Repression of IFN Regulatory Factor 8 by DNA Methylation Is a Molecular Determinant of Apoptotic Resistance and Metastatic Phenotype in Metastatic Tumor Cells

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Abstract

Apoptotic resistance is often associated with metastatic phenotype in tumor cells and is considered a hallmark of tumor progression. In this study, IFN regulatory factor 8 (IRF8) expression was found to be inversely correlated with an apoptotic-resistant and metastatic phenotype in human colon carcinoma cell lines *in vitro*. This inverse correlation was further extended to spontaneously arising primary mammary carcinoma and lung metastases in a mouse tumor model *in vivo*. Exogenous expression of IRF8 in the metastatic tumor cell line restored, at least partially, the sensitivity of the tumor cells to Fas-mediated apoptosis, and disruption of IRF8 function conferred the poorly metastatic tumors with enhanced apoptotic resistance and metastatic capability. DNA demethylation restored IRF8 expression and sensitized the metastatic tumor cells to Fas-mediated apoptosis. Analysis of genomic DNA isolated from both primary and metastatic tumor cells with methylation-sensitive PCR revealed hypermethylation of the IRF8 promoter in metastatic tumor cells but not in primary tumor cells. Taken together, our data suggest that IRF8 is both an essential regulator in Fas-mediated apoptosis pathway and a metastasis suppressor in solid tumors and that metastatic tumor cells use DNA hypermethylation to repress IRF8 expression to evade apoptotic cell death and to acquire a metastatic phenotype. [Cancer Res 2007;67(7):3301–9]

Introduction

Cancer cells often acquire genetic and epigenetic alterations that enable them to evade apoptosis (1). This phenomenon underpins resistance not only to γ-irradiation and chemotherapeutic agents (1–3) but also to innate or adaptive host immune effector mechanisms, including Fas-mediated cytotoxicity (4–7). Fas is a key death receptor that mediates apoptotic cell death in both immune and nonimmune cellular compartments (8, 9), including neoplastic cells. In humans, Fas is constitutively expressed at high levels along the entire colon tissue, but Fas expression is often diminished in colon carcinoma and is frequently absent in metastatic lesions (10). Furthermore, loss of Fas function is linked to immunoselection and tumor escape (7, 9, 11) and may be an important characteristic of immunoediting (9, 12). These findings suggest that acquisition of resistance to Fas-mediated apoptosis is a key determinant in tumor escape and progression (6, 7, 10, 13–15).

Although Fas expression is frequently diminished in tumor cells, especially in metastatic tumor cells, it has been shown that Fas expression could be up-regulated by exposure to IFN-γ (15–17). However, increased Fas levels were not always paralleled by sensitization of metastatic tumor cells to Fas-mediated apoptosis. We reasoned that loss of Fas expression, in combination with additional alterations of other key apoptosis regulators in the death pathway, is responsible for the apoptotic-resistant phenotype of metastatic tumor cells. To test this hypothesis, we carried out cDNA microarray analysis of differential gene expression between a matched pair of human primary and metastatic colon carcinoma cell lines and identified *IFN regulatory factor 8* (*IRF8*; also known as *IFN consensus sequence binding protein, ICSBP*) as a candidate gene whose expression is inversely correlated with an apoptotic-resistant phenotype (18).

IRF8 is a transcription factor of the IRF family, which also includes IRF1, IRF2, IRF3, IRF4/lymphoid-specific IRF/pip/ICSAT, IRF5, IRF6, IRF7, and ISGF3γ (19). In contrast to most of the IRFs that are expressed in a variety of cell types, IRF8 has long been thought to be exclusively expressed in cells of myeloid and lymphoid lineages (i.e., hemopoietic lineages; refs. 19–22). IRF8 is one of a cascade of factors whose level of expression is regulated by IFN-γ. IFN-γ is a pivotal cytokine produced by cells of the immune system that is crucial not only for the clearance of virally infected cells but also for the clearance of cancerous cells (12, 23–25). IRF8 has been characterized as a central element of the IFN-γ–signaling pathway (19, 22). Mice with a null mutation in IRF8 exhibit marked clonal expansion of undifferentiated granulocytes and macrophages that are resistant to apoptosis, and this expansion causes a syndrome similar to human chronic myelogenous leukemia (CML; refs. 26, 27). In patients with CML and acute myelogenous leukemia, IRF8 expression is decreased dramatically (28). Therefore, IRF8 is a pivotal regulator of apoptosis and leukemogenesis in myeloid cells.

In the present study, loss of IRF8 expression, not Fas expression, was found to be a limiting determinant of the apoptotic-resistant phenotype in solid (nonhemopoietic) metastatic tumor cells. We showed that IRF8 overexpression promoted apoptosis of metastatic tumor cells, whereas disruption of IRF8 function diminished primary tumor cell sensitivity to apoptosis and converted the poorly metastatic tumor to a metastatic phenotype. Furthermore,

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the expression of IRF8 is found to be repressed by DNA hypermethylation of IRF8 promoter in metastatic tumor cells. Taken together, our data suggest that IRF8 is both an apoptosis regulator and a metastasis suppressor in solid tumor cells, and transcriptional repression of IRF8 by DNA hypermethylation of the IRF8 promoter is a molecular mechanism responsible, at least in part, for the apoptotic-resistant and metastatic phenotype of solid tumors.

Materials and Methods

Cell lines. The human HLA-A2$^+$ colon carcinoma cell lines SW480 (CCL-228) and SW620 (CCL-227) were obtained from the American Type Culture Collection (Manassas, VA). SW480 and SW620 are two naturally occurring primary and metastatic colon adenocarcinoma cell lines established from the same patient. The SW620 cell line was derived as a lymph node metastasis identified 6 months later during disease relapse. Both cell lines were isolated from the patient without any prior chemotherapy. The mouse sarcoma cell line CMS4, kindly provided by Dr. A. Deleo (University of Pittsburgh, Pittsburgh, PA), is of BALB/c (H-2^d) origin.

Mice. Transgenic mice with spontaneously arising primary and metastatic mammary carcinoma, now backcrossed on to a C57BL/6 (H-2^b) background, were kindly provided by Dr. S. Gendler (Mayo Clinic, Scottsdale, AZ). This transgenic mouse model was originally produced by expression of the polyomavirus middle T antigen via germ-line introduction of the middle T oncogene under the transcriptional control of the mouse mammary tumor virus promoter/enhancer. Tumors and metastases-bearing lung tissues were excised from three mice, respectively. Female BALB/c (H-2^d) mice were obtained from Charles River Laboratories (Wilmington, MA). All mice were used at age ≥6 weeks. Tumor cells were resuspended in HBSS and injected i.v. into the lateral tail vein (2.5×10^5 cells in 100 μL of total volume) of naive, immunocompetent female BALB/c mice. Mice were euthanized 14 days later. For enumeration of lung metastases, lungs were injected with a 15% solution of Indian ink, resected, and fixed in Fekete's solution as described (29). All experiments involving mice were conducted in accordance with protocols approved by NIH and the Institutional Animal Care and Use Committee at the Medical College of Georgia.

