[Frontiers in Bioscience 12, 4855-4863, September 1, 2007]

IFI16 inhibits tumorigenicity and cell proliferation of bone and cartilage tumor cells

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1. ABSTRACT

IFI16 is a member of the interferon-inducible p200-protein family, capable of modulating cell proliferation, and cellular senescence. In this study, these effects of IFI16 were studied in tumor cells derived from bone and cartilage. The level of IFI16 was markedly lower in human osteosarcomas as compared with its level in normal bone. Overexpression of functional IFI16 in human osteosarcoma and chondrosarcoma cell lines markedly inhibited colony formation, and significantly inhibited cell growth, an effect that could be reversed by introduction of gene specific siRNA into tumor cells. These inhibitory effects of IFI16 were associated with upregulation of p21 and inhibition of cyclin E, cyclin D1, c-Myc and Ras. In addition, ectopic expression of IFI16 in tumor cells increased senescence-associated beta-galactosidase and induced a senescence-like phenotype. In view of such effects, IFI16 might be a suitable target for therapeutic intervention in osteosarcoma and chondrosarcoma.

2. INTRODUCTION

Osteosarcomas are malignant connective tissue tumors that encompass neoplastic cells undergoing osteoblastic differentiation. Osteosarcomas are the most common type of malignant bone tumors, accounting for 35% of primary bone tumors. They are the sixth most common tumor in children, with a second peak occurring in the elderly. In the latter group, the tumor is usually associated with underlying bone pathology such as Paget's disease, medullary infarct or prior irradiation (1). Chondrosarcomas are cartilage tumors comprising a rare malignant condition that can affect any age group and mostly involve pelvic and shoulder bones and bones of the upper arms and legs (2). At present, the molecular events involved in the etiology of both osteosarcomas and chondrosarcomas are largely unknown (3).

The interferon-inducible p200 (IFI-200) family of proteins is a group of highly homologous proteins that

possess antimicrobial, cell growth regulatory, differential regulatory and immunomodulatory properties (4, 5). The IFI-200 family includes four human proteins— IFI16 (6), IFIX (7), MNDA (8), and AIM2 (6)—and five murine—p202a (9), p202b (10), p203 (11), p204 (12), and D3/p205 (13). IFI16 (encoded by the IFI16 gene) is a phosphoprotein (80–85 kDa), which contains two repeats of 200 amino acids (one a-type and one b-type) that are separated by a serine–threonine–proline (S/T/P)-rich spacer region (14). The size of the spacer region in IFI16 is regulated by mRNA splicing and can contain one, two, or three copies of a highly conserved, 56-amino-acid S/T/P domain encoded by distinct exons. Thus, IFI16 is found in three different isoforms: A, B, and C (14).

IFI16 was first detected in human lymphoid cells (15) and has subsequently been found expressed in the spleen, thymus, lymph node, trachea, gastrointestinal tract, skin, and testis (16-18), as well as the heart (Jin and Lengyel, unpublished data). IFI16 has been found to be an important transcription modulator regulating cell proliferation, differentiation, cell cycle, apoptosis and cellular senescence (19, 20). The strongest evidence of the importance of IFI16 in disease is that IFI16 has been identified as a specific autoantigen in immunological disorders, including Sjogren's syndrome (21), systemic lupus erythematosus (22) and rheumatoid arthritis (23). Evidence linking IFI16 to tumorigenesis is also accumulating: IFI16 was isolated as a molecule that is differentially regulated by p210 BCR/ABL1 fusion oncogene in a cDNA microarray (24) and as a BRCA1-associated protein involved in the p53-mediated apoptosis pathway (25). Moreover, IFI16 was found to play a role in cellular senescence of both human fibroblasts and prostate epithelial cells (26) and to inhibit tube morphogenesis and proliferation of primary, but not HPV16 E6/E7-immortalized, human endothelial cells (27). The findings that IFI16 binds specifically to E6/E7 (Jin and Lengyel, unpublished data), pRb and E2F1 and also augments the transactivation of the p53-responsive p21 promoter by enhancing the DNA-binding activity of p53 have provided insights into the molecular mechanism by which IFI16 mediates growth inhibition (26).

