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Silencing of the interferon-inducible gene *Ifi204/p204* induces resistance to interferon-γ-mediated cell growth arrest of tumor cells

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ABSTRACT

Many tumor cells escape from cancer immunosurveillance and resist treatment with interferons (IFNs). Although the mechanism underlying IFN resistance is mostly attributed to a deficiency of components of the IFN-signaling pathway, some types of tumor cells resist IFN-mediated cell growth arrest despite the presence of an intact JAK/STAT signaling pathway. However, the molecular mechanisms underlying the unresponsiveness to IFNs independent of the defective JAK/STAT pathway remain to be clarified. To elucidate the mechanisms underlying IFNγ resistance, we examined the anti-proliferative effect of IFNγ on mouse tumor cell lines. Mouse squamous cell carcinoma (SCCVII) cells were resistant to IFNγ-mediated cell growth arrest despite the presence of the IFNγ-induced STAT1-dependent signaling pathway, whereas IFNγ inhibited cell growth of B16/F1 cells, a well-known IFNγ-sensitive mouse melanoma cell line, at the G1 phase of the cell cycle. Treatment of SCCVII cells with IFNγ neither downregulated the expression of cyclin D1, cyclin A2, and cyclin E1 nor induced a hypo-phosphorylated, active form of retinoblastoma protein (pRb). Interestingly, the hyper-phosphorylated, inactive form of pRb was exclusively localized in the cytoplasm in SCCVII cells. The IFN-inducible 204 gene (*Ifi204*), whose gene product, p204, binds to pRb and exerts an anti-proliferative effect, was repressed in SCCVII cells. p204 overexpression in SCCVII significantly inhibited cell growth, and mutation of a pRb-binding LXCXE motif decreased the anti-proliferative effect. These results suggest that silencing of *Ifi204*/p204 induces resistance to IFNγ-mediated cell growth arrest in SCCVII cells.

1. Introduction

IFNγ is a pleiotropic cytokine produced by activated T and NK cells. It exerts a diverse array of biological activities involved in host defense and immunomodulatory functions, including anti-tumor effects [1–3]. The growth inhibitory activity is one of the major functions of type I (IFNα/β) and type II (IFNγ) IFNs and has been extensively studied in many cell types, including various types of tumor cells [4–6] (For review, see Ref. [7]). IFNs cause cell growth arrest mainly at the G0/G1 phase of the cell cycle by inducing cyclin-dependent kinase (Cdk) inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip}$ [8–11], which inhibit cyclin/Cdk activity, thereby downregulating the phosphorylation of retinoblastoma protein (pRb) [12,13]. In some tumor cells, the anti-proliferative effect of IFNγ is independent of the induction of the Cdk inhibitors, and the mechanisms underlying IFNγ anti-proliferative effects include the downregulation of cyclin A, cyclin E, Cdk2 [14,15], and E2F-1 [16] and the upregulation of the IFN-inducible p200 family proteins [17–19]. Thus, the anti-proliferative effects of IFNs are regulated by various molecular mechanisms depending on the tumor cell type.

The murine p200 family proteins consist of at least six members designated p202a, p202b, p203, p204, D3 (p205), and absent in melanoma 2 (Aim2) and encoded by a cluster of genes located on the q21-q23 region of chromosome 1 (the *Ifi200* gene family) [17,20–22]; (for review, see Refs. [23;24]). They contain one or two highly conserved 200-amino-acid domains, and the members of the p200 family proteins are multifunctional regulators of cell proliferation, differentiation, apoptosis, and senescence, mediating these effects by binding to various transcription factors and signaling molecules [23–25]. The p204 protein is a 72-kDa phosphoprotein induced by IFNs, which subsequently translocates to the nucleus and binds to the pRb, contributing to IFN-induced anti-proliferative activity [19,21,26].

The sensitivity of tumor cells to IFNγ is indispensable for host-

Abbreviations: 5-Aza-dC, 5-Aza-2′-deoxycytidine; Cdk, cyclin-dependent kinase; Ifi, interferon inducible; ISGs, IFN-stimulated genes; p-pRb, phosphorylated pRb; pRb, retinoblastoma protein; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SCC, squamous cell carcinoma

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protective antitumor responses and anti-proliferative activity [27,28]. However, many tumor cells evade IFN treatment [29–39] and resist cancer immunotherapy targeting immune checkpoint molecules [40,41]. The molecular mechanisms underlying the unresponsiveness to IFNs mainly involve deficiency and/or an inactivation of components of the proximal IFN-receptor/JAK/STAT signaling cascade, which include IFN receptors [30–32], JAK1 [33], STAT1 [34–38], STAT2 [34], and IRF-9/p48 [34,39]. Thus, the JAK/STAT signaling pathway is essential for the sensitivity of tumor cells to both type I and type II IFNs. However, some types of tumor cells resist the anti-proliferative effect of IFNs despite the presence of an intact JAK/STAT signaling pathway [15,42–44], suggesting that downstream signaling molecules and/or growth-regulatory molecules are deregulated in these tumor cells. The molecular mechanisms underlying resistance to IFNs independent of a defective JAK-STAT1 signaling pathway remain to be fully elucidated.

In the present study, we examined the mechanism underlying resistance to the IFNγ-mediated anti-proliferative effect in a mouse squamous cell carcinoma cell line, SCCVII, which has been widely used as a mouse tumor model [45–48], in comparison to that in a well-known IFNγ-sensitive mouse melanoma cell line, B16F1 [49–52], as a positive control for IFNγ-responsiveness. The results of this study revealed that the resistance to IFNγ-mediated growth arrest of SCCVII cells is not caused by a deficiency in STAT1-dependent signaling but rather results from silencing of the IFN-inducible p200 family genes. These results provide a novel mechanism underlying the resistance to IFNγ-mediated growth arrest of tumor cells.

2. Materials and methods

2.1. Cell culture and cell counting experiments

The mouse squamous cell carcinoma cell line SCCVII has been widely used as a mouse oral SCC model [45–48]. The cells were kindly provided by Dr. K. Ono and S. Masunaga (Radiation Oncology Research Laboratory, Research Reactor Institute, Kyoto University) [45] through the Cell Resource Center for Biomedical Research (Tohoku University, Japan). The mouse melanoma cell line B16F1 (RCB2649) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. These cells were grown in RPMI 1640 containing L-glutamine, penicillin–streptomycin, and 10% FBS. Before performing experiments, we optimized cell density and culture periods of these cell lines. For cell counting experiments and preparation of total mRNA, SCCVII and B16F1 cells were seeded in 6-well plates at a density of 3×10^4 and 1.5×10^5 cells/well, respectively, and grown for 20 h in complete medium before IFNγ treatment. After IFNγ treatment for various durations, cells were harvested with 0.5% trypsin-EDTA solution and counted in triplicate using a hemocytometer.

2.2. DNA synthesis

DNA synthesis was measured by monitoring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) using a cell proliferation ELISA kit (Roche Diagnostics, Basel, Switzerland). Briefly, SCCVII and B16F1 cells were seeded into 96-well plates at a density of 0.333×10^3 and 1×10^3 cells/well, respectively, and cultured for 20 h in complete medium before IFNγ treatment. After treatment with or without IFNγ for various durations, cells were pulse-labeled with 100 μM BrdU for the last 2 h of culture. The cells were then washed, fixed with FixDenat solution for 30 min, and incubated with monoclonal antibody against BrdU conjugated with peroxidase for 90 min. After washing the wells with the washing solution, 100 μL of the substrate solution was added in each well, and the light emission of the samples was measured using a microplate luminometer (CentroXS^3 LB960, Berthold, Pforzheim, Germany).

2.3. Cell cycle analysis

SCCVII and B16F1 cells were seeded in 10-cm dishes at a density of 8×10^4 and 3×10^5 cells/dish, respectively, and grown for 20 h in complete medium before treatment with IFNγ. Cells were harvested by trypsinization, washed with PBS, and fixed with 70% cold ethanol. Cells were then treated with 250 μg/mL of RNAse at 37 °C for 60 min, and cellular DNA was stained with 50 μg/mL of propidium iodide (PI). Cells (5×10^4) were analyzed on a flow cytometer (Epics Elite ESP, Coulter Electronics, Brea, CA, USA). The proportions of cells in different stages of the cell cycle were determined by using WinCycle software (Coulter Electronics).

