PI3K Stimulates DNA Synthesis and Cell-Cycle Progression via Its p55PIK Regulatory Subunit Interaction with PCNA

Guihua Wang1,2, Xiaonian Cao1, Senyan Lai1, Xuelai Luo1, Yongdong Feng2, Xianmin Xia1, Paul M. Yen3, Jianping Gong2, and Junbo Hu1

Abstract

Previously, we have shown that p55PIK, an isoform of class IA phosphoinositide 3-kinase (PI3K), specifically interacts with important cell-cycle regulators, such as retinoblastoma (Rb), to promote cell-cycle progression. Here, we used the glutathione S-transferase pull-down assay to discover other p55PIK-interacting proteins besides Rb in a Rb-deficient cell line and found that p55PIK interacted with proliferation cell nuclear antigen (PCNA), which plays a key role in coordinating both initiation of the leading strand DNA replication and discontinuous lagging strand synthesis. Overexpression of p55PIK increased, and knockdown decreased, DNA synthesis and DNA replication by modulating the binding of DNA polymerase δ (Polδ) to PCNA. Moreover, a cell-permeable peptide containing the N-terminal-binding domain of p55PIK (TAT–N24) disrupted the p55PIK–PCNA interaction in cancer cells, and also inhibited the DNA synthesis and tumor growth in cell culture and in vivo. Altogether, our results show that the p55PIK–PCNA interaction is important in regulating DNA synthesis and contributes to tumorigenesis. Furthermore, the p55PIK–PCNA interaction provides a potential new target for anticancer drug development.

Mol Cancer Ther; 12(10); 1–10. ©2013 AACR.

Introduction

Class IA phosphoinositide 3-kinase (PI3K) signaling critically regulates many cellular processes and is involved in the initiation and progression of tumors (1, 2). PI3K consists of a p110 catalytic subunit (α, β, or δ) that dimerizes with one of several regulatory subunits (p85α, p85β, p55α, p55γ, or p50α). There are three genes encoding these regulatory subunits: PIK3R1, PIK3R2, and PIK3R3. p85α, p55α, and p50α are products of PIK3R1, whereas p85β and p55γ (also named p55PIK) are products of PIK3R2 and PIK3R3, respectively (2, 3). Regulatory subunits have no enzymatic activity, but are needed to recruit p110 subunits to specific cellular sites and thus regulate the catalytic activity of PI3K (4, 5). All regulatory subunits contain two SH2 domains as well as an intermEDIATE SH2 domain that binds to the p110 catalytic subunit. Regulatory subunits have distinct N-terminal sequences that enable the specific regulation of different PI3K signaling pathways (6, 7).

The association of PI3K catalytic subunits (p110) with cancer development and progression is well documented (8). The PIK3CA gene is mutated in many cancers, including colon, thyroid, and breast cancers (8, 9). PIK3CB is the principal catalytic isoform involved in mediating PTEN-deficient tumorigenesis (10). PIK3CD has been used as a key therapeutic target for hematologic malignancies (11). In contrast, our current understanding of the role(s) of specific PI3K regulatory subunits in cancer is limited. So far, there have been only a few reports describing the possible roles of PIK3R1 (encoding p85α, p55α, and p50α) and PIK3R3 (encoded p55PIK) in tumorigenesis (12, 13).

Previously, we showed that the p55PIK regulatory subunit was important for cell proliferation and tumor growth, and was overexpressed in some cancers (14, 15). The unique 24-amino acid N-terminal domain of p55PIK (N24) specifically binds to important cell-cycle regulators such as retinoblastoma (Rb) protein. N24 peptide expression inhibits the cell-cycle progression and induces cell differentiation by blocking the p55PIK interaction with Rb (16–18). Recently, we showed that non-Rb proteins can also interact with p55PIK and regulate the cell-cycle progression, particularly during the S-phase, when DNA synthesis occurs in the cell cycle. In this article, we report that p55PIK specifically interacts with proliferation cell nuclear antigen (PCNA).
through the N-terminal end of p55PIK (N24) and this interaction regulates the DNA synthesis in cancer cells by modulating the PCNA interaction with DNA polymerase δ (Polδ). We also show that the p55PIK–PCNA interaction is important in tumorigenesis and its inhibition with a novel peptide inhibitor decreases tumor growth.

