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Osteopontin Regulates Ubiquitin-Dependent Degradation of Stat1 in Murine Mammary Epithelial Tumor Cells[1]

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Abstract

Background: **Osteopontin (OPN) is a secreted glycoprotein that mediates cell–matrix interactions and cellular signaling by binding with integrin (primarily $\alpha_v\beta_3$) and CD44 receptors. OPN regulates cell adhesion, chemotaxis, macrophage-directed IL-10 suppression, stress-dependent angiogenesis, apoptosis prevention, and anchorage-independent growth of tumor cells. However, the molecular mechanisms that define the role of OPN in tumor progression and metastasis are incompletely understood.** ***Methods:*** **In this study, we use a system of 4T1 and 4T07 murine mammary epithelial tumor cell lines that are divergent in both metastatic phenotype and OPN expression. 4T1 expresses OPN and hematogeneously metastasizes, whereas 4T07 does not express OPN and is highly tumorigenic but fails to metastasize.** ***Results:*** **Our results demonstrate that OPN regulates Stat1 protein degradation through the ubiquitin–proteasome pathway to alter interferon-γ–dependent growth inhibition and p21 expression. We identify Stat-interacting LIM protein as the critical Stat ubiquitin E3 ligase in this setting.** ***Conclusions:*** **OPN regulates Stat1-dependent functions, such as growth inhibition and p21 expression, in the murine mammary epithelial cells lines 4T1 and 4T07. This relationship between OPN and Stat1 in the context of tumor biology has not been previously examined.**
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Keywords: Ubiquitin, SLIM, proteasome, p21, interferon.

Introduction

Osteopontin (OPN) is a secreted glycoprotein that is rich in aspartate and sialic acid residues and contains functional domains for calcium binding, phosphorylation, glycosylation, and extracellular matrix adhesion [1]. OPN mediates cell–matrix interactions and cellular signaling by binding with integrin (primarily $\alpha_v\beta_3$) and CD44 receptors. OPN regulates cell adhesion, chemotaxis, macrophage-directed IL-10 suppression, stress-dependent angiogenesis, apoptosis prevention, and anchorage-independent growth of tumor cells [1–4]. Recently, a substantial body of data has linked OPN with the regulation of tumor growth and metastatic spread [5]. However, the molecular mechanisms that define the role of OPN in tumor progression and metastasis are incompletely understood. In a previous study using endotoxin-stimulated murine macrophages, we demonstrated that OPN increases Stat1 ubiquitination and subsequent 26S proteasome–mediated degradation to inhibit Stat1-dependent cellular functions [6]. Stat-interacting LIM (SLIM) protein is the Stat ubiquitin (Ub) E3 ligase critical for Stat1 degradation. OPN had not been previously linked to Stat1 degradation, and our results suggest OPN to be a unique and as yet poorly characterized transactivator of Stat1 degradation by the Ub–proteasome system.

In a similar fashion, the potential relationship between OPN and Stat1 has not been previously studied in tumor biology. Stat1 is thought to possess tumor-suppressor functions. In the present study, we examine OPN-dependent Stat1 expression in the murine mammary epithelial tumor cell lines 4T1 and 4T07. Our results indicate that OPN regulates Stat1-dependent functions by mediating Stat1 degradation through the SLIM–Ub–proteasome system.

Materials and Methods

Materials

N-Carbobenzoxyl-L-leucinyl-L-norleucinal (MG132) and M2 anti-FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) agarose were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant mouse interferon γ (IFN-γ) and anti–mouse OPN antibody were purchased from R&D Systems (Minneapolis, MN). Antibodies against Stat1 p84/p91, actin, p21, and Ub were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Hemagglutinin (HA) monoclonal antibody (3F10) was obtained from Roche Applied Science (Indianapolis, IN). Rabbit anti–mouse SLIM serum has been previously described [7].

Cell Lines

Mouse mammary tumor cell lines 4T1 and 4T07 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and

Abbreviations: IFN, interferon-γ; SLIM, Stat-interacting LIM; OPN, osteopontin; Ub, ubiquitin

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streptomycin (100 μg/ml), and maintained at 37°C in a humidified atmosphere of 5% CO_2. These cells are thioguanine-resistant sublines derived from the parental population of 410.4 cells from Balb/cfC3H mice [8]. They are heterogeneous in their metastatic behavior and expression of OPN. 4T1 expresses OPN and hematogeneously metastasizes to the lung, liver, bone, and brain, whereas 4T07 does not express OPN and is highly tumorigenic but fails to metastasize.

Plasmid Constructs

Full-length Stat1 cDNA (U06924) was obtained by reverse transcription–polymerase chain reaction (RT-PCR) with primers Stat1-F 5′-ACGAAGCTTATGTCACAGTGG-TTCGAGCTTCAG-3′ and Stat1-R 5′-ACGAAGCTTTTA-CACTTCAGACACAGAAATCAAC-3′; the 2250-bp fragment was inserted into the pCMV-FLAG2 vector (Sigma-Aldrich). OPN cDNA (J04806) was amplified by RT-PCR with primers OPN-F 5′-CGCGAATTCCATGAGATTGGCAGTGATTTG-3′ and OPN-R 5′-CGCGGATCCTTAGTTGACCTCAG-AAGATG-3′; the 885-bp fragment was inserted into the mammalian expression vector pcDNA3.1/HisB (Invitrogen, Carlsbad, CA). HA/Ub plasmid was kindly provided by Dr. Bohmann (University of Rochester, Rochester, NY). PathDetect GAS *cis*-reporting vector was purchased from Stratagene (La Jolla, CA).