Measurement of apoptotic cell death. Apoptotic cell death was measured by propidium iodide staining as described earlier (15, 30). Briefly, tumor cells were incubated at 37°C for 24 h in the absence or presence of recombinant human FasL (PeproTech, Rocky Hill, NJ). Adherent and suspended cells were then harvested and incubated with propidium iodide/RNase solution (R&D Systems, Minneapolis, MN) for 10 min at room temperature and analyzed immediately by flow cytometry. The percentage of cell death was calculated by the following formula: % cell death = % propidium iodide–positive cells with FasL − % propidium iodide–positive cells without FasL.

Immunohistochemistry. Tissue sections were cut from formalin-fixed, paraffin-embedded tissues taken from primary (mammary glands) and metastatic (lungs) sites of tumor growth of independent transgenic mice. Specimens were blocked with normal goat serum (1.5%) and then incubated with an antigen affinity-purified, rabbit anti-IRF8 polyclonal antibody (1:1,000 dilution; Aviva, San Diego, CA) for 30 min followed by rinsing and staining with a goat anti-rabbit biotinylated antibody (1:2,000) for another 30 min. Color was developed by incubation with 3,3′-diaminobenzidine solution (Sigma, St. Louis, MO) followed by rinsing and counterstaining with hematoxylin.

Cell surface marker analysis. Untreated or IFN-γ–pretreated cells [250 units/mL for 18–24 h as described previously (15, 30)] were incubated with a FITC-conjugated anti-human Fas monoclonal antibody (mAb; clone DX-2, PharMingen, San Diego, CA) or isotype-matched control. Cells were then analyzed by flow cytometry.

Tumor cell irradiation. The SW480 and SW620 cells were harvested while in log growth phase. These cells were in suspension in culture medium while the irradiation was done. Tumor cells were placed on ice and irradiated (0–50 Gy) by exposure to a Cs-137 source (Gammacell-1000; AECL/Nordion, Kanata, Ontario, Canada) at a dose rate of 740 Rad/min. The irradiated cells were washed once with medium and cultured for 24 h before use in apoptosis and other assays.

Cell treatments. Tumor cells were harvested while in log growth phase, seeded in culture flasks, and cultured at 37°C overnight. For demethylation treatment with 5-azacytidine, cells were treated for 24 h on days 2 and 5 with 5-azacytidine (Sigma) at 0, 0.1, 0.5, and 1 μg/mL. After the second treatment (i.e., on day 6), medium was changed and recombinant human IFN-γ was added to the culture to a final concentration of 250 units/mL. Twenty-four hours later, cells were harvested for analysis for IRF8 and Fas expression and sensitivity to Fas-mediated apoptosis.

RT-PCR analysis. Total RNA was isolated from cells using RNA STAT-60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer's instructions and used for first-strand cDNA synthesis using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). The cDNA was then used as template for PCR amplification of the indicated transcripts as described previously (17, 18).

Western blotting analysis. Western blotting analysis was carried out as described previously (31). Briefly, tumor cells were lysed in lysis buffer containing 20 mmol/L HEPES (pH 7.4), 20 mmol/L NaCl, 10% glycerol, 1% Triton X-100, and a protease inhibitor cocktail (Calbiochem, La Jolla, CA). Cellular proteins were separated by 12% SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA), probed with anti-IRF8 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution, and detected using the Enhanced Chemiluminescence Plus Western Detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were stripped and probed again with anti-β-actin mAb (Sigma) at a 1:5,000 dilution.

Sodium bisulfite treatment and methylation-sensitive PCR analysis. Genomic DNA was purified from tumor cells using DNeasy Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Sodium bisulfite treatment of genomic DNA to convert unmethylated cytosine to thymidine was carried out using CpGenome Universal DNA Modification kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. Methylation-sensitive PCR primers were designed using CpG Ware program (Chemicon). The PCR sequences are as follows: unmethylated forward primer, 5′-CCATCCCCATAAAATAACACACAACAAA-3′; unmethylated reverse primer, 5′-GATGGTGTAGATGTGTGTTTGTGGTTT-3′; methylated forward primer, 5′-TCCCCGTAAAATAACGCGCGACGAA-3′; and methylated reverse primer, 5′-CGGTGTAGACGTGCGTTTGCGGTTT-3′.

Stable transfection of tumor cells. SW620 cells were transfected with the mammalian expression plasmid pcDNA (Invitrogen) containing the human *IRF8* gene, kindly provided by Dr. Levi (Tel Aviv University, Haifa, Israel) via Dr. Eklund (University of Chicago, Chicago, IL). CMS4 cells were transfected with mammalian expression plasmid pcDNA containing mouse IRF8 mutants. Transfections were done using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were propagated and maintained in culture medium containing geneticin (0.75 mg/mL; Invitrogen).

Results

Differential regulation of IRF8 and Fas by IFN-γ and ionizing radiation in human colon carcinoma cell lines. The primary (SW480) and metastatic (SW620) colon carcinoma cells were treated with IFN-γ and analyzed for Fas and IRF8 expression. RT-PCR analysis revealed that Fas was detectable in both cell lines. IFN-γ treatment increased Fas transcript levels in both cell lines (Fig. 1*A*). At the protein level, Fas level was higher in SW480 cells than in SW620 cells. The relative mean fluorescence intensity (MFI) of Fas is 6.1 in SW480 cells and 3.33 in SW620 cells (Fig. 1*A*). Consistent with what was observed at the transcript level, IFN-γ treatment increased the cell surface Fas protein levels in both cell lines. The MFI of Fas is 9.25 in IFN-γ–treated SW480 cells and 6.99 in IFN-γ–treated SW620 cells (Fig. 1*A*). IRF8 transcripts were very low or undetectable in untreated SW480 and SW620 cells (Fig. 1*A*).

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In contrast to Fas, exposure of the tumor cells to IFN-γ activated IRF8 transcription only in SW480 cells but not in SW620 cells (Fig. 1*A*). At the protein level, IRF8 expression was very low in untreated SW480 cells and undetectable in untreated SW620 cells (Fig. 1*A*). IFN-γ treatment increased IRF8 protein levels in SW480 cells but not in SW620 cells (Fig. 1*A*).