Materials and Methods

Cell culture, plasmid and adenoviral constructs, and transfection

The culture of thyroid carcinomas cell line FTC236 and colon cancer cell line HT29 was described previously (17) and all cell lines were authenticated. Adenovirus GFP and p55PIK–GFP were constructed as following. The pTrack-cytomegalovirus (CMV), Ad-Easy1, and Escherichia coli bacteria BJ5183 were purchased from Stratagene. The cDNA-encoding p55PIK was first amplified from p-FLAG-CMV 4 (Sigma) using primers CCGGTACCAGGCTACTGGCTGA and reverse: CCCTCGAGTTATCTGCACAAGCGAGGGCAT and then cloned into the EcoR V site of the pTrack-CMV vector. The Pmol-linearized pTrack-CMV plasmid was mixed with adenoviral backbone AdEasy1 to cotransform into E. coli BJ5183. The recombinant adenovirus vector expressing p55PIK was purified to transfect HEK293A cells, and the production of adenovirus particles was confirmed by visualizing the GFP signals in the cells. The amplification and purification of recombinant virus was conducted as described previously (17).

The vector expressing p55PIK was constructed in pcDNA™6/myc-His by cloning cDNA encoding p55PIK using primers (forward: CCGCTAGCATGTACAATACGGTGTGG and reverse: CCCTCGAGCTAAGATCCTTCGTAAACCCGAGGCAT) and the vector expressing PCNA in bacteria was constructed in pGEX-4T-1 using primers for PCNA (forward: CGGGATCCATGTTCGAGGCGCGCTTTTGCA and reverse: CCTTGAGTTATCCTGCAAAAGCCAGGGCAT) and the vector expressing PCNA proteins, bacteria were lysed in 8 mol/L urea and the affinity purification of His-p55PIK and glutathione S-transferase (GST)–PCNA was carried out according to the standard protocols. The purified fusion proteins were dialyzed against PBS (pH 9.5), filtered, and stored in aliquots at 4°C.

Flow cytometry for cell-cycle analysis and BrdUrd incorporation

For the flow cytometry analysis, cells were detached by trypsinization, washed twice with cold PBS, and resuspended in 80% ethanol for at least 30 minutes at −20°C. Then, cells were washed once with PBS and incubated on ice for 10 minutes with propidium iodide (PI; 50 µg/mL). Finally, the stained cells were analyzed using FACS Calibur from BD Biosciences. Aggregated cells revealed by forward scattering were filtered out of the dataset before the analysis. To determine G0–G1, S, and G2–M populations, the settings for 2N and 4N peaks were defined within each experiment and applied to all samples within a given experiment.

Bromodeoxyuridine (BrdUrd) incorporation was done as described previously (17). Briefly, cells were cultured in a 6-well plate overnight, then infected with Ad-GFP or Ad-p55PIK–GFP adenovirus for 48 hours, followed by 30-minute incubation of BrdUrd at 10µg/mL. After aspirating the medium, the cells were immediately fixed for more than 8 hours at −20°C. After immunostaining using the BrdUrd antibody, the DNA synthesis rate was assessed by calculating the percentage of BrdUrd+ cells out of the total cell count. Three biologic repeats were carried out for the final statistical analysis.