In this study, we demonstrated that the expression of IFI16 is dramatically reduced in human osteosarcomas and that overexpression of IFI16 inhibits growth and tumorigenicity of both osteosarcoma and chondrosarcoma cells. Furthermore, IFI16 expression can sensitize sarcoma cells to senescence, a phenomenon accompanied by increases in p21 protein levels and decreases in the levels of cyclin E, cyclin D1, c-Myc and Ras.

3. MATERIALS AND METHODS

3.1. Real-time PCR assays

Total RNA from cells was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA (1μg) was reverse-transcribed into cDNA and PCR-amplified with an ABI 7300 System using the SYBR Green PCR kit according to the manufacturer's protocol. The sequences of primers were: sense 5' TGCCACCAACAACTCCATCC 3', antisense 5' TGGAGGCATCTGAGGAGTGT 3' (IFI16). We selected GAPDH as the endogenous control for the real-time PCR relative quantification analysis. RT-PCR cycling conditions were as follows: initial incubation step of 2 min at 50°C and 94°C for 2 min, followed by 40 cycles of 15 s at 95°C for denaturation and 2 min at 62°C for annealing and elongation.

3.2. Western blotting assays

Protein lysates prepared with extraction buffer containing 0.05M Tris (pH 8.0), 0.15M NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1% NP-40 were subjected to 10% SDS-PAGE and electrotransferred to the membrane. Goat anti-IFI16 (Cat# sc-6050, Santa Cruz Biotechnology, Santa Cruz, CA) was added at a dilution of 1:1000 and incubated 1 h at room temperature. The secondary antibody, horseradish peroxidase-conjugated antigoat IgG antibody, was used with a dilution of 1:2000. The signal was visualized using enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

3.3. Generation of IFI16 overexpression stable cell lines

To obtain rat chondrosarcoma (RCS) and Saos-2 osteosarcoma cell lines stably expressing IFI16, cells were transfected with either a pcDNA3.1 (used as a control line) or pcDNA-IFI16 expression plasmid and selected with 1 mg/ml G418 for 15 days. The G418-resistant clones were pooled and the level of IFI16 was examined using a western blotting assay with IFI16 antibody (Santa Cruz Biotechnology).

3.4. Determination of cell growth and transformation

MTT and viable cell counts were employed to assess cell growth in RCS and Saos-2 stable cells and corresponding control cells. Briefly, cells were seeded at a density of 4000 cells/well in 96-well plates; control cells remained subconfluent and in exponential phase growth for the duration of the assay. All experiments were performed in sextuplicate. After 1, 2, 3, and 4 days, MTT was added (50 μg/well) for 4 h. Formazan products were solubilized with DSMO and optical density measured at 560 nm. For viable cell counts, cells were plated in 24-well tissue culture plates at a density of 1.5×10^3 cells/well. Cells in each treatment group were plated in sextuplicate and cultured for 5 days. Cells were harvested by trypsinization at 24-h intervals and stained with 0.4% trypan blue (Gibco, Grand Island, NY) to reveal the dead cells. Viable cells were then counted with a hemocytometer.

Cell transformation was assessed using a colony-forming assay. 1×10^3 cells were seeded in 60-mm plates in a culture medium containing 0.35% low-melting agarose over a 0.7% agarose base layer and incubated at 37°C. The number of colonies was determined with an inverted phase-contrast microscope at 100× magnification; a group of >10 cells was counted as a colony. Recorded data were expressed as means ± SE of four independent wells at optimum time of 14 days after initiation of cell seeding.

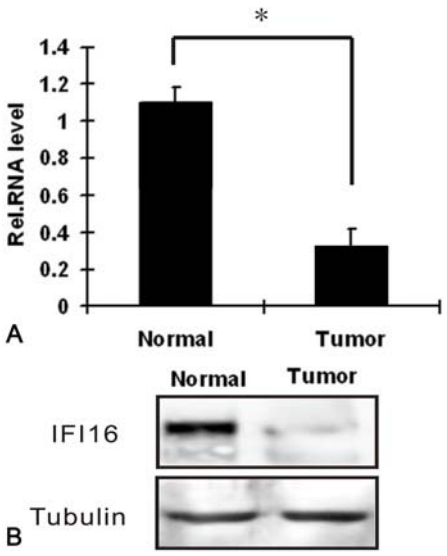

Figure 1. Reduced expression of IFI16 in human osteosarcoma. (A) Reduction of IFI16 mRNA levels in human osteosarcomas: The IFI16 mRNA levels in normal bone and in osteosarcomas were determined by real-time-PCR using primers specific to IFI16. GAPDH was used as control for RNA quality and quantity. (B) Reduction of IFI16 protein in human osteosarcomas: Tissue lysate was detected by western blot assay using specific IFI16 antibody. Tubulin protein levels served as loading controls.

