Loss of the Promyelocytic Leukemia Protein in Gastric Cancer: Implications for IP-10 Expression and Tumor-Infiltrating Lymphocytes

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Abstract

Gastric cancer is one of the most common causes of cancer-related mortality worldwide. Expression of the tumor suppressor, promyelocytic leukemia (PML) protein, is reduced or abolished in gastric carcinomas, in association with an increased level of lymphatic invasion, development of higher pTNM staging, and unfavorable prognosis. Herein, we investigated the relationship between the extent of tumor-infiltrating lymphocytes and the status of PML protein expression in advanced gastric carcinoma. We observed higher numbers of infiltrating T-cells in gastric carcinoma tissues in which PML expression was reduced or abolished, compared to tissues positive for PML. The extent of T-cell migration toward culture supernatants obtained from interferon-gamma (IFN-γ)-stimulated gastric carcinoma cell lines was additionally affected by expression of PML in vitro. Interferon-gamma-inducible protein 10 (IP-10/CXCL10) expression was increased in gastric carcinoma tissues displaying reduced PML levels. Moreover, both Pml knockout and knockdown cells displayed enhanced IP-10 mRNA and protein expression in the presence of IFN-γ. PML knockdown increased IFN-γ-mediated Signal Transducer and Activator of Transcription-1 (STAT-1) binding to the IP-10 promoter, resulting in elevated transcription of the IP-10 gene. Conversely, PML IV protein expression suppressed IP-10 promoter activation. Based on these results, we propose that loss of PML protein expression in gastric cancer cells contributes to increased IP-10 transcription via enhancement of STAT-1 activity, which, in turn, promotes lymphocyte trafficking within tumor regions.

Introduction

Gastric cancer is one of the most frequent causes of cancer-related deaths worldwide. Recent advances in genetics have facilitated the detection of events occurring during the course of gastric carcinogenesis, including activation of oncogenes, silencing of tumor suppressor genes, and mutation of genes involved in DNA repair [1]. Among these genetic events, it is known that tumor suppressor promyelocytic leukemia (PML) protein expression is reduced or abolished in gastric cancer, and that this is associated with more extensive lymphatic invasion, higher pTNM staging, and unfavorable prognosis, suggesting that PML loss is linked to carcinogenesis and gastric carcinoma progression [2]. Earlier studies implicated Pml dysfunction in progression of a variety of cancers, and reduced or abolished expression of PML has been reported in prostate, breast, CNS, colon, lung, and gastric cancers [2,3].

The Pml gene was originally identified by fusion with the retinoic acid receptor involved in the t(15;17) chromosomal translocation associated with acute promyelocytic leukemia (APL) [4,5,6,7,8]. PML is a tumor suppressor protein that regulates cell cycle progression, gene transcription, transformation suppression, and apoptosis. Pml knockout mice are considerably more sensitive to tumor formation after exposure to carcinogens than are wild-type controls, and Pml-deficient cells are less likely to undergo apoptosis after application of particular types of cellular stress [9]. In addition to reports focusing on the tumor suppressor role played by PML, several studies have confirmed that the protein acts as a transcriptional regulator, via association with the co-activator CREB-binding protein; co-repressors including HDAC, N-CoR, and mSin3A; and the transcription factors Nur77, AP-1, myc, p53, and STAT-1z [10,11,12,13,14,15,16,17].

Progressive mutation of and genetic alterations in several tumor suppressor genes promote tumor development and progression [18]. In addition to genetic alterations within tumor cells, both inflammatory cells and mediators contribute to formation of particular tumor microenvironments [19,20].
fibroblasts (TAFs) and macrophages (TAMs) are also involved in modulation of the tumor microenvironment via production of cytokines, chemokines, interferons, and other biologically active factors [21,22]. Cross-talk among tumor cells, TAFs, TAMs, and lymphocytes, is significant in tumor development and progression, and contributes to establishment of tumor microenvironments enriched in a variety of biologically active factors [23,24,25,26,27].