Real-time PCR, Western blot analysis, immunostaining, and immunoprecipitation

Procedures involving human subjects were approved by the Huazhong University of Science and Technology Ethics Committee and the formal form was explained to each subject for full understanding and consent. All animal experiments were carried out by following the Animal Study Guidelines of Huazhong University of Science and Technology (Wuhan, China). Human tissue samples were pathologically separated as normal or tumor tissues. Total RNA was isolated using the TRIzol method by following the manufacturer’s protocols. Reverse transcription and real-time PCR were conducted as described previously (17).

The Western blot analysis was conducted as described previously (17). Antibodies to Akt1/2/3, p-Akt(Thr308), p55PIK, p85α, p85β, p110α, p110β, α-tubulin, lamin B, BrdUrd, anti-mouse immunoglobulin G (IgG), and PCNA were purchased from Santa Cruz Biotechnology. Antibodies to Ki-67 and to DNA Polδ were from BD and Sigma, respectively.

Immunostaining was carried out by Guge Biotech. Briefly, cells were seeded onto a coverslip overnight. The coverslips were then washed twice with PBS and fixed in 4% paraformaldehyde for 30 minutes at room temperature. Immunostained images were acquired and merged by confocal laser scanning microscopy (LSM-410; Carl Zeiss).

For immunoprecipitation (IP), total protein extracted from 1 × 106 cultured cells was incubated for 1 hour at 4°C with 10 µL Protein A/G beads. Then, beads were washed and anti-p55PIK or anti-PCNA antibodies (Santa Cruz Biotechnology; 1:100 mouse) and mouse anti-IgG antibodies (Santa Cruz Biotechnology; 1:100) were added to incubate overnight at 4°C. Next, 50 µL protein A/G beads were added to incubate for additional 5 hours. Finally, beads were collected, washed five times with PBS, and mixed with 2× SDS-PAGE sample buffer, and the collected supernatant was boiled for 5 minutes for the Western blot analysis to detect p85α, p85β, p110α, p110β, and DNA Polδ. For quantitative immunoprecipitation, different amounts of total protein were loaded for the Western blot analysis.
Animal study

Four- to six-weeks athymic female nude mice weighing 15 to 20 g were obtained from Shanghai Laboratory Animal Center. Mice were fed under specific pathogen-free conditions in a temperature- and humidity-controlled environment. All animal experiments were in accordance with the Institutional Animal Research Guidelines approved by the Ethics Committee. For tumor-growth studies, HT29 cells with minimal expression of Rb (HT29Rb−) were established by stably transfecting HT29 cells with pcDNA–p55PIK vectors; control HT29 cells were stably transfected with pcDNA vectors. Cells were collected, washed, and resuspended in the culture medium and then injected subcutaneously into each mouse. Tumor size was measured every other day from the day tumors were visible. Mice were sacrificed at indicated times after inoculation and the tumors were removed for analysis.

The expression and purification of TAT–N24 and control TAT–N24M fusion proteins used in the animal study have been described previously (18).

Lipid emulsion injection was formulated from equal volumes of TAT–N24 or TAT–N24M proteins and injectable lipid emulsion (30% Intralipid; Sino-Swed Pharmaceutical Corp, Ltd.). For experiments investigating the TAT–N24 effect on the xenograft tumor growth, mice were injected with 200 μL TAT–N24 or TAT–N24M lipid emulsion via tail vein on the same day shortly after tumor inoculation. The mice in each group, consisting of 4 to 6 animals, received injections of 200 μL protein–lipid emulsion. Injections were repeated every 2 days for 10 to 20 days.

Statistical analysis

The data are presented as mean ± SD. P values were calculated using an unpaired Student t test.

Results

PI3K interacts with PCNA through the N-terminal ends of p55PIK (N24 domain)

We used the GST pull-down assay to identify proteins other than Rb that interacted with the N24 domain of p55PIK. For this purpose, we incubated the cell lysates from FTC236 cells that do not express detectable Rb (17) with His-tagged TAT–N24 fusion proteins (18), and pulled down bound proteins with Ni2+–agarose bead columns. One of the identified proteins was PCNA, which is known to play important roles in the DNA synthesis (19, 20). The interaction of PCNA with N24 was specific as the mutant N24 did not bind PCNA (Fig. 1A). In addition, the PCNA interaction with N24 was direct as the purified GST–PCNA protein could bind to N24 in vitro (Fig. 1B).

