

Programmed cell death 4 inhibits leptin-induced breast cancer cell invasion

VIANEY GONZÁLEZ-VILLASANA^{1,2}, RENÉ NIEVES-ALICEA³,
VANITY McMURTRY², YOLANDA GUTIÉRREZ-PUENTE¹ and ANA M. TARI^{2,4}

¹Departamento de Bioquímica, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, N.L., México; ²Department of Experimental Therapeutics, University of Texas M.D. Anderson Cancer Center, Houston, TX; ³Department of Nanomedicine and Biomedical Engineering, University of Texas Health Science Center, Houston, TX; ⁴Division of Hematology-Oncology, University of Florida, Gainesville, FL, USA

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Abstract. Obesity is a significant risk factor for post-menopausal women to develop and die from breast cancer. Leptin, an adipokine is produced in high levels in obese individuals, and its receptor is overexpressed in breast tumors and lymph node metastases. Previously, we demonstrated that leptin stimulates breast cancer cell invasion, which is correlated with breast cancer metastasis. Programmed cell death 4 (PDCD4) has been shown to block cancer cell invasion. However, whether PDCD4 blocks leptin-induced breast cancer cell invasion is not known. Here, we report the novel findings that leptin failed to induce invasion in MCF-7 breast cancer cells overexpressing PDCD4 (MCF-7/PDCD4). Tissue inhibitor of metalloproteinase-2 (TIMP-2) was essential to the anti-invasive effect of PDCD4, as leptin stimulated the invasion of MCF-7/PDCD4 cells pretreated with TIMP-2 siRNA. Furthermore, TIMP-2 knockdown allowed leptin to augment phosphorylation of extracellular signal-regulated kinases 1,2 and signal transducer and activator of transcription 3, but not that of Jun N-terminal kinases. These data indicate that PDCD4 utilizes TIMP-2 to exert its anti-invasive effect by suppressing leptin-induced activation of extracellular signal-regulated kinases 1,2 and signal transducer and activator of transcription 3. Novel therapeutic strategies aiming at enhancing PDCD4 expression in breast tumors may be able to stop obesity-related breast tumor progression and prolong the life of patients.

Introduction

Breast cancer is the most frequently diagnosed cancer in women. In 2010, about 207,090 new cases of invasive breast cancer were expected to be diagnosed among women, and about 39,840 women were expected to die from breast cancer. One of the major risk factors for postmenopausal women to develop breast cancer is obesity. Tumors from breast cancer patients with high body mass index are typically of the hormone-responsive type (1-3). Leptin, a 16-kDa hormone that was discovered as a regulator of body weight and energy balance acting in the hypothalamus (4), is an important hormonal link between obesity and breast cancer. As body weight and fat mass increase, the level of circulating leptin increases (5,6).

High expression of leptin and the long/signaling form of leptin receptor have been found in breast tumors (7-11). Leptin-deficient mice have decreased incidence of oncogene-induced mammary tumors (12). An inhibitor targeted to the leptin receptor suppresses breast tumor formation *in vivo* (13). High levels of leptin in breast tumors have been correlated with poorer patient prognosis and increased incidence of metastasis (7,10,11). Breast cancer metastasis is directly associated with breast cancer cell invasion. Recently, we showed that leptin stimulates invasion of MCF-7 breast cancer cells by activating Jun N-terminal kinases (JNK), which increase matrix metalloproteinase-2 activity (14).

Leptin has also been associated with the development of anti-estrogen resistance. Leptin is a potent modulator of the estrogen receptor signaling pathway (15-17). Leptin stimulates the production of estrogen by increasing aromatase expression. Leptin activates estrogen receptor in a ligand-independent manner. Further, leptin stabilizes the estrogen receptor by interfering with the proteasome-mediated degradation of this receptor. Therefore, high expression of leptin may be associated with poorer prognosis in breast cancer patients because it stimulates invasion and anti-estrogen resistance in breast cancer cells. Novel therapeutic strategies aiming at disrupting leptin signaling in breast tumors may be able to impede obesity-related breast tumor progression and prolong patients' lives.

