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p21 WAF1 Is Involved in Interferon-β- Induced Attenuation of Telomerase Activity and Human Telomerase Reverse Transcriptase (hTERT) Expression in Ovarian Cancer

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Telomerase activation is a key step in the development of human cancers. Interferon-β (IFN-β) signaling induces growth arrest in many tumors but the anticancer mechanism of IFN-β is poorly understood. In the present study, we show that IFN-β signaling represses telomerase activity and human telomerase reverse transcriptase (hTERT) transcription in ovarian cancer and suggest that this signaling is mediated by $p21^{WAF1}$. IFN-β triggered down-regulation of telomerase activity and hTERT mRNA expression and also induced p21 expression, independently of p53 induction. Ectopic expression of p21 attenuated hTERT promoter activity. Murine embryonic fibroblasts (MEFs) genetically deficient in p21 (p21-/-) showed elevated (> 15 times) hTERT promoter activity compared to wild-type MEFs. Overexpression of p21 reduced the hTERT promoter activity of p21-/- MEFs and hTERT mRNA expression in HCT116 $p21^{WAF1}$ null cell. These findings provide evidence that p21 is a potential mediator of IFN-β-induced attenuation of telomerase activity and tumor suppression.

INTRODUCTION

Ovarian cancer is one of the most lethal gynecological carcinoma. The five-year survival rate for ovarian cancer is generally no better than 35% (Jemal et al., 2006). Like other processes of human tumorigenesis, ovarian carcinogenesis results from the accumulation of multiple genetic changes. Expression of mutant tumor suppressor gene p53 (Henriksen et al., 1994), mutation of protooncogene KRas (Ichikawa et al., 1994; Varras et al., 1995) overexpression of c-erb-B2 (Meden et al., 1994), and activation of human telomerase reverse transcriptase (Kyo et al., 1999; Wan et al., 1997) have been reported in ovarian cancers. Telomerase, an RNA-dependent DNA polymerase that consists of an RNA and protein subunits, prevents telomere erosion at each round of DNA replication. The protein subunit, human telomerase reverse transcriptase (hTERT), plays an important role in many cancers, including gynecological cancers. A high level of telomerase activity has been reported in 85% of malignant human cells. In comparison, telomerase activity is undetectable in most human somatic cells, except regenerating tissues (Shay and Bacchetti, 1997). Significantly higher telomerase activity is also observed in ovarian cancer compared to normal ovarian tissues (Kar et al., 2007). Furthermore, the telomerase activity correlates with the clinical stage and the tumor aggressiveness of ovarian cancer (Counter et al., 1994). It is generally accepted that there is a link between cellular proliferation or senescence and telomerase activity. Moreover, inhibition of hTERT expression in cancer cells leads to drastic telomere shortening, senescence, and apoptosis (Kondo et al., 1998).

IFNs are a family of multifunctional cytokines that trigger immunomodulatory, anti-viral, anti-proliferative, and anti-tumor activities (De Maeyer et al., 1998; Deiss et al., 1995; Maciejewski et al., 1995). Previously, a novel anti-cancer mechanism of interferon-γ (IFN-γ)/interferon regulatory factor-1(IRF-1)/$p27^{kip1}$ signaling that down-regulates telomerase activity and hTERT expression in human cervical cancer was suggested (Lee et al., 2003; 2005). And Xu et al. (2000a) suggested that Interferon-α (IFN-α) inhibits telomerase activity and hTERT expression. IFNs stimulate antitumor activity and have been successfully used in the treatment of various cancer patients. IFN-β induces apoptosis in ovarian cancer cells (Morrison et al., 2001; 2005) and suppresses the growth of ovarian tumor xenografts in mice (Lindner et al., 1997; Odaka et al., 2001). Also, adenovirus-mediated IFN-β gene therapy improved survival (Hendren et al., 2004) and resulted in tumor cell killing (Odaka et al., 2002) in mouse models. And recent clinical studies focused on the use of IFN-β against human carcinomas. In previous studies, IFN-β induced $p21^{WAF1}$ (Katayama et al., 2007) and p21 inhibited tumor cell growth (el-Deiry et al., 1993) and induced apoptosis (Giandomenico et al., 1998). p21 expression levels were associated with poor prognosis in ovarian cancer (Anttila et al., 1999; Buchynska et al., 2007).

