Depletion of PI3K p85α induces cell cycle arrest and apoptosis in colorectal cancer cells

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Abstract. Colorectal cancer is one of the most common malignancies in the world. Overactivity of phosphatidylinositol 3-kinase (PI3K) is frequently detected in colorectal carcinoma. PI3K signaling plays a pivotal role in intracellular signal transduction pathways involved in cell growth, cellular transformation, and tumorigenesis. To specifically inhibit PI3K activity in colorectal cancer cells, we constructed a siRNA against the PI3K regulatory subunit p85α and transfected it into LoVo and SW480 cells. In the present study, treatment of colorectal cancer cells with PI3K p85α-specific siRNA inhibited cell proliferation, induced G1 phase cell cycle arrest and sensitized colorectal cancer cells to 5-FU-induced apoptosis. Furthermore, depletion of PI3K p85α resulted in significant activation of three Forkhead box class O (FoxO) transcription factors, which inhibited the expression of cyclin D1, cdk4 and induced expression of p27/Kip1. Activation of FoxO transcription factors also increased the expression of FasL. Thus, our results indicate that siRNA-mediated gene silencing of PI3K p85α may be a useful therapeutic strategy for colorectal carcinoma.

Introduction

Colorectal cancer has the third highest incidence rate of malignant tumors in the world, and its associated morbidity and mortality have increased over the years. Conventional chemotherapy regimens for the treatment of colorectal cancer have limited efficacy and are associated with significant toxicity (1). Therefore, increasing knowledge on the genetic control of cellular proliferation and modulation of signaling pathways that are aberrant in colorectal cancer have the potential to provide an effective and improved approach for its management (2).

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases involved in tyrosine kinase receptor-mediated signal transduction and comprise a complex family with three classes,

each with multiple subunits and isoforms (3). The class I PI3Ks are heterodimers composed of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110). The regulatory p85 subunit is essential for the stability of the p110 catalytic subunit and for its recruitment to activated growth factor receptors (4). The regulatory subunits p85α, p50α and p55α are encoded by the *PIK3R1* gene, p85α is the most abundantly expressed regulatory isoform of PI3K, and p55α and p50α are two additional minor alternative splicing isoforms (5). Class I PI3Ks play important roles in multiple cellular processes, such as mediating survival, apoptosis, mitogenic responses, insulin signalling and cell motility (6).

In addition to the regulation of normal cell processes, the promotion of cell survival by the activation of PI3K occurs by the inhibition of proapoptotic signals and the induction of survival signals, which contribute to the malignant transformation and tumor progression (7). Disturbances in the activation of PI3K signaling are associated with many cancers. In this regard, there is growing body of evidence to support the notion that the activation of PI3K/Akt is associated with colorectal tumors (8,9), and that class I PI3Ks are expressed in colonic epithelial carcinoma cell lines (10). They are therefore attractive cancer therapeutic targets.

In a previous study (11), we demonstrated by immohisto-chemical analysis that the expression level of PI3K p85α gradually increased when comparing normal colorectal tissue to colorectal adenoma and primary colorectal carcinoma. In this study, we investigated whether inhibition of PI3K p85α by siRNA induces anti-oncogenic properties in human colorectal carcinoma cells. Here, we show that depletion of PI3K p85α significantly inhibited cell proliferation, induced cell cycle arrest, and sensitized colorectal cancer cells to 5-FU-induced apoptosis. We have demonstrated that depletion of PI3K p85α activated three FoxO transcription factors in colorectal cancer cells, which regulated the expression of cell cycle-associated proteins and apoptosis-associated protein. These results suggest that RNAi directed against PI3K p85α might be a potential therapeutic strategy for colorectal carcinoma.

Materials and methods

Cell culture. The human colorectal cancer cell lines LoVo and SW480 were grown in RPMI-1640 supplemented with 10% fetal bovine serum under standard cell culture conditions (37°C, 5% CO_2 in a humidified incubator).

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siRNA and plasmid constructs. Oligonucleotides encoding 21-mer hairpin sequences were designed, and the sequences of small interfering RNA constructs targeting PI3K p85α were as follows: GCUCAGUAUAAUCCCAAAUUG (sense) and CAAUUUGGGAUUAUACUGAGC (antisense). A scrambled siRNA containing the same nucleotide composition as the p85α siRNA but lacking significant sequence homology to the genome was also designed as a control. The oligonucleotides were synthesized, annealed and ligated into the linearized pGPU6/Neo siRNA Expression Vector (GenePharma, China).

Selecting a population of cells that stably express siRNA. LoVo and SW480 cells were seeded in 24-well plates at a density of $5x10^4$ per well. Twenty-four hours later, 1 μg of p85α siRNA plasmid or control siRNA plasmid was transfected into colorectal cancer cells using Lipofectamine 2000 (Invitrogen, USA), and cultured for an additional 24 h without antibiotic selection. The cells were then cultured in medium containing 1000 μg/ml G418 (Merck, USA) until all of the cells in the non-transfected control culture were killed and antibiotic-resistant clones were formed in the transfected cells. The antibiotic-resistant cells were pooled and passaged in medium containing 500 μg/ml G418 as needed.