Programmed cell death 4 (PDCD4), originally identified as an inhibitor of murine cellular transformation, is a novel

Correspondence to: Dr Ana M. Tari, Division of Hematology and Oncology, University of Florida, 2033 Mowry Road, P.O. Box 103633, Gainesville, FL 32611-3633, USA
E-mail: ana.tari@medicine.ufl.edu

Abbreviations: ERK1,2, extracellular signal-regulated kinases 1,2; JNK, Jun N-terminal kinases; PDCD4, programmed cell death 4; STAT3, signal transducers and activators of transcription 3; TIMP-2, tissue inhibitor of metalloproteinase-2

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tumor suppressor. Expression of PDCD4 is decreased or lost in several tumors, including invasive ductal breast tumors (18). We showed that by increasing tissue inhibitor of metalloproteinase-2 (TIMP-2) expression, PDCD4 overexpression suppresses breast cancer cell invasion mediated by prostaglandin E₂ or interleukin-8 (19). However, it is not known whether PDCD4 could suppress leptin-induced breast cancer cell invasion.

In the present study, we report the novel finding that PDCD4 overexpression blocks leptin-induced invasion of MCF-7 breast cancer cells. We also report that PDCD4 overexpression utilizes TIMP-2 to block leptin-induced invasion by decreasing activities of extracellular signal-regulated kinases 1,2 (ERK1,2), and signal transducer and activator of transcription 3 (STAT3).

Materials and methods

Cell lines and culture conditions. Parental MCF-7 (MCF-7/WT) human breast cancer cells were stably transfected with empty pcDNA3.1 plasmid vector (MCF-7/Vector) or with pcDNA3.1 plasmid encoding the human PDCD4 gene (MCF-7/PDCD4) (19). MCF-7/Vector and MCF-7/PDCD4 cells were maintained in Dulbecco's modified Eagle's medium (DMEM/F12) (Invitrogen Corp.) supplemented with 5% fetal bovine serum (FBS) and 500 µg/ml of geneticin.

Matrigel invasion. The invasiveness of MCF-7 and MCF-7/PDCD4 cells treated with leptin was determined as previously described (14,19) by counting the number of cells that invaded through transwell inserts coated with the Matrigel artificial basement membrane. Briefly, MCF-7 and MCF-7/PDCD4 cells were collected and washed with serum-free media. Cells (4×10^5) were resuspended in 1 ml of serum-free DMEM/F-12 medium and added into 6-well plate transwell inserts (8 µm pore-size; Fisher Scientific, Middleton, VA, USA) coated with a Matrigel basement membrane (0.7 mg/ml; BD Biosciences, Bedford, MA, USA). The lower chambers were filled with 2 ml of DMEM/F-12 medium supplemented with 10% FBS. Cells in the transwell inserts were then treated with 0, 10 and 100 ng/ml of leptin in serum-free media. Seventy-two hours later, non-invading cells on the upper surface of the filter were removed with cotton swabs. Cells that invaded through the Matrigel onto the lower side of the filter were fixed, stained with the Hema-3 Stain System (Fisher Scientific), and photographed. For each filter, the number of invaded cells was counted in nine fields, and expressed as the mean number of cells of triplicate measurements. Two-sample t-tests were used to compare the number of invaded cells.

siRNA transfection. MCF-7/PDCD4 cells were plated at a density of 2×10^5 cells/well in 6-well plates. The next day, cells were transfected with 200 nM human TIMP-2 (Thermo Scientific) or non-silencing control siRNA (Qiagen) using HiPerFect® transfection reagent (Qiagen, Valencia, CA, USA). Forty-eight hours later, cells were harvested, plated at a density of 1×10^5 cells/well and treated with 100 ng/ml leptin in DMEM/F12 medium containing 5% FBS for an additional 72 h. Cells were collected for RT-PCR and Western blot analysis. Similarly, after cells were transfected with TIMP-2

or non-silencing control siRNA, cells were collected and invasion assays were performed as described above.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). To confirm that the level of TIMP-2 mRNA was reduced in cells transfected with TIMP-2 siRNA, total RNA was extracted using the RNeasy Mini kit (Qiagen). RNA (1 µg) was used in the SuperScript III one-step RT-PCR System (Invitrogen Corp.). TIMP-2 and GAPDH primer sequences have been previously described (19). GAPDH was used as loading control. TIMP-2: forward, 5'-GGT CTC GCT GGA CGT TGG AG-3' and reverse, 5'-GGA GCC GTC ACT TCT CTT G-3'. GAPDH: forward, 5'-GCC AAG GTC ATC CAT GAC AAC-3' and reverse, 5'-GTC CAC CAC CCT GTT GCT GTA-3'. The PCR conditions for all reactions were performed as described (19): 55°C for 30 min, 94°C for 2 min, 30 cycles of 94°C for 15 sec, 57°C for 30 sec, 68°C for 1 min, and 68°C for 5 min.

