

Mutant *PIK3CA*-Bearing Colon Cancer Cells Display Increased Metastasis in an Orthotopic Model

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Abstract

Mutations in the *PIK3CA* gene are common in human cancers, including colon cancer. We compared two pairs of colon cancer cells (HCT116 and DLD1) bearing only the wild-type (WT) or mutant (MUT) *PIK3CA* allele for their survival capacity under stress conditions *in vitro* as well as their metastatic properties in an *in vivo* orthotopic model. When subjected to growth factor deprivation stress (GFDS), the MUT *PIK3CA* cells displayed resistance to GFDS-induced apoptosis relative to the WT cells. Phosphatidylinositol 3-kinase (PI3K) and its downstream effector AKT were constitutively activated during stress conditions in the MUT *PIK3CA* cells but not in the WT cells. The MUT cells showed hypersensitivity to PI3K inhibition. Moreover, the proapoptotic protein Bax was expressed at a very high level in the WT *PIK3CA* cells, whereas it was almost undetectable in the MUT cells. Inhibition of Bax expression by small interfering RNA protected the WT *PIK3CA* cells from GFDS-induced apoptosis, suggesting an important role of Bax in GFDS-induced apoptosis. These results indicated that the MUT PI3K confers resistance to GFDS-induced apoptosis and that the MUT cells are more dependent on the PI3K pathway for survival. *In vivo* studies showed that the MUT *PIK3CA*-bearing cells were more metastatic than the WT cells in an orthotopic model of colon cancer. Taken together, these results suggest that MUT PI3K imparts a more aggressive phenotype in colon cancer cells and could be a potential therapeutic target for treatment of colon cancer patients bearing *PIK3CA* mutations. [Cancer Res 2007;67(12):5851–8]

Introduction

The phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases that are involved in a wide range of cancer-related signaling pathways, including proliferation, survival, motility, differentiation, cytoskeletal rearrangement, and angiogenesis (1–3). PI3Ks are categorized into three families according to their subunit structure, regulation, and substrate selectivity (4). Class IA PI3K, which comprises a 110-kDa catalytic subunit and a regulatory subunit of 85, 55, or 50 kDa, plays an important role in regulating proliferation, motility, and survival (5, 6). AKT, the major downstream effector of PI3K, is activated by phosphoinositide-dependent protein kinase 1, which is recruited and phosphorylated by activation of PI3K. The

PI3K/AKT signaling pathway regulates several transcriptional factors, including the forkhead transcription factor FKHR and nuclear factor- κ B, which are involved in cell cycle control and apoptosis (7–11). The PI3K/AKT pathway has also been reported to regulate multiple downstream signaling effectors relevant to apoptosis, including the Bcl-2 family member Bad, Bax, caspase-9, c-myc, and YAP (12, 13). Furthermore, the PI3K/AKT pathway has been shown to inhibit apoptotic processes, although inhibition of PI3K/AKT signaling sensitizes cells to apoptotic stimuli, such as drug treatment or cellular stress (14, 15). Thus, these studies show an important role of PI3K/AKT signaling in cell survival/apoptosis.

Bax, one of the downstream substrates of AKT, is an important proapoptotic protein. Recent studies suggested that Bax promotes apoptosis through several interdependent mechanisms, including translocation of Bax from the cytoplasm to the mitochondria, Bax conformational changes, and oligomerization of Bax and Bak (13, 16–18). Bax expression is closely related to cellular sensitivity to apoptosis. Human colon cancer cells lacking Bax expression display resistance to curcumin-induced apoptosis, whereas overexpression of Bax sensitizes prostate cancer cells to transforming growth factor- β -induced apoptosis (19, 20).