2.4. Preparation of RNA and quantitative real-time RT-PCR (qRT-PCR)

SCCVII and B16F1 cells were seeded in 6-well plates at a density of 3×10^4 and 1.5×10^5 cells/well, respectively, and grown for 20 h in complete medium before treatment with IFNγ. After treatment with or without IFNγ, cells were washed with ice cold-PBS, and total RNAs were prepared using the Nucleospin RNA kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. cDNAs were synthesized from the purified total RNA using a high-capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA). Real-time PCR probes and primers were selected using the Universal Probe Library Assay Design Center (Roche Diagnostics) and are listed in Supplemental Tables 1 and 2. Aliquots of cDNA were amplified using a LightCycler 480 Real-Time PCR System (Roche Diagnostics) and TaqMan Gene Expression Master Mix (Life Technologies) according to the manufacturers' instructions. The PCR cycling conditions were as follows: 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 50 °C for 30 s. The transcript levels were calculated relative to the levels of 18S rRNA used as an internal control.

2.5. Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic extracts were prepared using a modification of the method of Dignam et al [53] as described previously [54]. After incubation with IFNγ, the cells were washed with ice-cold PBS, harvested, and resuspended with 300 μL of hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 10 μg/mL of leupeptin, antipain, aprotinin, and pepstatin) for 10 min on ice. The cells were lysed in 0.6% NP-40 by vortexing for 10 s. The nuclei were separated from the cytosol by centrifugation at 12,000*g* for 1 min, and the supernatant was harvested as a cytosolic fraction. The residual nuclei were washed with 600 μL of buffer A, resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 10 μg/mL of leupeptin, antipain, aprotinin, and pepstatin), and briefly sonicated on ice. Nuclear extracts were obtained by centrifugation at 12,000*g* for 10 min; the protein concentration was measured using the Bradford method [55] with a protein dye reagent (Bio-Rad, Hercules, CA, USA).

2.6. Western blotting analysis

The extracts were resolved in SDS-PAGE sample buffer (62.5 mM Tris, pH 6.8, containing 2% SDS, 20% glycerol, 5% β-mercaptoethanol, and 0.2% bromophenol blue) and separated by SDS-PAGE in a 7.5% polyacrylamide gel. Proteins were transferred to polyvinylidene

fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by semi-dry blotting. The membranes were blocked with 5% non-fat milk in TBS-T [50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.1% Tween-20], incubated overnight with primary antibodies listed in Supplemental Table 3 at 4 °C, and washed three times with TBS-T. After washes with TBS-T, the blots were incubated for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase and washed again with TBS-T. The blots were developed using a SuperSignal West Pico chemiluminescence substrate kit (Thermo Fisher Scientific, Waltham, MA, USA) and exposed on X-ray films (Kodak, Rochester, NY, USA). In some experiments, chemiluminescence signals were captured and imaged on the ChemiDoc imaging system (Bio-Rad). The levels of protein expression were quantified by measuring the chemiluminescent signal of the blots.

2.7. Construction of the Ifi200 family member expression vectors

Plasmids containing cDNAs for mouse *Ifi203* [56] (IMAGE:3257492), *Ifi204* [57] (IMAGE:4018506), and *Ifi205* [22] (IMAGE:3466347) were obtained from OriGene (Rockville, MD, USA). The coding regions of these *Ifi200* family members were amplified by PCR using KOD plus DNA polymerase (Toyobo, Osaka, Japan) for the Gateway cloning system (Life Technologies). The attB1 and attB2 primers for the PCR are listed in Supplemental Table 4. The PCR products were purified and cloned into pDONR221 (Life Technologies) by BP recombination reactions using BP Clonase according to the manufacturer's instructions. The gene cassettes for *Ifi203*, *Ifi204*, and *Ifi205* were then transferred into the expression vector pLenti6/V5 (Life Technologies) by LR recombination reactions using LR Clonase. The resultant expression vectors were designated as pL-Ifi203, pL-Ifi204, and pL-Ifi205, which contain the V5-epitope tag at the C-terminus of the coding region. Mutations of the proximal and distal pRb-binding motif LXCXE in p204 were performed by substitution with LXGXK using the PrimeSTAR mutagenesis kit (Takara). Primers for the mutagenesis are listed in Supplemental Table 5. The resultant mutant expression vectors were designated as pL-mRb1 and pL-mRb2, respectively. The nucleotide sequences of the cloned genes were confirmed.

2.8. Transient transfection of the Ifi200 expression vectors and DNA synthesis assay

SCCVII cells were seeded into 96-well plates at a density of 1×10^3 cells/well and cultured for 20 h prior to transfection. The cells were then transiently transfected with 0.2 μg/well of either empty vector, the pL-Ifi203, pL-Ifi204, or pL-Ifi205 expression vector, or the mutant pL-mRb1 or pL-mRb2 expression vector using Lipofectamine 2000 (Life Technologies). The rate of DNA synthesis of the transfected cells was assessed by measuring BrdU incorporation at 24, 36, 48, and 72 h after transfection using the cell proliferation ELISA kit (Roche Diagnostics) according to the manufacturer's instructions.

For the analysis of protein expression, SCCVII cells were seeded into 6-well plates at a density of 3×10^4 cells/well and cultured for 20 h prior to transfection. The cells were then transiently transfected with 4 μg/well of either empty vector or the expression vector using Lipofectamine 2000 (Life Technologies) and cultured for 36 h. The cells were washed with PBS and resuspended in RIPA buffer, and the supernatants were used as total cell lysates for western blotting analysis using anti-V5 epitope tag antibody (Life Technologies). In some experiments, nuclear extracts were prepared for assessment of pRb protein by western blotting analysis.

2.9. Statistics

Student's *t* test for paired data and one-way ANOVA for multiple data were used to test for significant difference using Prism 5 software (GraphPad, San Diego, CA, USA) and a p-value < 0.05 was considered statistically significant.

3. Results

3.1. Resistance to IFNγ-mediated cell growth arrest in a mouse squamous cell carcinoma cell line, SCCVII

To examine the effects of IFNγ on cell proliferation of mouse squamous cell carcinoma cell line SCCVII and mouse melanoma cell line B16F1, an IFN-sensitive cell line used as a positive control for IFNγ-mediated cell growth arrest, exponentially growing cells were treated with IFNγ for the indicated times, and cell numbers were measured by direct cell counting (Fig. 1A). Although treatment of B16F1 cells with IFNγ almost completely inhibited cell growth, no growth inhibitory effect of IFNγ was observed in SCCVII cells at any time point. To determine the rate of DNA synthesis in these cells, incorporation of BrdU was analyzed (Fig. 1B). As observed in the cell growth assay, IFNγ significantly inhibited the incorporation of BrdU in B16F1 cells but not in SCCVII cells. Cell cycle analysis revealed that IFNγ treatment of B16F1 cells increased the percentage of cells in the G1 phase of the cell cycle (from 46.7% to 87.1%; Fig. 1C and D), though no significant change in cell cycle distribution was observed in IFNγ-treated SCCVII cells (Fig. 1E and F).

3.2. Impaired growth inhibitory effect of IFNγ in SCCVII is not due to a defect in the STAT1-dependent signaling pathway

IFNγ-mediated biological activities are dependent on the STAT1-mediated signaling pathway ([58,59]; for reviews, see Refs. [60;61]). To determine whether the impaired inhibitory effect of IFNγ was due to a defect of the STAT1-mediated signaling pathway, we examined STAT1 activation and the expression of STAT1-regulated genes in these cells after IFNγ stimulation. Initially, we assessed STAT1 protein levels in total lysates from B16F1 and SCCVII cells by western blot analysis (Fig. 2A and B). STAT1 protein levels in both B16F1 and SCCVII cells were upregulated after 12 h of IFNγ treatment. The tyrosine residue at 701 and serine residue at 727 of STAT1 were phosphorylated after IFNγ treatment in both cell types, and kinetics of STAT1 phosphorylation were similar in these cells. These results indicated that IFNγ-induced STAT1 activation is intact in SCCVII cells.

Next, we examined the expression of STAT1-regulated genes, *Cxcl9* [62], *Cxcl10* [63], and *Cxcl11* [64], which are IFN-inducible chemokines, in B16F1 and SCCVII cells (Fig. 2C–E). These cells were treated with IFNγ for 24 h, and total RNA was prepared. The expression of these genes was analyzed by qRT-PCR. Although levels of these chemokine mRNAs were low in untreated cells, IFNγ markedly upregulated these chemokine mRNA levels in SCCVII cells. We also examined other IFNγ-inducible genes in B16F1 and SCCVII cells (Fig. 2F-H). Interestingly, although *Givin1*, a member of the GTPase family [65], was markedly upregulated in IFNγ-treaded SCCVII cells (Fig. 2F), expression of *Irf1* and *Ifr7* was downregulated in SCCVII cells (Fig. 2G and H). Similarly, several other IFNγ-inducible genes were repressed in SCCVII cells (Supplemental Fig. 1). Taken together, these results indicated that although the proximal STAT1-dependent signaling pathway is intact in SCCVII cells, downstream of STAT1 activation and/or other IFNγ-inducible signaling pathways are impaired in SCCVII cells.