Western blotting. Western blotting was performed as described (14). Cells were harvested with cold PBS, and lysed with ice-cold lysis buffer and incubated on ice for 30 min. Lysates were centrifuged, supernatants were collected, and protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Samples were electrophoresed on 10% polyacrylamide gels (Bio-Rad Laboratories), and then transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked in Tris-buffered saline (20 mM Tris pH 7.6, 150 mM NaCl) with 0.1% Tween-20 containing 5% non-fat dry milk at room temperature for 30 min. After washing, primary antibodies, such as phosphorylated ERK1,2 (T²⁰²/Y²⁰⁴), total ERK1,2, phosphorylated STAT3 (Y⁷⁰⁵), and total STAT3, phosphorylated Jun N-terminal kinases (JNK) (T¹⁸³/Y¹⁸⁵), and total JNK, were added to membranes at 1:1,000 dilution. After overnight incubation at 4°C, membranes were washed and incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase. Proteins bands were detected via enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). Images were scanned by an AlphaImager densitometer (Alpha Innotech Corp., San Leandro, CA). β-actin or Grb-2 were used as loading controls. Membranes were incubated with anti-β-actin antibody (1:10,000 dilution) or anti-Grb-2 antibody (1:5,000 dilution) for 30 min at room temperature, washed, and incubated with anti-mouse secondary antibody (1:10,000 dilution) for another 30 min at room temperature.

Results

PDCD4 inhibits leptin-induced breast cancer cell invasion. We used Western blot analysis to confirm that MCF-7/PDCD4 cells express higher levels of PDCD4 protein than control MCF-7 cells. MCF-7/PDCD4 cells expressed 1.5-fold higher PDCD4 levels than control cells (Fig. 1A). We next performed Matrigel invasion assays after treating control MCF-7 cells and MCF-7/PDCD4 cells with 0, 10 and 100 ng/ml of leptin. In the absence of leptin, the number of invaded MCF-7 cells averaged 55 (Fig. 1B). In the presence of 10 and 100 ng/ml leptin, the number of invaded cells averaged 100 and 140, respectively (Fig. 1B). This confirmed our earlier report that