The anticancer mechanism of IFN-β is poorly understood al-

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though the antitumor effects of IFN-β on ovarian cancer have been tested on cell lines and mouse models. In the present study, we investigated the regulation of telomerase activity and hTERT mRNA expression by IFN-β in human ovarian cancer cells. Furthermore, we provide evidence that p21 plays an important role in mediating IFN-β-induced inhibition of hTERT expression and telomerase activity.

MATERIALS AND METHODS

Cell culture and mice

SKOV3 and OVCAR3 are human ovarian cancer cell lines. These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (Life Technologies Inc., USA), Fungizon (Life Technologies Inc., USA), anti-PPLO (Life Technologies Inc., USA), streptomycin, and penicillin G at 37°C in a humidified atmosphere with 5% CO_2. All mice were housed in cages with filter tops in a laminar-flow hood, fed food and acid water *ad libitum*, and bred in a SPF facility. Light: dark ratio was 12:12 h. Mice with targeted mutations in the p21 gene were backcrossed to C57BL/6 seven to ten times. Primary mouse embryonic fibroblasts (MEFs) from p21 heterozygote mating were prepared from embryos at day 13.5 of development (E13.5), and cultured in DMEM with 10% FBS.

Plasmids and cell transfections and growth-inhibition studies

Human telomerase 3396-bp promoter subcloned into pGL3-luc (Promega, USA) was provided by T.K. Kim (Won et al., 2002). MEFs were used at early passage (3-5 passages) for transfection in 6-well plates. MEF transfection experiments were carried out using Effectene (Qiagen) according to the manufacturer's instructions. Cells were co-transfected with SV40-pRL (Promega) to normalize the transfection efficiency. Cell growth was measured by MTT assay (Boehringer-Mannheim, Germany) according to the manufacturer's instructions.

RT-PCR Analysis of hTERT mRNA

Expression of hTERT mRNA was analyzed by reverse transcription (RT)-PCR and real-time PCR amplification. Total RNA was prepared from cell lines using TRIzol (Gibco-BRL) according to the manufacturer's protocol. One μg of total RNA was reverse transcribed at 37°C for 45 min in the presence of random hexamers and Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). Expression of the telomerase subunit was analyzed by RT-PCR as described previously (Nakamura et al., 1997). A 145-bp hTERT fragment was amplified using the primer pair 5′-CGGAAGAGTGTCTGGAGCAA-3′ and 5′-GGATGAAGCGG AGTCTGGA-3′.

Real-time PCR

Total RNA was isolated as described above. cDNA was synthesized from 1 μg of RNA using the iScriptTM Select cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time PCR was performed with a iQTM SYBR Green SUpermix (Bio-Rad). Primers used for hTERT were 5′-CG GAAGAGTGTCTGGAGCAA-3′ and 5′-GGATGAAGCGGAGT CTGGA-3′. Primers for real-time PCR for GAPDH were 5′-GAGTCCACTGGCGTCTTCAC-3′ and 5′-GTTCACACCCATG ACGAACA-3′. Primers for real-time PCR for mTERT were 5′-TGGGTCTCCCCTGTACCAAAT-3′ and 5′-GGCCTGTAACTA GCGGAACA-3′. Primers for GAPDH were 5′-TGTCCGTCG TGGATCTGAC-3′ and 5′CCTGCTTCACCACCTTCTTG. After an initial denaturation at 95°C for 10 min, the cDNA samples were subjected to 40 cycles of RT-PCR (95°C for 10 s, and 56 °C for 15 s, and 72°C 15 s). PCR reactions were performed in triplicate.

Telomerase assay

Extracts from ovarian cancers were prepared using CHAPS lysis buffer. Telomerase activity was detected by TRAPEZE Telomerase Detection Kit (Intergen Co.) according to the manufacturer's protocol. Telomerase activity was calculated by computing the ratio of the entire ladder to the signal of the amplified internal control. We used 0.5 μg of protein extract for each sample and a 36-bp internal standard was used as an internal control.

Luciferase assay

Transient transfection of luciferase reporter plasmids was performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's protocol. Luciferase assays were performed using the standard luciferase assay system (Promega). All experiments were performed in triplicate.

