Aberrant Phosphorylation of SMAD4 Thr277-Mediated USP9x–SMAD4 Interaction by Free Fatty Acids Promotes Breast Cancer Metastasis

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Abstract

Obesity increases the risk of distant metastatic recurrence and reduces breast cancer survival. However, the mechanisms behind this pathology and identification of relevant therapeutic targets are poorly defined. Plasma free fatty acids (FFA) levels are elevated in obese individuals. Here we report that TGFβ transiently activates ERK and subsequently phosphorylates SMAD4 at Thr277, which facilitates a SMAD4–USP9x interaction, SMAD4 nuclear retention, and stimulates TGFβ/SMAD3–mediated transcription of Twist and Snail. USP9x inhibited the E3 ubiquitin-protein ligase TIF1γ from binding and monoubiquitinating SMAD4, hence maintaining the SMAD4 nuclear retention. FFA further facilitated TGFβ-induced ERK activation, SMAD4 phosphorylation, and nuclear retention, promoting TGFβ-dependent cancer progression. Inhibition of ERK and USP9x suppressed obesity-induced metastasis. In addition, clinical data indicated that phospho-ERK and -SMAD4 levels correlate with activated TGFβ signaling and metastasis in overweight/obese patient breast cancer specimens. Altogether, we demonstrate the vital interaction of USP9x and SMAD4 for governing TGFβ signaling and dyslipidemia-induced aberrant TGFβ activation during breast cancer metastasis.

Cancer Res; 77(6); 1383-94. ©2017 AACR.

Introduction

Breast cancer is the most common cancer in women and one of the top five cancers causing overall cancer mortality globally, with a continuously rising incidence (1, 2). Recent estimates suggest that up to 35% of cases may be avertible via lifestyle and diet alteration (2). More recent studies have revealed obesity to be an established risk factor for breast cancer. It has also been related to increased incidence and mortality, poorer prognosis, a more aggressive tumor phenotype (3, 4). Being overweight/obese for a woman diagnosed with breast cancer increases the risk of developing distant metastatic recurrence, and reduces survival irrespective of treatment factors (5). The link between obesity and survival does not vary by menopause or tumor hormone receptor (HR) status. Obesity in rodents is also related to augmented incidence of spontaneous and chemically induced cancers (6). In addition, it has been proposed that breast cancer is associated with consumption of a high fat diet (HFD; ref. 7). A meta-analysis of substantial rodent breast cancer models demonstrated that HFD enhances susceptibility to mammary tumors (8). Although the precise mechanisms remain to be illuminated, dietary factors have been involved in nearly 35% of cancer-related deaths (9).

Overweight/obesity and HFD in both humans and rodents is characterized by elevated free fatty acids (FFA) levels (10, 11). Increasing evidence points to FFA signaling playing an important role in tumorigenesis and breast cancer development and progression. The mean levels of total FFA, two of the saturated fatty acids, and one unsaturated fatty acid [palmitic acid C16:0, stearic acid C18:0, and linoleic acid (ω6) C18:2] in the serum are remarkably higher in the breast cancer patients than the benign and the control groups and they have been identified as possible biomarkers for breast cancer (12). As for individual fatty acids, palmitate has been implicated in an increase in breast cancer risk in postmenopausal women cohort studies (13). Furthermore, Shannon and colleagues (14) reported a significant direct association between erythrocyte palmitic acid and the risk of breast cancer. Fatty acid synthase (FAS), which catalyzes the synthesis of palmitic acid, is also commonly overexpressed in breast cancer and other cancers (15, 16). Louie and colleagues (17) demonstrated that cancer cells strongly incorporate and remodel exogenous palmitate into structural...
and oncogenic glycerophospholipids, sphingolipids, and ether lipids, suggesting that cancer cells are addicted to FFA and utilize exogenous FFA for producing lipids required for proliferation and protumorigenic lipid signaling and energy production. By measuring the membrane lipid composition of breast cancer tissue, Hilvo and colleagues (18) reveal elevated levels of palmitate-containing phosphatidylcholine species in breast cancer relative to normal adjacent tissue. This trend is in accordance with breast cancer progression, predicted reduced survival, and more prominent in high histologic grade cancers. Moreover, breast cancer and other cancer cells can be rescued from the proapoptotic effect of fatty acid synthase (FASN) suppression by the exposure to exogenous palmitate (19). In spite of the physiologic significance of FFA in the breast cancer biology, the precise molecular mechanisms by which FFA, especially palmitate, might influence cancer development and progression, still remain to be elucidated.