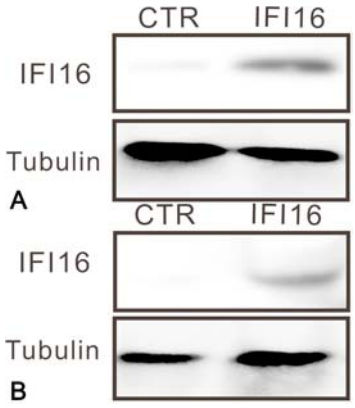

Figure 2. Establishing IFI16 stable cell lines in RCS and Saos-2 cells. Establishing IFI16 stable expression stable cell line in RCS (A) and Saos-2 (B) cells. Cell lysate from IFI16 stable cells and control cells were detected by western blot assay using IFI16-specific antibody. Tubulin protein levels served as loading controls.

3.5. IFI16 siRNA (IFI16-siRNA) efficiently knocked down the expression of IFI16

In the IFI16 siRNA assay, stable cells were transfected with either pSuper-IFI16, which produced a 19-nucleotide gene-specific sequence to IFI16 (GAAACCUACCAAAUUAAUC), or a pSuper control plasmid. Cell growth was assessed as described above. Immunofluorescence staining was also used to examine IFI16 protein levels in stable cell lines. Briefly, cultures plated on chamber slides (Nalge Nunc International, Naperville, IL) were fixed in cold 100% methanol and air-dried. After re-hydration in PBS and blocking with 30% goat serum for 30 min, the cells were incubated with primary antibodies against IFI16 for 1 hr. Secondary antibodies against goat IgG conjugated with FITC diluted 1:100 (Santa Cruz Biotechnology) were applied for 45 min, followed by incubation with 0.5 mg of 49,69-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min. The specimens were observed under an inverted fluorescent microscope (Olympus America Inc, Center Valley, PA) with appropriate optical filters. Microscopic images were captured using IPLab (BD Biosciences, Rockville, MD).

3.6. Beta-Galactosidase (beta-gal) staining

Senescence-associated beta-galactosidase assays were performed essentially as described previously (26). In brief, cells plated in 96-well plates were fixed using the *In situ* beta-galactosidase Staining Kit (Stratagene, La Jolla, CA). Fixed cells were incubated with the staining solution prepared in a buffer (pH 6.0) overnight at 37°C, and a Model 550 Microplate Reader (Bio-Rad, Hercules, CA) was used to measure the absorbance at λ 540 nm.

4. RESULTS

4.1. IFI16 is downregulated in human osteosarcomas

IFI16 has been linked to several kinds of carcinogenesis (24-26). We investigated whether expression of IFI16 is altered in human osteosarcomas. We first performed quantitative real-time PCR using primers specific to IFI16 to examine the mRNA level of IFI16. As shown in Figure 1A, IFI16 mRNA was significantly reduced in tumor tissues collected from human osteosarcomas compared to matched normal bone tissues. Next we measured the protein level of IFI16 using a western blotting assay and observed an even higher difference between tumor and control tissue; IFI16 was hardly detectable in the osteosarcoma (Figure 1B). These findings suggest that the posttranscriptional processes (e.g., translation and protein turnover) are also involved in the altered expression of IFI16 in osteosarcomas. Considering that aberrant expression and frequent mutations of pRb and p53 tumor suppressors have been found in musculoskeletal tumors, dramatic downregulation of IFI16 in human osteosarcomas suggests that IFI16, an interferon-inducible protein that associates with both pRb and p53, is a previously unknown molecule in the musculoskeletal system and may act as a novel suppressor of musculoskeletal tumors.