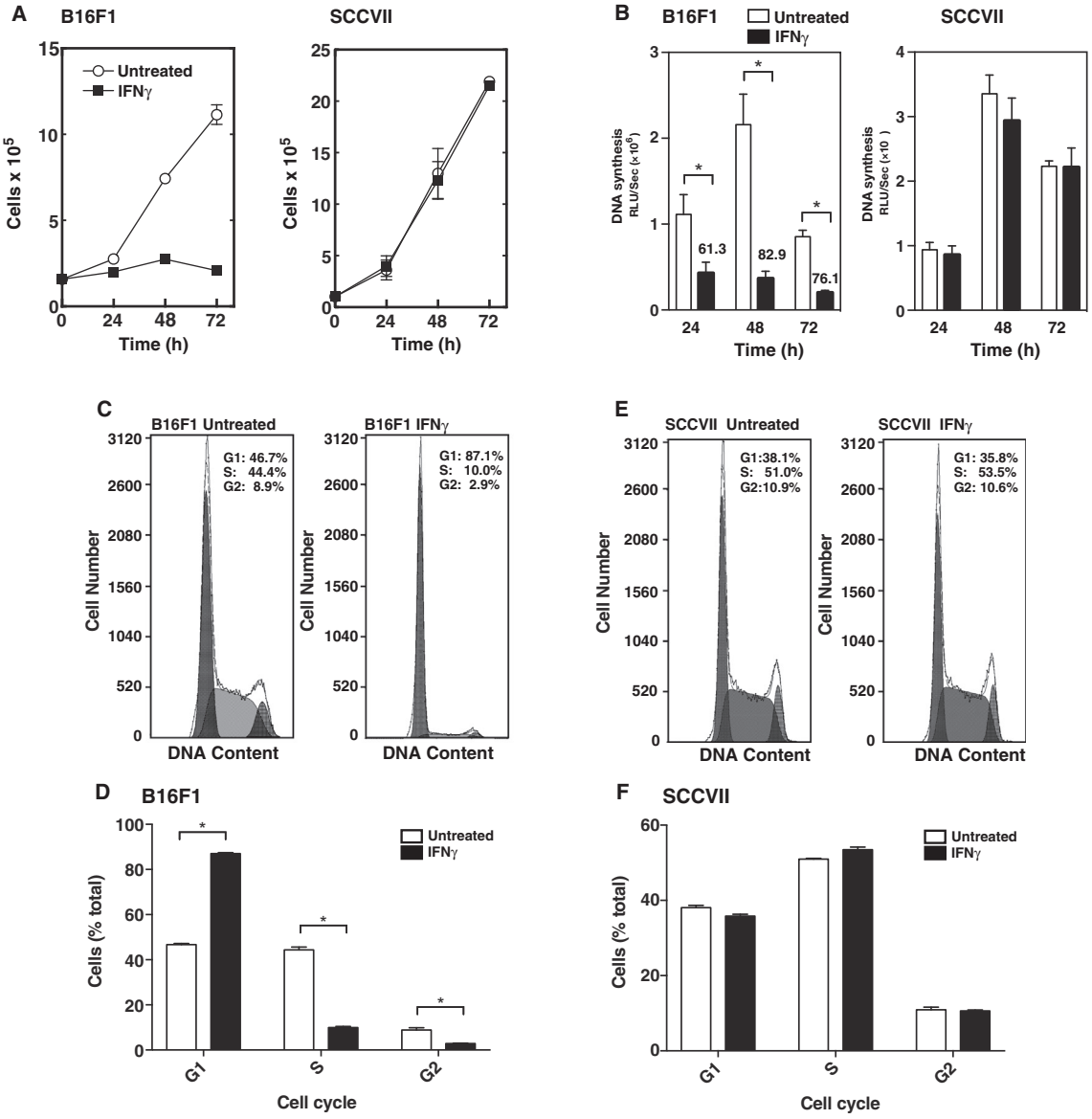

Fig. 1. Impaired cell growth arrest and DNA synthesis by IFNγ in mouse squamous cell carcinoma SCCVII cells.

SCCVII cells resist IFNγ-induced cell growth arrest (A). Cells were either left untreated or treated with IFNγ (10 ng/mL) for the indicated times. After culture, cells were detached by trypsin, and cell numbers were determined using a hemocytometer. Data represent the mean ± SD of triplicate experiments. Similar results were obtained in three separate experiments.

SCCVII cells resist IFNγ-induced inhibition of DNA synthesis (B). DNA synthesis was measured by monitoring of BrdU incorporation. Significance for the inhibition of DNA synthesis between untreated and IFNγ − treated samples is indicated ($^{*}p < 0.05$, Student's *t*-test). The percentage of inhibition of DNA synthesis is shown above the columns.

Effect of IFNγ on cell cycle phase distribution in B16F1 (C, D) and SCCVII (E, F) cells. Asynchronously growing cells were treated with or without IFNγ (10 ng/mL) for 48 h. Flow cytometric data were analyzed using WinCycle software to determine the proportion of cells in each cell cycle phase (D, F). Each column and bar represents the mean ± SD of triplicate cultures. Significance for the proportion of cells in each cell cycle phase between untreated and IFNγ − treated samples is indicated (*p < 0.05, Student's *t*-test).

3.3. Impaired regulation of IFNγ-induced cell cycle regulators in SCCVII cells

IFNγ is known to induce Cdk inhibitors such as $p21^{Cip1/Waf1}$ and $p27^{Kip}$ [8,9,11] and to inhibit the expression of cell cycle regulatory molecules such as cyclin A2, cyclin D1, and cyclin E1 in various cell types [14–16]. To examine the expression of these cell cycle regulators in response to IFNγ in B16F1 and SCCVII cells, cells were treated with IFNγ for the indicated times, and total RNA was prepared and used to assess the expression of these genes by qRT-PCR. As shown in Fig. 3, IFNγ significantly inhibited the expression of cyclin D1 (*Ccnd1*), cyclin A2 (*Ccna2*), and cyclin E1 (*Ccne1*) in B16F1 cells (Fig. 3A, C, and E), but did not in SCCVII cells (Fig. 3B, D, and F). Although IFNγ marginally upregulated (about 1.6-fold) the gene encoding Cdk inhibitor $p27^{Kip}$ (*Cdkn1b*) (Fig. 3I), no significant upregulation of $p21^{Cip1/Waf1}$ (*Cdkn1a*) expression was observed in both cell types (Fig. 3G and H). The

