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PTEN- and p53-Mediated Apoptosis and Cell Cycle Arrest by FTY720 in Gastric Cancer Cells and Nude Mice

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ABSTRACT

FTY720, a new immunosuppressant, derived from ISP-1, has been studied for its putative anti-cancer properties in the recent years. In this study, we have reported that FTY720 greatly inhibited gastric cancer cell proliferation for the first time, and found this effect was associated with G1 phase cell cycle arrest and apoptosis. Results from our Western blotting and Real-time PCR showed that FTY720 induced obvious PTEN expression in a p53-independent way, consistent with a substantial decrease in p-Akt and MDM2. FTY720 dramatically increased the expression of Cip1/p21, p27, and BH3-only proteins through the accumulation of p53 by PTEN-mediated inhibition of the PI3K/Akt/MDM2 signaling. Suppression of PTEN expression with siRNA significantly reduced the p53 and p21 levels and activated Akt, resulting in decreased apoptosis and increased cell survival. Furthermore, we have observed an additive effect of FTY720 in killing gastric cancer cells when in combination with Cisplatin, partly through PTEN-mediated Akt/MDM2 inhibition. In vivo study has also shown that tumor growth was significantly suppressed after FTY720 treatment. In conclusion, our results suggest that FTY720 induces a significant increase of PTEN, which inhibits p-Akt and MDM2, and then increases the level of p53, thereby inducing G1 phase arrest and apoptosis. We have characterized a novel immunosuppressant, for the first time, which shows potential anti-tumor effects on gastric cancer by PTEN activation through p53-independent mechanism, especially in combination with Cisplatin. This PTEN target-based therapy is worth further investigation and warrants clinical evaluation. J. Cell. Biochem. 111: 218–228, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: FTY720; GASTRIC CANCER; PTEN; AKT; P53

Gastric cancer is one of the most frequently occurring malignancies in the world and the mortality from gastric cancer is second only to lung cancer [Greenlee et al., 2000; Dvory-Sobol et al., 2007]. Chemotherapy in advanced gastric cancer is important, despite advances in conventional cancer chemotherapy, the prognosis for patients with gastric cancer is poor [Ajani, 1998; Mezhir et al., 2010]. Effects of chemotherapy are often discouraging because of chemo resistance and toxicity to normal cells [Oki et al., 2005]. Therefore, discovery of new effective and safer drugs for the treatment of gastric cancer is crucial.

FTY720 was originally derived as a novel immunosuppressant for use in organ transplantation to prolong graft survival [Fujita et al., 1994; Brinkmann et al., 2001], and it is currently undergoing clinical trials for the prevention of kidney graft rejection [Tedesco-Silva et al., 2004] and the treatment of relapsing multiple sclerosis [Kappos et al., 2006; O'Connor et al., 2009]. Previous researches have indicated that the effect of FTY720 on prolonging the survival of allograft is attributable to the ability to induce apoptosis of infiltrated lymphocytes [Li et al., 2001; Graler and Goetzl, 2004]. Recently, FTY720 has also been reported to induce apoptosis of cancer cells [Lee et al., 2004; Chua et al., 2005; Ho et al., 2005; Yasui et al., 2005; Shen et al., 2007; Liu et al., 2008]. To date, various signaling pathways have been raised to account for the ability of FTY720 to induce apoptosis in different cancer cells, including those mediated by mitogen-activated protein kinases [Permpongkosol et al., 2002], phosphatase 2A (PP2A) inhibition [Neviani et al., 2007], Rho-GTPase [Zhou et al., 2006], and Bcl-xL [Yasui et al., 2005]. However, the precise mechanisms of FTY720 on cancer cells have not been completely comprehended.

Abbreviations used: CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; MDM2, murine double minute-2; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PI3K, phosphatidylinositol 3-kinase; PBS, phospho-buffered saline; PCR, polymerase chain reaction; siRNA, small interfering RNA.

Tongsen Zheng, Xianzhi Meng and JiaBei Wang contributed equally to this work.

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To the best of our knowledge, the fact that the feasibility of using this drug in gastric cancer treatment yet has not been studied drew our attention. Therefore, the objective of the current study is to investigate the in vivo and in vitro anti-cancer potential of FTY720 and delineate the underlying mechanisms.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

AGS (gastric adenocarcinoma; wt-p53) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). MGC803 (gastric adenocarcinoma; wt-p53) was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultivated in DMEM medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml), in a CO_2 incubator (Heal Force). Wortmannin was purchased from Sigma (St. Louis, MO). FTY720 (Cayman chemical company, Ann Arbor, MI) was dissolved in normal saline (0.9%, w/v sodium chloride) and prepared in stock solution of 1 mg/ml and stored at 4°C.

CELL PROLIFERATION ASSAY

Cell proliferation was assayed using the Cell Counting Kit-8 (CCK-8; Dojindo Corp., Kumamoto, Japan) following the manufacturer's protocol. All the experiments were performed in triplicate.

CELL CYCLE ANALYSIS

Cells were treated with different drugs and then 10^6 cells were fixed in 80% ethanol at −20°C for 24 h. Fixed cells were stained as the protocol of Cycle TESTTM PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA) and analyzed by flow cytometry (Beckman Coulter FC 500). The experiment was done repeated thrice under the same conditions.

QUANTIFICATION OF APOPTOTIC CELLS

Cells treated with drugs were harvested, washed twice with pre-chilled PBS and resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. One hundred microliters of such solution (1×10^5 cells) was mixed with 5 μl of Annexin V-FITC and 5 μl of propidium iodide (BD Biosciences) according to the manufacturer's instruction. The mixed solution was gently vortexed and incubated in the dark at room temperature (25°C) for 15 min. Four hundred microliters of 1× dilution buffer were then added to each tube and cell apoptosis analysis was performed by flow cytometry (BD FACS Calibur) within 1 h.

WESTERN BLOTTING ANALYSIS

SDS–PAGE and Western blots were performed as previously described [Lee et al., 2004]. The primary antibodies against Phospho-Akt (473), Akt, PUMA, Cip1/p21, Kip1/p27, and poly (ADP-ribose) polymerase (PARP) were from Cell Signaling Technology; antibodies against Bcl-2, Bax, p53, PTEN, caspase-3, and caspase-9, CDK2, CDK4, cyclin D1, cyclin E, Rb, E2F1, and β-actin were purchased from Santa Cruz Biotechnology, Inc.; MDM2 and NOXA were from Abcam. The secondary antibodies, horseradish peroxidase-linked anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G, were also purchased from Santa Cruz Biotechnology. All immunoblots were repeated at least twice to confirm the results.