TAMs are found to correlate with angiogenesis and an unfavorable prognosis in several types of cancer, including gastric cancer [22,23]. Mast cell produces many angiogenic factors and a variety of cytokines, and its density has been reported to highly correlate with the extent of tumor neoangiogenesis and poor prognosis [29,30]. CD4⁺ and CD8⁺ lymphocytes are also found in a variety of solid cancer tissues. Until now, the precise molecular mechanisms by which the type and the number of tumor infiltrating cells are regulated are largely unclear. There are several controversial reports with regard to the number and function of tumor-infiltrating lymphocytes. Especially, CD8⁺ T lymphocytes, while their function is known to eliminate nascent tumor cells actively participates in the establishment of tumor microenvironments, and injury. To explore whether the PML level in gastric cancer cells influences T-cell infiltration, conditioned media from SNU-638 cells transiently transfected with either Pml siRNA or PML IV expression vector, then treated with IFN-γ was utilized in transwell migration assays. SNU-638 gastric cancer cells expressed high levels of PML protein, consistent with previous findings [2], whereas SNU-638 cells transfected with Pml siRNA displayed reduced levels of endogenous PML expression (~46–56%), as confirmed by immunoblotting (Figure 2A). IFN-γ induced a modest increase in PML protein expression, in agreement with previous results [38]. Migration of Jurkat T-cells toward conditioned medium from Pml siRNA-transfected SNU-638 cells was significantly enhanced, compared to that of control siRNA-transfected cells, after treatment with IFN-γ (Figure 2B). Upon transfection of SNU-638 with PML IV expression vector, migration of Jurkat T-cells toward conditioned medium from PML IV overexpressed cells was reduced compared to that of empty vector (Figure 2C). These results suggest that PML regulates the levels of secretory molecules produced by and released from IFN-γ-stimulated gastric cancer cells, and also influences the migration of T-lymphocytes.

Results

Loss of PML protein is associated with increased infiltration of CD8⁺ T-cells into advanced gastric carcinoma tissue

Immunohistochemical staining for PML and CD8 was performed to explore whether the extent of lymphocyte infiltration was associated with PML expression status in advanced gastric cancer. CD8-immunopositive cells were enumerated as described in Materials and Methods. In total, 35 samples (71.4%) showed partial-to-complete loss of PML in the tumor cell nuclei of stage IV advanced gastric carcinomas. Samples from 14 advanced gastric carcinoma patients were diffusely positive for PML, and contained an average of 32.7 CD8⁺ T-cells per field (Figure 1A). We identified 19 instances of advanced gastric carcinoma exhibiting focal positivity for PML, with an average of 47.2 CD8⁺ T-cells per field. Moreover, complete loss of PML was observed in 16 advanced gastric carcinoma patients; the samples contained an average number of 52.8 CD8⁺ T-cells per field. Samples displaying focal positivity or complete loss for PML showed a more marked increase in CD8⁺ T-cell number than did specimens exhibiting diffuse positivity for PML. Representative images are shown in Figure 1B. Although PML protein expression was reduced or abolished in tumor cells, all of normal gastric mucosal glands, stromal tissues, and lymphoid cells, exhibited diffuse moderate-to-strong nuclear immunopositivity for PML.

PML influences T-cell infiltration/migration in vitro

In view of the loss of PML protein expression and the increase in infiltration of CD8⁺ T-lymphocytes into advanced gastric carcinoma tissues, we hypothesized that PML contributed to T-cell infiltration/migration in gastric cancer. To explore whether the PML level in gastric cancer cells influences T-cell migration, conditioned media from SNU-638 cells transiently transfected with either Pml siRNA or PML IV expression vector, then treated with IFN-γ was utilized in transwell migration assays. SNU-638 gastric cancer cells expressed high levels of PML protein, consistent with previous findings [2], whereas SNU-638 cells transfected with Pml siRNA displayed reduced levels of endogenous PML expression (~46–56%), as confirmed by immunoblotting (Figure 2A). IFN-γ induced a modest increase in PML protein expression, in agreement with previous results [38]. Migration of Jurkat T-cells toward conditioned medium from Pml siRNA-transfected SNU-638 cells was significantly enhanced, compared to that of control siRNA-transfected cells, after treatment with IFN-γ (Figure 2B). Upon transfection of SNU-638 with PML IV expression vector, migration of Jurkat T-cells toward conditioned medium from PML IV overexpressed cells was reduced compared to that of empty vector (Figure 2C). These results suggest that PML regulates the levels of secretory molecules produced by and released from IFN-γ-stimulated gastric cancer cells, and also influences the migration of T-lymphocytes.