Western blot analysis

Cells were lysed and equal amounts of cell extracts (20 μg) were electrophoresed on 12% SDS polyacrylamide gel, electrotransferred onto nitrocellulose membrane, and probed with antibodies. Antibodies for p21, cyclin-E, and c-myc were purchased from Santa Cruz Biotechnology, Inc.; P53 was from Neomarkers. Proteins were detected using the enhanced chemiluminescence system (Amersham).

Statistical analysis

hTERT promoter activity data were analyzed by one-way ANOVA and the difference between hTERT promoter activities in OVCAR and SKOV3 cells was analyzed by two-way ANOVA.

RESULTS

IFN-β suppresses telomerase activity and induces apoptosis in human ovarian cancer cells

Previous studies have demonstrated that telomerase activity is down-regulated by IFNs in cancer cells. Ovarian cancer cell lines have high levels of telomerase activity. To examine the effect of IFNs in the regulation of telomerase activity, OVCAR3 ovarian cancer cells were treated with IFNs. After 48-h exposure of OVCAR3 cells to 250 U/ml IFNs, telomerase activity was examined. As shown in Fig. 1A, telomerase activity declined dramatically in IFN-β treated cells. Telomerase activity was also down-regulated in IFN-γ-treated cells but to a lesser extent than in IFN-β-treated cells. Next we examined whether IFN-β suppressed hTERT expression in SKOV3 and OVCAR3 cells by reverse transcription (RT) - PCR (Fig. 1Ba) and real-time PCR (Fig. 1Bb). Significant decreases in hTERT mRNA level were observed at 24 h after IFN-β treatment. To explore the role of IFN-β in ovarian cancer cells, SKOV3 and OVCAR3 cells were treated with IFN-β. IFN-β treatment caused a dramatic reduction in cell growth (Fig. 1C). To confirm that the reduction in cell number represented apoptosis, we examined the morphology of DAPI-stained nuclei in untreated and IFN-β-treated OVCAR3 cells. IFN-β-treated cells had fragmented nuclei, which are characteristic of apoptosis, whereas untreated cells had normal nuclei (Data not shown).

IFN-β increases p21 protein level

There is strong relationship between cell cycle arrest and telomerase activity (Akiyama et al., 1999; Shiratsuchi et al., 1999).