Cell proliferation assays. Cell proliferation was determined using the CCCK-8 solution (Beyotime Inst Biotech, China) according to the manufacturer's instructions. Briefly, $4x10^3$ cells/well were seeded in 96-well plates and cultured for 24, 48, 72 or 96 h. Subsequently, 10 μl CCK-8 solution was added to each well. The cells were then incubated at 37˚C for 2 h and the absorbance was finally determined at 450 nm using a spectrophotometer (MDC, USA).

Cell cycle analysis. The cells were trypsinized and pelleted by centrifugation. After washing the pellet with PBS, the cells were counted, and $1x10^6$ cells were fixed in 70% ethanol at -20˚C for 24 h. The fixed cells were then washed with PBS, and incubated with RNase A (0.25 mg/ml) for 30 min at 37˚C, and 5 μl of propidium iodide (KeyGen, China) was then added to the cell suspension. The mixture was incubated at RT for 30 min in the dark. The suspended cells were analyzed for cell cycle using the FACSCalibur Flow Cytometer (BD, USA).

Apoptosis assays. After stable transfection, LoVo and SW480 cells were treated with 5-FU for 48 h at final concentrations of 0.01 and 0.03 μmol/ml, respectively. Cells were then trypsinized and washed twice with PBS. A total of $5x10^5$ cells were resuspended in 500 μl of binding buffer (KeyGen). Five microliters of Annexin V-FITC (KeyGen) was then added to the cell suspension, which was counterstained with 5 μl of propidium iodide. The mixture was incubated at RT for 10 min in the dark, followed by apoptosis analysis using the FACSCalibur Flow Cytometer.

Preparation of protein lysates
Total protein lysate. Colorectal cancer cells stably expressing p85α siRNA or control siRNA were seeded in 60-mm dishes at a density of $1x10^5$ cells/ml. They were cultured until they reached 80% confluence. After removal of the medium, the cells were washed twice with ice-cold PBS, followed by a wash with ice-cold cell lysis buffer (Beyotime Inst Biotech), which also contained freshly added 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 1 mM NaSVO$_4$ (Sigma, USA), and 1 mM NaF (Sigma). The lysates were collected by scraping from the plates and centrifuging at 10000 x g for 5 min at 4˚C. The supernatant, which contained the total cell lysate, was either used immediately or stored at -70˚C.

Nuclear and cytoplasmic protein lysates. The Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Inst Biotech) was used according to the manufacturer's instructions. Briefly, cells were washed with ice-cold PBS and pelleted by centrifugation. Three hundred microliters of ice-cold cytoplasmic lysis buffer containing freshly added 1 mM PMSF, 1 mM NaSVO$_4$ and 1 mM NaF was then added to the pellet. The pellet was incubated on ice for 15 min with occasional vortexing. The cell lysates were centrifuged at 15000 x g for 5 min at 4˚C, and the supernatant, which contained the cytoplasmic protein lysate, was stored at -70˚C until needed for Western blot analysis. The cell pellet was resuspended in ice-cold nuclear lysis buffer containing freshly added 1 mM PMSF. The pellet was incubated on ice for 30 min with vigorous shaking. The cell lysates were centrifuged at 15000 x g for 10 min at 4˚C, and the supernatant, which contained the nuclear protein lysate, was either used immediately or stored at -70˚C.

Western blot analysis. The protein concentration was determined using the BCA Protein Assay Kit (Beyotime Inst Biotech). Either 30 or 50 μg of protein was separated by electrophoresis in 8-15% SDS-PAGE gels and transferred to PVDF membranes by semi-dry transfer. The membranes were blocked in 5% BSA or 5% non-fat milk in PBS-T containing 0.05% Tween-20. The membranes were then probed with the appropriate primary antibody, followed by a secondary horseradish peroxidase-conjugated antibody. The bands on the membrane specific for the protein of interest were developed with ECL solution (Pierce, USA). The following antibodies were used: primary antibodies against PI3K p85α, cyclin D1, cdk4 and ß-actin were purchased from Santa Cruz Biotechnology (USA); primary antibodies against Akt, Phospho-Akt (Ser473), FoxO1 (FKHR), Phospho-FoxO1 (FKHR, Ser256), FoxO3a (FKHRL1), Phospho-FoxO3a (FKHRL1, Ser253), FoxO4 (AFX), Phospho-FoxO4 (AFX, Ser193), p27/Kip1, FasL and Histone H3 were purchased from Cell Signaling Technology (USA). Quantification of the protein within the bands of interest was performed using Image-pro plus 6.0 software.