RNAi for OPN and SLIM

Using GenBank sequence NM_009263 for murine OPN cDNA and computer analysis software (Ambion, Austin, TX), we selected a candidate sequence in the OPN cDNA sequence for RNAi, 5′-AAGTCAGCTGGATGAACCAAG-3′. This 21-nt sequence exhibits no homology with other known mouse genes. Synthetic annealed siRNA oligonucleotides were synthesized chemically, gel electrophoresis–purified, and used during transient transfection experiments. In a similar fashion, siRNA was selected for SLIM (NM_145978): 5′-AAGAUCCGACAGAGCGCCUCA-3′. A mismatch (MM) hairpin siRNA with limited homology to mouse genes served as the siRNA negative control.

Transient Transfection and Luciferase Assay

DNA transfections were carried out in 12-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Briefly, 1×10^6 4T1 and 4T07 cells were plated on a 12-well plate and allowed to grow for 24 hours before transfection. Two micrograms of PathDetect GAS *cis*-reporting plasmid diluted in OPTI-DMEM and 3 μl of Lipofectamine 2000 diluted in OPTI-DMEM were combined and incubated at room temperature for 20 minutes. Cells with transfection reagents were incubated for 4 hours at 37°C in a CO_2 incubator. Transfection medium was then replaced with DMEM containing 10% fetal bovine serum. At least 24 hours later, the medium was changed, and cells were stimulated with 500 U/ml IFN-γ for 12 hours. To control transfection efficiency between groups, 0.1 μg of pRL-TK was added to each well. Cells were harvested in 0.4 ml of reporter lysis buffer (Promega, Madison, WI), and dual-luciferase reporter assays were performed by following the protocol provided by the manufacturer.

Immunoblot Analysis

4T1 and 4T07 cells were lysed in buffer [0.8% NaCl, 0.02 KCl, 1% sodium dodecyl sulfate (SDS), 10% Triton X-100, 0.5% sodium deoxycholic acid, 0.144% Na_2HPO_4, and 0.024% KH_2PO_4, pH 7.4] and centrifuged at 12,000*g* for 10 minutes at 4°C. Protein concentration was determined by absorbance at 650 nm using a protein assay reagent. Cell lysates (50 μg/lane) were separated by SDS–12% polyacrylamide gel electrophoresis, and products were electrotransferred to polyvinylidene difluoride membrane (Amersham Pharmacia, Piscataway, NJ). The membrane was blocked with 5% skim milk PBS–0.05% Tween for 1 hour at room temperature. After being washed thrice, blocked membranes were incubated with primary antibody for 1 hour at room temperature, washed thrice in PBS–0.05% Tween, and incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. After three additional washings, bound peroxidase activity was detected by the ECL detection system (Amersham Pharmacia).

Coimmunoprecipitation (co-IP)

4T1 and 4T07 cells were transfected with FLAG/Stat1 expression plasmid and/or HA/Ub expression plasmid; in some cases, OPN and SLIM siRNA were transfected. After 36 hours of transfection, cells were stimulated with 10 μM MG132 or DMSO control for 2 hours, washed with cold phosphate-buffered saline, and lysed for 15 minutes on ice in RIPA lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycho late, and 0.1% SDS) containing protease inhibitors. Cell lysates were cleared by centrifugation (4°C, 20 minutes, 13,000 rpm). Immunoprecipitation was performed with 1000 μg of cell lysate and 40 μl of M2 anti-FLAG agarose, according the method provided by the manufacturer (Sigma-Aldrich). After five washings with lysis buffer, immunoblotting was performed with HA monoclonal antibody or Ub antibody, as described.

Proliferation Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, 4T1 and 4T07 cells transfected with various plasmids or siRNA were plated in a 96-well plate at 2×10^3 cells/well. The cells were treated with 1000 U/ml IFN-γ. Three days after treatment, an MTT assay was performed.

Statistical Analysis

Data are expressed as mean ± SEM. Analysis was performed using two-tailed Student's *t* test. $P < .05$ was considered significant.

Results

Differential Expression of OPN, Ub-Stat1, and Total Stat1 Protein between 4T1 and 4T07 Cells

Stat1 protein expression in 4T1 and 4T07 cells was determined (Figure 1*A*). In 4T1 cells, Stat1 protein was not