We next examined whether the full-length p55PIK binds to PCNA in the cell culture and in vivo. First, we incubated purified GST–PCNA proteins with His-p55PIK and determined that indeed, GST–PCNA interacts with p55PIK in an in vitro binding assay (Fig. 1C). Because there are several isoforms of PI3K regulatory subunits, we examined whether the p55PIK–PCNA interaction was specific for p55PIK. Only p55PIK interacted with PCNA as other members of PI3K regulatory subunit family, such as p85α and p85β, did not bind with PCNA in FTC236 cells, consistent with the notion that p55PIK interaction with PCNA was mediated by its unique N24 domain in p55PIK (Fig. 1D). Moreover, the presence of p110β catalytic subunit of PI3K among the PCNA-bound proteins complexes strongly suggests that dimerized p110/P55 PI3K interacts with PCNA in cells. Furthermore, immunofluorescence staining using specific antibodies confirmed the colocalization of PCNA and p55PIK in the nuclei of cultured cancer cells (Fig. 1E).

Overexpression of p55PIK increased DNA Polδ bound with PCNA and stimulated DNA synthesis

To identify the roles of p55PIK–PCNA interaction in cell-cycle progression, we overexpressed p55PIK in Rb-deficient FTC236 cells to prevent any concomitant p55PIK–Rb interaction. We transformed FTC236 cells using an adenovirus construct (Ad-p55PIK–GFP) that separately expressed GFP and full-length p55PIK under the control of two CMV promoters. The adenovirus construct expressing only GFP (Ad-GFP) was used as a vector control. We analyzed cells by fluorescence-activated cell sorting 48 hours after the infection, and found little change in the cell-cycle distribution of cells infected with Ad-GFP compared with noninfected control cells. In contrast, treatment of cells with Ad-p55PIK–GFP caused a significant increase in the cell number at S-phase (33.8% S-phase cells in vector control group vs. 40% in cells overexpressing p55PIK). More importantly, the expression of p55PIK increased the number of cells that incorporated BrdUrd (Fig. 2A), showing that p55PIK increases the DNA synthesis.

PCNA plays a central role in coordinating both initiation of the leading strand DNA replication and the discontinuous lagging strand synthesis during the DNA synthesis. During the DNA synthesis process, the amount of DNA Polδ binding to PCNA determines the rate of the DNA synthesis (19). Accordingly, we examined the association of Polδ to PCNA in FTC236 cells that overexpressed p55PIK. Overexpression of p55PIK had no effects on the protein expression of PCNA and Polδ (Fig. 2B). However, p55PIK significantly increased the Polδ binding to PCNA (Fig. 2C), indicating that the p55PIK–PCNA interaction may enhance the Polδ association with PCNA, leading to increased DNA synthesis in cells overexpressing p55PIK. Consistent with these observations, p21 protein, which inhibits the cell proliferation during the cell cycle, also was decreased in FTC236 cells overexpressing p55PIK (Fig. 2B).

Knockdown of p55PIK inhibited cell-cycle progression by blocking DNA synthesis

Next, we examined the effects of p55PIK on cell-cycle distribution in Rb-deficient FTC236 cells after the knockdown by siRNA against p55PIK. Data in Fig. 3A showed an increased number of cells in the S-phase. Results from
BrdUrd incorporation assay also showed that the DNA synthesis in p55PIK knockdown cells was inhibited. Data from the cell-cycle distribution analysis showed that the knockdown of either p55PIK or PCNA in FTC236 cells led to cell-cycle arrest at S and G2–M phases (Fig. 3A and B). Of note, the knockdown of PCNA increased the cell death and apoptosis, whereas p55PIK did not significantly affect these processes (Fig. 3A and B). These results are consistent with our previous observations that p55PIK signaling did not play any significant role in the apoptosis (17).