Loss of PML enhances IFN-γ-induced IP-10 expression

Chemokines, a family of chemotactic cytokines, promote migration of circulating leukocytes/lymphocytes to sites of inflammation and injury. To explore whether chemokine levels were affected by Pml genetic status, chemokine gene expression was examined using ribonuclease protection assay (RPA). Pml⁻/⁻ and Pml⁺/⁺ mouse embryonic fibroblast (MEF) cells were incubated in the absence or presence of IFN-γ for 0-24 h, total mRNA was collected at various timepoints, and subjected to RPA. In Pml⁻/⁻ MEFs, IP-10 mRNA levels increased 1.5-fold at 2 h, and became markedly elevated (8-fold) by 4 h (Figure 3A). RANTES mRNA levels were increased 3.2-fold at 0.5 h, and remained elevated, but to a lower extent (1.3-fold), 8 h post-IFN-γ treatment in Pml⁻/⁻ MEFs. Other chemokine genes, including MIP-1, MIP-2, and MCP-1, were not detectably expressed in Pml⁻/⁻ or Pml⁺/⁺ MEFs (data not shown). STAT-1 expression was increased after exposure to IFN-γ in both cell types, but no difference was evident when IFN-γ levels were compared between Pml⁻/⁻ and Pml⁺/⁺ cells. As transcription of the IP-10 gene was substantially elevated in IFN-γ-stimulated Pml⁻/⁻ MEFs, we measured relevant protein levels in culture supernatants of Pml⁻/⁻ and Pml⁺/⁺ MEFs, using an enzyme-linked immunosorbent assay (ELISA). Consistent with the RPA results,
higher levels of IP-10 protein were evident in conditioned medium from IFN-γ-treated Pml−/− MEFs (Figure 3B). Also, IP-10 mRNA expression in Pml siRNA-transfected NIH3T3 cells was enhanced, compared to that of control siRNA-transfected cells, following IFN-γ treatment (Figure 3C). The reduction in expression of PML protein in Pml siRNA-transfected NIH3T3 cells was confirmed by immunoblotting.

Reduced PML expression is inversely correlated with IP-10 protein levels in gastric cancer tissue and SNU-638 cells

Next, we examined the relationship between IP-10 and PML protein expression in advanced gastric carcinoma tissue. Cancer tissues were immunohistochemically stained for PML and IP-10, and the intensity of IP-10 immunopositivity was measured as described in Materials and Methods. The average intensity of IP-10 was 1.2 in 14 samples showing diffuse positivity (DP) for PML, 2.2 in 19 instances of focal positivity (FP), and 2.0 in 16 samples in which there was complete loss (CL) of PML expression (Figure 4A, left panel). Significant increases in IP-10 expression were observed in samples showing local positivity of PML (P = 0.034), compared to those showing diffuse positive PML expression. An increase in IP-10 expression was observed when complete loss of PML was evident, compared with what was noted when PML was diffusely expressed; however, this difference did not reach statistical significance. Representative examples of variations in PML protein status that correlated with different IP-10 expression levels in tumor cells from advanced gastric carcinomas are shown (Figure 4A, right). To further examine the relationship between IP-10 and PML protein expression in gastric carcinoma cell lines, IP-10 protein levels were measured in culture supernatants from SNU-638 cells transiently transfected with either Pml siRNA or PML IV expression vector. Cells were grown in the absence or presence of IFN-γ for 8 h, supernatants collected, and IP-10 levels quantified therein (by ELISA). IFN-γ-induced IP-10 secretion was significantly enhanced in SNU-638-Pml siRNA-transfected cells, compared with that of SNU-638 cells transfected with control siRNA (Figure 4B). Transfection of SNU-638 with PML IV expression vector suppressed IFN-γ-induced IP-10 secretion (Fig 4C). The results clearly show that secretion of IP-10 from gastric cancer cells is increased when PML expression is reduced.