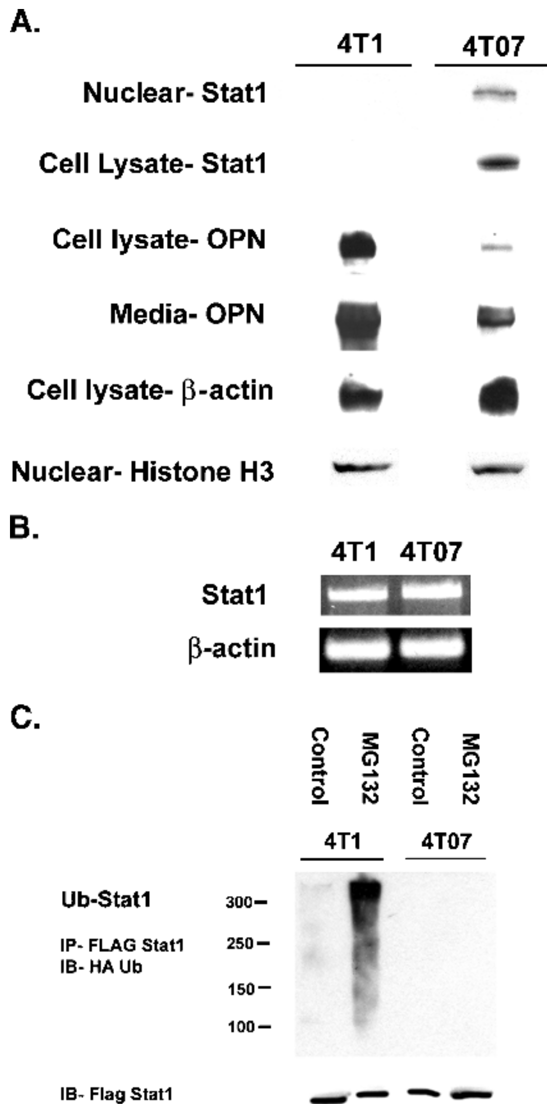

Figure 1. *(A) OPN and Stat1 protein expression in 4T1 and 4T07 cell lysates and culture media. The mouse mammary tumor cell lines 4T1 and 4T07 were cultured. Cells were lysed and centrifuged. For media analysis, serum-free DMEM was centrifuged to remove cellular materials and was concentrated 100-fold. Cell lysates (50 μg/lane) were separated by SDS–12% polyacrylamide gel electrophoresis, and products were electrotransferred to polyvinylidene difluoride membrane. After blocking and washing, membranes were incubated with primary antibody, washed, and incubated with horseradish peroxidase–conjugated secondary antibody. Bound peroxidase activity was detected. The blot is representative of three experiments. (B) Steady-state Stat1 mRNA expression in 4T1 and 4T07 cells. Total RNA was isolated and reverse-transcribed into cDNA. cDNA were used in subsequent PCRs; the primers for Stat1 were 5′-CTTATTCCATGGACAAGGTTTTG-3′ (forward) and 5′-GGTGCTTCTTAATGAGCTCTAGG-3′ (reverse). The gel is representative of three experiments. (C) co-IP of Ub-Stat1 in 4T1 and 4T07 cells. 4T1 and 4T07 cells were transfected with FLAG/Stat1 expression plasmid, HA/Ub expression plasmid, and/or empty expression plasmids. Full-length Stat1 cDNA (U06924) was obtained by RT-PCR with the following primers: Stat1-F 5′-ACGAAGCTTATGTCACAGTGGTTCGAGCTTCAG-3′ and Stat1-R 5′-ACGAAGCTTTTACACTTCAGACACAGAAATCAAC-3′; the 2250-bp fragment was inserted into the pCMV-FLAG2 vector (Sigma-Aldrich). HA/Ub plasmid was kindly provided by Dr. Bohmann (University of Rochester). After 36 hours of transfection, cells were stimulated with 10 μM MG132, an inhibitor of 26S proteasome function, or DMSO control for 2 hours; washed; and lysed for 15 minutes on ice in RIPA lysis buffer. Immunoprecipitation were performed with 1000 μg of cell lysate and 40 μl of M2 anti-FLAG agarose (Sigma-Aldrich). Immunoblotting was performed with HA or Ub antibody, as described. Blots are representative of three experiments.*

detected in either cell lysate or nuclear fraction. In contrast, Stat1 protein was found in 4T07 nuclear and cell lysate fractions. When samples were then probed for OPN protein, 4T1 cells expressed a large amount of OPN in both cell lysate and culture medium. In 4T07, OPN protein was > 10-fold less than that seen in 4T1 cells. Stat1 steady-state mRNA expression was equivalent between the two cell types (Figure 1*B*). As the Ub–proteasome system is one pathway by which Stat1 protein is degraded, co-IP studies were performed to assess the extent of Ub-associated Stat1 (Figure 1*C*). Cells were transiently transfected with FLAG/Stat1 and HA/Ub; MG132, a 26S proteasome inhibitor, was also added. In control 4T1 cells, trace amounts of Ub-Stat1 are found; in the presence of MG132, there is a 25-fold increase in Ub-Stat1. In contrast, Ub-Stat1 is not found in 4T07 cells in the absence or in the presence of MG132. Inhibitors of calpains (calpastatin, 5 μM) and lysosomal enzymes (leupeptin, 50 μM) had no effect on Ub-Stat1 in 4T1 and 4T07 cells (data not shown). These results indicate that 4T1 expresses more OPN, more Ub-Stat1, and less total Stat1 in comparison to 4T07. The differences between 4T1 and 4T07 Stat1 expression may result from differences in Ub–proteasome–mediated Stat1 degradation.

OPN Regulates Ub-Dependent Stat1 Degradation through CD44

We then proceeded to examine the potential relationship between OPN and Stat1 expression. 4T1 cells were treated with siRNA to OPN or MM siRNA control, and co-IP assay was performed (Figure 2*A*). When compared to MM-treated cells, inhibition of OPN expression significantly decreased the levels of Ub-Stat1 in both the presence and the absence of MG132. Western blot analysis of 4T1 cell lysates confirms the efficacy of OPN siRNA in the ablation of OPN expression (Figure 2*B*). A converse experiment was performed in 4T07 cells, which do not typically express OPN. 4T07 cells were transiently transfected with an OPN expression vector or an empty vector (Figure 2*C*). In 4T07 cells, the presence of OPN increased Ub-Stat1 by > 10-fold in the presence of MG132. These data indicate an essential role for OPN in the formation of Ub-Stat1. To delineate the cell surface receptor that mediates this effect, co-IP studies were performed in 4T1 cells in the presence of a blocking antibody to CD44 or a competitive inhibitor $\alpha_V\beta_3$ integrin receptor binding, RGD (Figure 2*D*). Blockade of CD44 binding restored the Ub-Stat1 profile to that seen with untreated control 4T1 cells, suggesting that extracellular OPN mediates Stat1 ubiquitination through the CD44 cell surface receptor.