4.2. IFI16 overexpression inhibits growth and transformation activity of musculoskeletal tumor cells

We next sought to determine the role of IFI16 in musculoskeletal tumors. For this purpose, we first generated IFI16 expression stable cell line in both osteosarcoma Saos-2 and chondrosarcoma RCS cells (Figure 2A,B) and performed cell proliferation assays (Figure 3). Both cell number counting and MTT assays indicated that cell growth and cell proliferation rates of

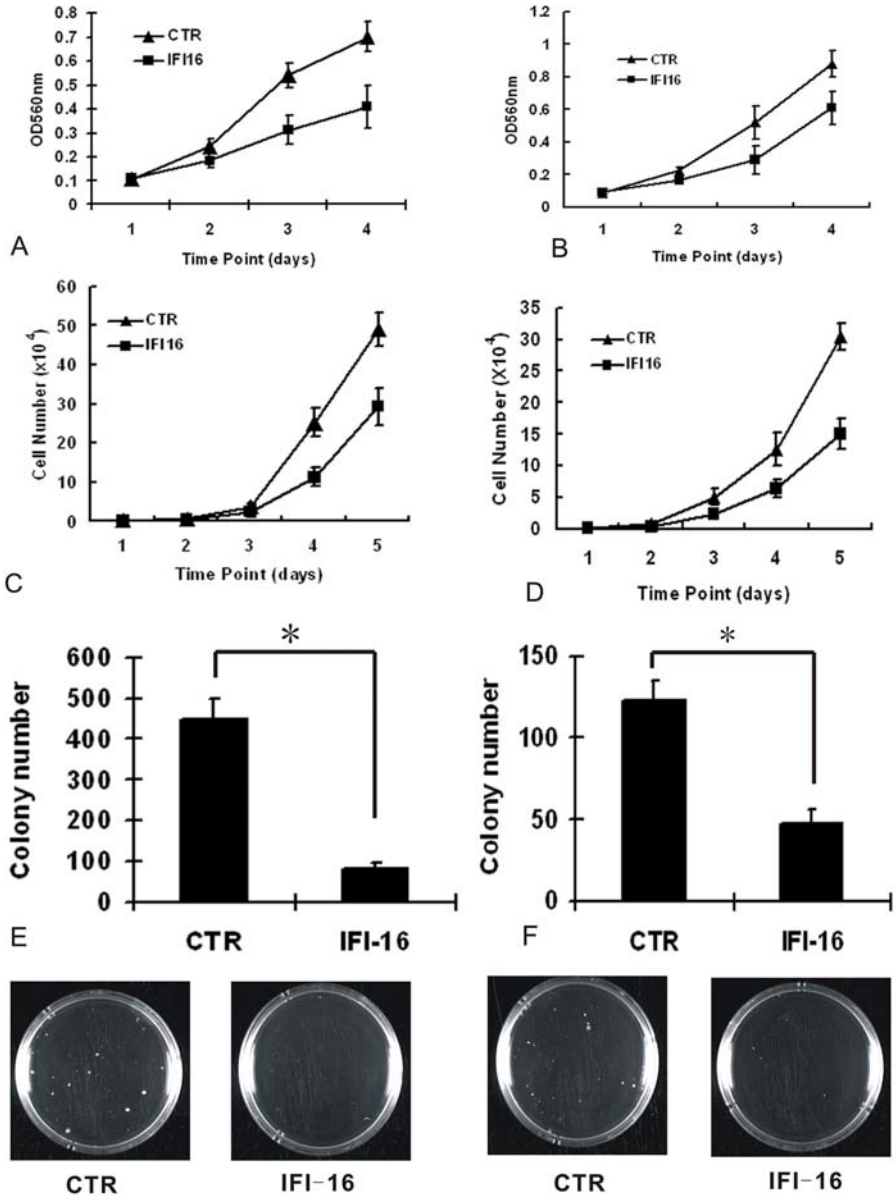

Figure 3. Suppression of growth and tumorigenicity in bone sarcoma cells by IFI16. (A-D) Reduced growth rates in IFI16 stable cell lines: The growth rate of the IFI16 stable cell line and control cells was measured daily by MTT assay (A for RCS, B for Saos-2) and viable cell counts (C for RCS, D for Saos-2). Each measurement was made in sextuplicate. (E, F) Suppression of *in vitro* transformation by IFI16: The IFI16 stable cell line or empty cell line derived from RCS (E) and Saos-2 (F) cells were seeded in soft agar and the colony number scored at 2 weeks after seeding.