Studies suggest that ionizing radiation mediates multiple biological or immunologic consequences against neoplastic cells, including up-regulating Fas and sensitizing tumor cells to apoptotic cell death (32). To further examine the relationship between IRF8, Fas, and an apoptotic-resistant phenotype, we analyzed the effects of ionizing radiation on SW480 and SW620 cells. RT-PCR analysis indicated that Fas expression levels were increased in both cell lines at both the transcript and cell surface protein levels. The MFI is 5.5 in untreated SW480 cells and 2.69 in untreated SW620 cells. The MFI is 7.35 in irradiated SW480 cells and 5.45 in irradiated SW620 cells (Fig. 1*B*). Similar to treatment with IFN-γ, radiation induced IRF8 expression in SW480 cells but not in SW620 cells (Fig. 1*B*).

The above *in vitro* studies clearly suggest an inverse correlation between IRF8 expression level and metastatic phenotype. To determine whether this finding is physiologically relevant *in vivo*, we extended our study to a mouse mammary carcinoma tumor model. The MTAG mouse is a transgenic mouse expressing the polyoma virus middle T antigen by germ-line introduction of the middle T oncogene under the control of the mouse mammary tumor virus promoter/enhancer. The transgenic mouse develops multifocal mammary adenocarcinoma palpable by 5 weeks of age. By 6 months of age, almost all mice form lung metastases. Tumor cell lines derived from the primary mammary carcinoma exhibited sensitivity to Fas-mediated apoptosis, whereas tumor cell lines derived from the lung metastases showed much lower sensitivity to apoptosis (17). We reasoned that if loss of, or alterations in, IRF8 expression is an important determinant of metastasis, then spontaneously arising primary and metastatic tumors should express differential levels of IRF8. Immunohistochemical staining revealed that IRF8 was indeed expressed in the primary mammary carcinoma cells in all three mice examined (Fig. 2, *1a, 2a*, and *3a*). However, IRF8 was not uniformly expressed in all tumor cells; instead, IRF8-positive tumor cells are sporadically located throughout the tumor mass. It is of interest that, although IRF8 is strongly expressed in the nuclei of the tumor cells, weak IRF8 expression in the cytoplasm was also detectable (Fig. 2, *1a, 2a*, and *3a*). In contrast, IRF8 protein was not detected in the metastatic foci in the lungs (Fig. 2, *1b, 2b*, and *3b*). No dramatic difference was noted among the three mice (Fig. 2). These observations thus confirm an inverse correlation between IRF8 level and metastatic phenotype in an *in vivo* mouse tumor model of spontaneously developed mammary carcinoma and lung metastases. The patterns of IRF8 protein levels in both primary and metastatic mammary carcinoma

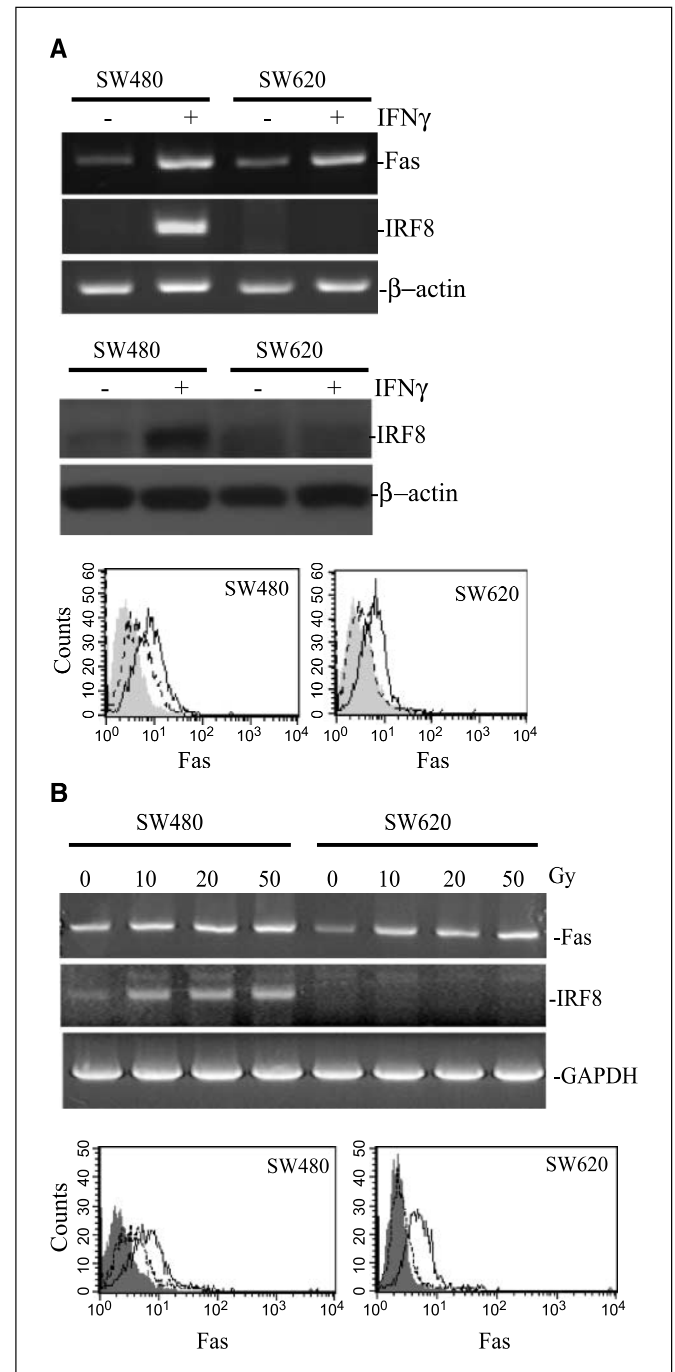

Figure 1. Inverse correlation of IRF8 expression with apoptotic resistance and metastatic phenotype in colon carcinoma cell lines. *A,* differential regulation of IRF8 expression by IFN-γ in primary and metastatic colon carcinoma cell lines. *Top,* tumor cells were treated with IFN-γ for ~24 h and then used for RNA isolation and RT-PCR analysis of Fas and IRF8. β-Actin was used as a normalization control. *Middle,* tumor cells were also used for total cell lysate preparation and Western blotting analysis of IRF8 protein. *Bottom,* to determine cell surface Fas protein levels, tumor cells were stained with human Fas-specific mAb and analyzed by flow cytometry. *Dotted lines,* histograms of untreated cells; *solid lines,* histograms of IFN-γ–treated cells. *Gray-filled areas,* isotype control staining for IFN-γ–treated cells. The Fas MFI is 6.1 ± 0.95 in untreated SW480 cells and 3.33 ± 0.74 in untreated SW620 cells. The Fas MFI is 9.25 ± 1.95 in IFN-γ–treated SW480 cells and 6.99 ± 0.93 in IFN-γ–treated SW620 cells. *B,* tumor irradiation differentially influences Fas and IRF8 expression in the primary and metastatic tumor cell lines. Tumor cells were irradiated by exposure to a γ-irradiator with the doses indicated and then cultured for 24 h. *Top,* total RNA was isolated and used for RT-PCR analysis of Fas and IRF8. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as normalization control. *Bottom,* tumor cells that were irradiated with 20 Gy dose of γ-ray were also analyzed for their cell surface Fas levels by flow cytometry. *Dotted lines,* histograms of untreated cells; *solid lines,* histograms of irradiated cells. *Gray-filled areas,* isotype control staining for the irradiated cells. The Fas MFI is 5.5 ± 0.44 in untreated SW480 cells and 2.69 ± 0.35 in untreated SW620 cells. The Fas MFI is 7.35 ± 0.35 in irradiated SW480 cells and 5.45 ± 0.02 in irradiated SW620 cells.