Active TGFβ and related factors have various regulatory activities that affect cell proliferation, differentiation, apoptosis, migration, adhesion, survival, development, tissue repair, tumorigenesis, immune defense, and inflammation (20). Hence, TGFβ family members are important in maintaining the homeostasis of adult cells and tissues. Ablative TGFβ signaling results in many human diseases, for example, cancer and fibrosis (21, 22). Importantly, the TGFβ pathway is involved in various metastatic processes and intensely influences the ability of cancer cells to spread throughout the body (23), nonetheless little is known about its molecular mechanism(s) or regulation. An essential step in TGFβ signaling transduction is dependent on the translocation of the SMADs from the cytoplasm to the nucleus (24). Upon binding with ligands, the TGFβ type I receptors are activated and directly phosphorylate the receptor-regulated SMADs (R-SMAD), for example, SMAD2/SMAD3, which subsequently form complexes with SMAD4, then together accumulate in the nucleus, where they are implicated in the regulation of transcription of target genes (25, 26).

As palmitate is the most abundant free saturated fatty acid in human serum and in the diet (27), we examined the effects of palmitate on the TGFβ signaling pathway, which plays crucial roles in the pathogenesis of cancer (28), in human breast cancer cells. Here, we demonstrate that palmitate facilitates TGFβ-induced ERK activation and SAMD4 nuclear retention, thus promoting TGFβ-dependent cancer invasion. Using a HFD-induced obese animal xenograft model of breast cancer, our results further explicated the therapeutic targets of the obesity-induced breast cancer metastasis and exhibited pronounced promise to efficiently thwart obesity-related breast cancer progression. In addition, tissue microarray analysis of overweight/obese breast cancer patient specimens further substantiated that phospho-ERK levels correlated with activated TGFβ signaling and metastasis. This study not only explains the crucial molecular mechanism by which FFA promotes the TGFβ signaling but also provides molecular characterization of the high FFA-induced breast cancer metastasis.

Materials and Methods

Cell culture, treatment, and standard assays

Human breast cancer cell lines, MCF-7, MDA-MB-231, BT549, and MDA-MB-468 cells were obtained directly from the ATCC. MCF7-HER2 and MCF7-neo cells (MCF-7 cells transfected with empty vector) were kind gift from Dr. Kent Osborne (Baylor College of Medicine, Houston, TX). These cells were authenticated by Bio-Synthesis, Inc., by short tandem repeat (STR) profiling and monitoring cell morphology and biologic behavior, and tested to exclude mycoplasma contamination before experiments. Cells were cultured not more than 3 months after resuscitation. Palmitic acid was added into the cell culture medium as palmitate–BSA complex as described in our previous work (29). Control groups were incubated with fatty acid-free BSA. Standard cell culture, immunoprecipitation and immunoblotting analysis, immunostaining analysis, quantitative real-time RT-PCR, in vitro invasion assay, wound-healing assay were carried out as described previously (30–33).

Nuclear and cytoplasmic fractionation

Subcellular fractionation was performed as described in our previous work (27). Nuclear and cytoplasmic fractions were assessed by immunoblotting of histone H3 and vinculin, GAPDH, or actin, respectively, which were used as loading controls.

Plasmids and shRNAs

Expression plasmids for wild-type SMAD4 and two SMAD4 mutants T277A and T277D were obtained from Shanghai HarriPort Biotech Co., Ltd. Plasmids were transfected with Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche). Human USP9x shRNAs and control shRNA lentiviral particles were obtained from Santa Cruz Biotechnology. For optimal shRNA plasmid transfection efficiency, Santa Cruz Biotechnology’s shRNA plasmid transfection reagent and shRNA plasmid transfection medium were used according to the manufacturer’s transfection protocol.

In vivo model of obesity-induced metastases

Female athymic nude mice (4 weeks of age) were anesthetized and ovarioctomized as described previously (34) and allowed to recover for 2 weeks. Diet-induced obesity was induced by feeding mice with a 45% kcal fat diet containing primarily lard with 17% sucrose (high fat sucrose diet, HFSMD) for an indicated time (n = 8/group). Control groups were fed with a standard chow-based diet (Chow). The mice were fed the aforementioned diets for 6 weeks and the body weight was measured every week. The cultured 4TO7 cells labeled with luciferase (4TO7-Luc) in the logarithmic phase were collected and diluted to 1 × 10⁷ cells/mL. Then, 100-µL cell suspension (in Matrigel) was inoculated into the second mammary fat pad on the right side of mice. PD0325901 (Sigma-Aldrich) was dissolved originally in DMSO as a stock solution (50 mg/mL; ref. 35). The stock solution was then diluted in water containing 0.05% (hydroxypropyl) methylcellulose and 0.02% Tween 80. The 250 µL PD0325901 formulation was administered to mice (25 mg/kg dose) by gavage 3 times a week for the duration of each study. Control mice were treated with vehicle by the same route. Tumor-bearing mice were administered with 100 µL of WP1130 suspension (40 mg/kg, i.p.) every other day. Treatments began 1 week after cell inoculations. Prior to tumor observation, mice were intra-peritoneally injected with luciferin (1.5 mg/10 g). After anesthesia with saturated Avertin (0.18 mL/10 g, i.p.), mice were placed in a small-animal in vivo imaging system and the images were acquired. All mouse experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University.
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Statistical analysis