However, because the knockdown of PCNA significantly increased the apoptosis and cell death, PCNA is likely to be involved in other pathways, in addition to the pathway(s) mediated by the p55PIK–PCNA interaction, to regulate the cell-cycle progression and/or prevent cell death.

**p55PIK stimulates tumorigenesis in vivo**

We initially created a cell line that had minimal expression of Rb protein in HT29 colon cancer cells using short hairpin RNA (shRNA) against Rb (HT29Rb− cells). To further examine the role of p55PIK-mediated Rb-independent interactions in tumorigenesis, we established a cell line stably overexpressing p55PIK by transforming HT29Rb− cells with Ad-p55PIK in HT29Rb−/p55PIK; (Fig. 4A).

We then injected HT29Rb−/p55PIK cells subcutaneously into athymic nude mice and tumors were harvested after 12 days. Mice injected with HT29Rb−/p55PIK cells had larger tumors than mice injected with control HT29Rb− cells (Fig. 4B). The immunohistochemical analysis of tumors confirmed the increased expression of p55PIK and showed

---

**Figure 1.** PI3K interacts with PCNA directly through the N-terminal ends of p55PIK (N24 domain). **A,** association of PCNA with N24. Lysates from FTC236 cells were incubated with TAT–N24 or TAT–N24M, fusion proteins. Ni++ agarose-bound proteins were analyzed by Western blotting (WB) using a monoclonal anti-PCNA antibody. **B,** interaction of GST-PCNA with TAT–N24. Purified GST-PCNA was incubated with TAT–N24 or TAT–N24M for 2 hours, Ni+++ agarose was added and pull-down proteins were analyzed by Western blotting using a monoclonal anti-PCNA antibody. **C,** interaction of recombinant His-p55PIK fusion protein with GST-PCNA *in vitro.* E. coli–expressed and purified fusion proteins His-p55PIK and GST-PCNA were mixed and incubated for 2 hours; Ni++ agarose pull-down proteins were analyzed by Western blotting using monoclonal anti-PCNA antibody and anti-p55PIK antibody. **D,** specific interaction of p55PIK and PCNA in cultured cells. FTC236 cell lysates were immunoprecipitated (IP) with an anti-PCNA antibody or nonspecific mouse IgG. Immunoprecipitated proteins were analyzed by Western blotting using anti-p55PIK antibody, anti-p85α antibody, anti-p85β antibody or anti-p110β antibody as indicated. **E,** colocalization of p55PIK and PCNA in cultured cells. FTC236 cells on coverslips were stained with a monoclonal anti-PCNA antibody and fluorescein-labeled rabbit anti-mouse IgG (green) and with an anti-p55PIK antibody and phycoerythrin-labeled sheep anti-goat IgG (red). The images were taken on a laser-scanning confocal microscope as described. Areas of colocalization appear yellow.
that p55PIK overexpression increased Ki-67, a marker of cell proliferation (Fig. 4C).