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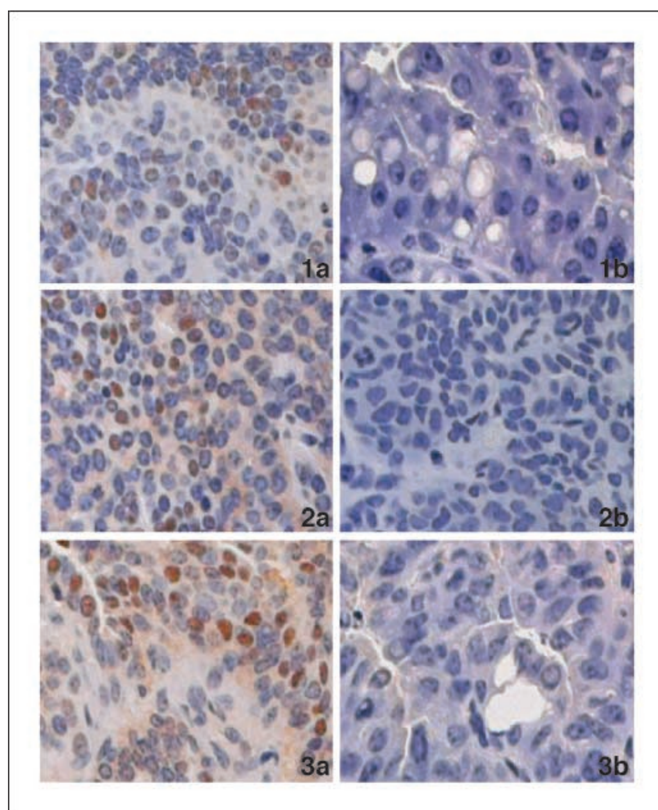

Figure 2. Immunohistochemical staining of IRF8 in primary and metastatic lesions derived from a mouse model of mammary carcinoma. Primary mammary tumors and metastases-containing lungs were excised from mice, fixed in formalin, and paraffin embedded. The sections were stained as described in Materials and Methods. Both primary tumors (*a*) and metastases-containing lungs (*b*) were obtained from three independent transgenic mice and are designated as *1a, 2a*, and *3a* and *1b, 2b*, and *3b*, respectively. Anti-IRF8 immunoreactivity is shown as the brown-stained cells, whereas cells that are unreactive to the anti-IRF8 antibody are indicated by the blue (hematoxylin) counterstain. Magnification, ×400.

cells correlated well with their Fas protein levels. Immunohistochemical staining indicated that Fas was also highly expressed in all primary mammary carcinoma examined, whereas Fas expression was considerably less in metastatic foci in the lung (data not shown; ref. 17).

IRF8 is the limiting determinant of an apoptotic-resistant phenotype in the metastatic tumor cell line. As described above, we have shown an inverse correlation between IRF8 expression and a metastatic phenotype in a matched pair of human primary and metastatic colon carcinoma cell lines *in vitro* as well as in spontaneously developed mouse mammary carcinoma and lung metastases *in vivo* (Figs. 1 and 2). To determine whether IRF8 expression level is functionally linked to an apoptotic-resistant phenotype, we next compared the IFN-γ and irradiated SW480 and SW620 cells to Fas-mediated apoptosis. Treatment of SW480 cells with IFN-γ or low dose of ionizing radiation did not induce detectable cell death (data not shown). Incubation of IFN-γ–pretreated SW480 cells with recombinant FasL induced approximately 40% to 50% cell death. However, FasL failed to induce an appreciable amount of cell death in SW620 cells (Fig. 3*A*). Similar results were obtained in irradiated SW480 and SW620 cells (Fig. 3*A*). Therefore, it is clear that the sensitivity of the tumor cells to Fas-mediated apoptosis is positively correlated with IRF8 expression, and IRF8 might be a limiting determinant of the apoptotic-resistant phenotype in these tumor cell lines.

Based on the above observations, we reasoned that if IRF8 is a limiting determinant of an apoptotic-resistant phenotype, forced expression of IRF8 in SW620 cells would sensitize SW620 cells to Fas-mediated apoptosis. To test this hypothesis, we transfected SW620 cells with a mammalian expression vector containing the full-length human IRF8 cDNA and established a stable subline. The transfected SW620 sublines were treated with IFN-γ and incubated with recombinant FasL. We observed that forced expression of IRF8 significantly increased Fas-mediated apoptosis of SW620 cells compared with parental and vector-transfected cells that do not express IRF8 under these conditions (Fig. 3*B*).

To further determine the role of IRF8 in the regulation of apoptosis, the apoptosis-sensitive mouse sarcoma tumor cell line CMS4 was stably transfected with a mouse IRF8 mutant construct that harbors a point mutation in its DNA-binding domain (K to E switch at amino acid 79). This mutant has been extensively characterized and has been shown to specifically block IRF8 functions in myeloid cells (33). We first analyzed the IRF8 transcript and protein levels in nontransfected and IRF8 mutant K79E-expressing CMS4 tumor cells (CMS4.K79E). RT-PCR analysis indicated that IRF8 transcript level is detectable but weak in untreated CMS4 and vector-transfected CMS4 cells (CMS4.Vector), whereas CMS4.K79E cells expressed a much higher level of IRF8 (Fig. 3*C*). The IRF8 transcript level increased 4 h after IFN-γ treatment in both CMS4 and CMS4.Vector cells. Because CMS4.K79E cells already expressed high level of IRF8, IRF8 level remained constantly high before and after IFN-γ treatment in CMS4.K79E cells (Fig. 3*C*). IRF8 protein is weak to undetectable in untreated CMS4 and CMS4.Vector cells. Consistent with the RNA level, IRF8 protein level is high in CMS4.K79E cells (Fig. 3*C*). IFN-γ treatment increased IRF8 protein levels in CMS4 and CMS4.Vector cells. IRF8 protein is constantly at a high level before and after IFN-γ treatment in CMS4.K79E cells (Fig. 3*C*).