Statistical analysis in this study was calculated with SPSS version 18.0 software (SPSS Inc.). Data were expressed as "mean value ± SD." The significance of mean values between two groups was determined by Student t test. All differences were two-sided. The significance of the data from patient specimens was analyzed by the \( \chi^2 \) test or the Pearson correlation coefficient test. A P value less than 0.05 was considered statistically significant.

Results

FFA treatment promotes TGF\( \beta \)-induced ERK activation, SMAD4 nuclear accumulation, and gene expression

First, we investigated the fate of ERK and SMAD4 after prolonged TGF\( \beta \) signaling. Nuclear and cytoplasmic extracts were prepared from MCF-7 cells incubated with TGF\( \beta \) for different times. Nuclear and cytoplasmic fractions were assessed by Western blotting of histone H3 and vinculin, respectively, which were used as loading controls. Control experiments demonstrated that there was no cross-contamination of nuclei with cytoplasm or vice versa (Fig. 1A; Supplementary Fig. S1A). The levels of p-ERK1/2 increased at early times after TGF\( \beta \) treatment and subsided to its basal levels after 5 hours of treatment. The transient increase induced by TGF\( \beta \) is coincident with nuclear SMAD4. As shown in Fig. 1A and B, SMAD4 levels start to increase in the nuclear fraction after a 30-minute treatment with TGF\( \beta \). As the SMAD4 levels elevate in the nuclear extracts, it correspondingly drops in the cytoplasmic extracts. After prolonged TGF\( \beta \) treatment (5 and 8 hours), levels of SMAD4 in the cytoplasm augment again to approximately the levels observed in unstimulated cells (Fig. 1A and B). Therefore,  

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the dynamics of nuclear SMAD4 could be due to its import into the nucleus from cytoplasm at the early stage of TGFβ stimulation and export of SMAD4 to the cytoplasm after prolonged TGFβ stimulation. The same experiment was performed in MDA-MB-231 cells (Supplementary Fig. S1). The levels of p-ERK and nuclear SMAD4 exhibit basically the same pattern of increase and decrease in MDA-MB-231 cells as it was observed in MCF-7 cells.

To investigate the effect of FFA on p-ERK and nuclear SMAD4 levels, we treated MCF-7 cells with 400 μmol/L palmitate, a concentration that mimics hyperlipidemia condition, for various durations. As shown in Fig. 1C and Supplementary Fig. S1C, incubation with palmitate further increases p-ERK and nuclear SMAD4 levels and maintains their high levels at the late stage of TGFβ exposure (5–8 hours). Our data indicate that palmitate facilitates and stabilizes ERK activation, which may lead to SMAD4 nuclear accumulation in human breast cancer cells.

To determine whether TGFβ target genes are further induced in response to palmitate, we performed qRT-PCR (Fig. 1D). The expression of Twist and Snail mRNA is induced by TGFβ1. Palmitate significantly promotes TGFβ-induced Twist mRNA expression with a maximal induction of 3.7-fold when compared with untreated controls. Palmitate treatment alone does not significantly increase Twist mRNA levels.

**ERK activation promotes TGFβ-induced SMAD4 nuclear accumulation via USP9x–SMAD4 interaction and nuclear SMAD4 deubiquitination**

Nucleocytoplasmic shuttling of SMADs is a critical regulatory step in TGFβ signaling and plays an important role in controlling gene expression (36). In certain cases, this intracellular trafficking is regulated frequently by posttranslational modifications such as phosphorylation and ubiquitination (37). Next, we determined whether ERK, an upstream kinase of SMADs (38), and USP9x, a deubiquitinating enzyme essential for TGFβ signaling and SMAD4 monoubiquitination status (39), plays a role in TGFβ-induced SMAD4 nuclear accumulation. As indicated in Fig. 1E and Supplementary Fig. S1E, inhibition of ERK by AZD6244, a potent and selective ERK inhibitor, significantly abolishes palmitate-induced SMAD4 nuclear accumulation in breast cancer cells. Similar effects were also observed when cells were treated with USP9x DUB activity inhibitor WP1130. These results suggest that ERK and USP9x signalings are upstream events of SMAD4 nuclear accumulation.