**Cell-permeable TAT–N24 blocked the binding of p55PIK with PCNA and inhibited DNA synthesis and tumor growth in vivo**

The unique N-terminal end (N24) of p55PIK is the interaction site for both Rb and PCNA. Previously, we showed that the cell-permeable TAT–N24 fusion protein inhibited cell proliferation and induced cell differentiation in cancer and leukemia cells expressing Rb by blocking p55PIK-mediated signaling pathways (18). We thus examined whether TAT–N24 could affect the binding of p55PIK and PCNA, and modulate the signaling pathways mediated by p55PIK and PCNA interaction. TAT–N24 was able to specifically block the binding of p55PIK with PCNA and decreased the association of Polδ with PCNA in a dose-dependent manner (Fig. 5A and B). These data were consistent with the observations that TAT–N24 induced the cell-cycle arrest at
Figure 3. Knockdown of p55PIK and PCNA led to cell-cycle arrest at S-phases and inhibited DNA synthesis. A, left, downregulated p55PIK expression in FTC236 cells. FTC236 cells were transfected with siRNA against p55PIK (si-p55PIK) and control RNA (si-control). Lysates from parental cells (control), si-p55PIK-transfected cells, and si-control-transfected cells were loaded on a gel and p55PIK protein expression was assessed by Western blotting using anti-p55PIK antibody. Top, right, downregulated p55PIK induced the cell-cycle arrest at S and G2–M phases. Cell-cycle analyses were conducted on control FTC236 cells, cells transfected with si-p55PIK or si-control. Representative histograms from an individual experiment and similar results were obtained in three independent experiments. Bottom right, downregulated p55PIK decreased the DNA synthesis in FTC236 cells. FTC236 cells were transfected with the si-p55PIK or si-control. BrdUrd was added 48 hours after infection, and cells were cultured for an additional 30 minutes. Cells then were collected and immunostained for BrdUrd and counterstained for nucleic acid with PI. BrdUrd incorporation into DNA and DNA content in nuclei were determined by flow cytometry analysis. Representative data from an individual experiment; similar results were obtained in three independent experiments. Note that the percentage of cells in active DNA synthesis is defined as BrdUrd$^+$ cells/total cells (the events in R2 area/total events).

B, left, downregulation of PCNA in Rb-deficient cells. FTC236 cells were transfected with siRNA against PCNA (si-PCNA) or control small RNA (si-control). Lysates from parental cells (control), si-PCNA-transfected cells, and si-control-transfected cells were loaded on a gel and PCNA protein expression was assessed by Western blotting using anti-PCNA antibody. Top, right, downregulated PCNA induced cell-cycle arrest at S-phase and increased cell death in Rb-deficient cells. Cell-cycle analyses were conducted on FTC236 cells transfected with si-PCNA or si-control as described in Materials and Methods. Representative histograms from an individual experiment and similar results were obtained in three independent experiments; the data are presented quantitatively with error bars and appropriate statistical analysis (**, $P<0.01$; *, $P<0.05$). Bottom right, downregulated PCNA inhibited DNA synthesis. FTC236 cells were transfected with the si-p55PIK or si-control. BrdUrd was added 48 hours after infection, and cells were cultured for an additional 30 minutes. Cells then were collected and immunostained for BrdUrd and counterstained for nucleic acid with PI. BrdUrd incorporation into DNA and DNA content in nuclei were determined by flow cytometry analysis. Representative data from an individual experiment; similar results were obtained in three independent experiments; the data are presented quantitatively with error bars and appropriate statistical analysis (**, $P<0.01$; *, $P<0.05$). Note that the percentage of cells in active DNA synthesis is defined as BrdUrd$^+$ cells/total cells (the events in R2 area/total events).
S-phase and inhibited the DNA synthesis in FTC236 and HT29/Rb-p55PIK cells (data not shown), and suggests that TAT–N24 can inhibit p55PIK effects on non-Rb signaling pathways, including those mediated by the p55PIK–PCNA interaction.

We next examined the effects of TAT–N24 on the tumor growth of HT29/Rb-p55PIK cells in athymic nude mice. HT29/Rb-p55PIK cells were injected into mice subcutaneously, and 2 mg TAT–N24 (0.1 g/kg body weight) or control TAT–N24M were injected intravenously into tail veins of mice every 2 days for 20 days. The volumes of tumors were measured twice a week before mice were sacrificed and their tumors were weighed (Fig. 6A). The mean xenograft tumor weight decreased by 54% in mice receiving TAT–N24 compared with mice receiving TAT–N24M (Fig. 6A and B).