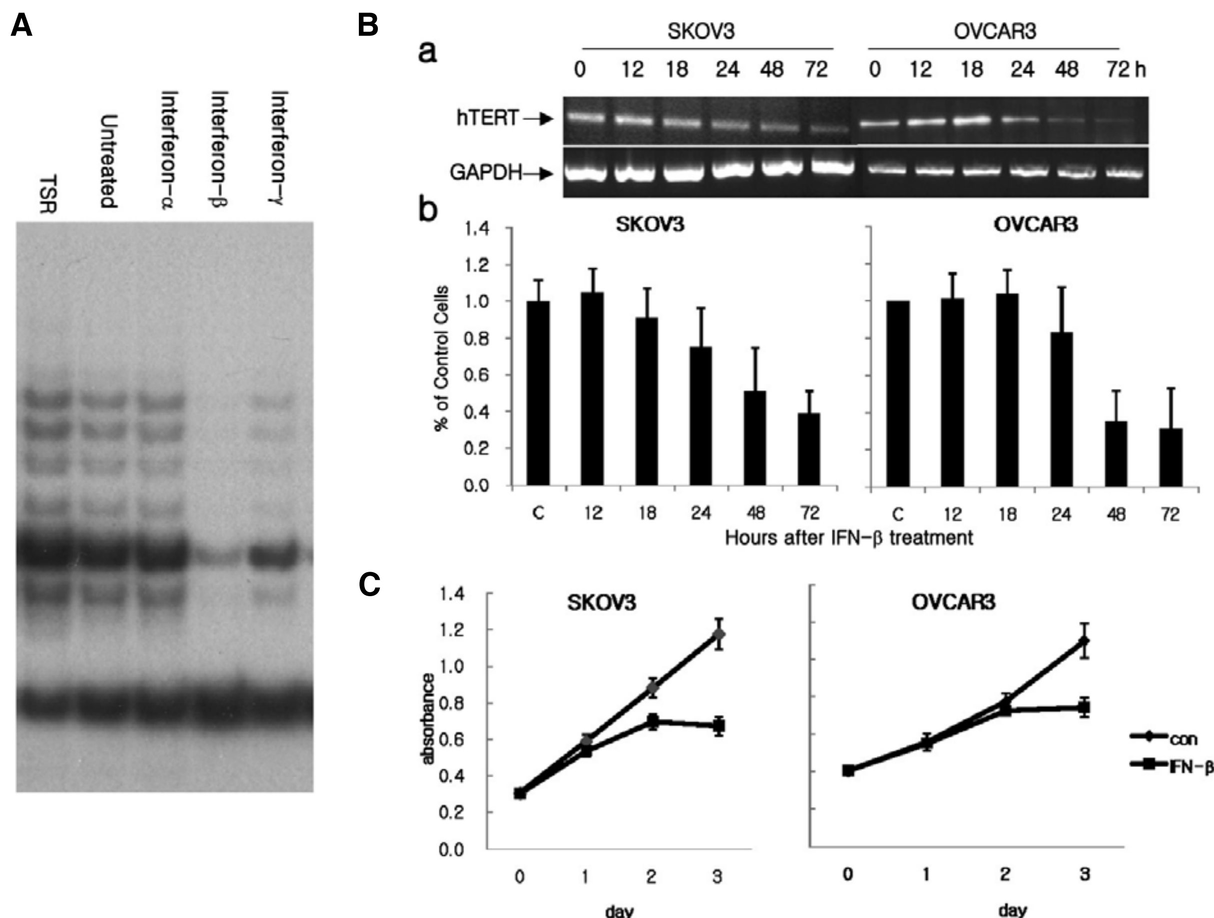

Fig. 1. Effects of IFNs on telomerase activity and IFN-β effect on hTERT mRNA expression and cell growth in ovarian cancer cell lines, OVCAR3 and SKOV3. (A) Cells were cultured in the presence of IFNs (250 U/ml) for 48 h. Telomerase activity was determined by TRAP assay. 1 μg of CHAPS extracts pretreated with RNase A (200 μg/ml) was used to inactivate the RNA component of telomerase. TSR, Quantitative Control. (B) Cells was cultured in the presence of IFN-β (250 U/ml) for the indicated times. hTERT mRNA expression was determined by using RT-PCR (Ba) and real-time RT-PCR (Bb). Data shown are representative of three independent experiments. (C) IFN-β effect on cell growth in the ovarian cancer cell lines, OVCAR and SKOV3. Cell growth rates were determined by MTT assay. At each time point, data were obtained from three separated cultures. Results are means ± SEM from two independent experiments.

While exploring cell cycle-related proteins, we identified p21 as a possible inhibitory mediator of telomerase activity. Human ovarian cancer cells, SKOV3, were incubated with 250 U/ml human IFN-β for the indicated times. Significantly increased p21 protein expression was observed without change of p53 expression (Fig. 2). c-Myc activates hTERT transcription by binding to the hTERT promoter (Wang et al., 1998; Wu et al., 1999). To determine whether c-Myc was involved in IFN-β-induced downregulation of telomerase activity in human ovarian cancers, we examined c-Myc expression levels after IFN-β treatment. Interestingly, the c-Myc protein level was not changed by IFN-β. Deregulation of cell cycle control is an important feature in cancer development. And cell cycle machinery regulates telomerase activity. p21 acts as universal inhibitor of cdk's with inhibition of a variety of cdk-cyclin complexes and causes G1 arrest. We examined the cyclin A, cyclin D1 and E expression level after by IFN-β treatment. Cyclin A protein level was decreased but the amount of cyclin D1 and E level was not changed. The CDK2 plays an important role in progression from G1 through S in dividing cells, as it is associated with cyclin D1 in late G1/early S phase, and with cylin A in the S-phase. This result indicates that in IFN-β treated ovarian cancer cells a prolongation of the S phase and reduced progression through the cell cycles occurs.