To further substantiate mechanisms underlying the nuclear retention of SMAD4 by TGFβ, we examined levels of USP9x–SMAD4 interaction and SMAD4 monoubiquitination. As indicated in Fig. 1F–H and Supplementary Fig. S1F–S1H, TGFβ treatment dramatically increases USP9x–SMAD4 interaction and suppresses SMAD4 monoubiquitination in nuclear fraction. These effects are significantly reversed by inhibition of ERK and USP9x with their corresponding inhibitors. Intriguingly, USP9x–SMAD4 interaction negatively correlates with TIF1γ–SMAD4 interaction, implying that USP9x-selective binding to SMAD4 in competition with TIF1γ facilitates nuclear SMAD4 deubiquitination and retention.

**Activation of ERK is required for FFA promotion of TGFβ-induced nuclear USP9x–SMAD4 interaction, SMAD4 deubiquitination, SMAD3–SMAD4 complex formation, and gene expression induction**

To substantiate the effects of FFA on TGFβ signaling pathway, we show that palmitate further promotes TGFβ-induced ERK phosphorylation and SMAD4 nuclear retention, and that these effects are completely blocked by AZD6244. In addition, inhibition of USP9x by WP1130 ablates palmitate promotion of TGFβ-induced SMAD4 nuclear retention (Fig. 2A). These results were further confirmed by immunofluorescence staining that palmitate further enhances TGFβ-induced SMAD4 nuclear retention and cells coincubated with palmitate and AZD6244 or WP1130 exhibited a decrease of nuclear SMAD4 in response to TGFβ (Fig. 2E; Supplementary Fig. S2B). To further explore the molecular mechanism(s) leading to this observation, we analyzed nuclear SMAD4 protein interaction and modification. We demonstrate that palmitate further increases TGFβ-induced nuclear USP9x–SMAD4 interaction, SMAD4 deubiquitination, and SMAD3–SMAD4 complex formation, and these effects completely blocked by either AZD6244 or WP1130 (Fig. 2B and C; Supplementary Fig. S2A). Furthermore, palmitate can further enhance TGFβ-induced expression of target genes, Twist and Snail, at protein and mRNA levels, which can be abolished by inhibition of ERK and USP9x with their pharmaceutical inhibitors (Fig. 2A and D). Taken together, these results suggest that ERK-induced nuclear USP9x–SMAD4 interaction and SMAD4 deubiquitination are affected by palmitate, resulting in the aggravated and sustained activation of TGFβ signaling and higher expression of downstream target genes. Such effects may enhance the risk of breast cancer development and metastasis.

**FFA enhances TGFβ-induced invasion and migration by activating ERK and USP9x**

The capability of cancer cells to undergo invasion and migration allows them to change location within the tissues and is crucial for cancer progression and metastasis. Because TGFβ/SMAD signaling is the primary pathway regulating invasion in breast cancer (40), next, we determined whether FFA has an effect on TGFβ1-induced invasion and migration of breast cancer cells. Palmitate treatment augments the TGFβ1-induced invasion and migration of MCF-7 and MDA-MB-231 cells (Fig. 3). To ascertain that the effect of FFA on the TGFβ1-induced invasion and migration is mainly dependent on ERK and USP9x, we inhibited ERK and USP9x using their pharmaceutical inhibitors. Inhibition of ERK and USP9x in MCF-7 and MDA-MB-231 cells significantly decreases the promoting effect of palmitate on the TGFβ1-induced invasive and migration ability of these cells (Fig. 3). Moreover, the scratch assays were also performed with one additional cell line, BT549 (Supplementary Fig. S3). Collectively, these results suggest that ERK and USP9x play critical roles in the regulation of TGFβ1-induced invasion and migration of breast cancer cells in response to high FFA.

**SMAD4 T277 phosphorylation mediates FFA promotion of TGFβ-induced USP9x–SMAD4 interaction, nuclear SMAD4 retention, and SMAD3–SMAD4 complex formation**