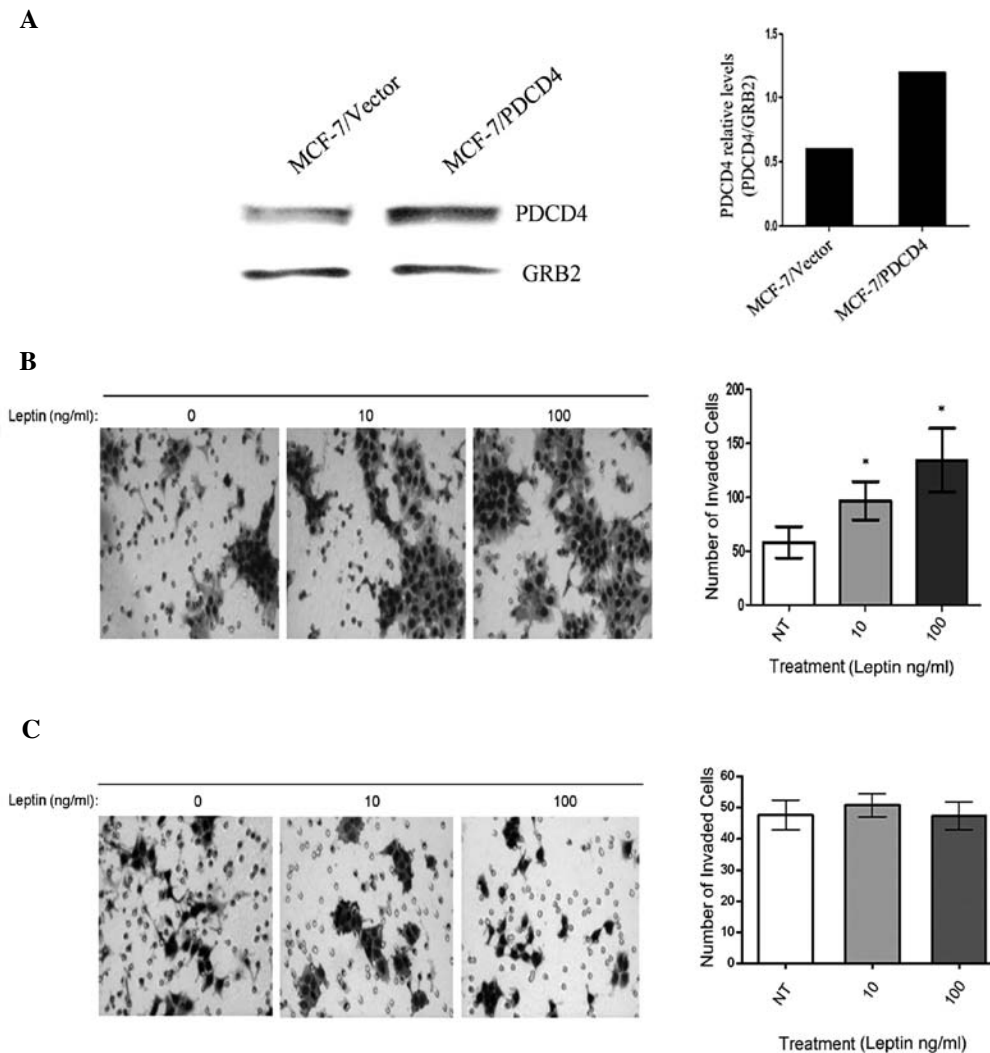


Figure 1. PDCD4 inhibits leptin-induced breast cancer cell invasion. (A) Western blot analysis was used to confirm higher PDCD4 expression in MCF-7/PDCD4 cells than in MCF-7/Vector cells. Matrigel invasion assay was performed to determine the effects of leptin on the invasiveness of (B) MCF-7/WT cells and (C) MCF-7/PDCD4 cells. The number of invaded cells was significantly higher in the leptin-treated vs. the untreated cells, * $p < 0.05$.

leptin stimulates the invasion of MCF-7 cells (14). On the other hand, leptin did not stimulate the invasion of MCF-7/PDCD4 cells; in the presence of 0, 10 and 100 ng/ml leptin, the average numbers of invaded MCF-7/PDCD4 cells were 48, 51, and 47, respectively (Fig. 1C). These data indicate that high level of PDCD4 blocks leptin-induced breast cancer cell invasion.

TIMP-2 is essential for PDCD4 to block leptin-induced breast cancer cell invasion. We were interested in determining whether TIMP-2 is essential to the anti-invasive effect of PDCD4. This is because previously we showed that PDCD4 overexpression blocks prostaglandin E_2 - or interleukin-8-induced invasion of breast cancer cells by increasing TIMP-2 expression (19). We used RT-PCR to determine the TIMP-2 mRNA levels in MCF-7/WT, MCF-7/Vector and MCF-7/PDCD4 cells. TIMP-2 mRNA levels were ~2.1- and 1.5-fold higher in MCF-7/PDCD4 cells than in MCF-7/WT and MCF-7/Vector cells, respectively (Fig. 2A). To determine whether TIMP-2 is essential for PDCD4 to block leptin-induced breast cancer cell invasion, we treated MCF-7/PDCD4 cells with TIMP-2 siRNA or with control siRNA (Fig. 2B), and then performed the Matrigel invasion assay.

When 100 ng/ml of leptin were used, the average numbers of untreated and control siRNA-treated MCF-7/PDCD4 cells that invaded through the Matrigel were 60 and 62, respectively (Fig. 2C). But under identical conditions, the average number of TIMP-2-treated MCF-7/PDCD4 cells that invaded through the Matrigel was 220 (Fig. 2C). These data demonstrate that when TIMP-2 expression is down-regulated, the invasiveness of leptin-induced MCF-7/PDCD4 cells increased significantly, indicating that TIMP-2 is essential for PDCD4 to block leptin-induced breast cancer cell invasion.