Functional Correlation of OPN with Stat1-Dependent Promoter Activity, p21 Expression, and Cell Proliferation

Using IFN-γ treatment, functional correlative studies examined the effect of this OPN–Stat1 pathway on IFN-regulated Stat1 (GAS)–dependent luciferase activity, p21 protein expression, and cell proliferation, as measured by MTT activity, in 4T1 and 4T07 cells (Figure 3). In 4T1 cells transfected with MM siRNA and a consensus GAS-luciferase reporter plasmid, exposure to IFN (1000 U/ml × 12 hours) increased luciferase activity by 10-fold (Figure 3*A*). When OPN

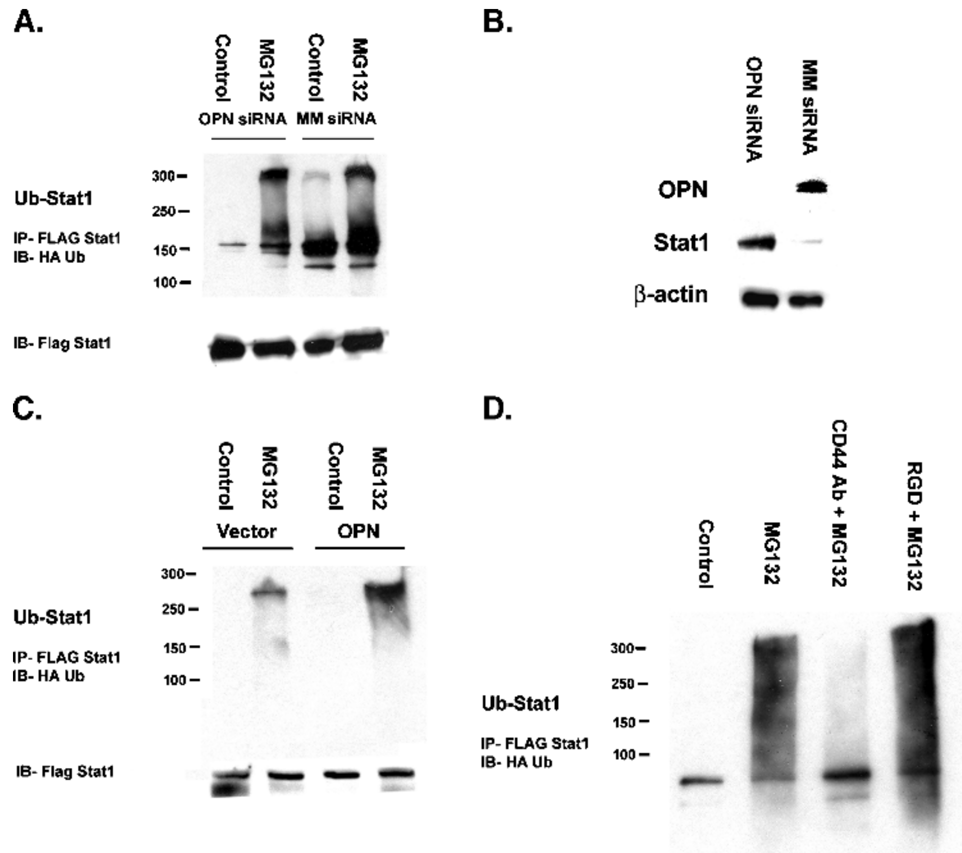

Figure 2. *(A) Co-IP of Ub-Stat1 in 4T1 cells. Co-IP was performed as previously described for Figure 1C. Cells were also transiently transfected with OPN siRNA. Using GenBank sequence NM_009263 for murine OPN cDNA, we selected a candidate sequence in the OPN cDNA sequence for RNAi, 5′-AAGTCAGCTGGATGAACCAAG-3′. This 21-nt sequence exhibits no homology with other known mouse genes. Synthetic annealed siRNA oligonucleotides were synthesized. MM hairpin (MM siRNA) with limited homology to mouse genes served as the siRNA negative control. Blots are representative of three experiments. (B) OPN and Stat1 protein expression in 4T1 cell lysate. Cells were transfected with OPN siRNA or MM siRNA, as described above. The blot is representative of three experiments. (C) Co-IP of Ub-Stat1 in 4T07 cells. Co-IP was performed as previously described for Figure 1C. Cells were also transiently transfected with an OPN expression vector. OPN cDNA was amplified by RT-PCR with primers OPN-F 5′-CGCGAATTCCATGAGATTGGCAGTGATTTG-3′ and OPN-R 5′-CGCGGATCCTTAGTTGACCTCAGAAGATG-3′; the 885-bp fragment was inserted into the mammalian expression vector pcDNA3.1/HisB (Invitrogen). Empty expression vector (Vector) was used as negative control. The blot is representative of three experiments. (D) Co-IP of Ub-Stat1 in 4T1 cells with blockade of CD44 or integrin receptors. Co-IP was performed as previously described for Figure 1C. 4T1 cells were treated with blocking antibody to CD44 or RGD, a competitive ligand inhibitor of integrin binding. The blot is representative of three experiments.*