It has been shown that SMAD4 can be constitutively phosphorylated (41), nonetheless the phosphorylation sites, the upstream kinases related to these phosphorylations and the implication of the phosphorylations in Homo sapiens have not yet been clarified. A study from Roelen and colleagues (42) suggests that MAP kinase can phosphorylate SMAD4 at threonine 276 (T276) in Sus scrofa kidney epithelial cells LLC-PK1, and that this phosphorylation is imperative for TGFβ-induced nuclear accumulation and transcriptional activity of SMAD4. Here, to determine the phosphorylation sites in human breast cancer cells
that is responsible for palmitate/ERK-induced SMAD4 nuclear accumulation and its transcriptional activity, we analyzed SMAD4 T277, the orthologous residue in human. Cellular SMAD4 phosphorylation at T277 was measured by IHC and immunofluorescence staining with anti-T277 antibody. As indicated in Fig. 4A, palmitate treatment significantly increases TGFβ-induced SMAD4 phosphorylation at T277, and the phosphorylated SMAD4 gathers mainly in nuclear region. AZD6244 can significantly inhibit TGFβ-induced SMAD4 phosphorylation and abolish palmitate promotion of TGFβ-induced SMAD4 phosphorylation. These results suggest that SMAD4 T277 is an important phosphorylation site that can be phosphorylated by ERK in human breast cancer cells and is associated with SMAD4 nuclear accumulation in response to FFA (Fig. 4A). SMAD4 T277 phosphorylation under different treatments was confirmed by Western blot analysis (Fig. 4B; Supplementary Fig. S4B). Because HER-2 overexpressing MCF7 cells (MCF-7-HER2) have a significantly increased ERK activation [43], we were interested to know whether this cell line has higher levels of SMAD4 phosphorylation at T277. As shown in Fig. 4C, MCF-7-HER2 cells have elevated levels of constitutive SMAD4 T277 phosphorylation and nuclear accumulation compared with its control MCF-7-neo cells.

To further test this notion directly, MCF-7 cells were transfected with SMAD4 phosphorylation defect mutant T277A. As shown in Fig. 4D and E and Supplementary Fig. S4E, IHC staining and Western blot analysis demonstrates that T277A transfection significantly inhibits TGFβ-induced SMAD4 phosphorylation and abolishes palmitate promotion of TGFβ-induced SMAD4

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**Figure 2.**

ERK activation is responsible for FFA promotion of TGFβ-induced USP9x–SMAD4 interaction, SMAD3–SMAD4 complex formation, nuclear SAMD4 retention, and gene expression. MCF-7 cells were treated with BSA or palmitate (PA) in the presence or absence of AZD6244 or WP1130 for 4 hours followed by treatment with or without 3 ng/mL of TGFβ for another 4 hours. A, Nuclear extracts were made to analyze nuclear SMAD4 levels. Whole-cell extracts were prepared and subjected to Western blot analysis using p-ERK, ERK, Twist, Snail, and actin antibodies. B, Nuclear extracts were made and coimmunoprecipitation of endogenous SMAD4 with USP9x or SMAD3 was performed. Data are presented as mean fold increases (±SD) in treated groups over basal values from three independent experiments. *, P < 0.01 versus controls; #, P < 0.01 versus BSA/−TGFβ; $, P < 0.01 versus palmitate/−TGFβ. C, Nuclear extracts were made and SMAD4 monoubiquitination was detected as described above. *, P < 0.01 versus controls (BSA/−TGFβ); #, P < 0.01 palmitate versus BSA; $, P < 0.01 palmitate + AZD versus palmitate, palmitate + WP1130 versus palmitate. D, total RNA was extracted and analyzed for Twist mRNA by real-time PCR. *, P < 0.05 versus controls; #, P < 0.01 versus TGFβ; $, P < 0.01 versus palmitate/−TGFβ. E, MCF-7 cells were treated with TGFβ in the presence or absence of palmitate, AZD6244, or WP1130 and processed for immunofluorescence with anti-SMAD4 antibody. The same cells were also stained with DAPI to visualize nuclei. Intensity of nuclear SMAD4 among these cells was quantified with Image-Pro Plus 6.0 software. The percentages of nuclear SMAD4 levels illustrated on the right represent the mean of three independent experiments, and error bars indicate the SD.
phosphorylation, as well as diminishes SMAD4 nuclear retention. This result was also substantiated by Western blot analysis showing that T277A dramatically reverses TGFβ-induced SMAD4 nuclear accumulation and abolishes palmitate promotion of TGFβ-induced SMAD4 nuclear accumulation, accompanied by decreased USP9x–SMAD4 interaction and increased TIF1γ–SMAD4 binding (Fig. 4F; Supplementary Fig. S4F). These results suggest that T277 phosphorylation may regulate USP9x–SMAD4 interaction and SMAD4 ubiquitination status, influencing SMAD4 subcellular localization.

Next, to further substantiate this possibility, SMAD4-null breast cancer cell line MDA-MB-468 was transfected with SMAD4 phosphorylation defect mutant T277A and phosphorylation mimic mutant T277D. As shown in Fig. 4G and Supplementary Fig. S4G, T277A undergoes less USP9x–SMAD4 interaction and SMAD4 monoubiquitination than WT protein, whereas T277D shows lower level of SMAD4 monoubiquitination, implying that SMAD4 T277 phosphorylation facilitates USP9x–SMAD4 interaction and reduces the chance of SMAD4 to bind with TIF1γ, thus decreasing SMAD4 monoubiquitination, which is important for its nuclear localization, combination with SMAD3, and transcriptional activity.