TIMP-2 knockdown increases leptin-induced phosphorylated ERK1,2 and phosphorylated STAT3 levels in MCF-7/PDCD4 cells. Leptin has been shown to activate ERK1,2, STAT3, and JNK in breast cancer (13-16,20-24). We proposed that TIMP-2 suppresses leptin-induced breast cancer cell invasion by reducing the activities of ERK1,2, STAT3, and/or JNK. Therefore, we expected TIMP-2 knockdown to increase leptin-induced ERK1,2, STAT3, and/or JNK phosphorylation. We pretreated MCF-7/PDCD4 cells with TIMP-2 siRNA or with control siRNA, before treating them with leptin. Western blot analysis was utilized to investigate the phosphorylated levels

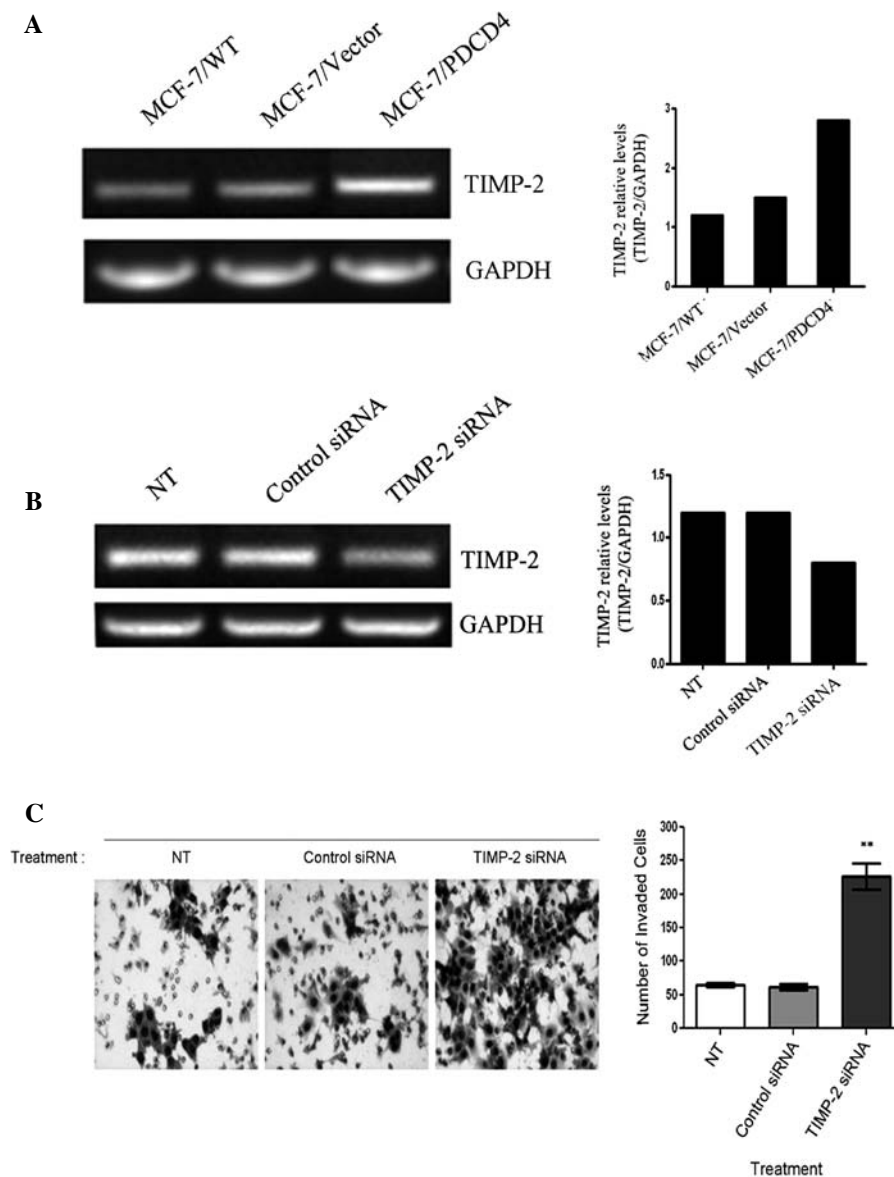


Figure 2. TIMP-2 is essential for PDCD4 to suppress leptin-induced breast cancer cell invasion. (A) RT-PCR was performed to determine *TIMP-2* mRNA levels in breast cancer cells. (B) RT-PCR was performed to confirm that *TIMP-2* siRNA reduced *TIMP-2* mRNA expression. (C) *TIMP-2* knockdown increased leptin-induced MCF-7/PDCD4 breast cancer cell invasion. * $p < 0.05$, number of invaded cells transfected with *TIMP-2* siRNA compared to cells transfected with control siRNA.

of ERK1,2, STAT3, and JNK. In the presence of leptin, MCF-7/PDCD4 cells treated with *TIMP-2* siRNA expressed 2-fold higher levels of phosphorylated ERK1,2 than control siRNA-treated cells (Fig. 3A), and 2.5-fold higher levels of phosphorylated STAT3 than control siRNA-treated cells (Fig. 3B). However, under identical conditions, there was no