IP-10 neutralization decreases migration of T-cells

As SNU-638-Pml siRNA-containing cells expressing reduced levels of PML protein showed enhancement of T-cell recruitment and IP-10 secretion in response to IFN-γ (Figures 2 and 4), we next examined whether an increase in IP-10 levels facilitated T-cell migration toward conditioned medium from SNU-638 gastric cancer cells stimulated by IFN-γ. SNU-638-Pml siRNA cells were grown in the absence or presence of IFN-γ for 8 h, and the collected supernatants were pretreated with an anti-IP-10 neutralizing antibody or control non-specific IgG for 1 h, prior to commencement of the transwell assay. Neutralizing antibody or IgG-containing supernatants were placed in the lower chambers, and migration of Jurkat cells from upper to lower chambers was analyzed by examining cells harvested from the lower chambers.
Inclusion of IP-10-neutralizing antibodies inhibited Jurkat T-cell migration toward conditioned medium from SNU-638-Pml siRNA cells (Figure 5A). These findings are consistent with data obtained from NIH3T3 cells; an IP-10-neutralizing antibody inhibited EL-4 T-cell migration toward conditioned medium from NIH3T3-Pml siRNA cells (Figure 5B).

PML knockdown enhances IP-10 promoter activation

As IP-10 expression was increased in PML knockdown cells, we further explored whether PML protein expression affected IP-10 promoter activity. The murine IP-10 promoter contains a putative GAS-like element lying between nts -246 and -238 (TTN5AA) (Figure 6A). We previously reported that PML negatively regulates
the transcriptional activity of STAT-1[17]. To ascertain whether PML affected IFN-γ-induced IP-10 gene expression via a mechanism involving STAT-1, we isolated and generated a reporter construct commencing at position -330 of the murine IP-10 promoter containing the putative GAS-like element, as described in Materials and Methods. The IP-10-luc reporter containing the GAS-like element was transiently transfected into NIH3T3 cells incubated with or without IFN-γ for 24 h, and subsequently assayed for luciferase activity. IFN-γ-induced IP-10 promoter activity was significantly enhanced in NIH3T3 cells transfected with Pml siRNA, compared to cells receiving control siRNA (Figure 6B). Upon transfection of NIH3T3 cells with the PML IV expression vector, IFN-γ-induced IP-10 promoter activation was significantly repressed. Downregulation of PML expression using Pml siRNA and overexpression of PML by PML IV expression vector were verified by immunoblotting. To determine whether the inhibitory effect of PML on IFN-γ-induced IP-10 promoter activity is occurred in gastric cancer cells lines, we...
performed similar experiments with SNU-638 gastric cancer cells. The patterns of increase and decrease in the luciferase activity in SNU-638 were similar to that of NIH3T3 cells (Figure 6C). The level of PML protein expression in SNU-638 cells transfected with either Pml siRNA or PML IV expression vector was verified by immunoblotting (data not shown).
PML knockdown increases IFN-γ-induced STAT-1 DNA binding to the IP-10 promoter

The effect of PML on IFN-γ-induced STAT-1 DNA binding to the IP-10 promoter was further examined. PML protein overexpression in NIH3T3 cells, after transient transfection with the PML IV expression vector, partially blocked IFN-γ-induced STAT-1 DNA binding to the IP-10 promoter, including the sequence at -246 to -238 (Figure 7A, compare lanes 3 and 5). Competition using a 100-molar excess unlabeled oligonucleotide encoding the IP-10 promoter abrogated complex formation (lane 6). The levels of PML and STAT-1 protein expression in whole-cell lysates (WCL) and the extent of STAT-1 tyrosine phosphorylation in nuclear extracts (NE) were verified by immunoblotting. Utilizing NE from NIH3T3-Pml siRNA cells obtained after 0.5 h of IFN-γ treatment, strong binding to the murine IP-10 promoter was observed (Figure 7B, top panel, lane 5), compared to binding seen in NIH3T3-control siRNA cells (lane 3). The same NEs were exposed to the STAT-1 consensus oligonucleotide, and STAT-1 DNA binding was enhanced in NE from NIH3T3-Pml siRNA cells (Figure 7B, middle panel, compare lanes 3 and 5). Using SNU-638 gastric cancer cells, we also found that STAT-1 DNA binding of Pml siRNA-transfected SNU-638 cells was enhanced compared to that of control siRNA-transfected cells, after treatment with IFN-γ (Figure 7C, compare lanes 3 and 5). These data indicate that PML partially inhibits STAT-1 binding to the IP-10 promoter, and that this effect is correlated with increased IFN-γ-induced IP-10 transcription in the absence of PML.

Discussion

Histopathologic studies have shown that the inflammatory microenvironment of tumors is characterized by the presence of mononuclear cell infiltrates, both in the supporting stroma and tumor regions. However, the precise mechanisms by which mononuclear cells infiltrate into and are sustained within cancer tissues are currently not fully understood. In the present study, we offer evidence that loss of expression of the tumor suppressor protein, PML, contributes to enhancement of lymphocyte infiltration into gastric cancer tissue, via regulation of IP-10 expression.