Statistical analyses. All experiments were performed three separate times. Data are expressed as means ± SD. Statistical analyses were carried out using the Student's t-test. P<0.05 was considered as statistically significant.

Results

Suppression of PI3K p85α overexpression by RNA interference. After p85α siRNA stable cell lines were established,
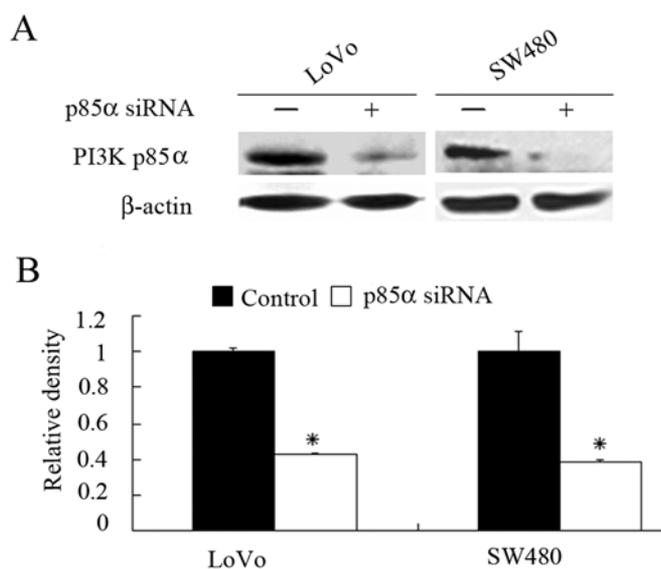
Figure 1. RNA interference effectively decreased PI3K p85α protein expression. (A) Following stable transfection, whole cell extracts from LoVo and SW480 cells were analyzed by Western blotting PI3K p85α protein. The corresponding ß-actin levels were used as loading controls. (B) Quantification of immunoblot data from at least three experiments, the amount of each protein was calculated relative to ß-actin and normalized to control cells. Columns, relative mean density; bars, +SD. *P<0.05, compared to those of control cells.

Figure 2. Down-regulation of PI3K p85α inhibited the proliferation of colorectal cancer cells. LoVo (A) and SW480 (B) cells expressing p85α siRNA or control siRNA were seeded and cultured. Cell proliferation was determined by the CCK-8 assay. The absorbance of cells at 450 nm was normalized to that in the control at 0 h. Proliferation assays were performed in triplicate. Columns, relative mean absorbance; bars, +SD. *P<0.05, compared to those of control cells.

the interference effect on p85α expression levels was evaluated by Western blot analysis. As shown in Fig. 1, the protein levels of PI3K p85α were significantly decreased in LoVo and SW480 cells transfected with p85α siRNA, compared to those of control cells. These results indicate that the siRNA could effectively suppress PI3K p85α protein overexpression.

Knockdown of PI3K p85α inhibited cell proliferation. To determine the effect of p85α knockdown on proliferation of colorectal cancer cells, the CCK-8 cell proliferation assay was performed. LoVo and SW480 cells that express either p85α siRNA or control siRNA were seeded and cultured until CCK-8 detection. As shown in Fig. 2, cells expressing the control siRNA grew very quickly, at a rate of 1.56- to 5.48-fold that of the 0 h control cells. In contrast, cells expressing the p85α siRNA exhibited a much slower proliferation rate, which was 1.13- to 2.66-fold that of 0 h control cells. These results indicate that PI3K p85α plays an important role in the proliferation of colorectal cancer cells.

Depletion of PI3K p85α induced cell cycle arrest. To examine if siRNA against p85α has an impact on cell cycle of colorectal cancer cells, flow cytometry analyses were performed, where the results showed that depletion of p85α caused a significant increase in the proportion (from 44.67 to 62.35%) of LoVo and SW480 cells at the G1 phase (Fig. 3A and B). The observed increase in G1 cell population in LoVo and SW480 cells was accompanied by a reduction (from 40.79 to 23.89%) of cells in the S phase (Fig. 3A and C). These findings suggest that depletion of PI3K p85α could obviously induce colorectal cancer cell cycle arrest.

PI3K p85α knockdown sensitized colorectal cancer cells to 5-FU-induced apoptosis. In order to evaluate the effect of p85α knockdown on induction of apoptosis, LoVo and SW480 cells were treated, after stable transfection, with 5-FU at final concentrations of 0.01 and 0.03 µmol/ml, respectively, for 48 h. The early apoptotic rate was then assessed using the Annexin V-FITC kit by means of flow cytometry analysis. As shown in Fig. 4, the proportion of cells that were positive for Annexin V was about 7-fold higher in p85α knockdown cells than in control cells. These results indicate that knockdown of PI3K p85α sensitized the colorectal cancer cells to 5-FU.