REAL-TIME PCR

Total RNA was extracted from AGS and MGC803 cells with Trizol Reagent (Invitrogen), and single-stranded cDNA was constructed using Superscript III polymerase (Invitrogen) and Oligo-dT Primers. Aliquots containing 5 μg of total RNA from each sample were converted to cDNA using Superscript II reverse transcriptase according to manufacturer's protocol. Real-time PCR was performed using the iCycler (Bio-Rad) and SYBR Green PCR master-mix reagents (Abgene). The following primers were used: human PTEN (F: 5′-AAGACCATAACCCACCACAGC-3′; R: 5′-TCATTACACCAGTTCGTCCCT-3′); human P53 (F: 5′-CCAGCCAAAGAAGAAACCAC-3′; R: 5′-CCTGGGCATCCTTGAGTTC-3′); human MDM2 (F: 5′-ATTCCCAACAAGCCCAACTAC-3′; R: 5′-CCAGGCACATCTGCTCCACT-3′); and human β-actin (F: 5′-CCTGTACGCCAACACAGTGC-3′; R: 5′-ATACTCCTGCTTGCTGATCC-3′).

PREPARATION AND TRANSFECTION OF siRNAs

The PTEN-specific siRNA (si-PTEN, 5′-GAUAUCAAGAGAUGGAUU-3′), p53-specific siRNA (si-p53, 5′-GACTCCAGTGGTAATCTACTT-3′), and scrambled control were synthesized by Gene Pharma company (Shanghai). Both gastric cancer cells were transfected with PTEN/53 siRNA and negative control siRNA respectively by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. One day after transfection, transfection complexes were removed and replaced with culture medium. After incubating with FTY720 in culture medium for 24 h, the cells were used for experiments.

TUMOR XENOGRAFT EXPERIMENTS

The nude mice (BALB/c-nu/nu) xenografts were established using MGC803 cell line. For the treatment group, FTY720 was administered by daily i.p. injection of 10 mg/kg/day for 20 days. The mice in both the treatment and control groups ($n = 15$ in each group) were sacrificed when snap-frozen and paraffin-embedded tumor tissue blocks had been obtained for further analysis. The body weight was recorded starting from the day of treatment, and tumor volumes were also calculated at the same time points using the following equation: tumor volume $=$ length $\times$ $(\text{width})^2 \times \pi/6$ and subsequently transformed into relative values (V; $V = V_t/V_0$, where V_0 is the tumor volume at initiation of treatment, whereas V_t is the tumor volume at any given day during entire treatment period) [Chua et al., 2005]. The study was approved by the Committee on the use of live animals in teaching and research of the Harbin Medical University, Harbin, China.

IMMUNOHISTOCHEMISTRY AND TUNEL STAINING

Immunohistochemistry and TUNEL assay was performed as described previously [Chua et al., 2005] using anti-mouse Ki-67 monoclonal antibody and a POD cell death detection kit (Boehringer, Mannheim).

STATISTICAL ANALYSIS

Data were presented as means ± SD for the indicated number of independently performed experiments. Student's *t*-test and one-way

ANOVA with Bonferroni post-tests were utilized for statistical analysis. For individual experiments and as groups based on condition and time point of analysis, the magnitude of within subject effects was assessed by Student's *t*-tests. In cases with more than two treatment groups analyzed, one-way ANOVA was performed with post hoc Bonferroni correction. The magnitude of treatment effect on bodyweight gain was assessed by comparison of the mean bodyweights between treated and control groups assayed at the same time point by Student's *t*-tests. For all comparisons, an alpha level of <0.05 was used as the criterion of significance.

RESULTS

FTY720 INHIBITS GASTRIC CANCER CELL PROLIFERATION IN A CONCENTRATION-DEPENDENT MANNER

AGS and MGC803 cells were treated with FTY720 at indicated concentrations for 12 and 24 h. The CCK-8 assay demonstrated that the FTY720 treatment have induced a dramatic reduction in cell viability in AGS and MGC803 (Fig. 1). For the AGS and MGC803 cell lines, IC50 was estimated to be 7.2 and 9.6 μM for 24 h, respectively. In summary, FTY720 is an effective inhibitor of gastric cancer cell growth in vitro, and the inhibition is concentration-dependent.

FTY720 INDUCES G1 PHASE ARREST IN GASTRIC CANCER CELLS

As we have observed a significant growth-inhibitory effect of FTY720 on gastric cancer cells, we further investigated whether FTY720 could inhibit the cell cycle progression. The treatment of AGS and MGC803 with FTY720 resulted in a higher number of cells in G1 phase at the concentrations of 5 and 10 μM, respectively, compared with control cells (Fig. 2B). The lowest concentration of FTY720 (5 μM) led to a modest increase in the number of cells in the G1 phase (48.33% vs. 67.45%, 47.94% vs. 64.29%), while higher concentration of FTY720 (10 μM) led to a greater G1 phase arrest (48.33% vs. 72.43%, 47.94% vs. 71.67%) in AGS and MGC803 cell