IRF8 is a transcription factor and binds to promoter regions of many IFN-γ–regulated genes to regulate transcription. However, IRF8 protein alone has no DNA-binding affinity and its function requires its association with other proteins (19). IRF8-interacting proteins bind to IRF8 through the IFN association domain of IRF8 to form protein complexes, which, in turn, activate IRF8. Therefore, it is the IRF8-binding protein that dictates IRF8 function. The IRF8.K79E mutant likely functions by competing with endogenous IRF8 to bind to IRF8-interacting proteins. Here, we showed that IRF8.K79E mutant protein is in high concentration in CMS4.K79E cells. Therefore, it is reasonable to postulate that this mutant form would act in a dominant-negative manner to disrupt endogenous IRF8 function by competing with IRF8-binding proteins from endogenous IRF8. If IRF8 is an essential regulator of apoptosis as we hypothesize, then disruption of IRF8 function will render tumor cells apoptotic resistance. To test this hypothesis, CMS4, CMS4.Vector, and CMS4.K79E cells were treated with IFN-γ and followed by incubation with FasL. Analysis of cell death by propidium iodide staining and flow cytometry revealed that the IRF8.K79E mutant indeed blocked Fas-mediated apoptosis in CMS4 cells (Fig. 3*C*). Thus, we concluded that IRF8 is an essential regulator of apoptosis, and loss of IRF8 expression is one of the limiting determinants of the apoptotic-resistant phenotype in the metastatic cell line.

IRF8 is also a metastasis suppressor. Because loss of IRF8 expression is linked to metastatic phenotype (Figs. 1 and 2), we

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next sought to determine whether IRF8 also functions as a metastasis suppressor. If IRF8 is a metastasis suppressor in solid tumor cells, then disrupting IRF8 function would confer primary tumor cells metastatic capability. CMS4.K79E cells were transplanted to naive mice by i.v. injection. Tumor metastasis was then examined in lungs of tumor-bearing mice. As expected, few tumor nodules were observed in lungs of mice, which received the poorly metastatic nontransfected and vector-transfected tumor cells. However, CMS4.K79E cells exhibited a significantly higher degree of metastatic capability as determined by tumor colonization in the lungs (Fig. 3*D*; $P = 0.00048$ and 0.00045, compared with parental CMS4 and CMS4.Vector, respectively).

The above observation is somewhat surprising because rendering poorly metastatic tumor cells apoptosis resistance did not confer a metastatic phenotype to these cells in an earlier study (17). However, the present observation suggests that IRF8 might play a direct role in suppression of metastasis that is independent of or in addition to apoptosis resistance. To dissect the functions of IRF8 in apoptosis and metastasis, we repeated the metastasis experiment with CMS4 cells stably expressing vFLIP, a viral form of FLIP that inhibits procaspase-8 associations with the Fas receptor complex and thereby blocks Fas-mediated apoptosis. It is clear that rendering the parental tumor cell line CMS4 apoptosis resistant by forced expression of vFLIP did not confer metastatic capacity to CMS4 cells (Fig. 3*D*). Therefore, we conclude that IRF8 is a novel metastasis suppressor and functions through a pathway that is independent of or in addition to Fas-mediated apoptosis.

DNA methylation modulates IRF8 expression and the apoptotic-resistant phenotype in metastatic colon tumor cells. To determine whether DNA methylation is responsible for the apoptotic resistance of SW620 cells, we treated SW620 cells with 5-azacytidine, a demethylating agent that has been widely used in inhibiting DNA methylation in tumor cells (34, 35), and analyzed IRF8 and Fas expression. RT-PCR analysis revealed that inhibition of DNA methylation dramatically up-regulated Fas and IRF8 expression in a dose-dependent manner in SW620 cells (Fig. 4*A*). Although both constitutive and IFN-γ–induced Fas expression were enhanced, inhibition of DNA methylation did not result in detectable IRF8 expression in the absence of IFN-γ. However, inhibition of DNA methylation sensitized SW620 cells to IRF8 induction by IFN-γ. At the protein level, 5-azacytidine treatment did not increase IRF8 translation in SW620 cells, but the treated