siRNA was substituted for MM siRNA, IFN increased luciferase activity by 40-fold in comparison ($P < .01$, 4T1 + IFN *vs* 4T1). In addition, luciferase activity in 4T1 + IFN with OPN siRNA was also four-fold greater than that seen in 4T1 + IFN with MM siRNA ($P < .01$). Converse experiments were then performed, in which 4T07 cells were transfected with an OPN expression vector. In this instance, IFN treatment increased GAS-luciferase activity by 10-fold in 4T07 cells transfected with control (empty) expression vector ($P < .01$). When transfected with an OPN expression vector, IFN-treated 4T07 cells exhibited a five-fold increase in GAS-luciferase activity ($P < .01$, 4T07 + IFN + OPN vector *vs* 4T07 + OPN vector). However, this level of luciferase activity was two-fold less than that seen with 4T07 + IFN + control vector ($P < .01$). In these gain-of-function and loss-of-function experiments, our results indicate that OPN expression is associated with decreased GAS promoter activation. We then proceeded with a Western blot analysis of p21 and Stat1 protein expression; p21-mediated growth inhibition is induced by activated Stat1 in response to IFN stimulation [9] (Figure 3*B*). In 4T1 cells, minimal p21 and Stat1 were seen in untreated cells; siRNA ablation of OPN expression was associated with increased p21 and Stat1 protein expressions in both the absence and the presence of IFN stimulation. In 4T07 cells, Stat1 and p21 were readily detected in the unstimulated state. Transfection of an OPN expression vector was associated with decreased Stat1 and p21 proteins in the absence and in the presence of IFN simulation. These data suggest that OPN regulates Stat1 and p21 expression. Finally, cell proliferation in 4T1 and 4T07 cells was measured by MTT activity (Figure 3*C*). In 4T1 cells, siRNA ablation of OPN expression did not alter the extent of 4T1 proliferation; however, in the setting of IFN stimulation, ablation of OPN in 4T1 cells is associated with a 40% decrease in proliferation ($P < .01$, 4T1 + IFN + OPN siRNA *vs* 4T1 + IFN + MM siRNA and 4T1 + OPN siRNA). In wild-type and control vector–transfected 4T07 cells, IFN treatment was associated with a 42% decrease in proliferation ($P < .01$, 4T07 + IFN *vs* 4T07). When transfected with an OPN expression vector, 4T07 proliferation was not altered when compared to 4T07 + control vector. However, in the presence of IFN, the proliferation of 4T07 + OPN vector cells was restored