PI3K p85α depletion activated FoxO transcription factors associated with regulating cell cycle-associated protein and apoptosis-associated protein expression. To further explore the mechanism that may be involved in the enhancement of cell cycle arrest and 5-FU-induced apoptosis by silencing p85α in LoVo and SW480 cells, we examined the expression levels of Akt, phospho-Akt, and three Forkhead box class O (FoxO) transcription factors in the nucleus and cytoplasm. Our results showed that p85α depletion led to a substantial reduction in the levels of Akt and phospho-Akt (Fig. 5). Consistent with this reduction in the level of phospho-Akt, Western blots revealed a significant decrease in the expression of phospho-FoxO1 (FKHR), phospho-FoxO3a (FKHRL1) and phospho-FoxO4 (AFX) in cytoplasm of p85α-knockdown cells (Fig. 6A and C). Prominent accumulation of FoxO1 (FKHR), FoxO3a (FKHRL1) and FoxO4 (AFX) in the nucleus
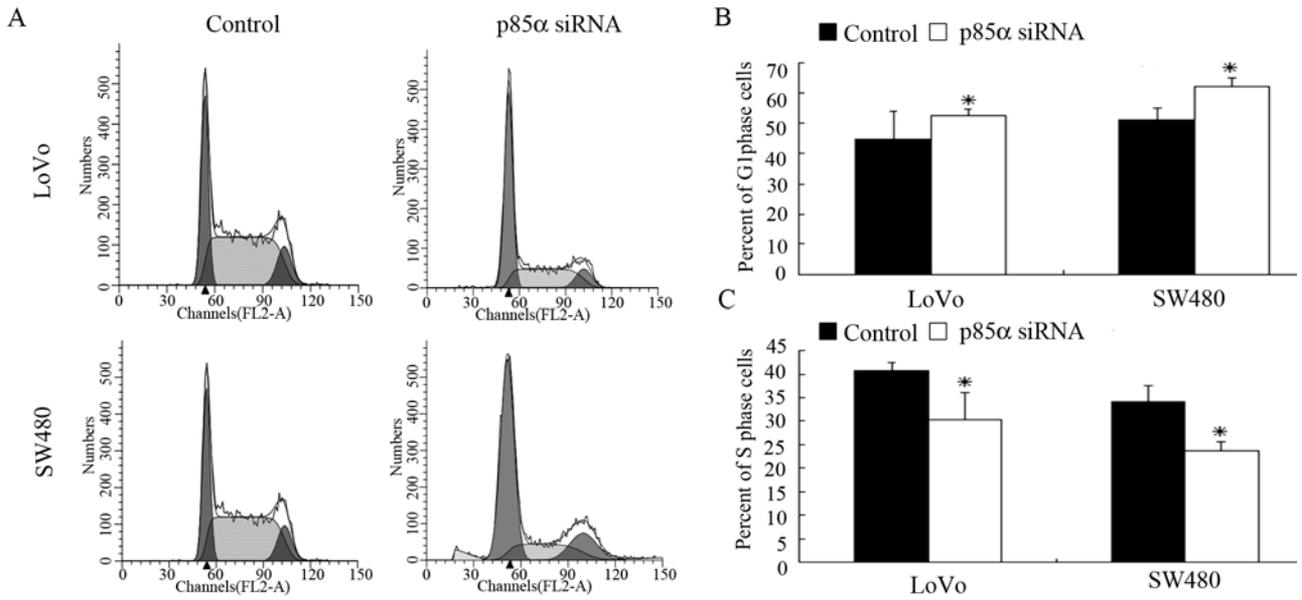
Figure 3. Depletion of PI3K p85α induced cell cycle arrest. (A) Flow cytometry analysis profiles revealed the positions of G1, S and G2/M phases of LoVo and SW480 cells after stable