P21 suppresses hTERT expression and telomerase activity

Next, we asked whether p21 suppressed hTERT expression and telomerase activity. As shown in Fig. 3A, telomerase activity was strongly suppressed at 48 h in SKOV3 and OVCAR3 cells after p21 transfection (Fig. 3A). hTERT expression was also markedly reduced by the introduction of p21, as determined by reverse transcription (RT) - PCR. Significant decreases in hTERT mRNA level were observed at 18 h (SKOV3) or 24 h (OVCAR3) after transient transfection (Fig. 3B). To confirm whether p21 could decrease the activity of the hTERT promoter, SKOV and OVCAR3 cells were cotransfected with 2 μg of a 396-bp hTERT promoter region reporter gene construct and various amounts of pcDNA-p21 (0, 0.5, 1, 1.5 and 2 μg). Transiently transfected p21 suppressed luciferase activity in a dose-dependent manner in both cell lines (Fig. 3C). The two-way ANOVA test revealed a statistically significant difference in hTERT promoter activities in SKOV3 and OVCAR3 cells ($P < 0.05$).

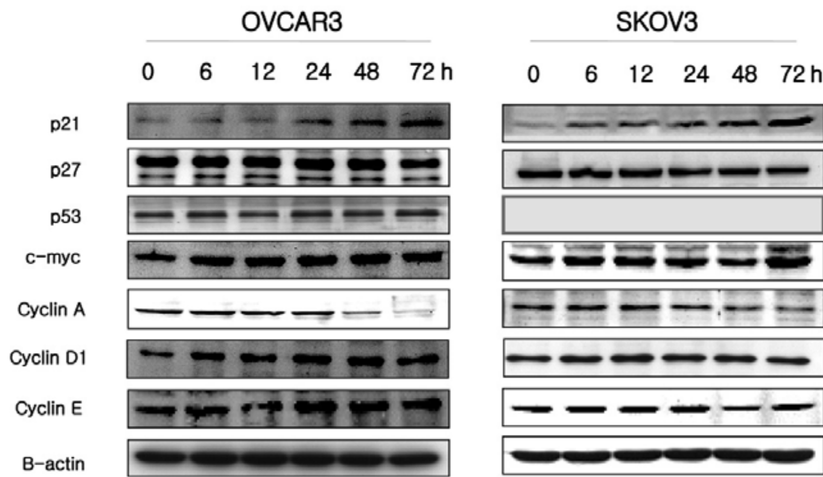

Fig. 2. Effects of IFN-β on expression of cell cycle-related proteins in OVCAR3 and SKOV3 cells. Western blot analysis was performed on total cell lysates (20 μg) using antiserum against represented protein products after IFN-β treatment. Cells were cultured in the presence of IFN-β (250 U/ml) for the indicated times.