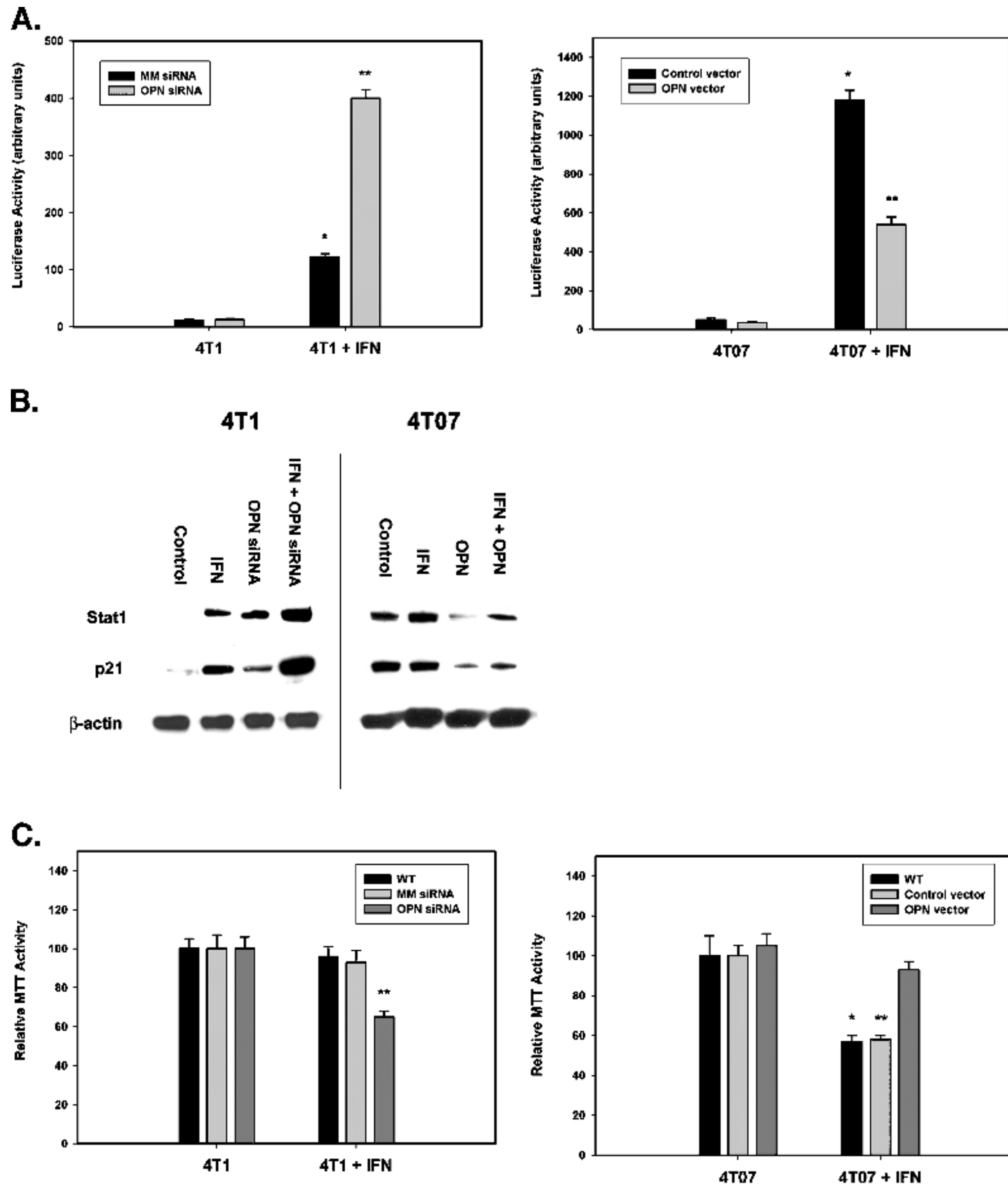

Figure 3. *(A) IFN-mediated Stat1 binding to GAS consensus-binding element in 4T1 and 4T07 cells. About 1 × 10^6 cells were plated on a 12-well plate and allowed to grow for 24 hours before transfection. In selected instances, cells were treated with IFN-γ (1000 U/ml) for 12 hours. Cells with transfection reagents were incubated for 4 hours. To control transfection efficiency between groups, 0.1 μg of pRL-TK was added to each well. Cells were harvested, and dual-luciferase reporter assays were performed. The PathDetect GAS* cis*-reporting vector was purchased from Stratagene. 4T1 cells were examined in the presence of OPN siRNA or MM siRNA; 4T07 cells were examined in the presence of an OPN expression vector or an empty control expression vector. Data are presented as the mean ± SEM of three separate experiments (*P < .01, 4T1 + IFN* vs *4T1 and 4T07 + IFN* vs *4T07; **P < .01, 4T1 + IFN + MM siRNA* vs *4T1 + IFN + OPN siRNA and 4T07 + IFN + MM siRNA* vs *4T07 + IFN + OPN siRNA). (B) IFN-mediated Stat1 and p21 protein expression in 4T1 and 4T07 cells. Stat1 and p21 protein expression was determined by Western blot analysis. Antibodies to Stat1 p84/p91, actin, and p21 were obtained from Santa Cruz Biotechnologies. 4T1 cells were examined in the presence of OPN siRNA or MM siRNA; 4T07 cells were examined in the presence of an OPN expression vector or an empty control expression vector. The blot is representative of three experiments. (C) IFN-mediated inhibition of cell proliferation in 4T1 and 4T07 cells. MTT assay was performed using the CellTiter One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, 4T1 and 4T07 cells and cells transfected with various plasmids or siRNA were plated on a 96-well plate at 2 × 10^3 cells/well. The cells were left untreated or were treated with 1000 U/ml IFN-γ. Three days after treatment, MTT assay was performed. Data are presented as the mean ± SEM of three separate experiments. (**P < .01, 4T1 + IFN + MM siRNA* vs *4T1 + IFN + OPN siRNA and 4T1 + OPN siRNA, 4T07 + IFN + MM siRNA* vs *4T07 + IFN + OPN siRNA; *P < .01, WT 4T07 + IFN* vs *WT 4T07).*

to a level equivalent to that noted in the absence of IFN. These results indicate that IFN-mediated inhibition of cell proliferation is downregulated by OPN expression in 4T1 and 4T07 cells. In total, these experiments indicate that OPN-regulated Stat1 protein expression carries functional implications for cellular proliferation, p21 expression, and Stat1-dependent promoter activation.

SLIM Regulates Ub-Dependent Stat1 Degradation

SLIM protein is a critical Ub E3 ligase for Stat1 degradation [7]. To determine the potential relationship between SLIM and OPN expression in our 4T1/4T07 model, Western blot analysis initially demonstrated that SLIM expression was equivalent in 4T1 *vs* 4T07 cells (data not shown). When siRNA was used to ablate SLIM in 4T1 cells, IFN-stimulated

Stat1 and p21 protein expression was significantly increased by more than 5-fold and 10-fold, respectively ($P < .01$, control *versus* IFN; Figure 4*A*). Co-IP studies were then performed in SLIM siRNA–treated 4T1 cells transiently transfected with FLAG/Stat1 and HA/Ub (Figure 4*B*). With increasing SLIM siRNA concentrations, the levels of Ub-Stat1 progressively decreased until none was noted in the presence of 100 nM SLIM siRNA. To assess the interplay between OPN and SLIM, co-IP studies were repeated in 4T07 cells that were transiently transfected with an OPN expression vector (Figure 4*C*). OPN expression was associated with increased Ub-Stat1, as expected. However, Ub-Stat1 signal is ablated in the presence of 100 nM SLIM siRNA. These data indicate that OPN-dependent SLIM activity regulates Ub-associated Stat1 degradation.

Discussion

OPN is a secreted glycoprotein that is rich in aspartate and sialic acid residues and contains functional domains for calcium binding, phosphorylation, glycosylation, and extracellular matrix adhesion [1]. OPN appears to mediate cell–matrix interactions and cellular signaling by binding with integrin (primarily $\alpha_v\beta_3$) and CD44 receptors. OPN is expressed in multiple species, including humans and rodents [10]. Cells that express OPN include osteoclasts; osteo-