We examined the function of PML with respect to lymphocyte recruitment using human gastric cancer tissue samples, Pml+/+ and Pml−/− MEFs, and PML knockdown in both NIH3T3 and SNU-638 cells. Data obtained from both human tissue tests and the transwell migration assays showed that loss of PML promotes lymphocyte trafficking; thus, we sought mechanisms that might possibly explain these observations. We show that an increase in IP-10 levels in cancer cells expressing low levels of PML contributes to lymphocyte recruitment. Additionally, IP-10 neutralizing antibody inhibited T-cell migration toward conditioned medium from NIH3T3 fibroblasts and SNU-638 gastric cancer cells in vitro, consistent with the results of several prior in vivo studies, which showed that neutralization of IP-10 suppressed T-cell recruitment [39,40]. To the best of our knowledge, this is the first study showing that the tumor suppressor, PML, regulates IP-10 chemokine transcription and lymphocyte trafficking in gastric cancer, by modulating the activity of the IFN-γ/STAT-1 signaling pathway.

IP-10, a lymphocyte-targeting CXC chemokine induced by type I and II IFNs, plays an important role in lymphocyte recruitment under several inflammatory conditions [41]. IP-10 is secreted by tumor-infiltrating immune cells, and also by tumoral and parenchymal cells in an interferon- and cytokine-enriched microenvironment. TAFs from lung cancer patients constitutively produce and secrete IP-10 [27]. Hepatocytes infected with the hepatitis C virus produce IP-10, and induce migration of activated T-cells to the liver parenchyma, indicating that IP-10 levels are correlated with histological severity and inflammation [42]. In our system, IP-10 secretion was observed in gastric cancer SNU-638 cells, NIH3T3 fibroblasts, and MEFs, in response to IFN-γ, and secretion was enhanced in the absence of PML protein expression. Since we found that there were no appreciable differences in the level of STAT-1 expression and phosphorylation in the absence or presence of PML upon IFN-γ (Figures 3 and 7), increased IFN-γ-induced IP-10 transcription in the absence of PML was caused mainly by enhanced STAT-1 DNA binding to IP-10 promoter. We suggest that PML-mediated inhibition of STAT-1 DNA binding leads to suppression of STAT-1-dependent IP-10 expression, in accord with our previous findings [17]. In the present study, we identify a potential regulatory region for PML in the IP-10 promoter, which is a putative STAT-1-binding site. Further, we
show that PML knockdown increases IFN-γ-induced STAT-1 DNA binding to the IP-10 promoter, and enhances IP-10 levels in the absence of PML.

Lymphocytes, neutrophils, macrophages, and mast cells are the major components of most tumor infiltrates. Tumor-infiltrating lymphocytes have been identified in a variety of solid cancer tissues. However, the specific functions of such cells within tumors, and the precise molecular mechanisms of lymphocyte recruitment into tumors, remain controversial. The status and functional relevance of CD8+ T-cell infiltration into tumors, and the association of such cells with patient prognosis, are also unclear. In renal cell carcinoma, infiltration of CD8+ T-cells is associated with favorable prognosis [43]. Conversely, such infiltration is linked to unfavorable prognosis in nonsmall cell lung cancer patients [33]. We here show that PML protein expression in tumor cells is inversely correlated with the extent of infiltration of CD8+ lymphocytes.
Figure 7. PML Expression Inhibits DNA Binding Activity of STAT-1 to the IP-10 Promoter. (A) Nuclear extracts (NE) from NIH3T3 cells transiently transfected with the PML IV protein expression vector and treated with IFN-γ (10 ng/ml) for 30 min were incubated in the presence of a radiolabeled DNA probe containing the GAS-like element of the IP-10 promoter, and subjected to electrophoretic mobility shift assay (EMSA). Shifted probe-protein complexes are indicated by the arrow. Increased PML protein expression in whole-cell lysates (WCL) transfected with PML IV expression vector was verified by immunoblotting. The amount of histone in NE is shown as a control. (B) NEs from NIH3T3 cells transiently transfected with either Pml siRNA or control siRNA and treated with IFN-γ (10 ng/ml) for 30 min were incubated in the presence of radiolabeled DNA probes containing the GAS-like element of the IP-10 promoter or a STAT-1 consensus sequence, and then subjected to EMSA. Binding specificity was tested...
by adding increasing concentrations of cold probe (competitor). Effective siRNA-mediated suppression of PML protein expression was verified for each assay by immunoblotting. (C) SNU-638 gastric cancer cells were transiently transfected with either Pml siRNA or control siRNA and treated with IFN-γ (10 ng/ml) for 30 min. NEs were obtained, incubated in the presence of radiolabeled DNA probes containing a STAT-1 consensus sequence, and then analyzed for EMSA as described in B. The levels of PML and STAT-1 protein expression in WCL and the extent of STAT-1 tyrosine phosphorylation in NE were verified by immunoblotting. Data shown are representative of at least three experiments.