IFI16 stable lines were significantly lower than those in both RCS and Saos-2 control cells (Figs. 3A-D). The soft agar assay was used to determine the effect of IFI16 on *in vitro* transformation property. As shown in Figure 3E,F, the number of colonies in the IFI16 stable cell line was consistently fewer than that of control cells; the reduction ranged from 81.25% (RCS cells) to 61.8% (Saos-2 cells). These results indicate that IFI16 suppressed the transformation phenotype of bone tumor cells and predicted a loss of tumorigenicity of IFI16 expression stable tumor cells.

We also determined whether reduction in the IFI16 level of IFI16 stable lines via a siRNA approach could overcome the inhibition observed in the overexpression assay described above. We generated a pSuper-IFI16 construct encoding a specific siRNA targeting a 19-nucleotide gene-specific sequence in the IFI16 gene. Immunocellstaining with IFI16 stable cells transfected with either pSuper-IFI16 or pSuper vector demonstrated that a siRNA encoded by pSuper-IFI16 efficiently knocked down the expression of IFI16 in the transfected cells (Figure 4A,B). Next we performed a cell

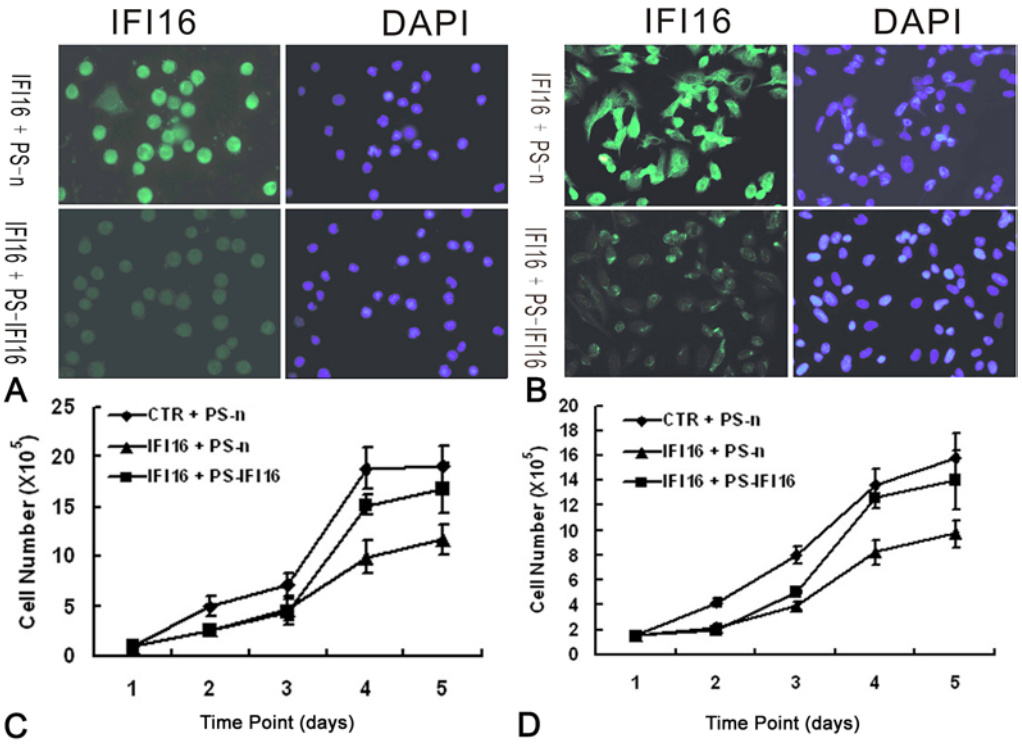

Figure 4. Growth inhibition was recovered in IFI16 stable cell lines in which IFI16 was knocked down by SiRNA. (A, B) Rb protein level was reduced, assayed by immunofluorescence cell staining: RCS (A) and Saos-2 (B) cells were stained with IFI16 antibody and the nuclei stained with DAPI. (C, D) Stable cells were transfected with pSuper-IFI16 plasmid or control plasmid. Viable cells were then counted with a hemocytometer (C for RCS, D for Saos-2). Each measurement was made in sextuplicate.

growth assay with IFI16 stable lines transfected with either pSuper-IFI16 or pSuper vector. As shown in Figure 4C,D, reduction in IFI16 via siRNA in both stable lines resulted in enhanced cell proliferation compared to control Saos-2 and RCS cells at days 4 and 5. It is worth noting that transfection of pSuper-IFI16 had no apparent effect on cell proliferation during the first 3 days after transfection, a result in keeping with the notion that siRNA does not give rise to significant knockdown of the target gene until 2 days after application.