USP9x-selective binding to SMAD4 in competition with TIF1γ promotes nuclear SMAD4 retention, SMAD3–SMAD4 complex formation, and target gene expression

To further substantiate the physiologic role of USP9x in cellular response to palmitate, we used shRNA-mediated knockdown of USP9x in MCF-7 cells. As with our USP9x knockdown studies, control shRNA was used as a control. Knockdown of USP9x was confirmed by Western blot analysis (Fig. 5A). USP9x shRNA delivery significantly inhibits TGFβ-induced SMAD4 nuclear retention and eliminates palmitate promotion of TGFβ-induced SMAD4 nuclear retention. Accordingly, USP9x knockdown could block TGFβ-induced Twist expression and inhibit the promoting effect of palmitate on TGFβ-induced Twist expression. Intriguingly, USP9x-deficient cancer cells exhibit higher levels of TGFβ-induced TIF1γ–SMAD4 interaction and SMAD4 monoubiquitination, and lower levels of SMAD3–SMAD4 interaction.
under the conditions of either BSA or palmitate exposure relative to control cells (Fig. 5B and C; Supplementary Fig. S5). Taken together, these results demonstrate that USP9x selectively binds to SMAD4 in competition with TIF1γ and deubiquitinates SMAD4, promoting nuclear SMAD4 retention, SMAD3–SMAD4 complex formation, and target gene expression. Our results, for the first time, directly show that SMAD4 posttranslational modifications including phosphorylation and ubiquitination regulate its nuclear–cytoplasmic shuttling.

**ERK and USP9x is responsible for obesity-related breast cancer metastasis**

We next ascertained whether the ERK and USP9x pathway observed in cell culture could modulate breast cancer metastasis in obese animal models. Plasma FFA levels are significantly increased in overweight/obese individuals (44) and hyperlipidemia in metabolic syndrome is characterized by an increase in FFA (45). To simulate such diet-induced metabolic abnormalities, a nude mouse model of diet-induced obesity was used in this study by feeding ovariectomized (OVX) athymic nude mice a HFSD (46). As indicated in Fig. 6A, the body weight is remarkably higher in mice fed the HFSD versus mice fed chow diet. The OVX athymic nude mice implanted with 4TO7 cells into the mammary fat pads do not produce spontaneous lung metastases when fed with chow diet (Fig. 6B). However, approximately 63% of the HFSD-induced obese mice produce lung metastases (Fig. 6B–D), suggesting that HFD and obesity are related to the metastatic phenotype. Importantly, the ability of the breast cancer cells to metastasize to the...
lung in nude mice and lung metastatic lesions were significantly repressed by inhibition of ERK and USP9x with administration of their inhibitors. These data suggest that ERK and USP9x are responsible for the metastasis formation in obese mice.

ERK and SMAD4 phosphorylation levels correlate with Twist expression and overweight/obesity-related metastasis in human breast cancer

In an attempt to investigate the clinical relevance of ERK and SMAD4 pathway in cancer progression and metastasis, we examined the expression levels of p-ERK, p-SMAD4, p-Twist, and Twist protein expression was analyzed by immunoblotting. Nuclear SMAD4, total USP9x, and Twist protein expression was analyzed by immunoblotting. Nuclear SMAD4-SMAD3 complex formation and target gene expression. A, MCF-7 cells stably expressing control shRNA or USP9x shRNA were incubated with BSA or palmitate (PA) in the presence or absence of TGFβ for 2 hours. Nuclear SMAD4, total USP9x, and Twist protein expression was analyzed by immunoblotting. B, Nuclear SMAD4–SMAD3 and SMAD4–TIF1γ interaction was determined by immunoprecipitation (IP) with SMAD4 antibody, followed by immunoblotting with SMAD3 or TIF1γ antibody. C, MCF-7 cells were transfected with WT-SMAD4, HA-ubiquitin, and the indicated shRNAs. The cells were treated with palmitate in the presence or absence of TGFβ for 2 hours and nuclear extracts were made. The SMAD4 monoubiquitination (Mono-Ub) was detected by anti-SMAD4 IP and immunoblot with HA-ubiquitin. *P < 0.01 versus shControl/−TGFβ/−palmitate; #P < 0.01 versus shControl/−palmitate; $P < 0.01 versus shControl/+ palmitate.

Figure 5.