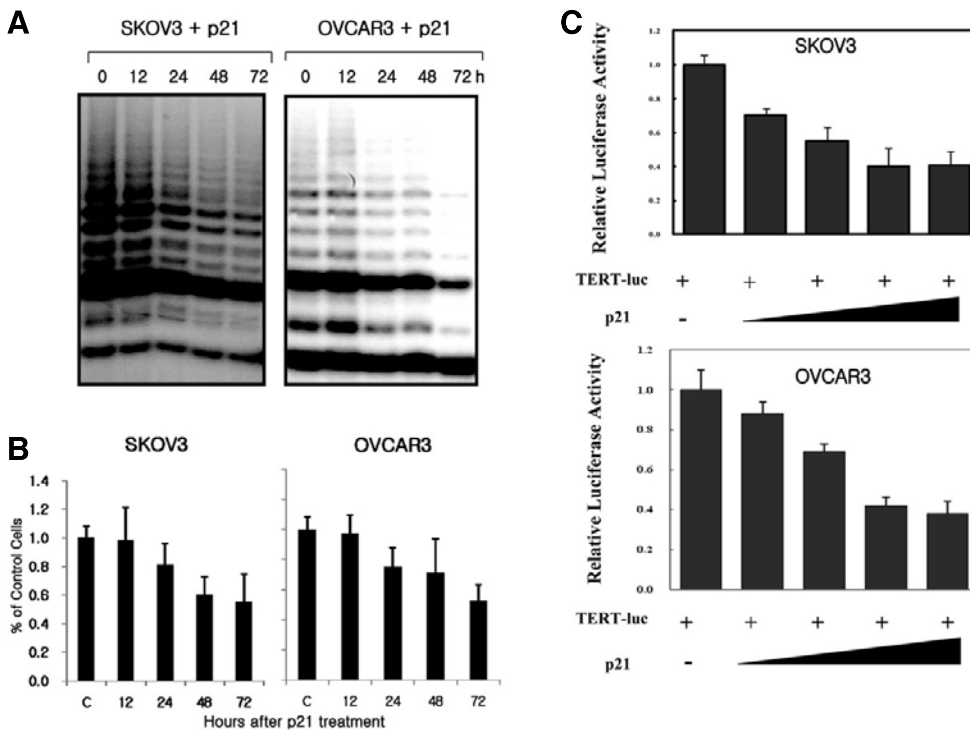

Fig. 3. Effects of p21 on telomerase activity, hTERT levels and hTERT promoter activity in OVCAR3 and SKOV3 cells. Cells were transfected with 10 μg of expression vector, pcDNA3.1-p21, for the indicated time. Telomerase activity (A) and hTERT expression (B) were determined using TRAP assay and RT-PCR, respectively. Luciferase activity was assessed 48 h after transfection and then normalized to β-gal activity (C). Values are mean ± S.D. of triplicate samples and are representative of two independent experiments.

Telomerase activity in p21-deficient MEFs

To confirm the suppression of telomerase activity caused by p21, wild-type and p21-deficient (p21-/-) MEF cells were used. To determine the basal level of hTERT promoter activity, a 3396-bp hTERT promoter region reporter construct was transfected into primary MEFs from wild-type and p21-deficient mice. As shown in Fig. 4A, p21 -/- MEFs showed hTERT promoter activity that was 15 times higher than that in wild-type MEFs. Furthermore hTERT promoter activity was determined after transient transfection of the p21 expression vector to the p21 -/- MEFs. As expected, overexpression of p21 reduced reporter activity (45%) (Fig. 4B) and also telomerase activity (Fig. 4Ca). And reduction of telomerase activity was more induced by IFN-β in wild-type MEFs compared with that in the p21 -/- MEFs (Fig. Cb). To confirm that p21 up-regulation is correlated with hTERT down-regulation, we check the expression of hTERT in p21 null genetic background cells upon interferon β or p21 treatment. p21 null HCT116 cells showed over 7 times higher hTERT mRNA expression than control HCT116 cells. And treatment of interferon β showed slight decrease but not notable change of hTERT expression, but overexpressed p21 dramatically inhibited hTERT mRNA expression in p21 null HCT116 cells (Fig. 4D). These results confirm that p21 is essential for the regulation of hTERT expression.

DISCUSSION

Telomerase activation is one of the hallmarks of cancers, and a strong correlation between telomerase activity and hTERT mRNA expression is observed in a variety of cancers (Meyerson et al., 1997; Takakura et al., 1998). We herein provide evidence that interferon-β induced p21 down-regulates telomerase