4.3. IFI16 overexpression results in a senescence-like phenotype and upregulates expression of p21, but downregulates expression of cyclin E, cyclin D1, c-Myc, and Ras

We next sought to elucidate the molecular mechanism by which IFI16 regulates cell growth and transforming activity in musculoskeletal tumors by examining the cellular senescence and expression of several molecules critical for carcinogenesis: cell cycle inhibitor p21, apoptosis-related enzyme caspase-3, caspase-8, oncogenic genes E6 and E7, cyclin E, cyclin D1, c-Myc and Ras. Overexpression of IFI16 in RCS and Saos-2 cells increased the number of large, flat cells as compared to control cells (not shown). Importantly, staining of cells for beta-galactosidase, a reliable senescence biomarker, revealed that overexpression of IFI16 significantly (75–90%) increased beta-gal-positive cells (Figure 5A,B) in two independent experiments.

Since levels of p21 increase in some human cell types when cells approach cellular senescence, we tested whether IFI16 regulates expression of p21 and the downstream genes cyclin E and cyclin D1. The data in Figure 5 indicated that this was the case: expression of IFI16 in RCS and Saos-2 stable cells resulted in upregulation of p21 and downregulation of cyclin E and cyclin D1 compared to control cells.

Intriguingly, the levels of c-Myc and Ras, two oncogenes involved in several cancers, were also markedly decreased in the IFI16 stable line compared to controls (Figure 5E,F). On the other hand, the apoptosis-related genes caspase-3 and caspase-8 and the oncogenic genes E6 and E7 were not significantly affected by overexpression of IFI16 (not shown). Collectively, these experiments indicated that IFI16-mediated inhibition of cell growth and transformation in musculoskeletal tumors are likely due in part to its regulation of the expression of tumor suppressor p21, cyclin E, cyclin D1, and the oncogenic genes c-Myc and Ras.

5. DISCUSSION

This study showed IFI16 expression to be markedly downregulated in tumor tissues as compared to matched normal bone tissue. The expression of IFI16 was originally identified in hematopoietic cells and was thought to be restricted in this cell type (19). There is growing