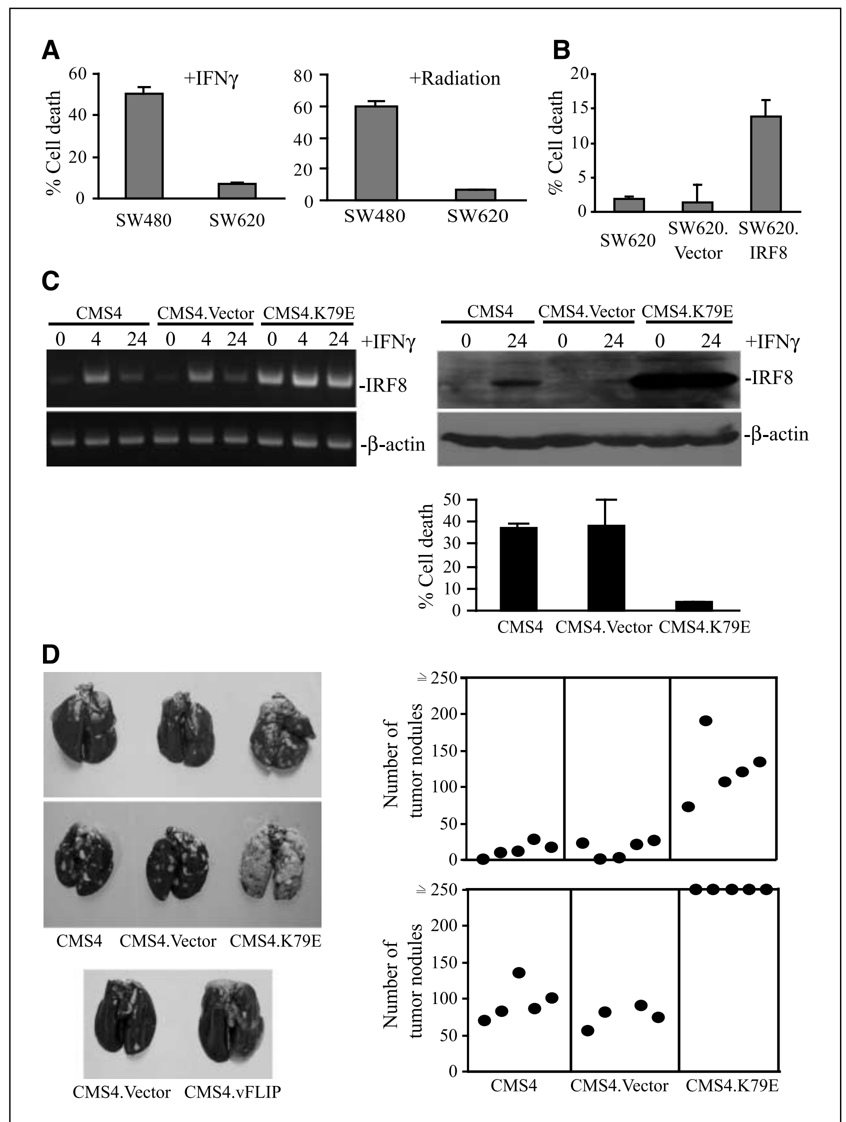

Figure 3. IRF8 is both an essential apoptosis regulator and a metastasis suppressor. *A,* sensitivity of SW480 and SW620 cells to Fas-mediated apoptosis. SW480 and SW620 cells were exposed to IFN-γ (*left*) or 20 Gy dose of γ-ray radiation (*right*) followed by incubation in the absence or presence of recombinant FasL for 24 h. To measure apoptotic cell death, cells were then stained with propidium iodide and analyzed by flow cytometry. *Columns,* mean of three experiments; *bars,* SD. *B,* exogenous expression of IRF8 restores sensitivity of the metastatic tumor cells to Fas-mediated apoptosis. The nontransfected tumor cells and tumor cells stably transfected with either control vector (SW620.Vector) or IRF8-expressing vector (SW620.IRF8) were treated with IFN-γ for 24 h and then incubated in the absence or presence of recombinant FasL for another 24 h. Apoptotic cells death was measured by propidium iodide staining and flow cytometry analysis. *Columns,* mean of three experiments; *bars,* SD. *C,* IRF8 mRNA and protein levels in CMS4 cells and a dominant-negative IRF8 mutant-transfected CMS4 subline. The nontransfected tumor cells and tumor cells stably transfected with control vector (CMS4.Vector) or IRF8 mutant-expressing vector (CMS4.K79E) were treated with IFN-γ. Cells were harvested 4 and 24 h after IFN-γ treatment, respectively, and used for RT-PCR analysis of IRF8 transcript levels (*left*) and for Western blotting analysis of IRF8 protein levels (*top right*). To assess the effects of disruption of IRF8 function on apoptosis, tumor cells were treated with IFN-γ overnight followed by incubation with recombinant FasL for 24 h. *Bottom right,* cell death was measured by staining with propidium iodide and analysis with flow cytometry. *Columns,* mean; *bars,* SD. *D,* disruption of IRF8 function conferred poorly metastatic tumor cells metastatic capacity. The parental primary sarcoma cell line CMS4 and sublines CMS4.Vector and CMS4.K79E were injected i.v. into naive mice [2.5×10^5 cells/mouse (*top left* and *top right*) and 3.5×10^5 cells/mouse (*middle left* and *bottom right*)]. Lungs were harvested 14 d later for examination of metastases and for enumeration of tumor nodules. *Right, dot,* total tumor nodule counts from a single mouse. *Top left* and *top middle,* representative tumor-bearing lungs derived from mice, which had received the indicated tumor cells; *bottom left,* resistance to apoptosis alone is insufficient for a metastatic phenotype. CMS4 tumor cells stably transfected with the vector (CMS4.Vector) or vFLIP (CMS4.vFLIP) were transplanted to mice by i.v. injection (2.5×10^5 cells/mouse). Lungs were excised from tumor-bearing mice 14 d later and examined for metastases. Lung results are from representative mice.

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cells responded to IFN-γ to express IRF8 protein (Fig. 4*A*). Consistent with the transcript level, quantification of cell surface Fas protein level in 5-azacytidine–treated SW620 cells indicated that both constitutively expressed Fas and IFN-γ–induced Fas protein were significantly increased in 5-azacytidine–treated cells (Fig. 4*B*; $P = 0.02$ and 0.03, respectively).

Next, the 5-azacytidine–treated cells were analyzed for their sensitivity to Fas-mediated apoptosis. As expected, cells that were not treated with 5-azacytidine exhibited a minimal degree of apoptotic cell death regardless of IFN-γ treatment (Fig. 4*C*). Although 5-azacytidine treatment alone did not sensitize SW620 cells to Fas-mediated cell death, the treated cells became significantly more sensitive to Fas-mediated apoptosis in a dose-dependent manner in the presence of IFN-γ (Fig. 4*D*). The *P* values for cells treated with 0.5 and 1 μg/mL of 5-azacytidine are both <0.0005.

IRF8 promoter region is hypermethylated in metastatic tumor cells. Aberrant focal CpG island hypermethylation, especially in the promoter region of a given gene, is often associated with gene silencing in tumor cells (36). Our data clearly indicated that DNA methylation is responsible for IRF8 repression and apoptosis resistance in the metastatic tumor cells (Fig. 4). Next, we sought to determine the methylation status of the IRF8 promoter in SW480 and SW620 cells.

Because the human IRF8 promoter has not been characterized, we first characterized the IRF8 promoter based on its DNA sequence from the human genome sequence database. Using BLAST search program (National Center for Biotechnology Information) and the human IRF8 cDNA sequence as bait, we identified two human genome sequences (Genbank accession nos. AC092723 and AC018695, respectively). We then used MacVector (MacVector Inc., Cary, NC) to align exon 1 of the *IRF8* gene with the genome sequence and identified the putative transcription initiation site (+1; Fig. 5*A*). The 5,000-bp DNA sequence upstream from the putative transcription initiation site was then downloaded to the MacVector program and analyzed for consensus DNA element GAS (*TTC*NNG*GAA*) that is the binding site of IFN-γ–activated transcription factors (GAF) and is responsible for IRF8 induction by IFN-γ (37, 38). The 5′ regulatory region was further analyzed with MethPrimer (Urogene, San Francisco, CA) and CpG