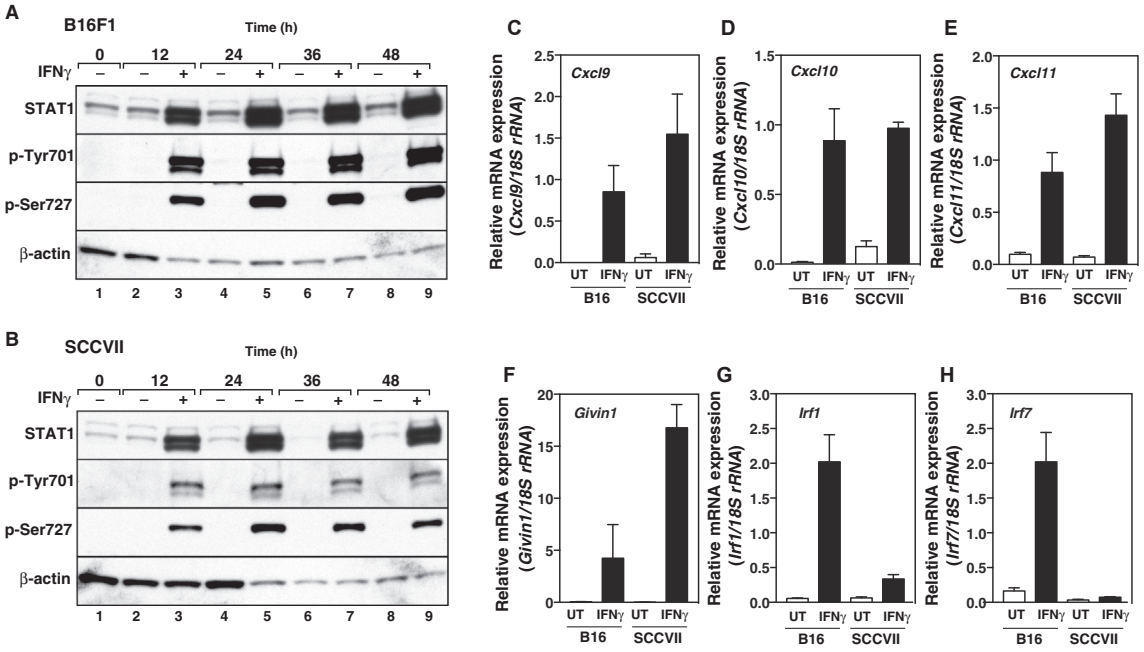

Fig. 2. IFNγ-induced STAT1-dependent signaling pathway is intact in SCCVII cells.
STAT1 activation in B16F1 (A) and SCCVII (B) cells. Cells were either left untreated or treated with IFNγ (10 ng/mL) for the indicated times before preparation of total cellular extracts. Equal amounts of protein (20 μg/lane) were loaded on the gel and analyzed by western blotting using antibodies against STAT1, phospho-STAT1 (Try701), phospho-STAT1 (Ser727), and β-actin (loading control).
IFNγ-inducible gene expression in B16F1 and SCCVII cells (C-H). Cells were either left untreated (UT) or treated with IFNγ (10 ng/mL) for 24 h before preparation of total RNA and analysis of specific mRNA levels by qRT-PCR. The relative mRNA expression levels are normalized to those of 18S rRNA used as an internal control. Each column and bar represents the mean ± SEM of three independent experiments.

expression of cyclin D2 (*Ccnd2*), cyclin D3 (*Ccnd3*), and *Cdk2* was not modulated by IFNγ in both cell types (Supplemental Fig. 2).

Protein levels of cyclin D1 and cyclin A2 were also analyzed by western blotting using cell lysates from B16F1 and SCCVII cells treated with IFNγ (Fig. 4A and B). Similar to mRNA levels, although IFNγ treatment for 12 h decreased cyclin A2 and cyclin D1 protein levels in B16F1 cells (Fig. 4A), IFNγ did not affect the expression of these cyclins in SCCVII cells (Fig. 4B). These results suggested that IFNγ does not alter the expression of cell cycle regulators in SCCVII cells, resulting in IFNγ resistance.

3.4. Deregulation of IFNγ-induced hypo-phosphorylated, active form of pRb and subcellular localization of pRb

The expression of cell cycle regulators for S-phase entry is induced by the transcription factor E2F-1 [12,13]. The transcriptional activity of E2F-1 is normally repressed by binding of the hypo-phosphorylated, active form of the tumor suppressor pRb during G0/G1 phase [12,13]. To determine whether IFNγ modulates the phosphorylation status of pRB, exponentially growing cells were treated with IFNγ for the indicated times, and cell lysates were prepared and used for western blotting (Fig. 5A and B). For B16F1 cells, a single band was observed in untreated cells (Fig. 5A, lane 1). When the cells were treated with IFNγ, an electrophoretically faster-migrating band appeared (Fig. 5A, lanes 3 and 5), which corresponds to the hypo-phosphorylated, transcriptionally repressive form of pRB [66]. The faster-migrating bands were gradually prominent as the time of IFNγ treatment increased (Fig. 5A, lanes 7 and 9). In SCCVII cells, however, only a single band was observed even after treatment with IFNγ (Fig. 5B, lanes 4, 6, 8, and 10), which corresponds to the hyper-phosphorylated, inactive form of pRB (Fig. 5B, lanes 1 and 2).

To further analyze the phosphorylation status and subcellular localization of pRb in IFNγ-treated cells, cytoplasmic and nuclear extracts were prepared from B16F1 and SCCVII cells treated with IFNγ, and the phosphorylation status of pRB was examined using an anti-phospho pRB (Ser807/811) antibody (Fig. 6). Phosphorylated pRB (p-pRb) in B16F1 cells was detected in cytoplasmic extracts from untreated cells (Fig. 6A, lane 2), and the levels of the p-pRb gradually declined in cells treated with IFNγ (Fig. 6A, lanes 8 and 10). Contrary to p-pRB, levels of hypo-phosphorylated pRb were low in nuclear extracts from untreated cells (Fig. 6B, lanes 2) but increased with IFNγ treatment (Fig. 6B, lanes 4, 6, 8, and 10). In contrast to B16F1 cells, high levels of p-pRb were observed in cytoplasmic extracts from SCCVII cells regardless of IFNγ treatment (Fig. 6C). Intriguingly, hypo-phosphorylated pRb was undetectable in nuclear extracts from IFNγ-treated SCCVII cells (Fig. 6D). These results suggested that the impaired responsiveness of SCCVII cells to IFNγ in terms of expression of cell cycle regulators is correlated with deregulation of the phosphorylation status of pRB; i.e., IFNγ does not induce the hypo-phosphorylated, active form of pRB in SCCVII cells.

3.5. Impaired expression of the Ifi200 members in SCCVII cells

To investigate the mechanism involved in the alteration of pRb phosphorylation in SCCVII cells, we focused on the interferon-inducible p200 protein family members (encoded by the *Ifi200* family genes) [23,24], which inhibit cell proliferation by interacting with pRb [18,26]. We initially examined the expression of members of the *Ifi200* family, including *Ifi202*, *Ifi203*, *Ifi204*, and *Ifi205*, in B16F1 and SCCVII cells by qRT-PCR. Although *Ifi202* expression was not detectable in both cell types, *Ifi203*, *Ifi204*, and *Ifi205* expression was observed in B16F1 cells in response to IFNγ (Fig. 7A–C). However, IFNγ treatment only marginally induced the expression of these *Ifi200* family genes in SCCVII cells (Fig. 7D–F).

3.6. Overexpression of p204 in SCCVII cells inhibits DNA synthesis

To determine whether the members of the p200 family proteins are capable of inhibiting SCCVII cell growth, we constructed vectors

expressing these p200 proteins. The vectors contained the V5-epitope tag fused in frame with the C-terminus region and were transiently transfected into SCCVII cells. Expression levels of these p200 proteins were assessed by western blotting using an anti-V5 antibody (Fig. 8A), and levels of protein expression were quantified by measuring the chemiluminescent signal (Fig. 8B). When SCCVII cells were transfected with these expression vectors, p205 was markedly expressed (Fig. 8A, lane 4), p204 was moderately expressed (lane 3), and p203 was marginally expressed (lane 2). We then investigated the effect of these p200 proteins on DNA synthesis in the transfected cells by assessing BrdU incorporation (Fig. 8C). Although the p203 protein had only a marginal effect on DNA synthesis in SCCVII cells, the p205 protein moderately (about 30%) inhibited DNA synthesis in SCCVII cells. Interestingly, the modest expression of p204 protein compared to p205 expression markedly (60–90%) inhibited DNA synthesis. Fig. 8D demonstrates that overexpression of p204 at 24 h after transfection led to an increased level of nuclear pRb protein (lane 4). These results indicated that the expression of p204 and, to some degree, that of p205 inhibits DNA synthesis and suggested that the impaired expression of these p200 proteins in SCCVII cells results in the resistance to IFNγ-mediated cell growth arrest.