difference in the levels of phosphorylated JNK between *TIMP-2* siRNA- and control siRNA-treated MCF-7/PDCD4 cells (Fig. 3C). These results show that *TIMP-2* down-regulation allows leptin to stimulate ERK1,2 and STAT3 activities in MCF-7/PDCD4 cells.

Interestingly, *TIMP-2* knockdown led to an increase in phosphorylated ERK1,2 levels but a decrease in total ERK1,2 levels. Yet, *TIMP-2* knockdown stimulated phosphorylated STAT3 levels without affecting total STAT3 levels. We speculate that a feedback mechanism may exist regulating the ratio of phosphorylated ERK1,2 to total ERK1,2 levels.

Discussion

Obesity is a mortality risk factor for women diagnosed with breast cancer; women with a body mass index of at least 40 have a 2.1-fold elevated risk of death from breast cancer (25). Leptin is a vital link between obesity and breast cancer, as high levels of leptin in breast tumors have been associated with poorer patient prognosis and increased incidence of metastasis (7,10,11). Novel therapeutic strategies are needed to impede leptin-mediated breast tumor progression.

Here, we show for the first time that PDCD4 inhibits leptin-induced breast cancer cell invasion. Similarly to our previous report (19), we observed that *TIMP-2* is critical to the anti-invasive effects of PDCD4. Leptin stimulated the invasion of MCF-7/PDCD4 cells when *TIMP-2* expression was knocked down. Interestingly, PDCD4 expression is only slightly decreased in ductal carcinoma *in situ* samples in comparison