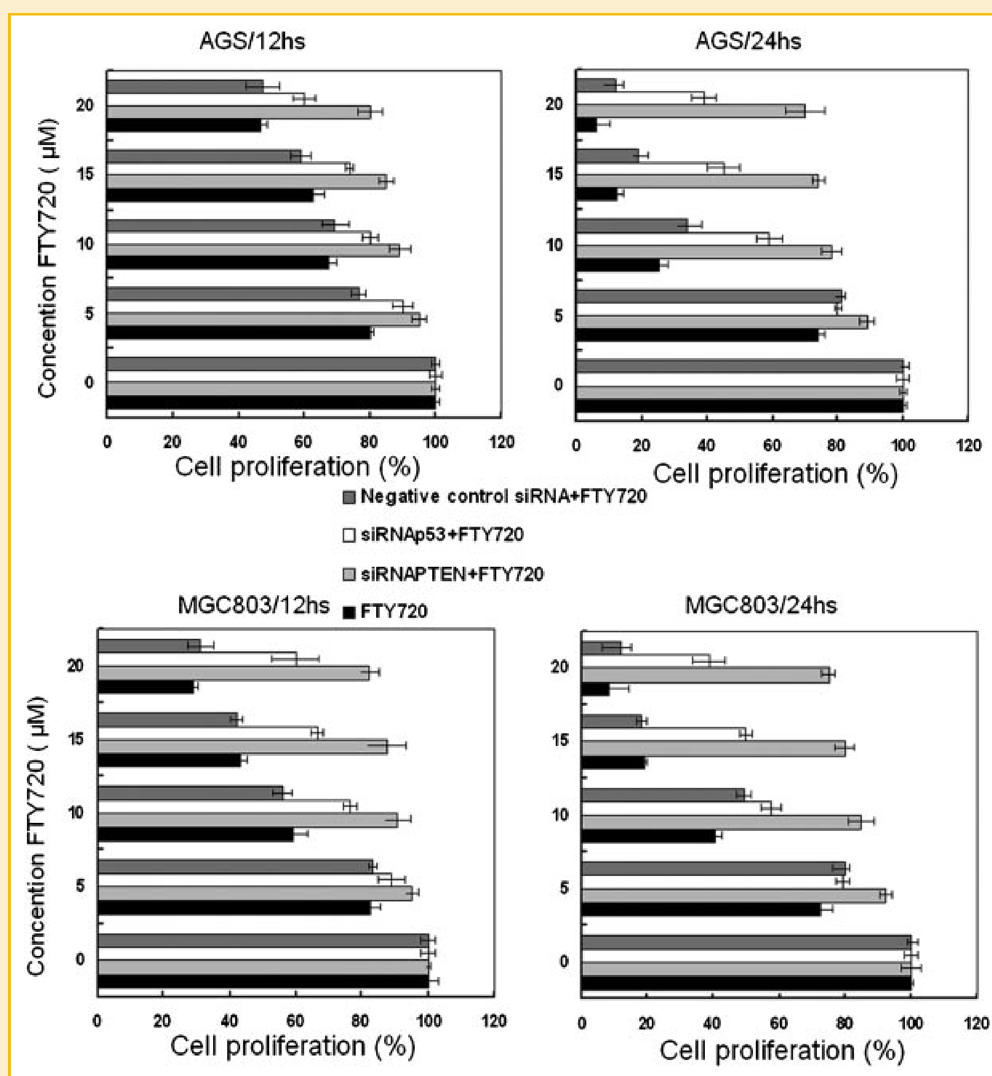

Fig. 1. FTY720 inhibits gastric cancer cell proliferation. AGS (gastric adenocarcinoma; wt-p53) and MGC803 (gastric adenocarcinoma; wt-p53) cells (including p53-silenced and PTEN-silenced gastric cancer cells) were incubated with increasing concentrations of FTY720 (0–20 μmol/L) for 12 and 24 h periods and analyzed for cell proliferation using CCK-8 assay. All assays were done in triplicate. Error bars represent the standard deviation of at least three independent experiments done in triplicates.

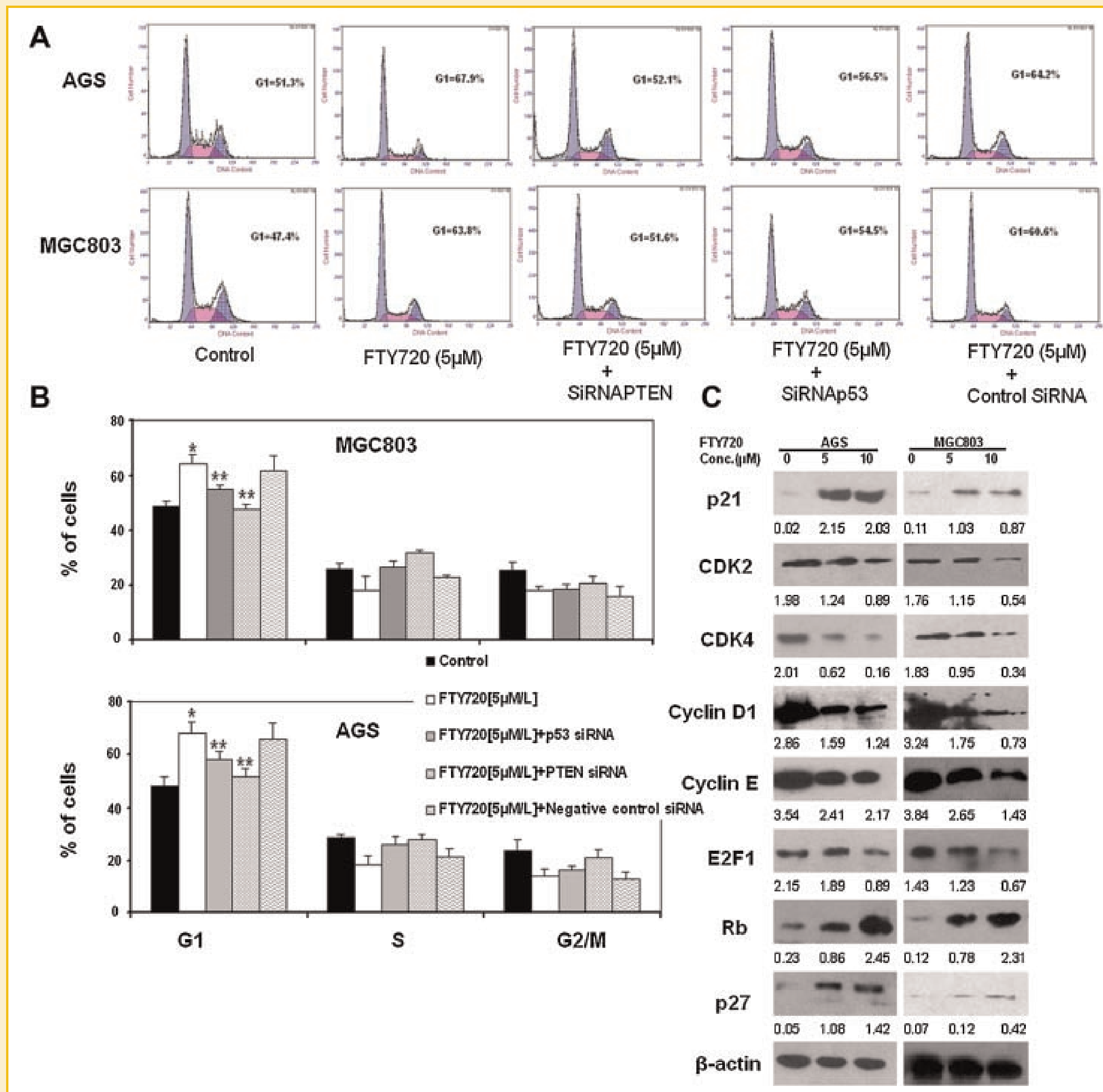

Fig. 2. Effects of FTY720 on cell cycle progression of human gastric cancer cells. A, B: AGS and MGC803 cells (including p53-silenced and PTEN-silenced gastric cancer cells) were exposed to indicate concentrations of FTY720 for 24 h followed by cell cycle distribution assay. Error bars represent the standard deviation of at least three independent experiments done in triplicates (*ANOVA $P < 0.05$; post hoc Student's *t*-tests, $P < 0.05$ vs. control; **ANOVA $P < 0.05$; post hoc Student's *t*-tests, $P < 0.05$ vs. negative siRNA-transfected cells treated with FTY720). C: The effects of the compound on the expression of cell cycle -related proteins were determined by Western blot analyses after AGS and MGC803 cells were exposed to various concentrations (5 and 10 μmol/L) of FTY720 for 24 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

lines, respectively. These data suggests that the inhibition of cell proliferation by FTY720 is associated with the induction of G1 phase arrest.

FTY720 MEDIATES PERTURBATIONS IN CELL CYCLE REGULATORY PROTEINS

Having observed a significant increase in the population of G1 phase of the cells treated with FTY720, we further evaluated the effects of FTY720 on the expression of cycle progression-related proteins. As is shown in Figure 2C, treatment with FTY720 resulted in a reduction of cyclin D1, cyclin E, CDK2, and CDK4 in a concentration-dependent manner. Analysis also indicates that FTY720 causes concentration-dependent increase in Kip1/p27 and Cip1/p21 expression and decreased E2F1 expression. The expression of Rb was also induced by FTY720 in MGC803 and AGS cells (Fig. 2C). These observations suggested that the increase of CDK inhibitors possibly may play an important role in the induction of G1 phase arrest in human gastric cancer cells, probably by the inhibition of CDK activity.