To evaluate the functional significance of the Rb-binding motif

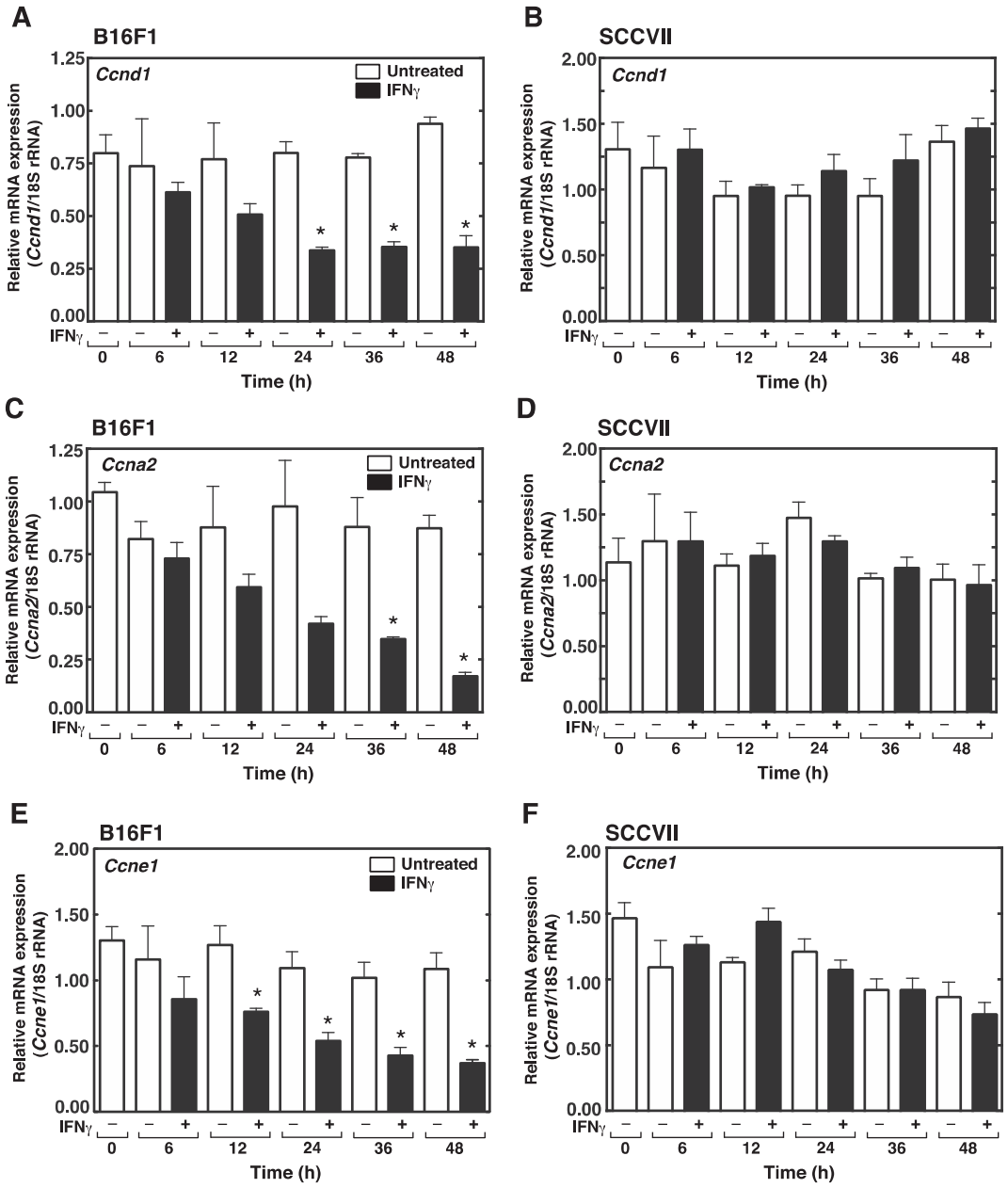

Fig. 3. IFNγ fails to downregulate the expression of *Ccnd1*, *Ccna2*, and *Ccne1* in SCCVII cells.
B16F1 (A, C, E, G, I) and SCCVII (B, D, F, H, J) cells were seeded in 6-well plates, incubated for 20 h (time 0), and either left untreated or treated with IFNγ (10 ng/mL) for the indicated times before preparation of total RNA and analysis of specific mRNA levels by qRT-PCR. The relative mRNA expression levels are normalized to that of 18S rRNA used as an internal control. Each column and bar represents the mean ± SEM of three independent experiments. Significance for the inhibition of mRNA expression between untreated and IFNγ−treated samples is indicated ($^{*}p < 0.05$, Student's *t*-test).

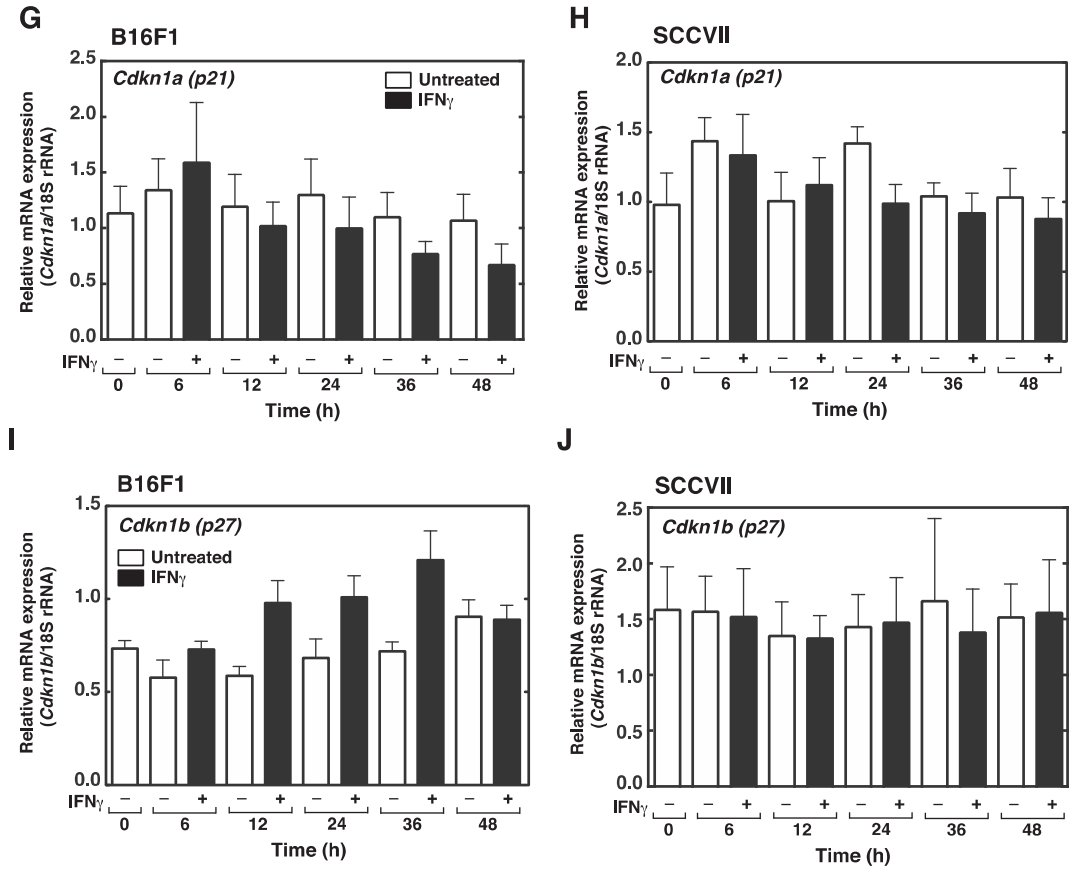

Fig. 3. (*continued*)

LXCXE in p204, we constructed mutant p204, in which two Rb-binding sites were individually mutated by site-directed mutagenesis (Fig. 9A) and transiently transfected into SCCVII cells. Expression levels of these mutant p204 proteins were assessed by western blotting using an anti-V5 antibody (Fig. 9B), and levels of protein expression were quantified. Although the level of protein expression in cells transfected with the mutant proximal LXCXE motif (mRb1) decreased by 70% (Fig. 9C), the inhibitory effect on DNA synthesis was comparable to that of wild-type p204 (Fig. 9D). This result suggested that the magnitude of inhibition of DNA synthesis does not correlate with the level of protein expression. Mutation of the distal LXCXE motif (mRb2) resulted in a comparable level of protein expression to that of wild-type p204 (Fig. 9C), and the inhibitory effect on DNA synthesis was significantly but not completely decreased (Fig. 9D). These results indicated that although the distal LXCXE motif is required for the inhibitory effect on DNA synthesis, other domains in p204 are also needed for full inhibition of cell proliferation.

4. Discussion

Many tumor cells are resistant to IFN treatment and escape from immunosurveillance by the host defense system. The resistance to IFNs is often associated with defective components of the JAK/STAT signaling pathway. However, some types of tumor cells resist growth arrest by IFNs despite presenting a functional JAK/STAT signaling pathway. The molecular mechanisms underlying resistance to IFNs and

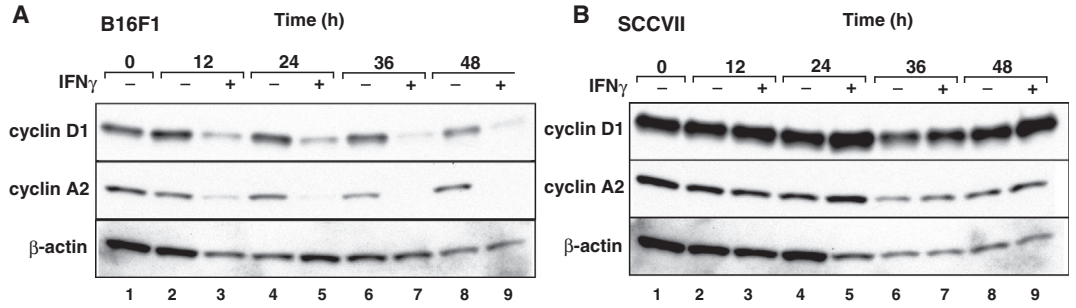

Fig. 4. IFNγ fails to downregulate the expression of cyclin D1 and cyclin A2 proteins in SCCVII cells.
B16F1 (A) and SCCVII (B) cells were seeded in 10-cm dishes, incubated for 20 h (time 0), and either left untreated or treated with IFNγ (10 ng/mL) for the indicated times before preparation of total cellular extracts. Equal amounts of protein (20 μg/lane) were loaded on the gel and then analyzed by western blotting using antibodies against cyclin D1, cyclin A2, and β-actin (loading control).