**Discussion**

In the current study, we used two Rb-deficient cell lines to show that the p55PIK regulatory subunit of PI3K can bind to PCNA in addition to Rb. This interaction enhanced the binding of Polδ to PCNA and was associated with the increased DNA synthesis. Furthermore, the unique N24 domain in p55PIK was sufficient for the binding of p55PIK to PCNA as cell-permeable TAT–N24 abolished the p55PIK–PCNA interaction. This, in turn, led to DNA synthesis inhibition and cell-cycle arrest at the S-phase. It is well known that multiple PI3K signaling pathways are involved in cell-cycle progression; however, the precise mechanism(s) used by PI3K to regulate the DNA synthesis is not well understood (21, 22). Our findings provide insight into a novel PI3K signaling mechanism that directly modulates the DNA synthesis and cell-cycle progression.

PCNA is an essential component of the chromosomal DNA replisome (19). In eukaryotes, both DNA leading strand replication and completion of Okazaki fragments synthesis require PCNA and DNA polymerases such as Polα, Polδ, and Polε (19, 23, 24). Our results showed that the overexpression of p55PIK increased the association of Polδ with PCNA and led to increased DNA synthesis. We currently are working on determining the precise mechanism for PI3K regulation of DNA synthesis. However, one major potential mechanism may involve p21Waf/Cip, the major protein that competes with Polδ for binding to PCNA, and inhibits DNA synthesis (25). It is possible
that p55PIK overexpression decreases the expression of p21\textsuperscript{Waf/Cip} during cell-cycle progression, leading to an increase of Polδ binding to PCNA. This hypothesis is supported by our findings that the overexpression of p55PIK decreases the expression of p21\textsuperscript{Waf/Cip}, whereas the inhibition of p55PIK signaling increases the expression of p21\textsuperscript{Waf/Cip} (Fig. 2B). In the N-terminal region of p55PIK (N24), there is no apparent PIP box or other motifs found in PCNA-binding proteins, suggesting that it may contain a new PCNA-binding domain (19). The finding that N24 was both necessary and sufficient for PCNA binding excludes the possibility that SH2, SH3, bcr, and proline-rich domains in isolated by the ability of its SH2 domains to bind phosphorylated tyrosine in signaling proteins (26, 27). However, its unique N24 domain specifically interacts with Rb and the p55PIK–Rb interaction is important for cell-cycle progression at G0–G1 phases (16). In our current study, we identified a new N24 interacting partner, PCNA, that is involved in cell-cycle progression at the S-phase. The fact that other PI3K regulatory subunits do not associate with Rb or PCNA suggests that each PI3K regulatory subunit may specify distinct functions and may help explain how PI3K can be involved in diverse, and sometimes opposing,

Figure 5. TAT–N24 blocked the binding of p55PIK with PCNA and decreased association of PCNA with Polδ. A, TAT–N24 blocked the binding of p55PIK with PCNA in cultured cells. FTC236 cells were incubated with TAT–N24 (200 μg/mL) or control TAT–N24M for 24 hours. Cellular lysates were immunoprecipitated (IP) with an anti-PCNA antibody. Immunoprecipitated proteins were analyzed by Western blotting using anti-Polδ antibody as indicated; PCNA was also analyzed with an appropriate antibody to show the equal amount of PCNA proteins in every group. B, TAT–N24 fusion proteins decreased the association of PCNA with Polδ in cultured cells. FTC236 cell were incubated with various concentrations of TAT–N24 or TAT–N24M (200 μg/mL) for 24 hours. Cellular lysates were immunoprecipitated with an anti-PCNA antibody. Immunoprecipitated proteins were analyzed by Western blotting using anti-Polδ and PCNA antibodies.