FTY720 INDUCES SIGNIFICANT APOPTOSIS IN GASTRIC CANCER CELLS

To further investigate the underlying mechanism of decreased cell proliferation observed in the CCK-8 assay, we examined the

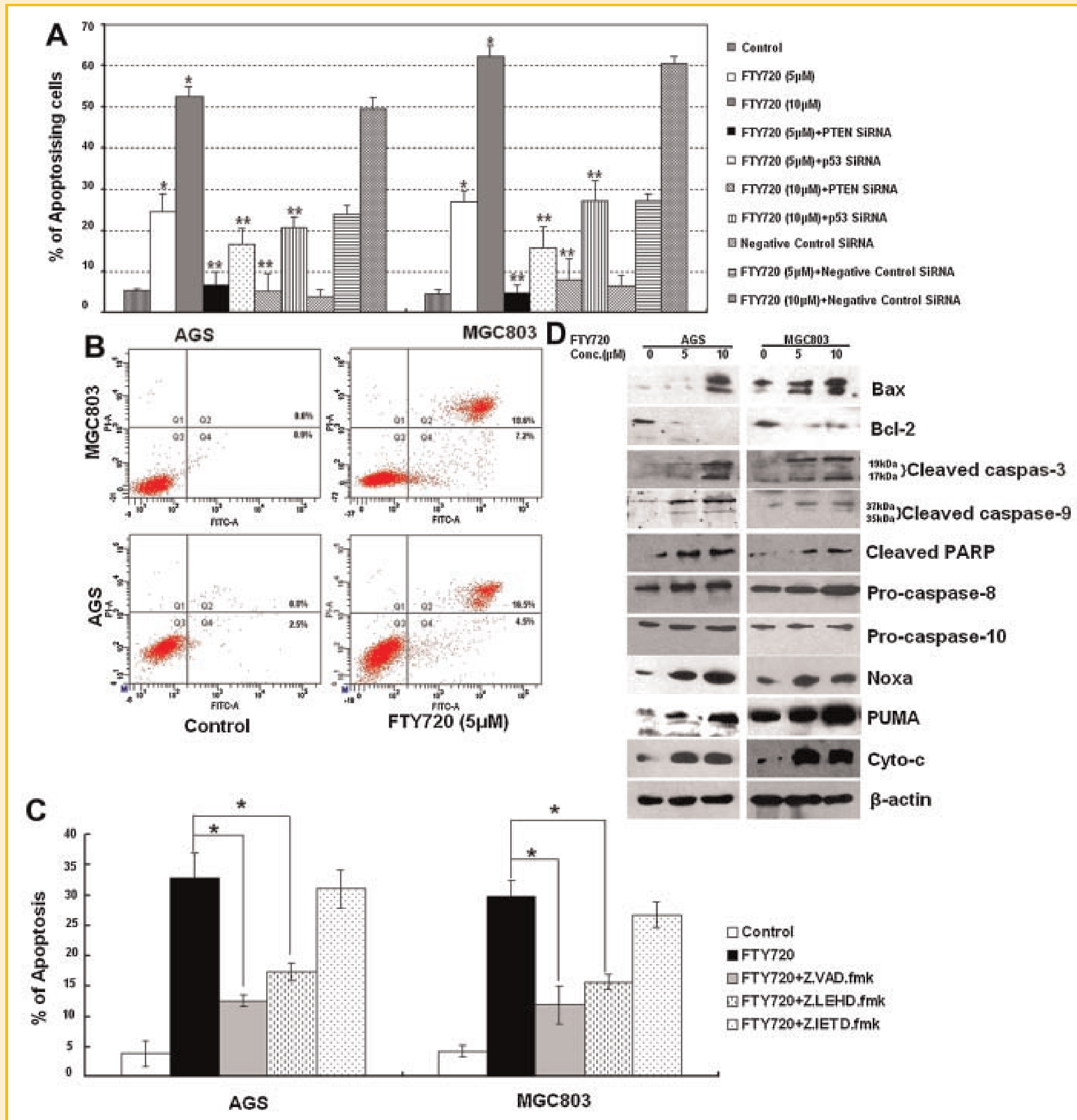

Fig. 3. FTY720 induces apoptosis in human gastric cancer cells in casepase-dependent manner and PTEN/p53 siRNA inhibits FTY720-induced cell death. A, B: Quantification of apoptotic cells (cell death) determined by flow cytometry. Error bars represent the standard deviation of at least three independent experiments done in triplicates (*ANOVA $P < 0.001$; post hoc Student's t-tests, $P < 0.001$vs. untreated control; **ANOVA $P < 0.001$; post hoc Student's t-tests, $P < 0.001$ vs. negative siRNA-transfected cells treated with FTY720). C: Cells were cultured with caspase-8 inhibitor (50 μmol/L), caspase-9 inhibitor (50 μmol/L), or pan caspase inhibitor (50 μmol/L) with or without FTY720 (ANOVA $P < 0.05$; *post hoc Student's t-tests, $P < 0.05$) D: AGS and MGC803 cells were exposed to various concentrations of FTY720 for 24 h, and the target proteins were detected by Western blot analyses. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

apoptosis effect on gastric cancer cells induced by FTY720 using Annexin V/propidium iodide assay as described in Materials and Methods Section. As shown in Figure 3A, both gastric cancer cells have shown a concentration-dependent apoptosis, including early as well as late apoptotic cell death. The analysis demonstrated >50% of the AGS cancer cells apoptosis within 24 h after initiation of FTY720 treatment (10 μM), whereas >60% of the cancer cells in MGC803. In contrast, the cells without FTY720 treatment demonstrated normal cell viability without considerable cell death.

FTY720 INDUCES CHANGES IN THE EXPRESSION OF APOPTOSIS-RELATED PROTEINS

As we have observed FTY720-induced apoptosis in gastric cancer cells, we further determined the levels of apoptosis-related proteins in cells treated with FTY720. As shown in Figure 3D, AGS and MGC803 cells exposed to FTY720 have shown a concentration-dependent reduction of Bcl-2, and a concomitant increase of Bax, compared with the control cells. Caspase-3 was cleaved into fragments after exposure to FTY720, and cleavage of caspase-3 became more intense with increased concentrations of FTY720; a

similar tendency was observed for caspase-9. Activation of caspase-3 was further confirmed by poly (ADP-ribose) polymerase (PARP) cleavage, a typical feature of caspase-dependent apoptosis. PARP was cleaved by FTY720 at 24 h as is shown in Figure 3. These results suggest that FTY720 induced the apoptosis of gastric cancer cells by activating caspases and promoting PARP cleavage. This suggests that the mitochondrial apoptotic path way is involved in the apoptosis. One of the critical mediators of the mitochondrial apoptotic pathway is p53 [Bykov and Wiman, 2003]. Treatment of cancer cells with FTY720 resulted in a concentration-dependent increase in p53 and a decrease in MDM2 (Fig. 4A), suggesting that the apoptosis induced by FTY720 might be related to the increasing level of p53. As p53 must be activated to induce apoptosis, and this is in part through selectively targeting genes. So, we have also analyzed the expression of several apoptosis-related targets of p53. As shown in Figure 3D, the p53 transcriptional target BH3-only proteins, PUMA, and NOXA, were induced in both AGS and MGC803 cells, suggesting FTY720 might possibly induce apoptosis partly by the activation of p53.