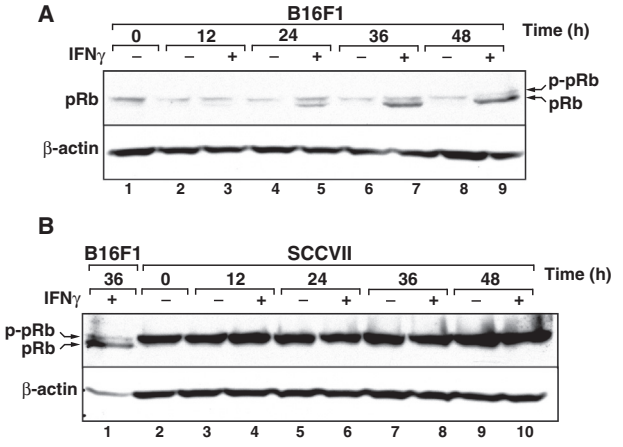

Fig. 5. IFNγ fails to induce the hypo-phosphorylated, active form of pRb in SCCVII cells.
B16F1 (A) and SCCVII (B) cells were seeded in 10-cm dishes, incubated for 20 h (time 0), and either left untreated or treated with IFNγ (10 ng/mL) for the indicated times before preparation of total cellular extracts. Equal amounts of protein (20 μg/lane) were loaded on the gel and then analyzed by western blotting using antibodies against pRb and β-actin (loading control). Total cellular extracts from B16F1 cells treated with IFNγ for 36 h were also loaded in the same gel (panel B: lane 1), and hyper-phosphorylated (p-pRb) and hypo-phosphorylated forms of Rb (pRb) are indicated.

the physiological significance of the partial sensitivity to IFNs remain to be fully elucidated. In the present study, we showed that the resistance to IFNγ-induced cell growth arrest results from impaired IFNγ-induced expression of the *Ifi200* family genes, in particular *Ifi204*, whose gene product p204 potently inhibits DNA synthesis in a murine squamous cell carcinoma cell line, SCCVII.

The analysis of the IFNγ-induced phosphorylation of STAT1 (Tyr701 and Ser272) and IFNγ-induced genes (*Cxcl9*, *Cxcl10*, *Cxcl11*, and *Givin1*) revealed that there was no significant difference in the activation of STAT1 and the expression of STAT1-dependent genes between IFNγ-resistant SCCVII and IFNγ-responsive B16F1 cells (Fig. 2). These results indicated that SCCVII cells harbor an intact STAT1-dependent signaling pathway and suggested that cell cycle regulatory molecules induced by IFNγ-activated STAT1 are deregulated in SCCVII cells. Examination of cell cycle regulators in SCCVII cells demonstrated that the expression of cyclin D1, cyclin E1, and cyclin A2 was refractory to IFNγ treatment (Figs. 3 and 4). These cyclins are essential for the G1/S transition and transcriptionally regulated by the pRb-E2F pathway [12,13]. IFNγ is known to induce the Cdk inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip}$ [8,9,11] (encoded by *Cdkna1* and *Cdkn1b*, respectively), which inhibit cyclin/Cdk activity, thereby downregulating the phosphorylation of pRb and repressing the transcription of E2F-dependent genes [12,13,67]. However, our data indicate that the expression of *Cdkna1* and *Cdknb1* was not significantly upregulated by IFNγ in both cell lines, suggesting that these Cdk inhibitors are likely to be less related to the IFNγ-induced cell growth arrest.

Cell growth arrest induced by IFNγ is closely associated with the appearance of the hypo-phosphorylated, active form of pRb (Fig. 5), which is known to repress E2F-dependent transcription required for the transition from G1 to S phase [12,13,67]. We focused on the *Ifi200* family genes, because their gene products, p200 family proteins, inhibit cell proliferation and induce the hypo-phosphorylated, active form of pRb [18,26]. Analysis of the expression of the *Ifi200* family genes showed that IFNγ-induced expression of *Ifi203*, *Ifi204*, *and Ifi205* was impaired in SCCVII cells (Fig. 7). Functional analysis of the p200 family proteins revealed that overexpression of p204 in SCCVII cells markedly inhibited DNA synthesis. p205 also had an inhibitory effect, but to a lesser extent (Fig. 8). These results indicated that the IFNγ-induced expression of p200 family proteins, notably p204, can inhibit cell growth arrest and suggested that the resistance to IFNγ-induced cell growth arrest in SCCVII cells results from the repression of the *Ifi200* family genes.

We observed cytoplasmic localization of phosphorylated pRB in B16F1 and SCCVII cells (Fig. 6), and IFNγ treatment restored nuclear localization of pRB only in B16F1 cells, which correlated with cell

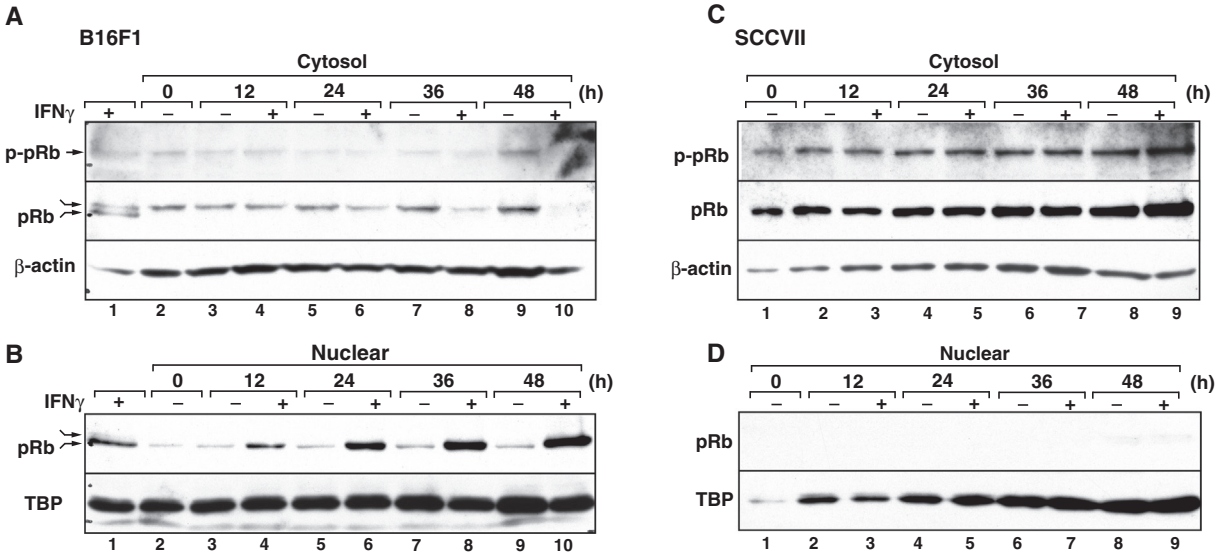

Fig. 6. Impaired IFNγ-induced sub-cellular localization and hypo-phosphorylation of pRb in SCCVII cells.
B16F1 (A, B) and SCCVII (C, D) cells were seeded in 10-cm dishes, incubated for 20 h (time 0), and either left untreated or treated with IFNγ (10 ng/mL) for the indicated hours before preparation of cytosolic and nuclear extracts. Equal amounts of protein (20 μg/lane) were loaded on the gel and then analyzed by western blotting using antibodies to pRb, phospho-pRb (Ser807/811), β-actin, and anti-TATA binding protein (TBP). Total cellular extracts from B16F1 cells treated with IFNγ for 36 h were also loaded in the same gel (panel A and B: lane 1), and hyper-phosphorylated (p-pRb) and hypo-phosphorylated forms of Rb (pRb) are indicated.