It has been shown that deregulation of the PI3K pathway is very common in tumors (21, 22) and that aberrant PI3K activation plays important roles in sustaining processes important to malignancy, including cell proliferation, adhesion, survival, and motility. *PIK3CA*, which encodes the p110 α catalytic subunit, has a high frequency of mutations in many human cancers, including colon, breast, brain, and stomach cancer (23–26). Sequencing studies showed that ~30% of colon cancer samples harbor mutations in *PIK3CA* (5, 24). These cancer-specific *PIK3CA* mutations were identified, and the majority of them were located to two hotspot regions: the helical domain encoded by exon 9 and the kinase domain encoded by exon 20 (26, 27). The two most frequently altered residues of p110 α in colon cancer are E545 in the helical domain and H1047 in the kinase domain. E545 is commonly mutated to lysine, whereas H1047 is frequently substituted with arginine. Both of these mutations cause a gain of enzyme function and significantly increase PI3K activity (26, 28). The gain-of-function nature of these *PIK3CA* mutations suggests that these mutations may be associated with “oncogenic addiction” and are, therefore, hypersensitive to PI3K inhibition similar to the gain-of-function mutations associated with epidermal growth factor receptor (EGFR; ref. 29). Thus, mutant (MUT) PI3K may be a potential target for PI3K small-molecule inhibitors. Determination of the roles of MUT PI3K in cell survival, tumorigenicity, and metastasis will help uncover mechanisms of tumor progression and perhaps facilitate development of drugs for cancer treatment.

Note: X-N. Guo and A. Rajput contributed equally to this work.

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We hypothesized that the aberrant activation of PI3K/AKT signaling by gain-of-function mutations is responsible for inappropriate cell survival by malignant cells in response to stress. We used a cell model system developed by Samuels et al. (30) to test our hypothesis. The model system is composed of two pairs of colon cancer cell lines HCT116 wild-type (WT) *PIK3CA* and MUT *PIK3CA* cells and DLD1 WT and MUT cells. The WT or MUT cells bear only the WT or MUT *PIK3CA* allele, respectively, as a result of asymmetrical knockout of the MUT or WT *PIK3CA* allele (30). We compared the WT and MUT cells for their survival capacity under stress conditions *in vitro* as well as their metastatic properties in an *in vivo* orthotopic model. We found that the MUT cells were more resistant to growth factor deprivation stress (GFDS)-induced apoptosis than the WT cells. We predicted that this was probably due to the constitutive PI3K activity during stress that leads to low Bax expression. The MUT cells were hypersensitive to a potent PI3K inhibitor LY294002 as reflected by significantly increased apoptosis compared with the WT cells when treated with this agent. *PIK3CA* small interfering RNA (siRNA) significantly induced apoptosis in the MUT cells but not in the WT cells. In contrast, Bax siRNA decreased GFDS-induced apoptosis in the WT cells, whereas there was little effect in the MUT cells because of the already low levels of this proapoptotic protein in these cells. In addition, *in vivo* studies revealed that the MUT cells possess greater potential to metastasize to distant organs compared with the WT cells. Our results indicate that MUT *PIK3CA* confers increased survival capacity to colon cancer cells, which might contribute to increased metastasis *in vivo*, and that the MUT cells display an increased sensitivity to inhibition of the PI3K signaling pathway. Therefore, constitutive PI3K/AKT signaling during stress might provide a therapeutic target for treatment of cancers bearing these mutations.

Samuels et al. (30) showed that *PIK3CA* mutations in these cells constitutively activated the PI3K/AKT pathway, which led to resistance to apoptosis and increased tumor formation in a mouse model when cells were *i.v.* injected. We have expended these observations and found that WT cells had much higher Bax expression than the PI3K MUT cells and that Bax played an important role in GFDS-induced apoptosis. More importantly, we showed for the first time in an orthotopic model that colon cancer cells bearing MUT *PIK3CA* are more metastatic than the WT cells as evidenced by metastatic spread to the liver and lungs. Metastasis is a complex, multistep process that requires a tumor cell to be able to fulfill two rate-limiting steps: invasion and distant colony formation (31). *In vivo* models, such as the tail vein injection described in the Samuels article, allow for the study of the ability of a tumor cell to form distant colonies. They do not, however, allow for the study of invasion, which is ultimately necessary before distant colony formation. In the absence of transgenic animal models of metastasis, this orthotopic model of colon cancer allows for the study of invasion in the bowel wall as well as distant colony formation in the liver and lungs. Metastatic spread to the liver and lungs recapitulates the pattern of colorectal cancer metastases in humans and thus shows the potential key role of gain-of-function *PIK3CA* mutations in colon cancer progression.