The selective caspase inhibitors were used to determine whether FTY720-induced apoptosis of gastric cancer cells was caspase-dependent. As is shown in Figure 3C, FTY720-induced apoptosis was inhibited by the pan-caspase inhibitor (zVAD.fmk) and a caspase-9 inhibitor (zLEDH.fmk) in both cell lines, but not the caspase-8 inhibitor (zIETD.fmk). Furthermore, to rule out the potential contribution of the extrinsic pathway of apoptosis, Western blotting was used to determine the caspases-8 and -10 activation status. As is shown in Figure 3D, there is no obvious change in procaspases-8 and -10, and the cleaved products have not been detected. These data indicate that FTY720 induces apoptosis

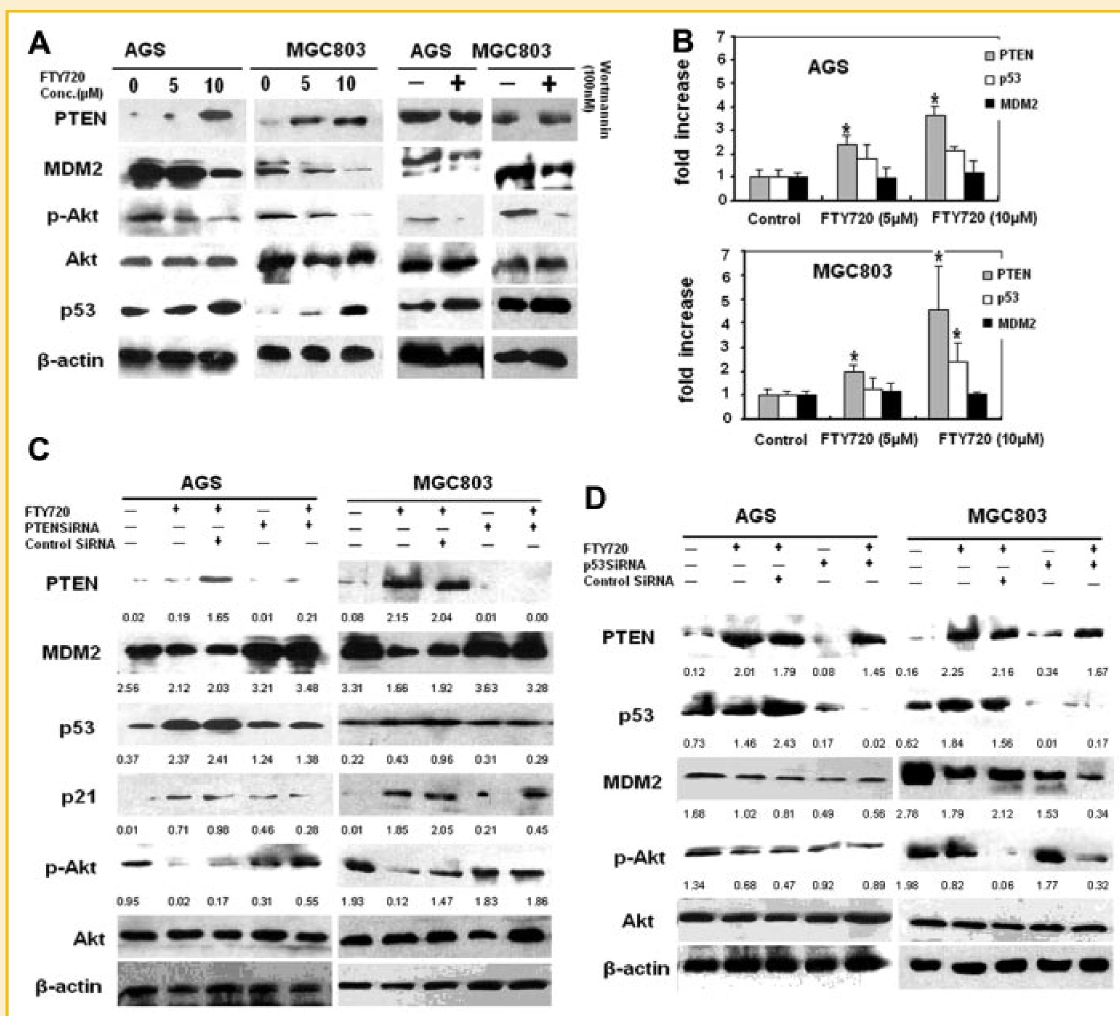

Fig. 4. A, B: The inhibition of Akt/MDM2 pathway and the increase of p53 protein mediated by FTY720-enhanced PTEN expression and PI3K inhibitor in both gastric cancer cells. (A) AGS and MGC803 cells were treated with 5 and 10 μmol/L FTY720 or Wortmannin 100 μmol/L, a PI-3K inhibitor, for 24 h, respectively. Western blot analysis was performed with antibodies recognizing human PTEN, phospho-Akt, Akt, MDM2, p53. β-actin was included as an internal control. In addition, PTEN, p53 and MDM2 were analyzed by Real-time PCR as shown in (B). Error bars represent the standard deviation of at least three independent experiments done in triplicates (*ANOVA $P < 0.05$; post hoc Student's *t*-tests, $P < 0.05$). C,D: Suppression of PTEN or p53 levels by RNA interference in FTY720-exposed gastric cancer cells. AGS and MGC803 gastric cancer cells were transfected with siRNA targeting PTEN (C), p53 (D) or negative control siRNA. AGS and MGC803 cells, PTEN/p53 siRNA-transfected cells, and negative control siRNA-transfected cells were then cultured for another 24 h in the presence or absence of FTY720 (5 μM/L). Western blot were prepared as described in Materials and Methods Section.

through the intrinsic apoptotic pathways, not the extrinsic pathway of apoptosis.