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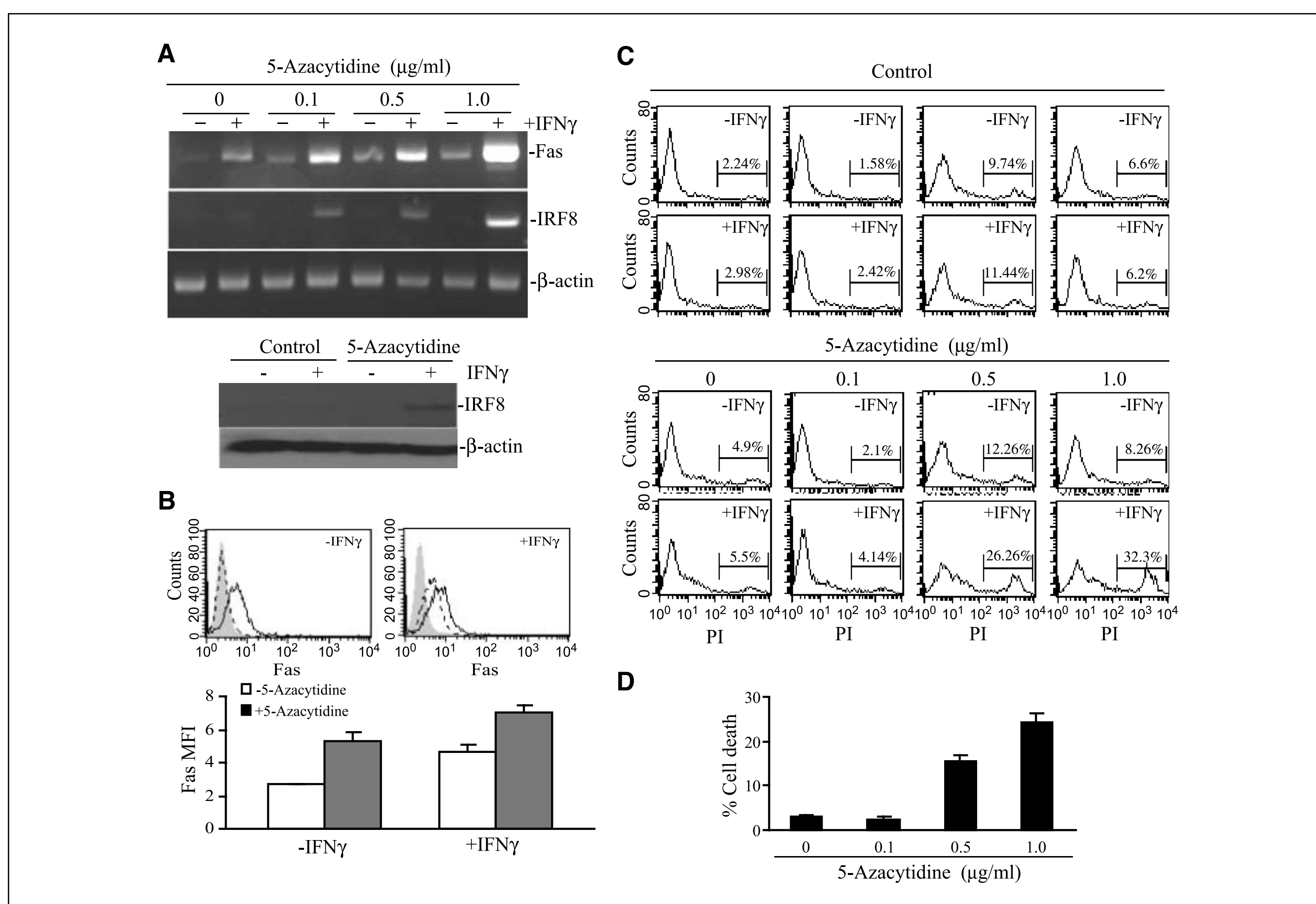

Figure 4. Repression of IRF8 expression in the metastatic tumor cells is modulated by DNA methylation. *A,* RT-PCR analysis of Fas and IRF8 transcripts and Western blotting analysis of IRF8 protein levels in SW620 cells after treatment with 5-azacytidine. *Top,* tumor cells were treated with different concentrations of 5-azacytidine as described in Materials and Methods and used for RT-PCR analysis. β-Actin was used as normalization control. *Bottom,* tumor cells that were treated with 1.0 μg/mL 5-azacytidine were also used for Western blotting analysis of IRF8 protein levels. β-Actin was used as normalization control. *B,* cell surface Fas expression in untreated and 5-azacytidine–treated (1.0 μg/mL) SW620 cells. Tumor cells were treated with 5-azacytidine followed by incubation in the absence (*top left*) or presence (*top right*) of IFN-γ for 24 h. *Bottom,* relative Fas levels were quantified by MFI. *Columns,* mean of three experiments; *bars,* SD. *C,* apoptotic cell death assay of SW620 cells after 5-azacytidine treatment. Tumor cells were either not treated (*top*) or treated (*bottom*) with 5-azacytidine followed by incubation in the absence (*−IFN-γ*) or presence (*+IFN-γ*) of IFN-γ for 24 h. Cells were then treated with recombinant FasL for another 24 h, stained with propidium iodide (*PI*), and analyzed by flow cytometry. *D,* quantification of apoptotic cell death as in (*C*). *Columns,* mean of three experiments; *bars,* SD.

Ware (Chemicon) programs for CpG islands. We identified a CpG island region located between +5 and −701 and a GAS motif (GATTTCTCGGAAAGC) located between −131 and −145 (Fig. 5*A*). The GAS motif sits inside the CpG island. The sequence of this human GAS element is almost identical to the reported mouse GAS (pIRE) element (GATTTCTCGGAAAGA) in the mouse IRF8 promoter region (38). No CpG islands and no GAS elements were identified beyond −701.

The existence of a CpG island and the fact that the GAF-binding motif (GAS) is located inside the CpG island suggest that DNA hypermethylation in this region of the human IRF8 promoter region might be the cause of failure of IFN-γ to activate IRF8 in metastatic colon carcinoma cells. Next, we isolated genomic DNA from SW480 and SW620 cells and modified the genomic DNA with a CpGenome Universal DNA Modification kit. The modified genomic DNA was then analyzed by methylation-sensitive PCR using unmethylated and methylated primers, respectively (39), to determine the methylation status of the CpG island. We observed that only unmethylated primers amplified IRF8 promoter DNA fragment from methylation-modified SW480 genomic DNA, whereas only methylated primers amplified methylation-modified genomic DNA isolated from SW620 (Fig. 5*B*). Therefore, we concluded that the CpG island within the human IRF8 promoter is not methylated in SW480 cells but is hypermethylated in the metastatic colon carcinoma cell line SW620.

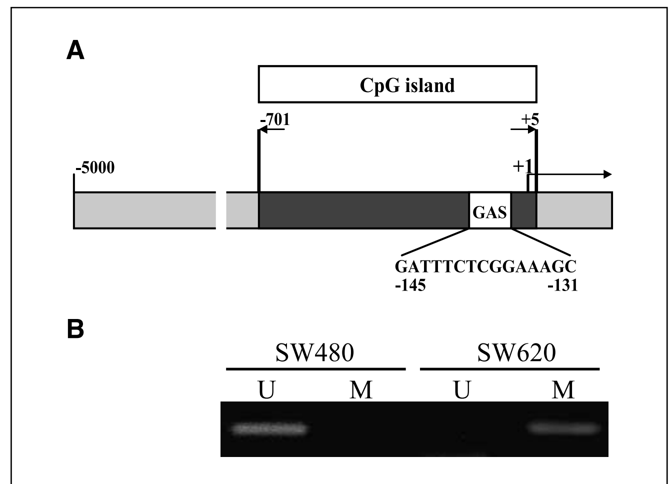

Figure 5. The 5′ regulatory region of the *IRF8* gene is methylated. *A,* genomic organization of the human IRF8 gene promoter region. The 5′ regulatory region of the human *IRF8* gene was deduced from the human genome DNA sequence. Organization of the 5′ untranslated region (exon 1) and 5,000 bp (−5,000) upstream from the putative transcription initiation site (+1). *Solid dark gray box,* location of the CpG island; *open box,* consensus GAF-binding element (GAS). *B,* methylation-sensitive PCR analysis of the IRF8 gene promoter region in the primary colon carcinoma SW480 cells and the metastatic colon carcinoma SW620 cells. Genomic DNA was isolated from these two tumor cell lines and modified using the CpGenome Universal DNA Modification kit as described in Materials and Methods. The modified genomic DNA was then used as template for methylation-sensitive PCR analysis using unmethylated (*U*) or methylated (*M*) primers.