Materials and Methods

Cell lines and cell culture. The WT and MUT *PIK3CA* cells of human colon cancer cell lines DLD1 and HCT116 were generated by Samuels et al. through asymmetrical knockout of the MUT or WT *PIK3CA* allele, respectively (30). They were kindly provided by Drs. Vogelstein and

Velculescu (The Johns Hopkins Kimmel Cancer Center, Baltimore, MD). Both the WT and MUT cells were cultured in McCoy's 5A medium (Sigma) supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Western blot analyses. Cells were grown to 80% to 90% confluence in six-well culture plates and then changed to growth factor and serum-free medium for 2 or 4 days. After rinsing with ice-cold PBS, cells were lysed in 0.1 mL of Laemmli buffer [50 mmol/L Tris-HCl (pH 6.8), 100 mmol/L DTT, 2% SDS, 10% glycerol, 1 mmol/L Na₃VO₄, 0.1% bromophenol blue]. The lysates were resolved by 8% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Life Sciences). The membranes were blocked for 1 h, probed with various primary antibodies, and then reacted with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent (Pierce). To determine the total protein level, membranes were stripped with reblot solution (Chemicon), reprobed with the appropriate antibody, and detected as described above. Antibodies against the following proteins were used in the work: phosphorylated AKT (Ser⁴⁷³) and AKT, PI3K p110 α , actin (Cell Signaling Technology), and Bax (Santa Cruz Biotechnology).

Inhibition of *PIK3CA* and Bax expression by siRNAs. siRNA down-regulation was done using siRNA duplexes against *PIK3CA* (Dharmacon Research, Inc.) or Bax (Cell Signaling Technology). A scrambled siRNA duplex (Qiagen, Inc.) was used as a control. Cells were seeded and grown to 50% to 60% confluence in 24-well culture plates. The cells were then transfected with 50 nmol/L *PIK3CA* siRNA or 100 nmol/L Bax siRNA using LipofectAMINE 2000 (Invitrogen). Twenty-four hours after transfection, the cells were changed to growth factor and serum-free medium for another 72 h and then lysed for Western blot analyses or apoptosis assays.

Apoptosis assays. Cells were seeded in 96-well culture plates and allowed to attach overnight. The cells were then treated with LY294002 (Biosource) or transfected with *PIK3CA* siRNA (50 nmol/L) or Bax siRNA (100 nmol/L). After deprivation of growth factors and serum for 72 h, the cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) for 2 h and dissolved in DMSO for absorbance measurements at 570 nmol/L. Apoptosis assays were done using a DNA fragmentation ELISA kit as instructed by the manufacturer's protocol (Invitrogen).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. Cells were seeded in 24-well culture plates and allowed to attach overnight. The cells were treated with various concentrations of LY294002 in serum-free medium for 72 h or transfected with Bax siRNA (100 nmol/L) or *PIK3CA* siRNA (50 nmol/L) for 24 h and then cultured in growth factor and serum-free medium for 72 h. The cells were harvested, washed with PBS, fixed in 4% paraformaldehyde (in PBS, pH 7.4) at room temperature for 1 h, and then transferred to permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 5 min on ice. The cells were then washed with PBS and incubated in the dark at 37°C for 1 h with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction mixture according to the manufacturer's instructions (Promega). Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma) for 1 min for visualization of total nuclear DNA. The samples were mounted on glass slides and photographed under fluorescent microscopy using selective band-pass filters.

Reverse transcription-PCR assays. Lung tissues were harvested and RNA was isolated using RNeasy Mini kits (Qiagen) according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) was carried out to detect the RNA level of human-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and nonspecific GAPDH using the SuperScript One-Step RT-PCR Systems (Invitrogen). The primers used for amplification included 5'-CGAGATCCCTCCAAAATCAA as a forward primer and 5'-AGGTCCACCACTGACACGTT as a reverse primer for human-specific GAPDH and 5'-AACGGATTGGTCGTATTGG as a forward primer and 5'-TTGTTCATGGATGACCTTGGC as a reverse primer for nonspecific GAPDH as control.