THE ACCUMULATION OF p53 THROUGH THE INHIBITION OF PI3K/Akt/MDM2 SIGNALING PATHWAY BY FTY720-INDUCED PTEN EXPRESSION

It has been reported that the PTEN can negatively regulate the PI3K/Akt-dependent cell survival [Mayo et al., 2002; Hou et al., 2008]. PTEN inhibits PI3K/Akt signaling, which promotes translocation of MDM2 in the nucleus, where it negatively regulates p53 [Mayo and Donner, 2001; Mayo et al., 2002]. As shown in Figure 4A, the levels of total Akt were comparable between the control and two FTY720-treated groups. However, obvious decrease of phospho-Akt (473) level was observed after FTY720 treatment at indicated concentrations in both gastric cancer cell lines, suggesting that the inhibition of Akt phohsphorylation might be related with the tumor cell survival. Thus, we further investigated whether or not FTY720 blocked the activation of PI3K/Akt signaling on MDM2 stabilization and promotes p53 function in gastric cancer cells. Hereby, we investigated the change of PTEN and MDM2 after FTY720 treatment. As evidenced by the Real-time PCR analysis, FTY720 resulted in the increase of PTEN mRNA level, when gastric cancer cells were exposed to different concentrations (0, 5, and 10 μM) of FTY720 as shown in Figure 4B. The induction of PTEN and p53 gene mRNA increased dramatically and moderately for periods of up to 24 h at a concentration of 10 μM respectively, however, the MDM2 mRNA almost remained unchanged. But the Western blotting data has shown that FTY720 up-regulated PTEN and p53 expression and down-regulated MDM2 expression. These data suggest that the treatment of FTY720 may result in the induction of p53 stability through PTEN/PI3K/Akt/MDM2 pathway. As p53 may induce G1 phase arrest through transcriptional activation of Cip1/p21 [Harper et al., 1993], we thus examined the effect of FTY720 on the expression of Cip1/p21 and found it was clearly induced in FTY720-treated gastric cancer cells as mentioned above (Fig. 2C). Moreover, PI3K inhibitor wortmannin induced MDM2 degradation by blocking Akt activation without regulation of PTEN expression (Fig. 4A). These results suggest that the induction of PTEN expression in FTY720-treated cells or PI3K inhibitor-treated cells induced a cell cycle arrest and apoptosis as well as the stability of p53 protein by facilitating MDM2 degradation through the inactivation of PI3K/Akt pathway.

EFFECTS OF siRNA TARGETING PTEN OR p53 ON THE GROWTH INHIBITION INDUCED BY FTY720

To evaluate the role of PTEN and p53 in the anti-proliferative response of cancer cells to FTY720, we used siRNA to knock down the expression of PTEN and p53. We have observed that siRNA knockdown of PTEN significantly attenuates the sensitivity of AGS and MGC803 cells to FTY720 (Fig. 1), including the apoptosis (Fig. 3A) and G1 phase arrest (Fig. 2B). These results suggested that up-regulation of PTEN plays a decisive role in FTY720-induced growth inhibition. In addition, we also observed siRNA p53 slightly blocks the response of AGS and MGC803 cells to FTY720 (Fig. 1), including the apoptotic response (Fig. 3A) and G1 phase arrest (Fig. 2B). These data indicated that up-regulation of p53, at least in part, accounting for the G1-cell cycle arrest and apoptosis caused by FTY720.

siRNA TARGETING PTEN GENE RESTORES INACTIVATED Akt/MDM2 PATHHWAY INDUCED BY FTY720

To investigate whether the siRNA targeting PTEN restore the inactivated PI3K/Akt pathway mediated by FTY720. Small interfering RNA (siRNA) targeting PTEN and control siRNA was utilized in FTY720-treated cells. As shown in Figure 4C, the expression of PTEN induced by FTY720 was repressed in cells transfected with siRNA targeting PTEN, compared to FTY720-exposed cells without PTEN siRNA or FTY720-treated cells transfected with control siRNA, as determined by Western blot analysis. Furthermore, the suppression of PTEN expression by PTEN siRNA in FTY720-treated cells resulted in the increase in phospho-Akt (473) compared to FTY720-treated cells without PTEN siRNA or FTY720-treated cells transfected with negative control siRNA. Moreover, PTEN siRNA restored the decreased level of MDM2 protein and increased level of p53 and Cip1/p21 protein through inhibiting the effect of PTEN on PI3K/Akt pathway modulated by FTY720.

FTY720 UP-REGULATES PTEN EXPRESSION THROUGH p53-INDEPENDENT MECHANISM

Previous studies have shown that transcriptional activation of PTEN gene is regulated by p53 protein [Chung et al., 2003]. We further investigated whether FTY720 induces p53-dependent transcription of PTEN gene in p53-silenced gastric cancer cells. As shown in Figure 4D, the level of p53 was repressed in cells transfected with siRNA targeting p53, compared to cells without p53 siRNA, or with negative control siRNA. However, FTY720 up-regulated PTEN expression in gastric cancer cells after p53 siRNA, these results might suggest that proliferation of gastric cancer cells was modulated by FTY720-stimulated PTEN expression through p53-independent mechanism.

FTY720 SENSITIZES GASTRIC CANCER CELLS TO CISPLATIN

Previous studies have shown that phospho-Akt might play certain roles in multi-drug resistance (MDR) of gastric cancer through P-glycoprotein signaling pathways [Han et al., 2007]. As we have shown that FTY720 induced a marked expression of PTEN, which blocked PI3K/Akt/MDM2 pathway, and then up-regulate p53. Considering this point, we further investigated the ability of low concentrations FTY720 treatment to synergize with Cisplatin to inhibit gastric cancer cell growth and cell survival. AGS and MGC803 cells were treated with increasing concentrations of Cisplatin in the absence or presence of 2 μM FTY720 and cell proliferation was determined 24 h later using CCK-8 assay. The combined treatment with Cisplatin and FTY720 produced profound decrease in cell growth (Fig. 5A) and induced higher apoptosis (Fig. 5B) in both cell lines, demonstrating that FTY720 enhances the cytotoxicity of Cisplatin in gastric cancer cells. It has been shown that hyperactivation of Akt exerts anti-apoptotic effects through phosphorylation of substrates that indirectly inhibit apoptosis, such as MDM2 [Zhou et al., 2001]. Also in the Western blotting results (Fig. 5C), Cisplatin was observed to induce up-regulation of phospho-Akt and MDM2. However, induced expression of PTEN