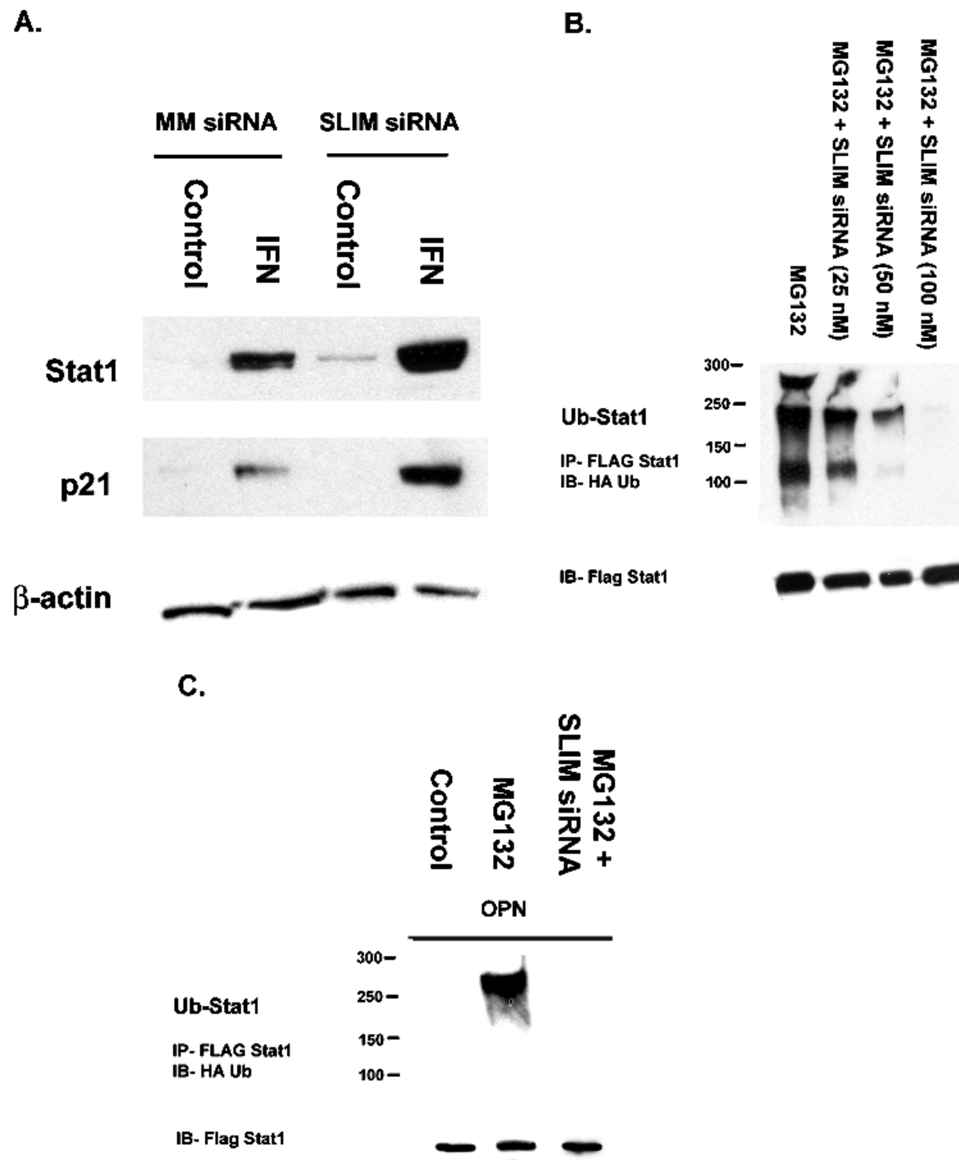

Figure 4. *(A) Effect of SLIM on Stat1 and p21 protein expression in IFN-treated 4T1 cells. Stat1 and p21 protein expression was determined by Western blot analysis. An siRNA was selected for SLIM (NM_145978): 5′-AAGAUCCGACAGAGCGCCUCA-3′. MM hairpin siRNA with limited homology to mouse genes served as the siRNA negative control. The blot is representative of three experiments. (B) The effect of SLIM on Ub-Stat1 in 4T1 cells. Co-IP was performed as previously described. Increasing concentrations of SLIM siRNA (25, 50, and 100 nM) were transfected. The blot is representative of three experiments. (C) The effect of SLIM and OPN in 4T07 cells. Co-IP was performed as previously described. 4T07 cells were transiently transfected with an OPN expression vector, and Ub-Stat1 was measured in the presence or in the absence of MG132. In selected instances, SLIM siRNA (100 nM) was added. The blot is representative of three experiments.*

blasts; kidney, breast, and skin epithelial cells; nerve cells; vascular smooth muscle cells; and endothelial cells [1,3,4, 11,12]. Activated immune cells such as T cells, NK cells, macrophages, and Kupffer cells also express OPN. The secreted OPN protein is widely distributed in plasma, urine, milk, and bile [13–15]. Constitutive expression of OPN exists in several cell types, but induced expression is detected in T lymphocytes, epidermal cells, bone cells, macrophages, and tumor cells in remodeling processes such as inflammation, ischemia–reperfusion, bone resorption, and tumor progression [1,3,4]. A variety of stimuli, including phorbol 12-myristate 13-acetate, 1,25-dihydroxyvitamin D, basic fibroblast growth factor, tumor necrosis factor-α, IL-1, IFN-γ, and lipopolysaccharide, appears to upregulate OPN expression [1–4]. OPN has multiple molecular functions that mediate cell adhesion, chemotaxis, macrophage-directed IL-10 suppression, stress-dependent angiogenesis, apoptosis prevention, and anchorage-independent growth of tumor cells [1–4]. With regard to our findings, we define OPN to be a unique and as yet poorly characterized transactivator of Stat1 degradation by the Ub–proteasome system. A relationship between OPN and Stat1 has not been previously identified.