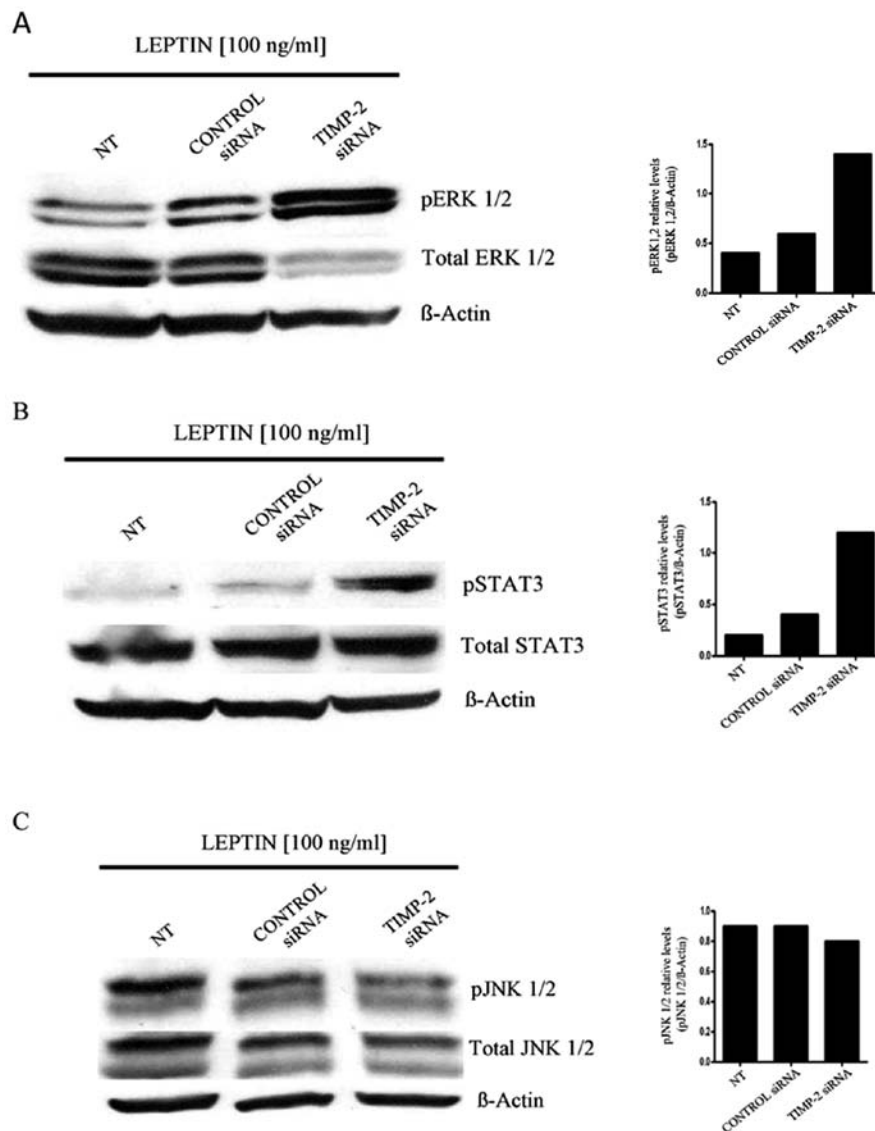


Figure 3. TIMP-2 knockdown increases leptin-induced phosphorylated ERK1,2 and STAT3 levels. MCF-7/PDCD4 cells were transfected with TIMP-2 or control siRNA for 48 h, before being treated with leptin. Western blot analysis was used to determine (A) phosphorylated ERK1,2 and total ERK1,2 levels, (B) phosphorylated STAT3 and total STAT3 levels, and (C) phosphorylated JNK and total JNK levels.

to normal breast epithelial cells, but is markedly decreased in invasive ductal carcinoma samples (26). Thus, PDCD4 may act as a breast tumor suppressor by blocking breast cancer cell invasion.

Here, we have also studied the mechanisms by which PDCD4 utilizes TIMP-2 to exert its anti-invasive effects. Leptin is known to stimulate ERK1,2, STAT3, and JNK phosphorylation in breast cancer cells (13-16,20-24). We found that TIMP-2 knockdown allows leptin to augment ERK1,2, and STAT3 phosphorylation in MCF-7/PDCD4 cells, indicating that PDCD4 utilizes TIMP-2 to counteract leptin-mediated activation of ERK1,2 and STAT3 to block invasion. Indeed, ERK1,2 and STAT3 are essential to leptin-stimulated invasion, as pharmacological inhibitors of JAK2/STAT3 and ERK1,2 blocked leptin-mediated invasion of hepatocellular and endometrial carcinoma cells (22,27). However, TIMP-2 knockdown did not change leptin-mediated JNK phosphorylation levels, indicating that TIMP-2 is not utilized by PDCD4 in the regulation of JNK activity. PDCD4 has been shown to

inhibit the transcription of mitogen-activated protein kinase kinase kinase 1 (MAP4K1) (28), a kinase upstream of JNK, thereby inhibiting c-Jun activation and AP-1-dependent transcription (28). Alternatively, PDCD4 could inhibit AP-1-dependent transcription by interfering with the recruitment of the coactivator p300 by c-Jun, and suppresses the phosphorylation of c-Jun by JNK (29).

In conclusion, our results demonstrate that overexpression of PDCD4 led to increased transcription of TIMP-2 which can overcome leptin-induced breast cancer cell invasion by blocking ERK1,2 and STAT3 activation. Novel therapeutic strategies aiming at enhancing PDCD4 expression in breast tumors may be able to impede obesity-related breast tumor progression and prolong patients' lives.

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