Orthotopic implantation. All animal procedures were conducted with approval of and in compliance with the Roswell Park Cancer Institute

Institutional Animal Care and Use Committee. Orthotopic implantation was done as described previously (32, 33). Briefly, green fluorescent protein (GFP)-labeled HCT116 MUT *PIK3CA*, HCT116 WT *PIK3CA*, DLD1 MUT *PIK3CA*, and DLD1 WT *PIK3CA* cells (5×10^6) were s.c. injected onto the dorsal surfaces of separate BALB/c nude male mice. Once xenografts were established, they were excised and minced into 1 mm^3 pieces. Two of these pieces were then orthotopically implanted into other BALB/c nude mice. Twenty-nine animals were implanted with HCT116 MUT cells, 28 animals with HCT116 WT cells, 30 animals with DLD1 MUT cells, and 27 animals with DLD1 WT cells.

For operative procedures, animals were anesthetized with isoflurane inhalation. A 1-cm laparotomy was done and the cecum and ascending colon were exteriorized. Using $\times 7$ magnification and microsurgical techniques, the serosa was disrupted in two locations. Pieces of xenograft (1 mm^3) were subserosally implanted using an 8-0 nylon suture at the disrupted serosal locations. The bowel was then returned to the peritoneal cavity and the abdomen was closed with 5-0 vicryl suture.

Fluorescence imaging was done weekly on the animals to follow tumor growth and progression (LightTools). Thirty-five days after implantation, animals were euthanized. Organs were explanted, imaged, and immediately placed in buffered 10% formalin. Tissues were then processed and embedded in paraffin. Slides were cut for H&E staining.

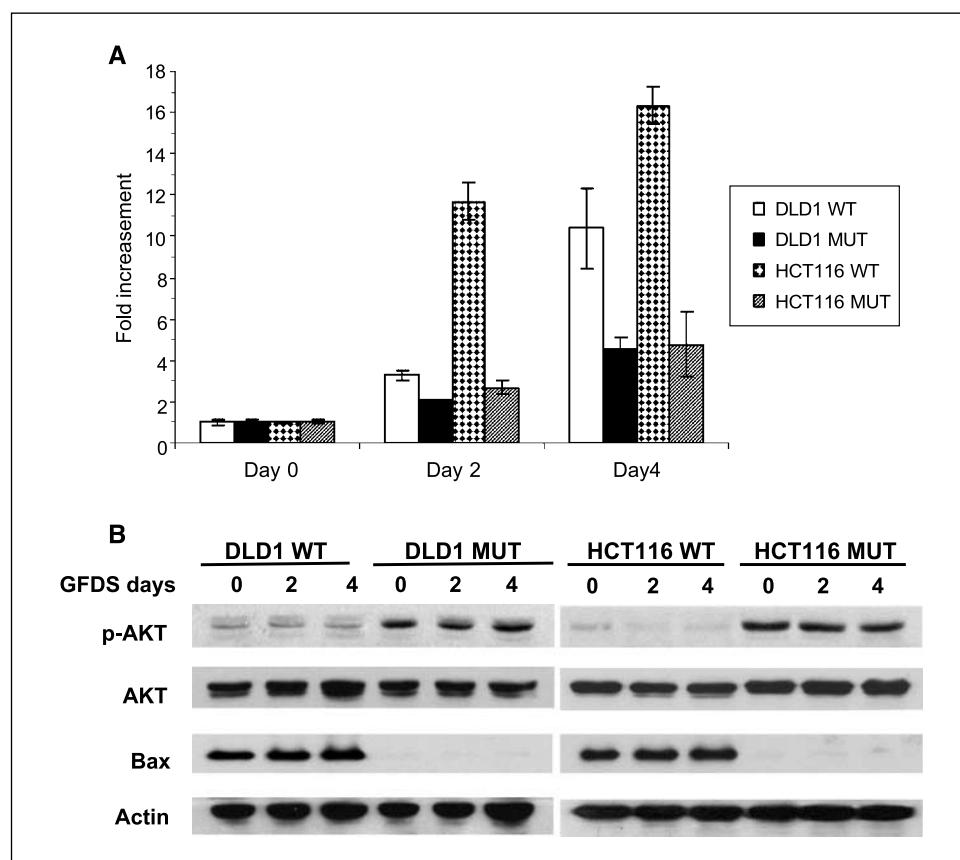
Results

PIK3CA mutation conferred resistance to GFDS-induced apoptosis. Human colon cancer cell lines DLD1 and HCT116 each harbor one of the "hotspot" gain-of-function mutations of *PIK3CA*. Because the mutations were heterozygous in both cell lines, WT or MUT *PIK3CA* allele-bearing haploid cells of the two cell lines were established by Samuels et al. using asymmetrical somatic knockout

