mice transplanted with WT MCF-7 cells. The results demonstrated that phosphorylation of WSTF^{Ser158} plays important role in tumorigenesis of human breast cancer.

Discussion

In this study, Ser158 was identified as a phosphorylation site both *in vitro* and *in vivo*. In addition, WSTF^{Ser158} was clarified as a pahosphorylation target of Ras-MAPK signaling pathway in breast cancer. The specific kinase within or downstream of Ras/MAPK signaling pathway responsible for WSFT Ser158 phosphorylation in breast cancer cells remains to be found. Since WSTF and P-ERK1/2 were co-localized in MEC-7 nuclei, and a positive correlation between P-ERK1/2 and P-WSTF^{S158} was demonstrated in human breast cancer tissues, P-ERK1/2 was speculated as the specific kinase responsible for phosphorylation of WSTF Ser158.

ERK1 and ERK2 are key proteins in Ras-MAPK pathway, which are dually phosphorylated on threonine and tyrosine residues by mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) [16]. Phosphorylation activated ERKl/2 translocate from cytoplasm to nucleus followed by activate a variety of substrates including transcription factors, protein kinases and phosphotyrosine protein phosphatases, leading to positive or negative regulation of signalling cascades [16]. However, the sequence "CDS158PSSDKENS" around the Ser158 of WSTF is not a typical substrate-sequence of ERK1/2 kinases, and the substrates of which often contain the motif PX(T/S)P [17]. We found that the sequence "CDS158PSSDKENS" is evolutionarily conserved (**Figure 3A**), which indicates its essential importance across species. The sequence may be a novel substrate-sequence of P-ERK1/2. Therefore, it is still need to be discussed that if WSTF^{S158} is a direct phosphorylation site of ERK1/2 kinases. In the future study, the association between ERK1/2 and WSTF should be confirmed by Glutathione S transferase (GST)-pull down, and protein kinase assay should be performed to analysis the extended identifiable region of ERK1/2 kinases on substrates.

Moreover, the *in vivo* tumorigenicity assay in this study suggested that the decreased level of P-WSTF^{Ser158} could inhibit the growth of breast cancer. Therefore, MAPK-dependent phosphorylation of WSTF probably play an important role in oncogenesis of breast cancer and the phosphorylation modification of WSTF maybe is a potential target of cancer treatment. Further studies should be performed to explore the mechanisms of how WSTF^{S158} phosphorylation mediated the transduction of Ras/MAPK

oncogenic signal, including the precise roles of $WSTF^{S158}$ phosphorylation in chromatin assembly, gene regulation, and DNA repair.

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Disclosure of conflict of interest

None.

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