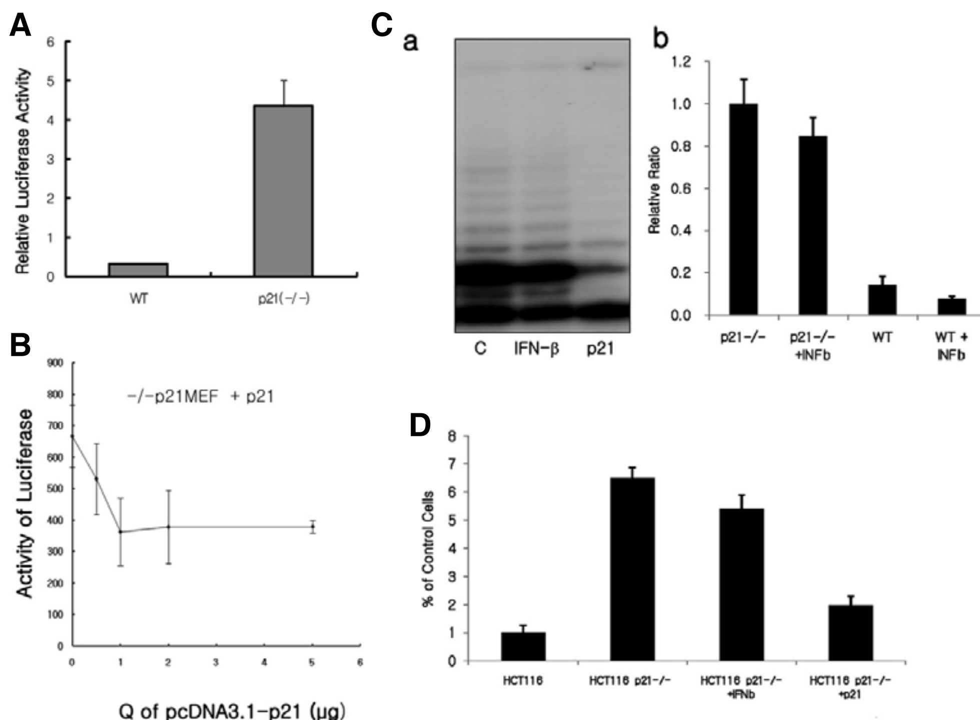

Fig. 4. Transcriptional activity of human telomerase promoter is up-regulated in $p21^{-/-}$ cells. (A) Human telomerase 3396bp promoter reporter gene construct (2 μg) was transfected into wild-type (WT) or $p21^{-/-}$ primary embryonic fibroblasts, and the basal level of hTERT promoter activity was then determined. (B) Cells were transfected with 2 μg of human telomerase 3396bp promoter reporter gene construct and various amounts of pcDNA-p21 (0, 0.5, 1, 2.and 5 μg). Luciferase activity was assessed 48 h after transfection and then normalized to β-gal activity. Values are means ± S.D. of triplicate samples and are representative of two independent experiments. (C) $p21^{-/-}$ primary embryonic fibro- blasts cells were treated with interferon-β (250 U/ml) or transfected with 10 μg of expression vector, pcDNA3.1-p21, for 72 h. Telomerase activity was determined using TRAP assay (Ca). Wild-type and $p21^{-/-}$ primary embryonic fibroblasts cells were treated with interferon-β (250 U/ml) and relative telomerase activity was determined using TRAP assay (Cb). (D) p21 null HCT116 cells were treated with IFN-β (250 U/ml) or transfected with 10 μg of pcDNA3.1-p21 and mTERT mRNA expression was determined by using real-time RT-PCR. Data shown are representative of three independent experiments.

activity and hTERT expression in ovarian cancer. p21 -/- MEFs showed a hTERT promoter activity that was 10 times higher than that present in wild-type MEFs. Consistent with this, increase of p21 by ectopic transfection suppressed hTERT promoter activity and hTERT gene expression.

One of the critical differences between normal and tumor cells lies in their state of telomerase activity. Interferons have been previously explored as antitumor agents for the treatment of ovarian cancer. In many malignant cell lines, IFN-β showed growth inhibitory and apoptotic effects and more effective antitumor activity than IFN-α (Chawla-Sarkar et al., 2001; Damdinsuren et al., 2003; Sanceau et al., 2000) Consistent with this, our results (Fig. 1) showed that IFN-β inhibited telomerase activity more effectively than IFN-α. In a previous study, inhibition of telomerase activity in human cancer cells forced them either into apoptosis or to differentiate (Kondo et al., 1998). Thus, the antitumor activity of IFNs may closely connected with telomerase activity.

Interferons are known to induce apoptosis in human malignancies. And in most malignant cell lines, IFN-β had greater growth inhibitory and apoptotic effects than other IFNs. IFN-β also suppresses ovarian cancer growth by inducing apoptosis and has been using ovarian cancer treatment but the underlying mechanism is not well understood. p21 is known as an inhibitor of the cyclin/CDK complexes, which block the G1-S transition. The function of p21 as an anti-oncogenic factor is also well known, although the underlying mechanism is not well understood. Several groups reported that IFN-β induces p21 and inhibits cancer cell growth (Giandomenico et al., 1998; Katayama et al., 2007). However no direct evidence was provided. In this study, we proposed that overexpression of p21 by IFN-β repressed hTERT mRNA expression and telomerase activity in human ovarian cancer cell lines. Our studies with p21 -/- MEFs transfected with the hTERT promoter reporter system indicate that p21 is critically involved in down-regulation of hTERT expression. Consistent with this, p21 KO mice have impaired tumor-suppression (Jackson et al., 2003). We also suggested that p21 null HCT116 cells showed over 7 times higher hTERT mRNA expression than control HCT116 cells. And treatment of interferon β showed no notable change of hTERT expression, but overexpressed p21 dramatically inhibited hTERT mRNA expression in p21 null HCT116 cells. Taken together, our findings suggest a mechanism in which p21 exerts its anti-oncogenic action by negatively regulating hTERT mRNA expression and telomerase activity.