of the MUT or WT *PIK3CA* allele, respectively (30). Considering the important role of PI3K in cell survival, we hypothesized that, compared with the WT *PIK3CA*-bearing cells, the MUT *PIK3CA*-bearing cells were more resistant to GFDS-induced apoptosis. To test this hypothesis, the cells were starved in serum-free medium for 2 or 4 days. DNA fragmentation assays were used to detect apoptosis of these cells. As shown in Fig. 1A, both HCT116 and DLD1 WT cells were more sensitive to GFDS-induced apoptosis than the MUT cells, suggesting that MUT PI3K might provide cancer cells with a survival advantage under environmental stresses, such as growth factor and nutrient deficiency.

We next examined the downstream signaling of PI3K that might be responsible for the differences in apoptosis between the WT and MUT cells under GFDS. As shown in Fig. 1B, phosphorylation of AKT was much higher in the MUT cells than in the WT cells. The phosphorylation level of AKT was sustained in the MUT cells even under GFDS for 2 or 4 days, whereas it remained at a low level in the WT cells during GFDS. These results indicated that AKT was constitutively activated in the MUT cells, which we hypothesized to contribute to the resistance of the MUT cells to GFDS-induced apoptosis. Another difference between the WT and MUT cells was the expression level of the proapoptotic protein Bax. The WT cells had high levels of Bax expression, whereas Bax was almost undetectable in the MUT cells (Fig. 1B). Bax is an important proapoptotic protein whose expression and translocation play an important role in apoptosis. A conformational change in Bax that exposes its COOH-terminal hydrophobic domain activates Bax by facilitating translocation of Bax from cytosol to mitochondria on induction of apoptosis (34–36). Translocation and accumulation of

Figure 1. MUT PI3K confers resistance to GFDS-induced apoptosis. **A**, the WT and MUT cells of HCT116 and DLD1 were seeded in 96-well culture plates and deprived of growth factors and serum for 0, 2, and 4 d. DNA fragmentation assays were done as described in Materials and Methods. **B**, the cells were grown to 80% to 90% confluence and deprived of growth factors and serum for 0, 2, and 4 d. Cells were then harvested and Western blot assays were done to analyze downstream PI3K signaling, including the effects on AKT and Bax. Actin was used as a loading control. *p*-AKT, phosphorylated AKT.