transfection. (B, C) Computational analysis of the cell population in G1 and S phase. Flow cytometry analyses were performed in triplicate. Columns, means; bars, +SD. *P<0.05, compared to those of control cells.
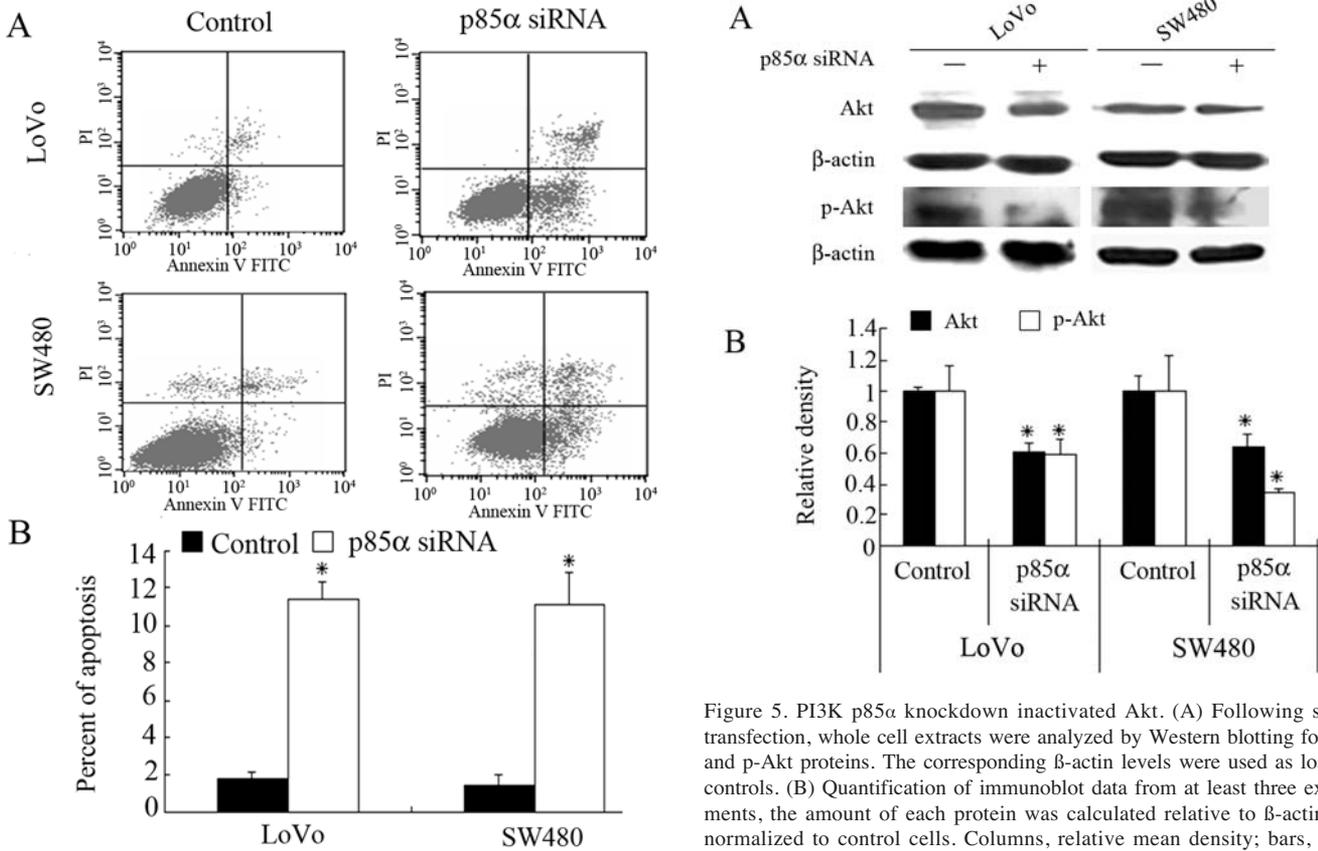
Figure 4. Depletion of PI3K p85α enhanced 5-FU-induced apoptosis. (A) Following stable transfection, the extent of 5-FU-induced apoptosis was evaluated by Annexin V staining and flow cytometry analysis. (B) Computational analysis of the apoptosis rate. Flow cytometry analyses were performed in triplicate. Columns, means; bars, +SD. *P<0.05, compared to those of control cells.
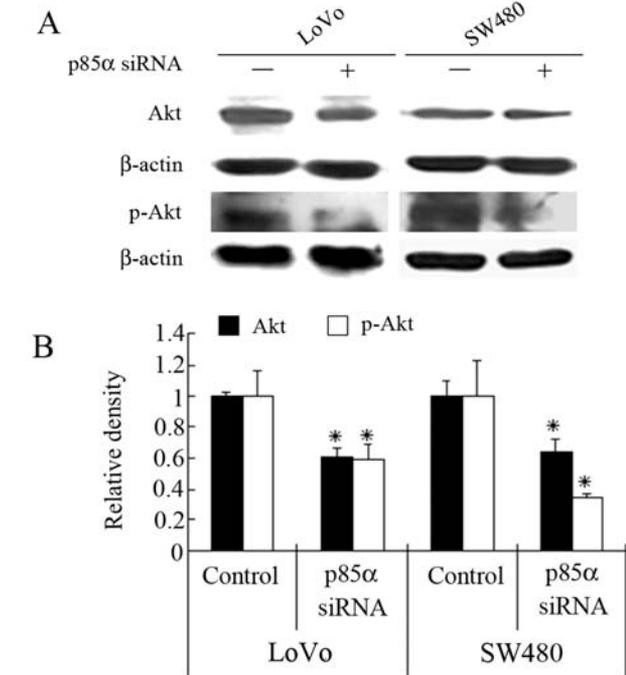
Figure 5. PI3K p85α knockdown inactivated Akt. (A) Following stable transfection, whole cell extracts were analyzed by Western blotting for Akt and p-Akt proteins. The corresponding ß-actin levels were used as loading controls. (B) Quantification of immunoblot data from at least three experiments, the amount of each protein was calculated relative to ß-actin and normalized to control cells. Columns, relative mean density; bars, +SD. *P<0.05, compared to those of control cells.