Many oncogenes and tumor suppressor genes, including p53, SP1, c-Myc, E2F-1 and Rb, have been implicated in the regulation of telomerase activity and hTERT expression (Crowe et al., 2001; Kyo et al., 2000; Oh et al., 2001; Xu et al., 2000b). In previous studies, c-Myc activates hTERT transcription by binding to the hTERT promoter (Wang et al., 1998; Wu et al., 1999). And IFN-β promotes c-Myc proteolysis in leukemic cells (Hu et al., 2005). However, we could not observed down-regulation of c-Myc protein level in IFN-β treated ovarian cancer cell lines. These results suggest that the down-regulation of telomerase activity and hTERT mRNA expression may not be caused by c-Myc expression.

There has been a controversy regarding the relation between cell cycle arrest and telomerase activity. Myeloma cells were arrested in G1 by IFN-α and showed lower telomerase activity compared to cultured myeloma cells without IFN-α, while in sorted S-phase cells the telomerase activity is higher (Shiratschi et al., 1999). Zhu et al. (1996) also reported cell cycle dependent modulation of telomerase activity in tumor cells.

Therefore it is possible that the p21 mediated suppression of telomerase activity is cell cycle dependent. In contrast to these findings, several groups have demonstrated that telomerase activity or hTERT mRNA expression was not dependent on cell cycle regulation. Xu et al. (2000) reported that there is rapid decline in hTERT mRNA and telomerase activity in human malignant and nonmalignant hematopoietic cells treated with IFN-α, and that cell cycle arrest in early S phase by aphidicholin does not inhibit telomerase activity. Similar results were reported in IFN-γ treated cervical cancer cell lines (Lee et al., 2003). Rapamycin inhibited growth of all the cervical and 3 of 4 ovarian cancer cell lines. And Rapamycin reduced hTERT mRNA expression in both rapamycin-sensitive and -resistant cell lines (Bae-Jump et al., 2006). Cyclin A is produced in late G1 and its expression accumulates during S and G2 phase. Inhibition of cdk2, for which Rb is thought to be a substrate, has been shown to block telomerase activity (Buchkovich and Greider, 1996). Cyclin A associates with and activates primarily cdk2. In the present study, IFN-β treated ovarian cancer cell lines, SKOV3 and OVCAR3 showed decreased expression of cyclin A. This result indicates that in IFN-β treated and p21 over-expressed cells a prolongation of the S phase and reduced progression through the cell cycles occurs. Telomerase activity is upregulated at cell cycle entry as cells progress through S-phase but telomerase activity and hTERT mRNA expression were down-regulated in IFN-β treated or p21 transiently transfected ovarian cancer cells. These results suggest that IFN-β induced down-regulation of telomerase activity may not caused by cell cycle arrest. However, decreased cyclin A may also indicate G1 arrest our data do not rule out a possibility that G1 arrest induced by p21 downregulates telomerase activity.

Shats et al. (2004) reported that repression of hTERT by p53 is mediated by p21 and E2F. But growth arrest and reduced tumorigenicity, as a consequence of telomerase inhibition, were also observed in several cell lines with inactivated p53 (Hahn et al., 1999; Zhang et al., 1999). And there is a significant loss of p21 expression in tumors that over-express p53 (Bali et al., 2004). Wang et al. (1998) also proposed that Ultraviolet radiation down-regulates p21 expression independently of p53. Consistent with these findings, our present results showed that p21 repressed telomerase activity and hTERT expression in OVCAR and SKOV3 (p53 null) cell lines without change of p53 expression (Fig. 2), which indicates that IFN-β/p21 signaling may down regulate hTERT expression and telomerase activity by a p53-independent pathway.

In conclusion, this study provides evidence that IFN-β induced p21 is a negative regulator of hTERT expression and telomerase activity in ovarian cancer cells. We propose that inhibition of telomerase activity is an important mechanism through which p21 exerts its tumor-suppressing effects.

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