Reagents and antibodies

Recombinant murine and human IFN-γ (mIFN-γ and hIFN-γ) and mIP-10 and hIP-10 were purchased from ProSpec (Rehovot, Israel). Antibodies to PML (mouse monoclonal) and STAT-1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA) and neutralizing antibody to IP-10 was purchased from R&D Systems (Minneapolis, MN, USA). Antibody to p-Y701-STAT-1 was purchased from Cell Signaling Technology (Beverly, MA). Antibodies to histone, Lamin B and PML (rabbit polyclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-tubulin antibody was purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA).

Immunohistochemistry, cell counts, and measurement of immunoreactive areas

Human gastric cancer tissues were processed for immunohistochemical analysis as described previously [2] with minor modifications. Primary antibodies for PML (1:200; Santa Cruz Biotechnology), CD8 (1:50; Thermo Fisher Scientific, Fremont, CA, USA), and IP-10 (1:50; R&D Systems) were utilized. All slides were counterstained with Mayer’s hematoxylin. Positive controls were obtained by omitting primary antibodies from all slides. Immunopositivity for PML protein was categorized as diffuse positivity (nuclear immunoreactivity in ≥50% of tumor cells), focal positivity (in 10% but <50%), or complete loss (in <10% of tumor cells). The numbers of CD8 immunopositive cells in one high power field (HPF, x40) of 0.25 mm² for five randomly selected tumor infiltrative borders were counted for each specimen, followed by calculation of the mean number and SD. Immunopositivity for IP-10 was defined as any nuclear or cytoplasmic positive expression in tumor cells. For determining the expressed level of each slide, digital images were obtained using a microscope-mounted digital camera. ImageJ software (http://rsb.info.nih.gov/ij), each image was converted to 8-bit gray scale and area densities were measured from the pixels in the region of interest; only pixels exceeding a certain level (>50 intensity value) were included to eliminate background error.

siRNA transfection

NIH3T3 cells were transiently transfected using Mirus transfection reagent (Mirus Bio LLC, Madison, WI, USA) or Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) with either 300 nmol of mPML siRNA or negative control siRNA (Samchully Pharm, Co. Ltd., Seoul, Korea), according to the manufacturer’s instructions. The human gastric carcinoma cell line SNU-638 was also transfected using Mirus reagent or Lipofectamine™ RNAiMAX with either 100 nmol of hPML siRNA (Santa Cruz Biotechnology) or negative control siRNA. Cells were allowed to recover for 48 h before treatment with 10 ng/ml of IFN-γ for various times, and then analyzed by immunoblotting and/or RT-PCR.
Immunoblotting

Cells were plated, incubated with IFN-γ (10 ng/ml) for 0–24 h, and then lysed with lysis buffer [17]. Twenty μg sample of total protein or 5 μg of nuclear protein was subjected to SDS/PAGE, and blots were probed with PML, histone or tubulin antibodies. After secondary antibody incubation, blots were developed using ECL chemiluminescence system (Amersham, Buckinghamshire, UK).

Transwell migration assay

In vitro migration assays were performed using Costar Transwell Inserts with a 5-μm pore size in 24-well plates (Corning Costar, Cambridge, MA, USA). SNU-638 cells were seeded in the upper chambers of transwell migration units. The upper chambers received 5 × 10^5 Jurkat or EL-4 cells in a volume of 100 μl and the transwell migration units were incubated at 37 °C for 2 h. The numbers of migrating cells obtained from the lower chambers were analyzed by fluorescent count-bead mediated counting on a flow cytometer (BD Biosciences, San Jose, CA, USA). For neutralization, the supernatants were pretreated with an anti-IP-10 neutralizing antibody or control non-specific IgG for 1 h, then placed in the lower chambers, and migrating cells harvested from the lower chamber were similarly determined.