was simultaneously observed (Fig. 6A and B). Because the potential downstream targets of FoxO transcription factors are cell cycle-associated proteins and apoptosis-associated
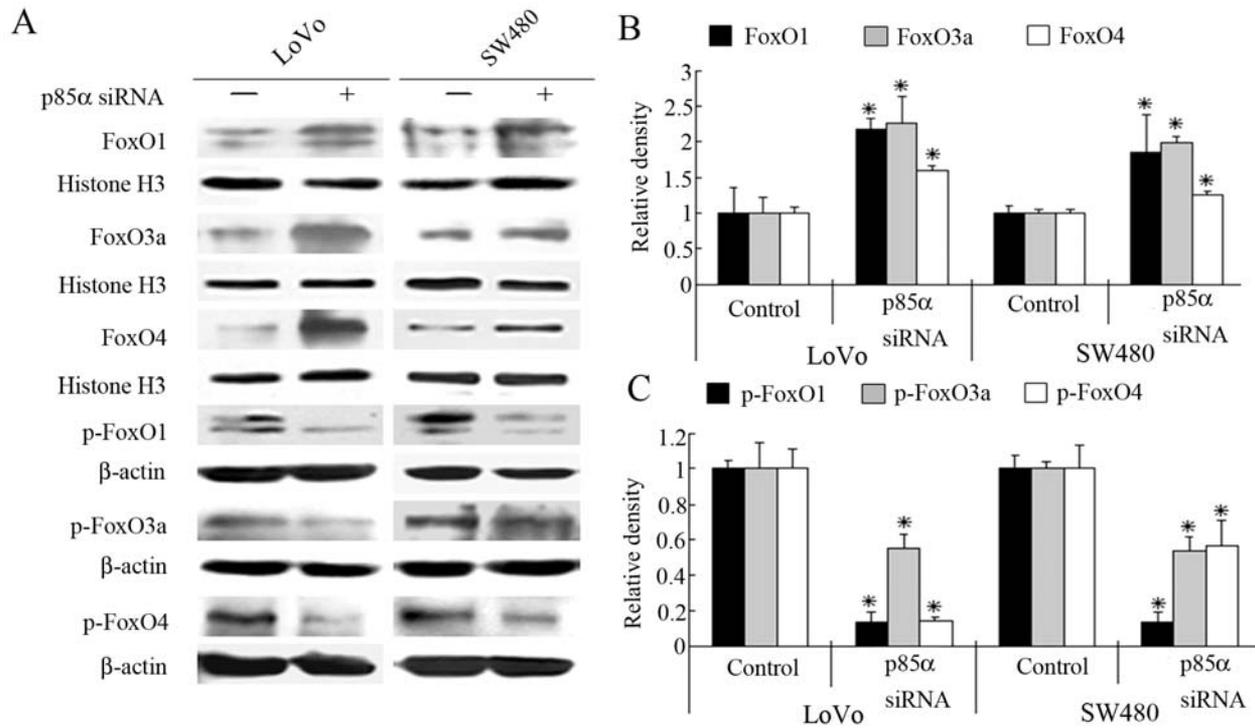
Figure 6. PI3K p85α knockdown activated FoxO transcription factors. (A) Following stable transfection, nuclear extracts and cytoplasmic extracts were analyzed by Western blots for FoxO proteins and phosphorylated FoxO proteins, respectively. The corresponding Histone H3 and ß-actin levels were used as loading controls. (B) Quantification of immunoblot data from at least three experiments, the amount of nuclear protein was calculated relative to Histone H3 and normalized to control cells, the amount of cytoplasmic protein was calculated relative to ß-actin and normalized to control cells. Columns, relative mean density; bars, +SD. *P<0.05, compared to those of control cells.
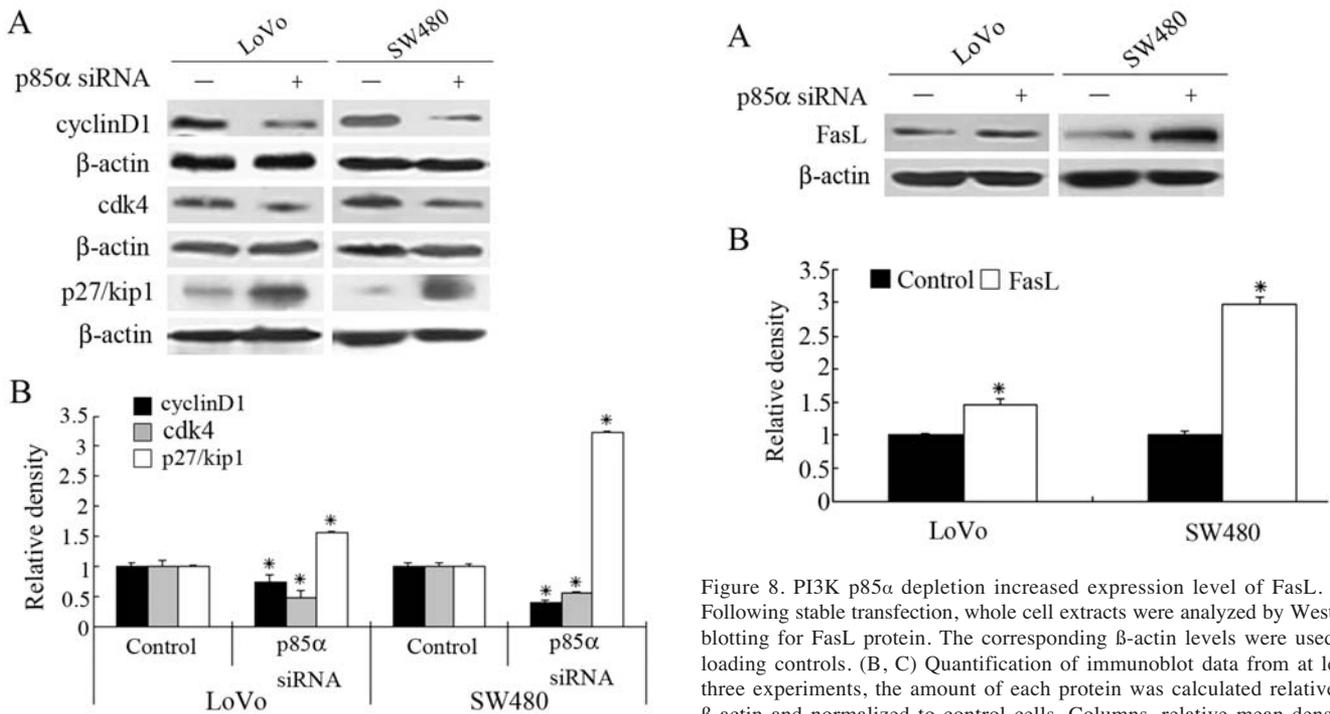
Figure 7. PI3K p85α depletion led to a change in cell cycle regulatory proteins. (A) Following stable transfection, whole cell extracts were analyzed by Western blotting for cyclin D1, cdk4 and p27/Kip1 proteins. The corresponding ß-actin levels were used as loading controls. (B, C) Quantification of immunoblot data from at least three experiments, the amount of each protein was calculated relative to ß-actin and normalized to control cells. Columns, relative mean density; bars, +SD. *P<0.05, compared to those of control cells.