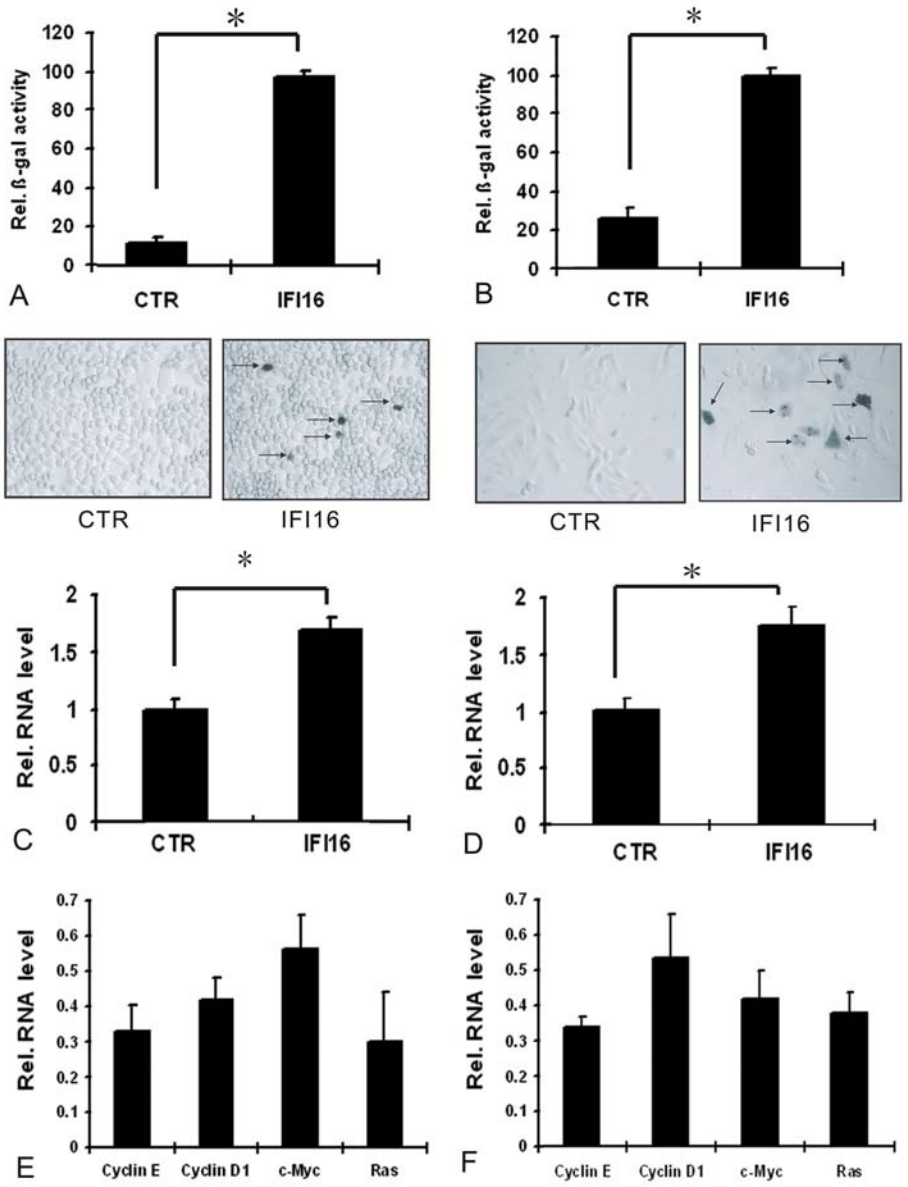

Figure 5. IFI16 overexpression induces cell senescence, upregulates p21 expression, and downregulates cyclin E, cyclin D1, c-Myc, and Ras expression. (A,B) IFI16 overexpression induced senescence-associated beta-galactosidase, assayed by *in situ* beta-gal staining in RCS cells (A) and Saos-2 cells (B). Cells were stained and the readers measured at λ540nm. The mean value was from six independent experiments. *$p < .05$. (C,D) IFI16 overexpression induced p21 expression in RCS cells (C) and Saos-2 cells (D). p21 gene expression was determined by real-time PCR. Expression of p21 was normalized against the GAPDH endogenous control. The normalized values were then calibrated against the control value. (E,F) IFI16 overexpression inhibited cyclin E, cyclin D1, c-Myc, and Ras expression in RCS cells (E) and Saos-2 cells (F). The same procedure was followed to detect the RNA expression of cyclin E, cyclin D1, c-Myc, and Ras. The normalized values were calibrated against control cell lines.

evidence, however, that IFI16 is expressed in several tissues in addition to lymphoid cells (17, 18). Our finding that IFI16 is expressed in normal bone tissue supports the notion that HIN-200 expression is not restricted to hematopoietic cells. Taken together, these observations suggest that IFI16 may play a role in maintaining the normal growth of bone cells and that the downregulation of IFI16 expression may contribute to uncontrolled cell growth and lead to tumorigenesis (26).

Compared to control cells, RCS (chondrosarcoma) and Saos-2 (osteosarcoma) derivatives that expressed exogenous IFI16 lead to inhibition of cell growth and form fewer colonies in soft agar, suggesting that IFI16 suppresses tumorigenicity. Moreover, IFI16-mediated inhibition of cell growth was completely overcome when exogenous IFI16 was knocked down by a specific siRNA of IFI16 (Fig 4). These results demonstrate that a certain level of IFI16 is crucial for precise control of

bone and cartilage tumor cell proliferation. They also suggest the feasibility of using IFI16 as an antitumor agent.