USP9x-selective binding to SMAD4 in competition with TIF1γ facilitated nuclear SMAD4 retention, formation of the nuclear SMAD3–SMAD4 complex, and target gene expression. A, MCF-7 cells stably expressing control shRNA or USP9x shRNA were incubated with BSA or palmitate (PA) in the presence or absence of TGFβ for 2 hours. Nuclear SMAD4, total USP9x, and Twist protein expression was analyzed by immunoblotting. B, Nuclear SMAD4–SMAD3 and SMAD4–TIF1γ interaction was determined by immunoprecipitation (IP) with SMAD4 antibody, followed by immunoblotting with SMAD3 or TIF1γ antibody. C, MCF-7 cells were transfected with WT-SMAD4, HA-ubiquitin, and the indicated shRNAs. The cells were treated with palmitate in the presence or absence of TGFβ for 2 hours and nuclear extracts were made. The SMAD4 monoubiquitination (Mono-Ub) was detected by anti-SMAD4 IP and immunoblot with HA-ubiquitin. *P < 0.01 versus shControl/−TGFβ/−palmitate; #P < 0.01 versus shControl/−palmitate; $P < 0.01 versus shControl/+ palmitate.

Discussion

In this study, we reveal that the USP9x–SMAD4 interaction represents a pivotal mechanism for regulating TGFβ signaling and breast cancer cell invasion and metastasis. TGFβ activates ERK and subsequently marks SMAD4 for phosphorylation at Thr277. In this study, for the first time, we showed that in human breast cancer cells, phosphorylation of SMAD4 at Thr277 facilitates USP9x–SMAD4 interaction. Such interaction contributes to low monoubiquitination status of SMAD4, SMAD4 nuclear retention, SMAD3–SMAD4 complex formation, and implement their transcription function. Under normal physiologic conditions, induction of signaling by TGFβ is not sustained for very long and the signaling cascade is terminated upon the restoration of ERK activity, leading to the reinstatement of SMAD4 Thr277 phosphorylation. Consequence of such event is USP9x–SMAD4 disassociation and then SMAD4–TIF1γ interaction, which marks SMAD4 for monoubiquitination, resulting in SMAD4 nuclear export. In both humans and rodents, consuming HFD or being...
overweight/obese is usually accompanied with high levels of plasma FFA. In this study, for the first time, we showed that high FFA intensifies TGFβ-induced ERK activation and SAMD4 phosphorylation, USP9x-SMAD4 interaction, and nuclear accumulation, thus promoting TGFβ-dependent cancer development and progression. However, inhibition of ERK and USP9x with pharmaceutical or genetic approach in breast cancer cells abolishes FFA promotion of TGFβ-induced SMAD4–USP9x interaction, SMAD4 nuclear retention, SMAD3–SMAD4 complex formation, and target gene expression, resulting in a decrease in cancer cell invasion and metastasis (Fig. 7C).

In vivo studies further substantiate that inhibition of ERK and USP9x suppressed obesity-induced metastasis. These findings are highlighted by the significant clinical observation that phospho-ERK levels positively correlated with activated TGFβ signaling and metastasis in overweight/obese breast cancer patients. These data explain not only how TGFβ signaling pathway functions under the physiologic and pathologic conditions, but also provides a novel mechanistic explanation of why overweight/obesity/high FFA increases the risk of developing distant metastatic recurrence and is associated with poor prognosis of breast cancer. In addition, we provide the evidence that ERK- and USP9x-specific inhibitors can be used as therapeutic targets for treatment of obesity-related breast cancer.

SAMD4 plays a crucial role in all TGFβ signaling pathways and has been identified as a tumor suppressor. The role of SMAD4-mediated TGFβ signaling in tumor progression and metastasis is contentious. Here, we demonstrate that SMAD4 nuclear–cytoplasm shuttling is dependent on its posttranslational modifications including phosphorylation and monoubiquitination, which play counterbalancing roles for maintaining a delicate and an accurate TGFβ physiologic signaling. We also found that FFA/ERK-induced SMAD4 T277 phosphorylation is indispensable in SMAD4–USP9x interaction and its deubiquitination as well as subsequent nuclear accumulation, which is required for TGFβ-induced breast cancer invasion and metastasis under the high FFA/obesity conditions. These findings significantly extend our understanding of molecular mechanisms underlying SMAD4-mediated TGFβ signaling in breast cancer progression, which might facilitate the development of effective therapies targeting TGFβ signaling for the treatment of human tumors.