Figure 6. TAT–N24 inhibited tumor growth in vivo. A, intravenous injection of TAT–N24 fusion proteins inhibited the HT29\textsuperscript{RB–p55PIK} tumor xenografts growth. Mice inoculated with HT29\textsuperscript{RB–p55PIK} cells (10\textsuperscript{6}/site) were divided into three groups receiving either 200 μL solvent–lipid emulsion, TAT–N24 (10 mg/mL emulsion) or TAT–N24M (10 mg/mL emulsion) via tail vein. The injection was repeated every 2 days. The size of tumors was measured (mean ± S.D; n = 5; \(*\), P < 0.05; \(**\), P < 0.01; TAT–N24–treated group compared with TAT–N24M–treated group). B, TAT–N24 inhibitory effects on the xenograft tumor weight in vivo. Shown are representative tumor xenografts and mean tumor weights after necropsy (mean ± S.D; n = 5; \(*\), P < 0.05; \(**\), P < 0.01; TAT–N24–treated group compared with TAT–N24M–treated group).
cellular processes. It is interesting to note that the down-regulation of p55PIK in Rb-deficient cells led to the cell-cycle arrest at the S-phase without any significant effects on apoptosis or cell death, suggesting that the p55PIK–PCNA signaling does not directly affect pathways involved in cell death (Fig. 3A). In contrast, the knockdown of PCNA in Rb-deficient cells not only inhibited the cell-cycle arrest at the S-phase, but also induced apoptosis (Fig. 3B). The different effects by p55PIK or PCNA knockdowns on cell death clearly indicate that while they may have overlapping functions on DNA replication, PCNA may also have other roles than p55PIK on cell survival.

Although PI3K is critical for normal cell function, dysregulated PI3K activities contribute to the development of cancer and immune-mediated pathologies. Several PI3K inhibitors are effective in cancer cell lines and animal models (28). However, their lack of signaling pathway specificity limits their clinical application, particularly as only a subset of the many cellular processes regulated by the PI3K pathway are directly involved in cell proliferation (28, 29). Thus, an important issue in the clinical application of PI3K inhibitors will be the development of drugs that can specifically block pathways involved in cell proliferation with minimal adverse side effects due to the inhibition of other PI3K-dependent cellular processes. Our previous and current studies have shown the essential and specific roles of p55PIK–PI3K on Rb- and/or PCNA-mediated signaling in cell-cycle progression and DNA synthesis. Furthermore, the blockade of p55PIK signaling by either siRNA or TAT–N24 did not significantly affect other PI3K signaling pathways as it had little systemic side effects in vivo. Our findings suggest that targeting specific PI3K regulatory subunits such as p55PIK could potentially facilitate the development of a new class of anticancer drugs that have advantages over present PI3K catalytic subunit inhibitors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: P.M. Yen, J. Gong, J. Hu
Development of methodology: G. Wang, X. Cao, X. Xia, J. Hu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Wang, X. Cao, S. Lai, X. Luo, J. Hu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Wang, X. Cao, X. Xia, J. Hu
Writing, review, and/or revision of the manuscript: G. Wang, Y. Feng, X. Xia, P.M. Yen, J. Hu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Wang, X. Luo
Study supervision: Y. Feng, X. Xia, J. Hu

Grant Support
J. Hu was supported by grants of foundation of “973” Program (no. 2009CB821802), National Natural Science foundation (nos. 30872472, 30973496, and 30800569) and PCSIRT1131. P.M. Yen was funded by faculty funds from the Ministry of Health, Ministry of Education, A’Star, and Duke-National University of Singapore Graduate Medical School, Singapore.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 19, 2012; revised July 1, 2013; accepted July 22, 2013; published OnlineFirst August 12, 2013.

References
Molecular Cancer Therapeutics

PI3K Stimulates DNA Synthesis and Cell-Cycle Progression via Its p55PIK Regulatory Subunit Interaction with PCNA

Guihua Wang, Xiaonian Cao, Senyan Lai, et al.

Mol Cancer Ther Published OnlineFirst August 12, 2013.

Updated version Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-12-0920

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.