Figure 8. PI3K p85α depletion increased expression level of FasL. (A) Following stable transfection, whole cell extracts were analyzed by Western blotting for FasL protein. The corresponding ß-actin levels were used as loading controls. (B, C) Quantification of immunoblot data from at least three experiments, the amount of each protein was calculated relative to ß-actin and normalized to control cells. Columns, relative mean density; bars, +SD. *P<0.05, compared to those of control cells.

proteins, the expression levels of cyclin D1, cdk4, p27/Kip1 and FasL were also determined. As shown in Fig. 7, the expression levels of cyclin D1 and cdk4 were significantly decreased in p85α depleted cells, while that of the cyclin

kinase inhibitor p27/Kip1 and FasL (Fig. 8) were markedly increased.

Discussion

Aberrant activation of PI3K/Akt pathway components has been observed in many cancers. Akt is overexpressed in a number of cancers, including colon, pancreatic, ovarian, and some steroid hormone-insensitive breast cancers (12,13). Loss of phosphatidylinositol 3-kinase and phosphatase and tensin homologue (PTEN) function, due to somatic deletions or mutations of this tumor suppressor gene, are frequently detected in many human cancers (14,15). The gene coding for p110α (*PIK3CA*) is amplified in ovarian and breast tumors (16), *PIK3CA* has somatic mutations in several other cancers, including 32% of colorectal cancers, 27% of glioblastomas, 25% of gastric cancers (17), implicating *PIK3CA* as a potential oncogene in these cancers. An oncogenic mutated form of PI3K p85α has also been described (18). Recent studies have also shown that p85α forms a complex with a protein network that is associated with oncogenic fusion tyrosine kinases (FTKs), resulting in constitutive activation of the p110 catalytic subunit of PI3K (19). Therefore, PI3K inhibitors could potentially provide useful information about the role of the PI3K/Akt pathway in human cancers. However, this pathway regulates a variety of cellular processes, some of which are necessary to maintain normal physiological functions. The most commonly used broad-spectrum PI3K inhibitors, LY294002 and wortmannin, may therefore potentially contribute to the undesirable side-effects.

Recent progress in the siRNA technique has demonstrated that it is an efficient method for silencing specific genes (20). For siRNA to be applied therapeutically, however, several problems need to be solved, including the delivery method and the short duration of siRNA-mediated silencing. Despite these problems, siRNA remains a potentially promising technique for the treatment of human cancer, especially given that it can be more selective and less toxic than traditional approaches. In our study, siRNA-mediated targeted depletion of PI3K p85α resulted in significant inhibition of PI3K p85α protein levels in LoVo and SW480 cells.