Discussion

Apoptotic resistance is often associated with a metastatic phenotype in tumor cells (40). Fas is a key death receptor that initiates apoptosis by both innate and adaptive immune cells (8). Thus, suppression of such a cell death pathway in neoplastic cells confers a survival advantage for tumor cells to escape from cellular destruction. Several functional studies support the notion that metastatic tumor cells use loss of Fas expression and function as a means to acquire enhanced metastatic capability (13–15, 30, 41). In this study, we examined the inverse correlation between IRF8 expression and apoptotic-resistant phenotype and revealed that loss of IRF8 expression is another key determinant of apoptotic resistance in addition to Fas receptor in the metastatic tumor cells. Therefore, it seems that metastatic tumor cells use loss of Fas expression in concert with repressed IRF8, a key proapoptotic regulator in the Fas-mediated death pathway, to acquire an enhanced apoptotic-resistant phenotype. This observation is of interest because it has been rarely observed that two key modulators in the same cell death pathway are down-regulated simultaneously to achieve an apoptotic-resistant phenotype by tumor cells. Alterations in more than one molecule of the same death signaling pathway might ensure the capability of tumor cells to evade destruction in the host if one defect is reversed by host mechanisms. For example, immune cells often secrete proinflammatory cytokines, such as IFN-γ and tumor necrosis factor-α, when activated by stimuli, including recognition of neoplastic cells (24). Proinflammatory cytokines often activate the expression of key apoptotic genes, including *Fas* (12). Alterations of multiple regulators in the Fas-mediated apoptosis pathway enable the tumor cells to resist apoptotic cell death induction even if the defect of one regulator (i.e., Fas) is reversed by IFN-γ. Therefore, simultaneous alterations in the expression patterns of multiple apoptosis modulators in the same death pathway dramatically increase the survival capability of the tumor cells. For the metastatic SW620 cells, although exposure to IFN-γ up-regulated Fas expression, the IRF8 expression was still repressed, and the tumor cells remained resistant to Fas-mediated apoptosis. Therefore, the metastatic SW620 cells maintained the apoptotic-resistant phenotype even if Fas is up-regulated.

Although loss of Fas expression is often associated with metastatic phenotype (10), and loss of Fas function has been implicated in enhancement of metastatic potential of tumor cells (14), rendering cells Fas resistant alone seems insufficient to convert the primary tumor cells into a metastatic phenotype in certain types of tumors (17). We observed that disruption of IRF8 function conferred the poorly metastatic tumor cells with a metastatic phenotype, whereas blocking apoptosis in the same primary tumor cell line did not change the metastatic capability (Fig. 3), suggesting that IRF8 plays a distinct role in inhibition of metastasis that is independent of or in addition to its role in regulation of apoptosis. Thus, IRF8 plays essential roles in both regulation of apoptosis and suppression of metastasis. However, although it seems that IRF8 functions to sensitize solid tumor cells to apoptosis and suppress metastasis through independent mechanisms, it is not clear whether these two pathways overlap. Furthermore, the role of IRF8 as a metastasis suppressor needs to be tested in other types of tumors and in spontaneously developed tumors.

The expression of apoptotic genes is regulated by multiple mechanisms, including both genetic and epigenetic events (42–44). Silencing of genes involved in apoptosis by epigenetic events is frequently associated with cancer development (34, 35, 45). It has been shown that transformed mouse embryonic fibroblast cells silence Fas expression by histone acetylation to evade natural killer cell–mediated killing (46), whereas DNA hypermethylation of tumor suppression genes is common in human colon neoplasia

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and other types of cancers (34, 35). What we observed is a unique pattern of epigenetic modulation of gene expression that is quite distinct from what has been previously reported in the literature. First, two of the key modulators in the same cell death pathway are coordinately down-regulated by DNA hypermethylation; second, it seems that the IRF8 promoter region in the primary tumor cells is not hypermethylated, whereas the metastatic tumor cells that were derived from the primary tumor cells developed an epigenetic mechanism (DNA methylation) to repress IRF8 expression. These observations suggest that epigenetic mechanisms that modulate the expression of apoptosis regulators might change in the course of tumor progression. This might be significant in terms of development of molecular mechanism-based therapeutic strategies. Switching between different regulatory mechanisms or developing additional strategies to repress apoptotic regulators as tumors progress might confer tumor cell resistance to cancer therapies that target one molecule or one mechanism at a certain stage of cancer.

Promoter DNA hypermethylation is often the course of DNA methylation-modulated gene silencing (47). We observed that a GAS element is located in the CpG island of the IRF8 promoter region, and the CpG island is hypermethylated in metastatic cancer cell lines. This observation explains the inability of metastatic tumor cells to undergo IFN-γ–mediated activation of IRF8 gene expression (Figs. 1 and 4). *IRF8* is a primary response gene regulated directly by the IFN-γ receptor-initiated signaling pathway (16). It has been well shown that IFN-γ binding to its receptor triggers signal transducers and activators of transcription 1 (STAT1) phosphorylation and dimerization. The dimerized STAT1 then translocates to the nucleus and binds directly to the IRF8 promoter element (the GAS element) to activate IRF8 transcription (37, 38, 48). Because IFN-γ up-regulates Fas expression in SW620 cells, it is reasonable to assume that the function of STAT1 in the IFN-γ receptor-initiated signaling pathway is not altered. No gene transcription is required in the signaling transduction pathway between IFN-γ receptor ligation and IRF8 transcription (16). Therefore, methylation of the IRF8 promoter region must be the cause of transcriptional repression of the *IRF8* gene, and our data support this notion (Fig. 5).

Although we have shown here that IRF8 is an essential regulator of apoptosis in solid tumor cells, and that metastatic tumor cells might use DNA hypermethylation to repress IRF8 expression to acquire apoptotic resistance, it is unlikely that IRF8 repression is the only mechanism for apoptotic resistance in metastatic tumor cells. Further studies are needed to identify other apoptosis regulators whose expression might also be altered by genetic or epigenetic events in metastatic tumor cells.

In summary, our data suggest that IRF8 functions as an essential regulator of apoptosis and metastasis suppressor. Metastatic tumor cells might use DNA hypermethylation of IRF8 promoter to silence IRF8 expression and thereby evade apoptotic cell death and acquire an enhanced metastatic capability. In the future, it will be important to determine the signaling pathway(s) that regulates IRF8 hypermethylation in solid tumor cells.

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