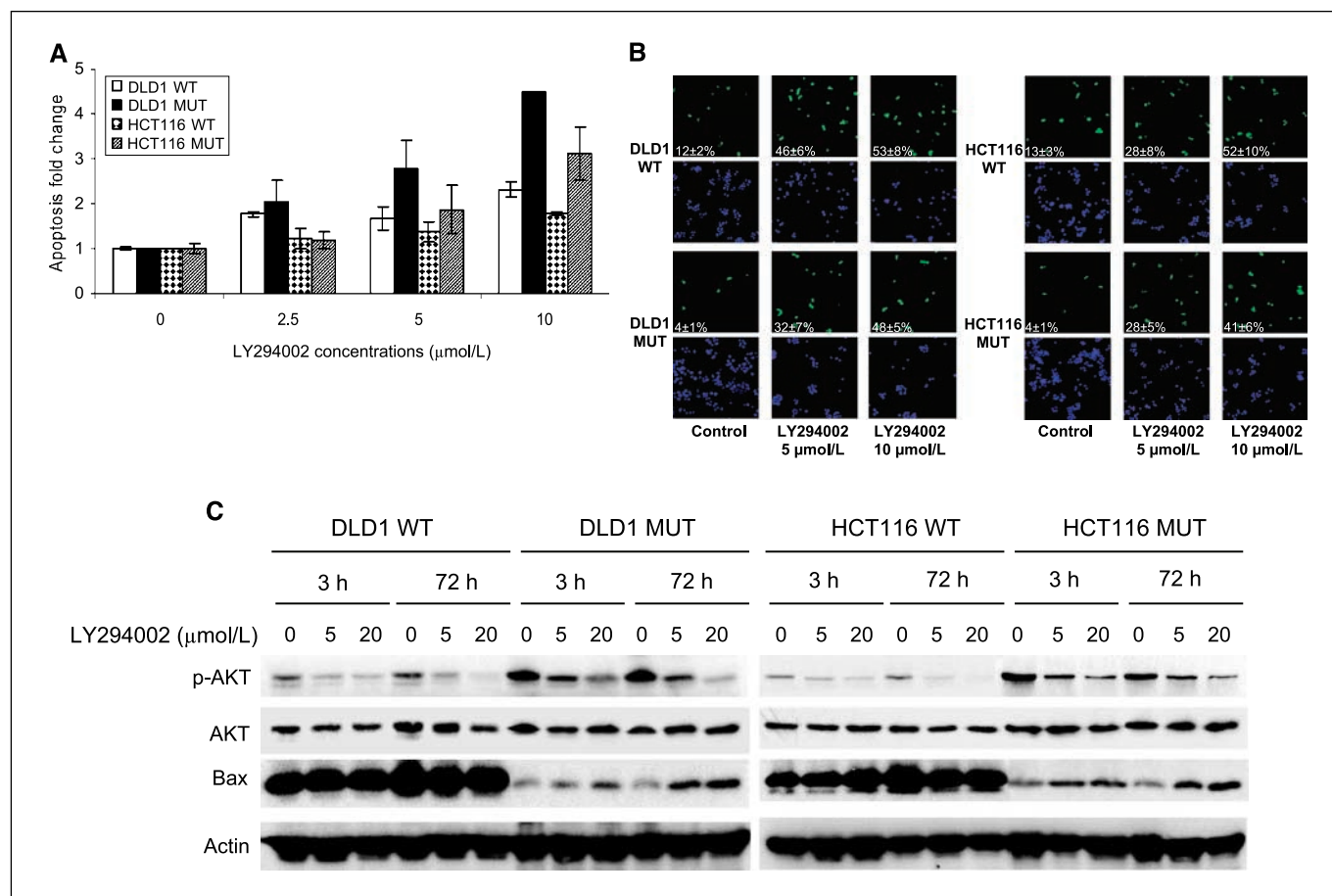


Figure 2. Effect of LY294002 on apoptosis of the WT and MUT cells under GFDS. **A**, the WT and MUT cells were seeded in 96-well culture plates and allowed to grow to 80% to 90% confluence. The cells were then deprived of growth factors and serum and exposed to various concentrations of LY294002 for 72 h. DNA fragmentation assays were done as described in Materials and Methods. **B**, the cells were grown to 50% to 60% confluence in 24-well culture plates and treated with LY294002 as described in (A). Apoptosis was detected by TUNEL assays ($\times 100$ magnification; *top*), whereas DAPI staining was used to identify total numbers of cells in a field ($\times 100$ magnification; *bottom*) as described in Materials and Methods. The numbers were the mean values of three different fields. **C**, the cells were seeded in six-well culture plates and treated with LY294002 as described in (A). The cells were then harvested and PI3K downstream signaling (AKT and Bax) was analyzed by Western blots. Actin was used as a loading control.

Bax in mitochondria initiates apoptotic pathways (36). Therefore, the striking difference in Bax expression between the WT and MUT cells may also contribute to the different sensitivity of the two cell types to GFDS-induced apoptosis.

The MUT cells were hypersensitive to PI3K inhibition. Because MUT PI3K provided the MUT cells a survival advantage under GFDS, we next explored whether the MUT cells were more dependent on PI3K signaling than the WT cells. LY294002 is a potent PI3K inhibitor that reversibly inhibits the activity of several different PI3Ks (37, 38). Both the WT and MUT cells were treated with LY294002 while deprived of growth factors. DNA fragmentation assays were used to detect apoptosis. The apoptosis of untreated WT and MUT cells was normalized to 1.0 and LY294002 treatment-induced apoptosis was calculated as fold change relative to the untreated condition. Consequently, whereas LY294002 induced apoptosis in both WT and MUT cells in a concentration-dependent manner, apoptosis was induced to a much greater degree in the MUT cells (1.5- to 4.5-fold) than in the WT cells (1.3- to 2.3-fold; Fig. 2A). TUNEL assays were then used to confirm these results. Although there were similar numbers of apoptotic cells in the WT and MUT cells after LY294002 treatment, there were less apoptotic cells in the MUT cells than in the WT cells under the

control condition (Fig. 2B). Consequently, there was a greater fold increase of apoptosis in the MUT cells than in the WT cells. The results indicated that the MUT cells were hypersensitive to PI3K inhibition.