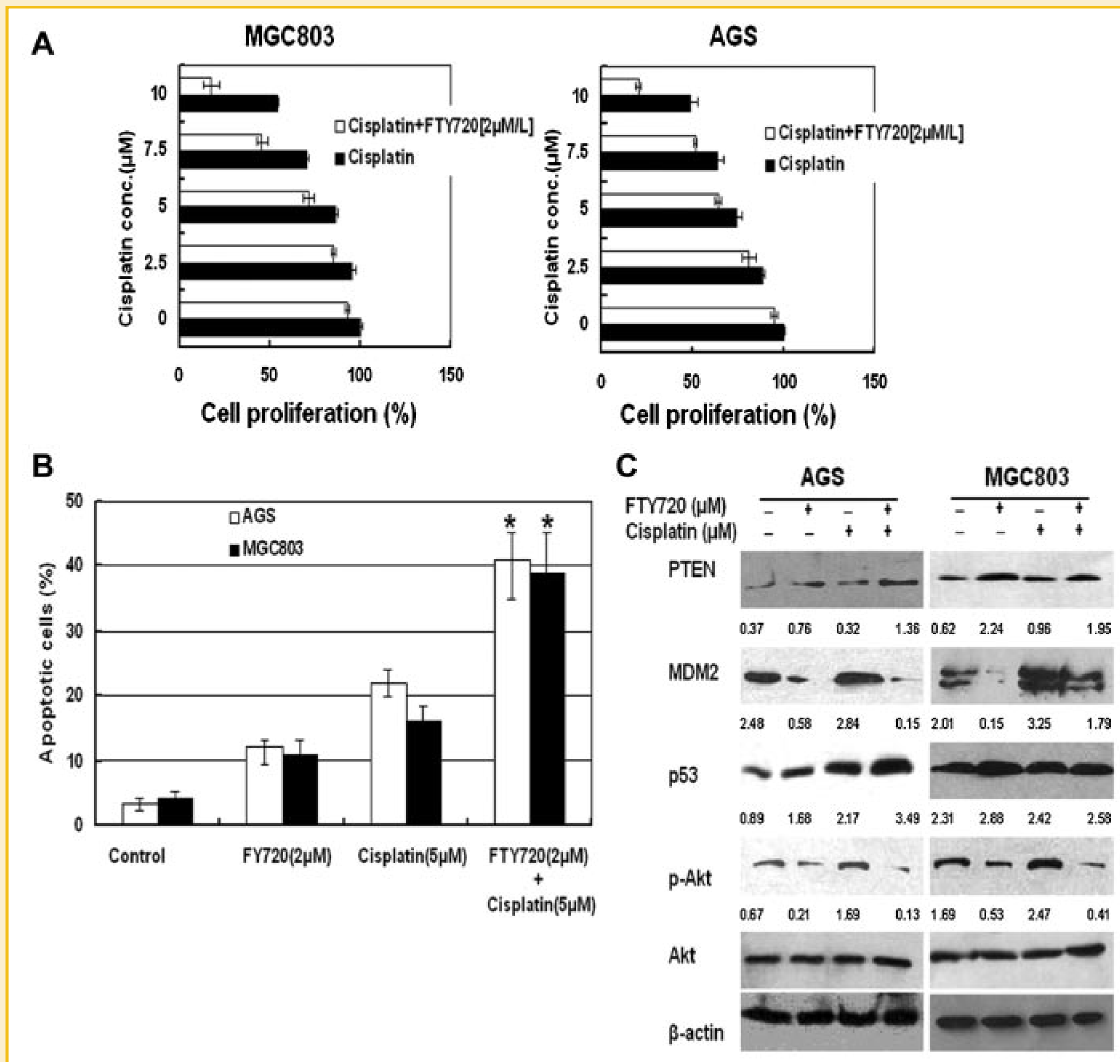

Fig. 5. FTY720 sensitized gastric cancer cells to Cisplatin by promoting growth inhibition and apoptosis. A: AGS and MGC803 cells were treated with the indicated concentrations of Cisplatin in the absence or presence of 2 μM/L FTY720. Cell growth was determined using CCK-8 assay 24 h after drug treatment. Cell growth was determined by measuring the percentage change in growth compared to mock-treated controls. Data represent means ± standard error (n = 3). B: FTY720 sensitizes gastric cancer cells to Cisplatin-induced apoptosis. Cells were treated with 5 μM/L Cisplatin, 2 μM/L FTY720 or both drugs for 48 h. Cells were analyzed for the onset of apoptosis using propidium iodide staining and FACS analysis (*ANOVA $P < 0.05$; post hoc Student's *t*-tests, $P < 0.05$ vs. untreated control) C: Western blot analysis of MGC803 and AGS cells treated with the indicated concentrations of FTY720, Cisplatin or both. Twenty-four hours post-treatment, cells were harvested and protein expression was determined by Western blot with the indicated antibodies.

by FTY720 could down-regulated the basal and Cisplatin-induced phospho-Akt and MDM2.

FTY720 INHIBITS IN VIVO TUMOR GROWTH

We examined the ability of FTY720 to suppress the growth of human gastric cancer cell xenografts in nude mice. The time course of tumor growth (V_t/V_0) is shown in Figure 6A. In general, the tumors in control group grew continuously during the experimental period whereas the tumor growth in the FTY720-treated mice was suppressed significantly. Ki-67 staining for cell proliferation was performed in tumors removed from the animals on day 21. The relative number of Ki-67 positive tumor cells was substantially less in tumors from mice treated with FTY720, when compared with control tumors (Fig. 6E,F). The apoptotic index was markedly increased in that of FTY720 group by TUNEL analysis (Fig. 6C,D). In addition, the body weight of mice from treated group was similar to the control group (Fig. 6B). These data imply that FTY720 is a potential therapeutic treatment of gastric cancers and that it is relatively non-toxic to nude mice.

DISCUSSION

Chemotherapy is important for advanced gastric cancer as for improving overall survival rate and quality of life for patients [Cunningham et al., 2006; Rivera et al., 2007]. However, therapies fail because of MDR either intrinsic or acquired after an initial round of treatment [Fan et al., 2005]. For this reason, more favorable