Enzyme-linked immunosorbent assay (ELISA)

Primary Pml^+/+ and Pml^-/- MEFs (5 × 10^5 cells/well) were seeded and cultured with IFN-γ (10 ng/ml) for 12 h, then the concentration of IP-10 in supernatants was determined using a mouse IP-10 ELISA Kit (R&D Systems), according to the manufacturer’s instructions. SNU-638 cells (2.4 × 10^5 cells/well) were incubated with either negative control siRNA or PML siRNA for 48 h, then treated with IFN-γ for 8 h. IP-10 protein levels in cell culture supernatants were measured using a human IP-10 BD optEIATM (BD Biosciences) and normalized by the cell protein concentration.

RNA isolation, RT-PCR, and RPA

Total cellular RNA was isolated from confluent monolayers of cells using easy-BLUE™ (IniRON Biotechnology, Seongnam, Korea) according to the manufacturer’s protocol. cDNA was synthesized from 5 μg of total RNA with reverse transcription and PCR was performed using 3 μl cDNA with a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The primer sequences were as follows: mouse IP-10, forward 5'-GATGGCTAGTCC-GTCACCAACTGG-3', reverse 5'-TCATCTGATTTCTCAAACAGCTCACG-3'; β-actin, forward 5’-GTCACCAACTGG-GACGACA-3', reverse 5’-TGGCCATCTTGCTCGAA-3'. For RPA, the mCK-5 multi-riboprobe was purchased from Pharmingen (BD Biosciences) and prepared following the manufacturer’s guidelines. Total RNA (15 μg) was hybridized with the riboprobe as described previously [17]. Quantification of the protected RNA fragments was performed by scanning with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). Values for mRNA expression were normalized to GAPDH mRNA levels for each experimental condition.

Plasmids, transient transfection, and reporter assays

The IP-10-luciferase reporter plasmids containing 330 bp of the murine IP-10 promoter were generated by PCR [55]. For overexpression of PML protein, the pCMV-Tag2B-PML-IV expression construct was used [53]. For reporter assays, 0.4 μg of the IP-10-luc construct was transiently transfected into 0.5 × 10^5 NIH3T3 cells in 12-well plates, with 0.1 μg of pCMV-β-galactosidase vector to normalize for transfection efficiency as described previously [17]. The PML IV expression vector (1 μg) was co-transfected and differences in the amount of DNA adjusted with empty vector. Cells recovered for 24 h before treatment IFN-γ (10 ng/ml) for 24 h, then luciferase activity was measured. The luciferase activity of each sample was normalized to β-galactosidase activity to yield relative luciferase activity (RLA).

Nuclear extracts and electrophoretic mobility shift assays (EMSA)

EMSA was performed with 10 μg of nuclear extracts as previously described [17]. Nuclear extracts from NIH3T3 cells treated with IFN-γ for 30 min were incubated with either the IP-10 sequence (5’-TCATCTGATTTCTCAAACAGCTCAGG-3’) or consensus STAT-1 sequence (SC-2573, Santa Cruz Biotechnology) which was end-labeled with [32P]ATP for 30 min. For competition experiments, a 100-molar excess of unlabeled oligonucleotide was added to the nuclear extracts for 30 min before addition of the labeled probe. Bound and free DNA were then resolved by electrophoresis through a 6% polyacrylamide gel and exposed for autoradiography.

Statistical analysis

Statistical analyses were performed using the Student’s t-test to compare between sample groups, and ANOVA was used to determine differences among multiple groups. Statistical significance of the data was at P<0.05.

Ethics statement

This study protocol was approved by the Institutional Review Board (IRB) of the Ewha Womans University Mokdong Hospital (IRB protocol number: ECT11-20-08). Informed consent had been obtained from each patient prior to surgery and was waived in this study by IRB due to the retrospective nature.

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Author Contributions

Conceived and designed the experiments: DES HJK ENB YHC. Performed the experiments: DES HJK SYL SHL SJL YHC. Analyzed the data: DES HJK JLLK SJL ENB YHC. Contributed reagents/materials/analysis tools: JLLK ENB. Wrote the paper: DES ENB YHC.

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