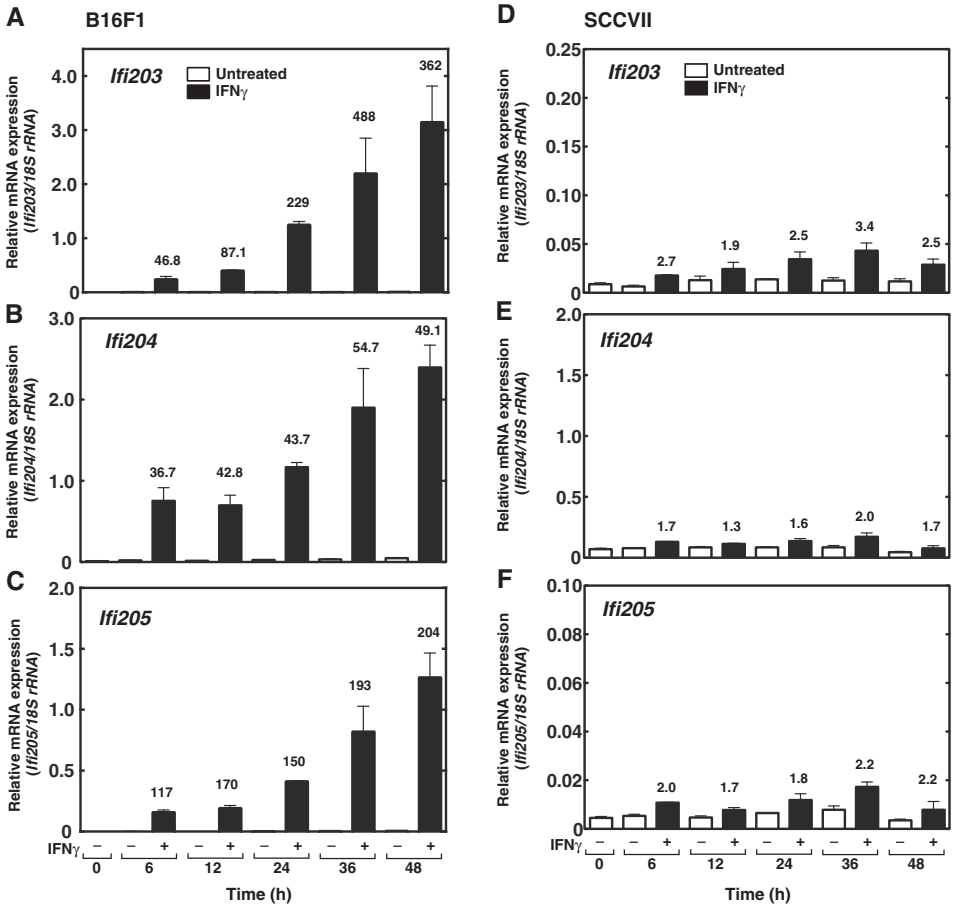

Fig. 7. Impaired expression of the IFNγ-induced *Ifi200* family genes in SCCVII cells.
B16F1 (A-C) and SCCVII (D-F) cells were seeded in 6-well plates, incubated for 20 h (time 0), and either left untreated or treated with IFNγ (10 ng/mL) for the indicated hours before preparation of total RNA and analysis of specific mRNA levels by qRT-PCR. The *Ifi203*, *Ifi204*, and *Ifi205* mRNA expression levels are normalized to 18S rRNA used as an internal control. Each column and bar represents the mean ± SEM of three independent experiments. Fold induction of the mRNA expression induced by IFNγ compared to that in untreated cells is shown above the columns.

growth arrest. This result is consistent with previous studies showing that the hyper-phosphorylated, inactive form of pRb was mislocalized to the cytoplasm in various types of tumor cells, and the re-localization of pRb to the nucleus by pharmacological inhibition of Cdk activity restored the tumor-suppressive function of pRb [68]. The cytoplasmic pRb is localized by nuclear export through the Cdk-dependent Exportin1, and the C-terminal phosphorylation residues of pRb, including Ser807 and Ser811, are required for the interaction of pRb with Exportin1 [69]. In the present study, marked phosphorylation of pRb was observed in SCCVII cells, and the hyper-phosphorylated form of pRb was exclusively localized in the cytoplasm (Fig. 6C). Whether the aberrant cytoplasmic localization of pRb is attributed to increased Cdk activity in SCCVII cells remains to be determined.

Our results are consistent with previous studies showing that p204 overexpression in mouse cell lines increases the hypo-phosphorylated, active form of pRb, which induces cell growth arrest [19]. In our study, enforced expression of p204 in SCCVII cells decreased the level of phosphorylated pRb in cytoplasmic extracts and increased hypo-phosphorylated pRb in nuclear extracts (Fig. 8D). The p204 protein contains two pRb-binding LXCXE motifs, and deletion of the binding motifs abrogated the growth inhibitory effect, suggesting that binding to pRb impairs the phosphorylation of pRb [26]. We observed that overexpression of p204 and p205 in SCCVII cells markedly (about 90%) and partially (about 30%), but significantly, inhibited DNA synthesis, respectively (Fig. 8C). The difference in the magnitude of the inhibitory activity of these p200 proteins may depend on the structural difference of the conserved 200-amino-acid-long motifs. While p205 contains one LXCXE motif, p204 presents two LXCXE motifs [70]. To determine the functional significance of the LXCXE motifs, we generated mutant p204, in which the proximal (Rb1) and distal (Rb2) LXCXE motifs were individually substituted with LXGXK (Fig. 9A). Although mutation of Rb1 (mRb1) had no effect on the inhibition of DNA synthesis (Fig. 9D), mutation of Rb2 (mRB2) significantly decreased the inhibitory effect. These results indicated that although the Rb2 motif is required for the inhibitory effect on DNA synthesis, other motifs in p204 are also needed for full inhibition of cell proliferation.

The mechanism involved in the repression of the *Ifi200* family genes in SCCVII cells is currently unknown. Genetic alterations such as mutation and/or deletion of the genes associated with the IFN signaling pathway have been reported [34,35,37,71]. Epigenetic gene silencing of the IFN signaling molecules is also observed in some types of tumor cells [33,38,72,73]. In both cases, IFN-stimulated genes (ISGs) regulated by the JAK/STAT signaling pathway should be broadly repressed. In the present study, SCCVII cells appeared to harbor an intact JAK/STAT signaling pathway, indicating that the unresponsiveness to IFNγ is primarily confined to some of the ISGs involved in cell growth arrest. In fact, some of the IFNγ-induced genes (*Irf1*, *Irf7*, *Isg20*, *Oas1g*, *Ddx58*, *Ccl5*, *Mx1*, and *Ciita*) were refractory to IFNγ in SCCVII cells (Supplemental Fig. 1). Methylation-mediated epigenetic gene silencing is commonly observed during the process of tumor formation and progression [74]. Our preliminary experiments showed that treatment with 5-Aza-2′-deoxycytidine (5-Aza-dC), an inhibitor of DNA methyltransferases (DNMTs), partially restored the IFNγ-induced expression of the *Ifi200* family genes, suggesting that the repression of the *Ifi200* family genes may result from methylation-mediated epigenetic silencing. Since 5-Aza-dC globally alters the methylation status of the genome, further studies are required to confirm this hypothesis.

Although the mechanism of the gene-specific repression of the ISGs associated with cell cycle regulation remains to be determined, it is reasonable to speculate that the residual sensitivity of tumor cells to IFNγ without its growth-suppressive effect is beneficial for the development and progression of tumor cells. A previous study on a mouse