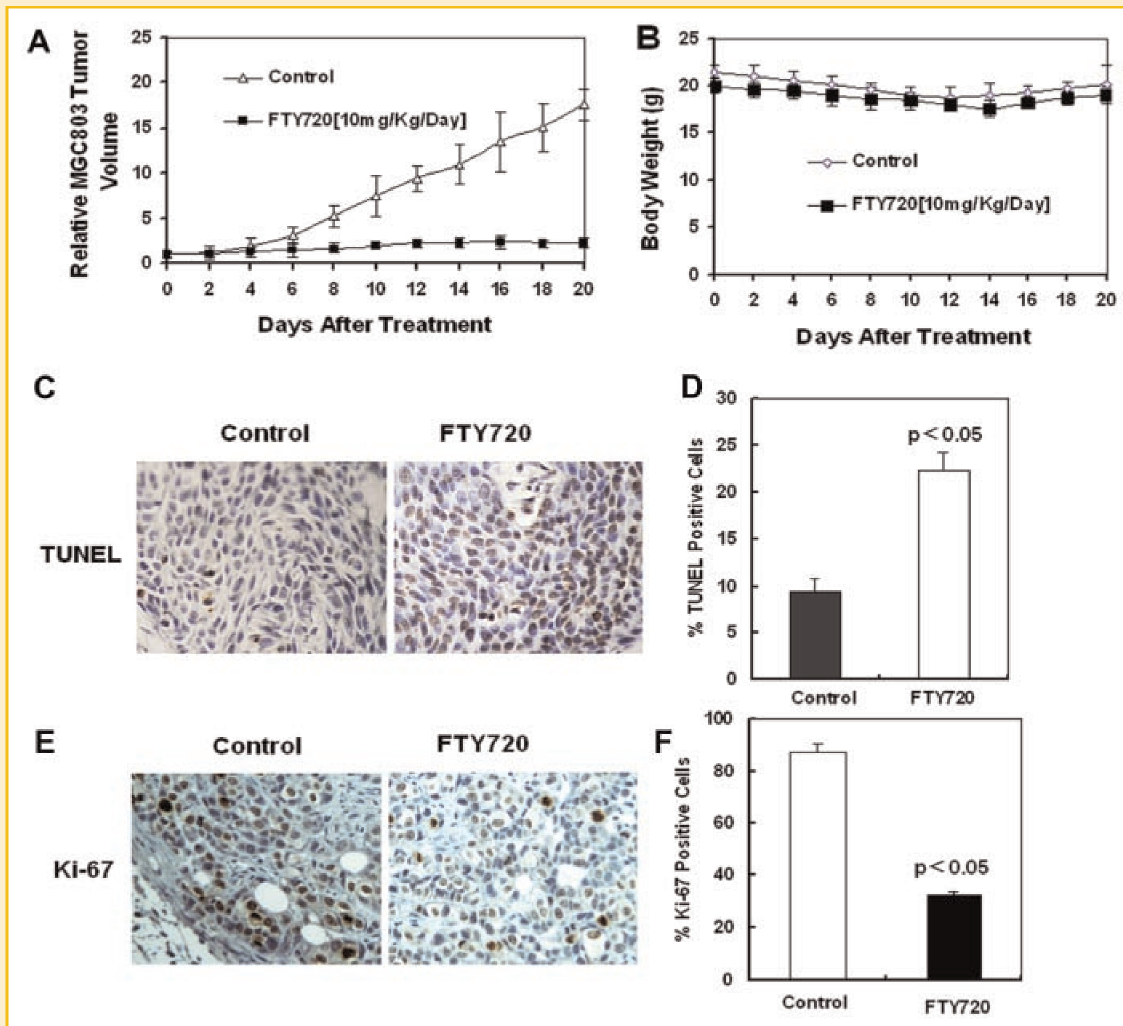

Fig. 6. In vivo effect of FTY720 on MGC803 gastric carcinoma xenografts grown in nude mice. A: Relative MGC803 derived tumor volumes of the nude mice treated with FTY720 and the controls. Tumor volumes were measured and transformed subsequently to relative tumor volume as detailed in Material and Methods Section. Relative tumor volume is shown as mean ± SEM. B: The graph showed the body weight of the animals in MGC803 derived tumors in the control and treatment groups throughout the treatment period. C: TUNEL-positive cells were rarely found in tumor sections from control mice, whereas many positive cells with marked nuclear fragmentation were present in the sections from mice treated with FTY720. D: Comparison of TUNEL-positive cells in FTY720-treated (open column) and the control (solid column) groups. E: Representative results of Ki-67 staining in the treated (panel 2) and the control (panel 1) tumors. F: Comparison in percentage of Ki-67-positive cells from the FTY720-treated group (solid column) and the controls (open column). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

agents for the treatment of gastric cancer are required. FTY720 is a chemical substance derived by modifying an immunosuppressive metabolite and has been shown to possess anti-cancer properties in various types of cancer [Liu et al., 2008]. Herein, we have demonstrated that FTY720 induces apoptosis and cell cycle arrest of gastric cancer cells and inhibits in vivo tumor growth in the nude mice model without notable side-effects. Additionally, the effect of FTY720 was augmented when used in conjunction with Cisplatin partly through PTEN-mediated inhibition of the PI3K/Akt/MDM2 survival signaling.

Our observation of decreases in cyclin D1, cyclin E, CDK2, CDK4, and E2F1 may explain the observed disruption of cell cycle progression and provide a mechanism by which FTY720 induces G1 phase arrest. The results show that the cell cycle arrest is mediated through the up-regulation of Cip1/p21 and Kip1/p27 proteins, which enhances the formation of complexes with the G1-S CDKS and cyclins, thereby inhibiting their activities [LaBaer et al., 1997]. As mentioned above, the up-regulation of Cip1/p21 might be due to the accumulation of p53, while the up-regulation of protein p27kip1 might be regulated directly by Akt dephosphorylation or indirectly by FKHR, which plays a substantial role in cell cycle progression by inhibition of p27kip1 transcription through PI3K-induced FKHR-L1 phosphorylation as reported in earlier researches [Lee et al., 2004]. It is important to elucidate the mechanisms by which FTY720 induces apoptosis in gastric cancer cells, particularly when in adjunction with other agents. Tumor suppressor p53 plays a legitimate role in regulating the response of cancer cells to stresses and damage [Wang et al., 2000]. Previous works have identified phosphorylation of serine 166 and 186 in the domain of MDM2 by Akt is obligate for translocation of MDM2 from the cytoplasm into the nucleus, where

it regulates the activity of p53 protein [Mayo and Donner, 2001], while PTEN can inhibit PI3K/Akt signaling, which could promote translocation of MDM2 into the nucleus [Mayo and Donner, 2002]. This ability of PTEN increases the cellular content and transactivation of the p53 [Mayo and Donner, 2001]. In this study, our Western blotting results have shown remarkable decrease of phospho-Akt level after FTY720 treatment and the level of p53 is increased in a concentration-dependent manner after FTY720 treatment; the expression of PTEN induced by FTY720 is effective with respect to p53 accumulation for cell cycle arrest and apoptosis by inactivating PI3K/Akt/MDM2. As the results have indicated, FTY720 treatment increased p53 and led to the up-regulation of the classic p53 target-Cip1/p21. This was accompanied by the up-regulation of the BH3-only proteins PUMA and NOXA and the activation of the intrinsic apoptotic pathway as evidenced by the increase of cleaved caspases-3 and -9, supporting the role of caspase in the FTY720-induced apoptosis. We also have found an increase in the expression of Bax protein and a decrease in the expression of Bcl-2 in gastric cancer cells. An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome and the cytosolic cytochrome c then binds to Apaf-1, leading to the activation of caspase-3 and PARP [Bossy-Wetzel and Green, 1999]. However, as per the previous studies are concerned, it is noteworthy that the mode of anti-tumor action of FTY720 may vary in different types of cancers [Schmid et al., 2007; Hung et al., 2008; Chua et al., 2009]. For instance, in prostate cancer cells, it was recently found that FTY720 treatment down-regulated Runx2 expression and its transcriptional activity, as well as inhibited its regulated downstream events [Chua et al., 2009], while in hematologic malignant cells, FTY720-induced apoptosis by activating PP2A signaling [Neviani et al., 2007]. A more recent study suggested that FTY720 induces apoptosis in hepatocellular carcinoma cells through activation of protein kinase C [Hung et al., 2008], as previous reports have found that PTEN could control the phosphorylation of protein kinase C [Parekh et al., 2000]. Therefore, in the future study, we hypothesize that it would also be worthy and interesting to investigate whether the induced expression of PTEN by FTY720 would adapt a similar mechanism in regulating the phosphorylation of protein kinase C in gastric cancer cells. The ability of FTY720 to target different clinically relevant signaling pathway mechanisms in different cancers might have therapeutic relevance in cancer treatment.

Earlier studies have indicated that chemotherapy reagents might activate Akt pathway and decreased the chemotherapy sensitivity of gastric cancer cell lines via suppressing the expression of p53 [Liu et al., 2006]. As we have shown that FTY720 induces a marked expression of PTEN, which blocks PI3K/Akt/MDM2. We next hypothesized that using FTY720 in conjunction with Cisplatin, which activate PI3K/Akt signaling pathway would overcome the chemo resistance conferred by Akt phosphorylation. As is shown in Figure 5, our CCK-8, apoptosis assays and the Western blotting results have confirmed our hypothesis that FTY720 certainly enhances gastric cancer cell death induced by Cisplatin.

In conclusion, we have provided two major findings in the current investigation: the first being the in vivo and in vitro anti-cancer effects of FTY720 on human gastric cancer and the second being PTEN identified as a potential therapeutic target and the possible involvement of the network of PTEN, Akt, MDM2, and p53 in the therapeutic effect of FTY720 on gastric cancer. The results of this study could have implications for incorporation of FTY720 into the prevention/therapeutic strategies against gastric and other cancers.

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