Comparison of the downstream signaling pathways of the WT and MUT cells after LY294002 treatment showed that phosphorylation of AKT was inhibited by LY294002 in a concentration-dependent manner in both cell types. Despite the constitutively high level of phosphorylated AKT in the MUT cells, it suppressed to a low level similar to that of the WT cells following LY294002 treatment (Fig. 2C). Of note, the total AKT level was not affected by the drug (Fig. 2C). Furthermore, LY294002 slightly increased Bax expression in the MUT cells after 72 h of treatment (Fig. 2C). Taken together, these results indicated that LY294002 sensitized the MUT cells to GFDS-induced apoptosis through inhibition of the PI3K/AKT signaling pathway and increased expression of proapoptotic protein Bax.

To show whether the MUT and WT cells are dependent on PI3K, they were challenged with *PIK3CA* siRNAs. Transfection of *PIK3CA* siRNA into both cell types resulted in significantly reduced expression of p110 α with a concomitant decrease of phosphorylation of AKT (Fig. 3A), indicating that *PIK3CA* siRNA knocked

down p110 α expression and inhibited PI3K downstream signaling. Interestingly, *PIK3CA* siRNA also increased Bax expression in the MUT cells compared with the scrambled siRNA (Fig. 3A), suggesting that inhibition of PI3K induces Bax expression.

If the survival of the MUT cells was more dependent on PI3K signaling, we would expect that *PIK3CA* siRNA would have more effect on the survival of MUT cells than the WT cells. DNA fragmentation assays showed that *PIK3CA* siRNA induced apoptosis in the MUT cells, but not in the WT cells, compared with the scrambled siRNA (Fig. 3B). These results were further confirmed by TUNEL assays. There were less apoptotic cells in the MUT cells than in the WT cells when untransfected or transfected with the scrambled siRNA. However, the increase of apoptotic cells in the MUT cells was much higher than that in the WT cells (4-fold versus 1.5-fold) when transfected with *PIK3CA* siRNA (Fig. 3C). Therefore, the MUT cells displayed higher sensitivity to GFDS-induced

apoptosis than the WT cells when PI3K signaling was inhibited. Taken together, these results showed that, compared with the WT cells, the MUT cells are hypersensitive to PI3K inhibition and display dependence on constitutive PI3K activity for survival under stress conditions.

Bax plays an important role in GFDS-induced apoptosis.

Previous results showed that Bax expression was much lower in the MUT cells than in WT cells and inhibition of PI3K increased Bax expression in the MUT cells after LY294002 treatment or *PIK3CA* siRNA transfection (Figs. 3 and 4). We therefore hypothesized that Bax plays an important role in GFDS-induced apoptosis. To test this hypothesis, the WT and MUT cells were transfected with Bax siRNA. Western blot analyses showed that Bax expression was knocked down significantly by Bax siRNA in the WT cells, whereas it was undetectable in the MUT cells (Fig. 4A). In addition, phosphorylation of AKT was not affected by Bax down-regulation (Fig. 4A).