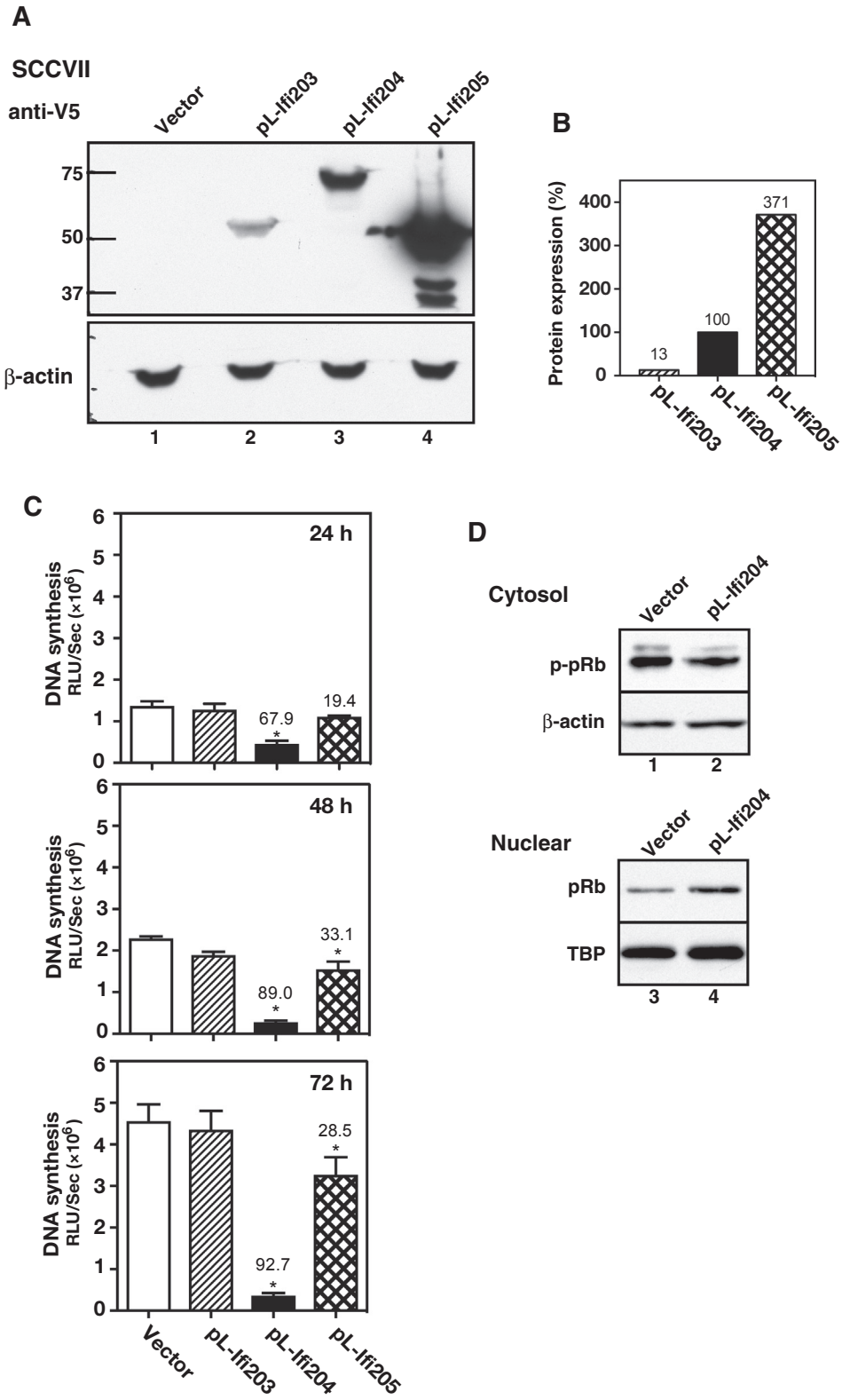

Fig. 8. Overexpression of p204 inhibits DNA synthesis in SCCVII cells.

Western blot analysis of the p200 family proteins in transfected SCCVII cells (A). Levels of the p200 family proteins were assessed by western blotting using antibodies against anti-V5 epitope, and the levels of protein expression were quantified by measuring the chemiluminescent signal using the ChemiDoc imaging system. Expression of p200 family proteins was normalized to that of β-action (B).

Inhibitory effect of the p200 family proteins on DNA synthesis in SCCVII cells (C). Each column and bar represents the mean ± SD of quintuplicate cultures. Significance for the inhibition of DNA synthesis by the Ifi200 expression vectors compared to empty vector is indicated ($^{*}p < 0.05$, one-way ANOVA). The percentage of inhibition of DNA synthesis is shown above the columns. Similar results were obtained in three independent experiments.

Western blot analysis of the pRb proteins in transfected SCCVII cells (D). Cells were transfected either empty vector (Vector) or the p204 expression vector (pL-Ifi204) and cultured for 24 h before preparation of nuclear extracts (lanes 1 and 2). Equal amounts of protein (20 μg/lane) were loaded on the gel and then analyzed by western blotting using antibodies to pRb and TBP.

melanoma model of adaptive T cell immunotherapy showed that the anti-tumor activity of cytotoxic T lymphocytes against B16 melanoma appears to be largely dependent on IFNγ-induced cell cycle arrest rather than on tumor cell lysis [52]. Therefore, deregulation of the IFNγ-mediated cell cycle arrest may contribute to the escape from cancer immunosurveillance. Furthermore, immunosuppressive molecules such as PD-L1 and indoleamine-2,3-dioxygenase (IDO) are upregulated by IFNγ in various types of tumor cells and in the tumor microenvironment *in vivo* [75,76]. Thus, tumor cells that are insensitive to IFNγ-mediated anti-proliferative effects but are capable of inducing the immunosuppressive molecules may effectively evade the anti-tumor response and resist immunotherapeutic intervention with PD-1/PD-L1 signal-blockade agents [77]. Therefore, the SCCVII cell line may be a useful tool in pre-clinical studies to elucidate the mechanisms

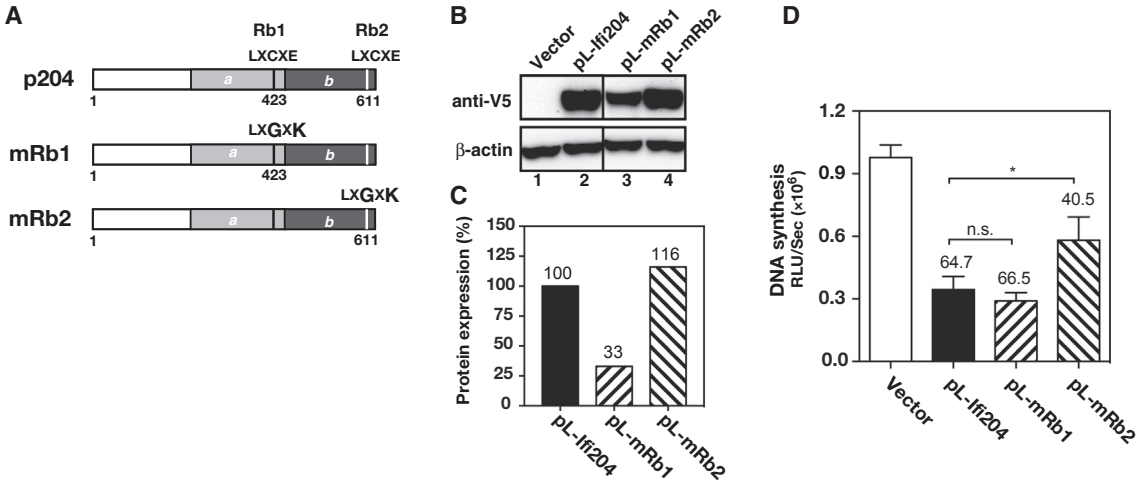

Fig. 9. Mutation of distal Rb-binding sites of p204 decreases inhibitory effect on DNA synthesis in SCCVII cells.
Schematic representation of wild-type and mutant p204 proteins (A). The conserved 200-amino-acid domain (type *a* and type *b* domain) and the proximal and distal Rb-binding motifs, Rb1 and Rb2, respectively, are shown (modified from ref. [26]). Wild-type Rb-binding motif LXCXE was substituted with LXGXK in mutant p204. Western blot analysis of the mutant p200 proteins in transfected SCCVII cells (B). Levels of the p204 proteins were assessed by western blotting using antibodies against anti-V5 epitope, and the levels of protein expression were quantified by measuring the chemiluminescent signal using the ChemiDoc imaging system. Expression of p200 family proteins was normalized to that of β-actin (C).
Inhibitory effect of the mutant p204 proteins on DNA synthesis in SCCVII cells (D). Each column and bar represents the mean ± SD of quintuplicate cultures. Significance for the inhibition of DNA synthesis by the p204 expression vectors compared to empty vector is indicated ($^{*}p < 0.01$, one-way ANOVA; n.s., not significant). The percentage of inhibition of DNA synthesis is shown above the columns. Similar results were obtained in three independent experiments.

underlying resistance to immunotherapy and to develop effective cancer immunotherapy.

5. Conclusion

Mouse squamous cell carcinoma (SCCVII) cells resist IFNγ-mediated cell growth arrest. The resistance of SCCVII cells to IFNγ-mediated cell growth arrest is not caused by a deficiency in the STAT1-dependent signaling but rather results from silencing of the IFN-inducible 204 gene (*Ifi204*), whose gene product, p204, binds to pRb and exerts an anti-proliferative effect. Overexpression of p204 in SCCVII rescues the anti-proliferative effect of IFNγ, and mutation of a pRb-binding motif reduces the anti-proliferative effect. These results suggest that silencing of *Ifi204*/p204 induces resistance to IFNγ-mediated cell growth arrest in SCCVII cells. Furthermore, the SCCVII cell line may be a useful tool in pre-clinical studies to elucidate the mechanisms underlying resistance to immunotherapy and to develop effective cancer immunotherapy.

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