Figure 6.
The ERK and USP9x inhibitors inhibits the stimulative effect of obesity on metastases in vivo. A, Diet-induced obesity in ovariectomized athymic nude mice that were fed with a HFSD. A standard chow-based diet (Chow) was used as the control diet (n = 8/group). The mice were fed the diets for 12 weeks and the body weight of each mouse was measured every week. B, Tumor growth and metastasis in diet-induced obese ovariectomized athymic nude mice versus the control animal group with the regular diet. After 6 weeks of feeding, luciferase-labeled 4T107 tumor cells were implanted into the mammary fat pad on the right side of mice. One week after cell inoculations, PD0325901 was administered to mice (25 mg/kg dose) by gavage 5 times a week. Control mice were treated with vehicle by the same route. Tumor-bearing mice were administered with WP1130 suspension (40 mg/kg, i.p.) every other day. Six weeks after inoculation, mice were intraperitoneally injected with fluorescein substrate (1.5 mg/10 g). After anesthesia with saturated avertin (0.18 mL/10 g, i.p.), mice were placed in a small-animal in vivo imaging system and images were acquired. C, The photon quantity in each mice group was analyzed (bottom). #, P < 0.05 versus Chow/Control; *, P < 0.05 versus HFSD/Control. D, Percentage of mice with lung metastases, as determined by lung bioluminescence.
The normal cellular protein ubiquitination levels are maintained by the ubiquitin ligases and deubiquitinating enzymes (DUB), two enzyme families with opposite activities (47). The X-linked deubiquitinase USP9X belongs to the family of DUB enzymes. It controls various cellular functions, for example, mediating cell survival (48), regulating cell adhesion molecules, apoptosis, cell polarity, chromosome segregation, NOTCH, mTOR, and TGFβ signaling (39, 49–51), by deubiquitinating and stabilizing its substrates. A decade ago, in a study Xenopus, Dupont and colleagues (52) identified USP9x as the SMAD4 deubiquitinase and described its counteracting protein, SMAD4 ubiquitin ligase TIF1γ. They also revealed that TIF1γ antagonizes TGFβ signals through binding to SMAD4 and enhancing its ubiquitylation and that USP9x is a TGFβ pathway component required for SMAD4 activity. In addition, studies in the fly wing suggested the epistatic relationship between USP9x and TIF1γ in the competition for SMAD4 (39). Consistent with these studies, we, for the first time, observed that in human breast cancer cells, USP9x selectively binds to SMAD4 in competition with TIF1γ. In these cells, USP9x facilitates nuclear SMAD4 deubiquitination and retention, which in turn promotes target gene transcription. Therefore, the counterbalancing activities of USP9x and TIF1γ fine tune SMAD4 function in response to TGFβ signals. More importantly, depletion of USP9x hinders motility of MDA-MB-231 metastatic breast cancer cells (53), which may be an indication for the role of this protein in tumor cells invasion and metastasis. Clinically, USP9x overexpression correlates with poor prognosis in human non–small cell lung cancer, multiple myeloma, and esophageal squamous cell carcinoma.
Moreover, tumors with low USP9x expression are particularly sensitive to some of the conventional therapeutic agents (48). Accordingly, here we demonstrate that the inhibition of USP9x in breast cancer cells abolishes FFA promotion of TGFβ-induced SMAD4 nuclear retention, SMAD3–SMAD4 complex formation, and target gene expression, resulting in suppression of cancer cell invasion and metastasis. Furthermore, inhibition of USP9x in vivo suppressed obesity-induced breast cancer metastasis.

In conclusion, our results indicate that TGFβ transiently activates ERK and subsequently marks SMAD4 for activation via Thr277 phosphorylation, which facilitates SMAD4–USP9x interaction, SMAD4 nuclear retention, SMAD3–SMAD4 complex, and promotes TGFβ-mediated target gene transcription. USP9x competitively inhibits TIF1γ from binding and monoubiquitinating SMAD4, thus maintains SMAD4 nuclear retention and stabilizes the SMAD3–SMAD4 complex in the nucleus. High FFA further intensifies TGFβ-induced ERK activation, SMAD4 phosphorylation, SMAD4 nuclear retention, and SMAD3/SMAD4 formation, thus overactivating this system and promoting TGFβ-dependent cancer progression (Fig. 7C). Our findings suggest that FFA overactivation of ERK stimulates cancer cell invasion and metastasis, whereas inhibition of ERK or USP9x hinders obesity/high FFA-associated breast cancer metastasis. These findings shed light on mechanisms of cancer progression and invasion and provide strategies for therapeutic intervention of cancer progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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Grant Support
This work was supported in part by NIH-NIMHD U54MD007598, NIH/NCH1U54CA14393, U56 CA101599-01; Department-of-Defense Breast Cancer Research Program grant B043180, NIH/NCATS CTSI U54 TR000124 to J.V. Vadgama, and Accelerating Excellence in Translational Science Pilot Grants G0812D05, NIH/NCI SC1CA200517 to Y. Wu.

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Received August 1, 2016, revised November 22, 2016, accepted December 10, 2016, published OnlineFirst January 23, 2017.
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