Stat1 is required for signaling by IFNs, which serve as potent inhibitors of growth and promoters of apoptosis. Although Stat1-deficient mice develop no spontaneous tumors, they are highly susceptible to chemical carcinogen–induced tumorigenesis. Crossing Stat1 mutation into a p53-deficient background yields animals that develop tumors more rapidly and possess a broader spectrum of tumor types than is seen with p53 single mutants. Because tumors from carcinogen-treated wild-type animals also grow far more rapidly when transplanted into Stat1-deficient animals than they do in a wild-type host, it appears that Stat1 is needed for the host's tumor-surveillance capabilities. The requirement of Stat1 for apoptosis and growth arrest in some cell types may be explained by its ability to upregulate caspases and the cdk inhibitor p21 [16,17]. It is well known that cells deficient in Stat1 are unable to induce growth arrest in response to IFN and that Stat1 activates the transcription of the cyclin D kinase inhibitor p21 [9,17–19]. As a result, Stat1 is considered to be a tumor suppressor. In the present study, our observation that OPN, a tumor progression and metastasis protein, accelerates Stat1 degradation is consistent with its growth-enhancing effects as an inhibitor of apoptosis.

Stat signaling is tightly regulated, and several mechanisms have been proposed to account for this control [20]. The suppressor of cytokine signaling (SOCS) and protein inhibitor of Stat (PIAS) families of proteins have been shown to bind to and inhibit either the cytokine receptor–associated Janus kinase (JAK) or activated Stat molecule, respectively. SOCS proteins are induced following cytokine stimulation, bind to JAK kinases, and inhibit activated JAK kinases from further phosphorylating Stat proteins [21,22]. As a result of inhibition by SOCS proteins, Stat signaling becomes a transient response, and levels of phosphorylated Stat proteins decrease within hours of activation. However, the SOCS feedback mechanism does not result in reduced levels of Stat proteins. Each member of the PIAS family has been shown to inhibit Stat-mediated gene activation. Another mechanism that is known to downregulate Stat signaling involves tyrosine phosphatases, such as SHP-1 and SHP-2. These tyrosine phosphatases have been shown to downregulate the activity of Stat1, Stat3, or Stat5 following activation by IFN-γ, leukemia-inhibitory factor, or IL-2 either by dephosphorylating Stat proteins directly or by dephosphorylating JAK kinases [23–25]. Like the SOCS feedback and PIAS mechanisms, downregulation through tyrosine phosphatases also does not lead to reduced levels of Stat proteins. Other posttranslational modifications of Stat proteins, such as arginine methylation, acetylation, and ubiquitination, have been suggested as important means to regulate Stat signaling. Polyubiquitination of substrates targets them for degradation by the 26S proteasome. E3 Ub ligases confer specificity to ubiquitination reaction. E3 ligase transfers Ub from the E2 enzyme to a lysine residue on a substrate protein, resulting in an isopeptide bond between the substrate lysine and the C-terminus of Ub. Ub ligation provides the key step of substrate selection and Ub transfer to the protein target. There are at least four classes of E3 ligases: HECT-type, RING-type, PHD-type, and U-box–containing. For a specific substrate, E3 ligase is the only member of the E1, E2, and E3 sequence that undergoes regulation [26,27]. SLIM contains a PDZ domain and a LIM domain, and interacts in the nucleus with tyrosine-phosphorylated Stat molecules [28]. The LIM domain forms a zinc-finger structure related to the RING finger and PHD structures; similar proteins have been shown to possess E3 ligase activity [28]. SLIM inhibits gene expression mediated by Stat4 or Stat1 by promoting ubiquitination and degradation of Stat4 and Stat1. The cDNA-encoding mouse SLIM is 1509 bp (GenBank BC024556) for mice, with an open reading frame of 349 amino acids [7,28]. It is expressed as a 38-kDa nuclear protein. The human transcript (GenBank NM_021630) is homologous to the mouse transcript, with 77% identity at the amino acid level and 79% identity at the cDNA level. It is highly expressed in lung, spleen, thymocyte, and primary hematopoietic cells, including macrophages. SLIM is the first identified Ub ligase with specificity for Stat proteins.

Using a system of lipopolysaccharide-treated RAW264.7 macrophages, we have previously demonstrated that OPN regulates the Ub-dependent degradation of Stat1 through the activity of SLIM as Ub E3 ligase [6]. This regulation of Stat1 degradation underlies OPN's effect as an inhibitor of inducible nitric oxide synthase gene transcription. These were novel findings that defined OPN as a unique and as yet poorly characterized transactivator of Stat1 degradation by the Ub–proteasome system. Initially characterized by Tanaka et al. [7], SLIM inhibits the gene expression mediated by Stat4 or Stat1 by promoting ubiquitination and degradation of Stat4 and Stat1. The cDNA-encoding mouse SLIM is 1509 bp (GenBank BC024556) for mice, with an open reading frame of 349 amino acids [7,28]. It is expressed as a 38-kDa nuclear protein. The human transcript (GenBank

NM_021630) is homologous to the mouse transcript, with 77% identity at the amino acid level and 79% identity at the cDNA level. It is highly expressed in lung, spleen, thymocyte, and primary hematopoietic cells, including macrophages. SLIM is the first identified Ub E3 ligase with specificity for Stat proteins. SLIM has not been previously examined in the context of OPN-regulated cancer biology.

The signal transduction pathway that links OPN and SLIM is unknown. There are two possibilities. In the first possibility, OPN may induce the expression of SLIM, which is constitutively active. A second and more likely possibility is that OPN may simply activate constitutively expressed SLIM by a posttranslational mechanism. Given our results indicating equivalent SLIM protein expression between 4T1 and 4T07, it appears that SLIM is indeed constitutively expressed and that OPN may therefore activate SLIM by posttranslational modification. As OPN is a secreted protein that binds to CD44 to ubiquitinate Stat1 in this system, future studies delineating OPN–SLIM relationships must begin at the level of OPN's interaction with CD44 at the cell surface.

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