PI3K activation is required for cancer progression because it promotes tumor growth through cell proliferation and cell cycle progression (21). As shown in the CCK-8 assays, we observed a significant decrease in cell proliferation in both colorectal carcinoma cell lines after treatment with p85α siRNA, which demonstrated that inhibition of PI3K p85α can affect tumor cell growth. Given that another cause of tumorigenesis is uncontrolled cell division, we also wanted to prove that p85α inhibition could affect cell proliferation through cell cycle regulation. Flow cytometry analyses showed that depletion of p85α resulted in a significant increase in the accumulation of LoVo and SW480 cells in the G1 phase of the cell cycle. The observed increase in the number of colorectal carcinoma cells that were in the G1 was accompanied by a reduction in the number of cancer cells in S phase. These results are consistent with observations in breast carcinoma cells and consistent with studies that involved the treatment of breast carcinoma BT-474 and osteosarcoma Saos-2 cells with PI3K inhibitor LY294002 (22-24).

One important function of activated PI3K in cells is inhibition of apoptosis. PI3K inhibition may be associated with an induction of apoptosis in cancer cells (25,26). Given that 5-FU is one of the most widely used agents in colorectal cancer chemotherapy, we used the Annexin V-FITC kit to determine the effect of p85α knockdown on 5-FU-induced apoptosis. Our data show that the proportion of cells that were positive for Annexin V was about 7-fold higher in p85α knockdown cells than in the control cells. The results indicate that the down-regulation of the PI3K/Akt pathway by PI3K p85α RNAi play a role in the sensitivity of colorectal cancer cells to 5-FU treatment. Our study suggested that the combination of 5-FU treatment and PI3K p85α depletion might be a potential strategy for cancer chemotherapy.

Studies have suggested that FoxO transcription factors, which belong to the Forkhead family of proteins, are controlled by PI3K/Akt signaling and involved in regulating cell cycle progression and cell death (27). Direct phosphorylation by Akt inhibits transcriptional activation of FoxO factors, which results in their displacement from the nucleus into the cytoplasm. In the absence of Akt activity, FoxO transcription factors are predominantly nuclear and are presumed to be active, inducing either cell cycle arrest or apoptosis via transcriptional modulation of a variety of genes (28). In order to further explore the mechanism that may be involved in the enhanced cell cycle arrest and 5-FU-induced apoptosis by p85α silencing in colorectal cells, we determined the effect of p85α knockdown on the regulation of FoxO transcription factors. We first evaluated the effect of p85α depletion on Akt activation, which directly phosphorylates and represses the FoxO transcription factors. As shown by Western blot analyses, p85α depletion led to a substantial reduction in the levels of Akt and phospho-Akt, indicating that treatment with p85α siRNA is similar to direct inhibition of Akt activation. In the following Western blot analyses, we found that targeted siRNA depletion of p85α in colorectal carcinoma cells resulted in a significant increase in nuclear FoxO1, FoxO3a and FoxO4 proteins. Our data also show that p85α depletion resulted in a significant decrease in the phosphorylated forms of FoxO1, FoxO3a and FoxO4 in the cytosol. These observations provide strong evidence that inhibition of PI3K p85α activated FoxO transcription factors in both LoVo and SW480 cells.

All three Forkhead protein families have been reported to inhibit cell cycle progression at the G1/S transition (29). Consequently, we studied a series of proteins that are related to G1/S cell cycle transition and transcriptionally regulated by activation of FoxO transcription factors. Western blot analyses showed that the expression levels of cyclin D1 and cdk4 were significantly decreased in p85α knockdown cells, while the expression levels of cyclin kinase inhibitor p27/Kip1 were markedly induced. These results are consistent with the findings obtained from ERα-positive breast carcinoma cells (22). Recent reports have indicated that upregulation of some pro-apoptotic genes are likely correlated with FoxO nuclear localization (28,30). Reports on FoxO target genes that could be responsible for the induction of cell death in colorectal cancer cells are rare. In the present study, expression level of FasL protein was significantly increased in p85α knockdown cells, which may be involved

in the enhanced sensitivity to 5-FU-induced apoptosis in p85α depletion cells.

In summary, our results demonstrate that targeted silencing of PI3K p85α by RNA interference in human colorectal cancer cells inhibited cell proliferation, induced G1 phase cell cycle arrest, and sensitized colorectal cancer cells to 5-FU-induced apoptosis. The mechanism that may be involved in these observations was an activation of FoxO transcription factors, which, in turn, activated transcription of certain target genes, such as those involved in cell cycle regulation and apoptosis. The present study suggests that development of gene thera-peutic approaches or pharmacologic inhibitors directed at PI3K p85α may be a feasible method for the management of colorectal cancer.

Acknowledgements

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