Proteins encoded by the Ifi200 gene family share at least one repeat of 200 amino acids, which appears to be important for protein–protein interactions (28, 29). Some proteins (including IFI16) also contain a newly identified pyrin/PAAD/DAPIN protein–protein interaction domain at their N-termini (29, 30). Based on the presence of protein–protein interaction domains, the p200 family proteins are thought to act as scaffolds for the assembly of protein complexes that regulate gene transcription (26, 29). IFI16 has been found to associate with several proteins—including p53 (31), pRb (26), BRCA1 (25), E2F1 (26), androgen receptor (32) and E6/E7 (27)—and to participate in related pathophysiological processes.

IFI16 has been shown to increase the expression of p21 and to inhibit E2F-stimulated transcription, which is critical for IFI16 to induce cellular senescence and suppress growth of prostate cancer cells (26). Consistent with that observation, the expression of IFI16 induces senescence and increases the expression of p21 in bone cancer cells. This observation suggests that IFI16 may mediate p21 upregulation in response to IFN. Given that IFI16 can upregulate p21 in Saos-2 cells in which the normal function of p53 and pRb is frequently lost, it is likely that the upregulation of p21 by IFI16 is independent of p53 and pRb. The p53-independent upregulation of p21 has been well documented (33-36). In particular, one recent study showed that ectopic expression of IFI16 in cancer cell lines, which do not express functional p53, upregulates p21 expression (26). This observation suggests that IFI16 can upregulate expression of p21 via a p53-independent pathway. Since the regulation of p21 expression may occur on transcriptional and/or posttranscriptional levels (37, 38), elucidating the mechanism underlying IFI16-mediated p21 upregulation will require further studies to determine the half-life of p21 mRNA and protein and the effect of IFI16 on p21 transcriptional activity.

Members of the p200 family are known to inhibit cell proliferation, at least in part through the p21/pRb/E2F pathway (28). Moreover, Xin *et al.* have reported that the protein IFI16 binds to both pRb and E2F and that forced expression of IFI16 inhibits E2F-mediated transcription (26). These observations support the hypothesis that IFI16 contributes to the senescence-associated irreversible arrest of cell growth by potentiating the pRb–E2F-mediated transcriptional repression of E2F-responsive genes. Since Saos-2 cells lack functional pRb, however, the inhibition of the E2F/pRb pathway by p21 cannot account for the mechanism of IFI16-mediated growth inhibition of Saos-2 cells. In the case of IFI16-expressing RCS cells, p21 upregulation leads to inactivation of pRb, resulting in cell senescence. Additionally, the upregulation of p21 leads to inactivation of p130, a retinoblastoma family protein, resulting in cell growth inhibition and senescence (39).

In conclusion, IFN-inducible IFI16 from the p200-protein family is associated with growth retardation and tumor transformation in bone tumors. It may be speculated that IFI16 inhibits the proliferation of sarcoma cells in part by upregulating expression of p21 to induce cell senescence. Identification of IFI16 as a novel critical mediator in musculoskeletal tumors as well as the elucidation of the molecular events involved provide fundamental insights into the processes of musculoskeletal tumors regulated by interferon and may highlight a new research direction in understanding pathological mechanisms for musculoskeletal cancer. Considering its potent inhibition of tumor growth, IFI16 seems to have great potential in being employed as a therapeutic target in the treatment of both osteosarcomas and chondrosarcomas.

6. ACKNOWLEDGMENTS

This study was supported in part by NIH research grants AR052022 and AR050620 and a Dorothy W. Goldstein Young Scholar Award from the Arthritis Foundation, New York Chapter (all C. J. Liu). The authors also wish to thank William Green and Jason Lee for their critical reading and editing of the manuscript.

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Abbreviations: pRb, retinoblastoma protein; HPV, human papillomavirus; IFNs, interferons; IFI16, interferon inducible protein 16; MNDA, myeloid cell nuclear differentiation antigen; AIM2, Absent In Melanoma, gene #2; SS, Sjogren's syndrome; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; BRCA1, breast cancer 1 gene; RT-PCR, reverse transcription-polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; MTT, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RCS, rat chondrocyte sarcoma; DAPI, 49,69-diamidino-2-phenylindole dihydrochloride; AR, androgen receptor; IFN, interferon.

Key Words: IFI16, Osteosarcoma, Chondrosarcoma, Musculoskeletal Tumor, Proliferation, Cellular Senescence

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