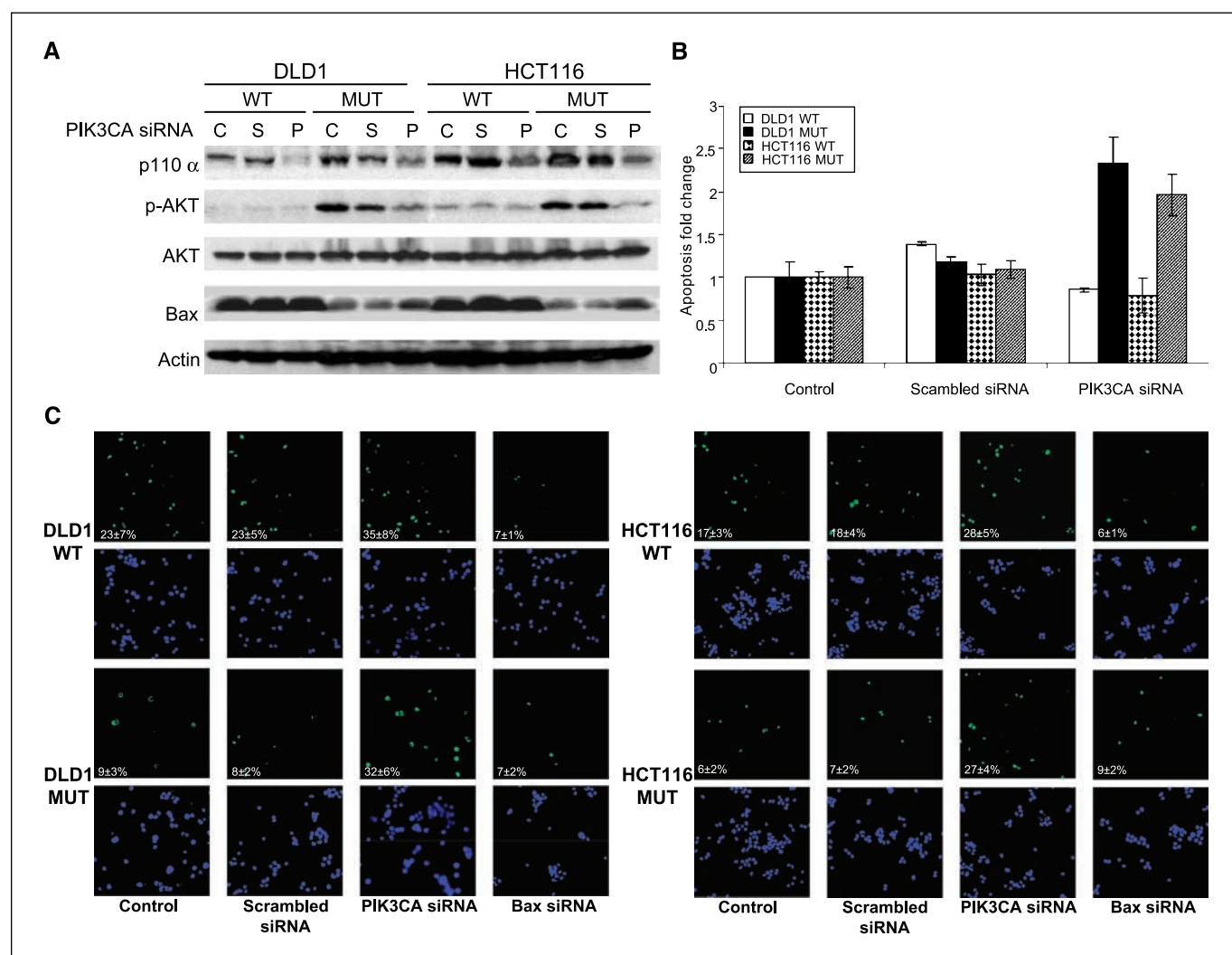


Figure 3. Effect of p110 α down-regulation on apoptosis. *A*, the WT and MUT cells were grown to 50% to 60% confluence in 24-well culture plates and transfected with *PIK3CA* siRNA (*P*) or scrambled siRNA (*S*) using LipofectAMINE 2000. Nontransfected cells were used as a control (*C*). The next day, cells were changed to growth factor and serum-free medium and starved for 72 h. Cells were then harvested and Western blot analyses were done with p110 α , phosphorylated AKT, AKT, or Bax antibodies. Actin was used as a loading control. *B*, the cells were seeded in 96-well culture plates and transfected with *PIK3CA* siRNA or scrambled siRNA as described in (*A*). The cells were then deprived of growth factors and serum for 72 h, and DNA fragmentation assays were done as described in Materials and Methods. *C*, the cells were grown to 50% to 60% confluence in 24-well culture plates and transfected with scrambled siRNA, *PIK3CA* siRNA, or Bax siRNA. The cells were then deprived of growth factor and serum for 72 h. Apoptosis was detected by TUNEL assays ($\times 100$ magnification; *top*) and total cell numbers were determined by DAPI staining ($\times 100$ magnification; *bottom*) as described in Materials and Methods. The numbers were the mean values of three different fields.

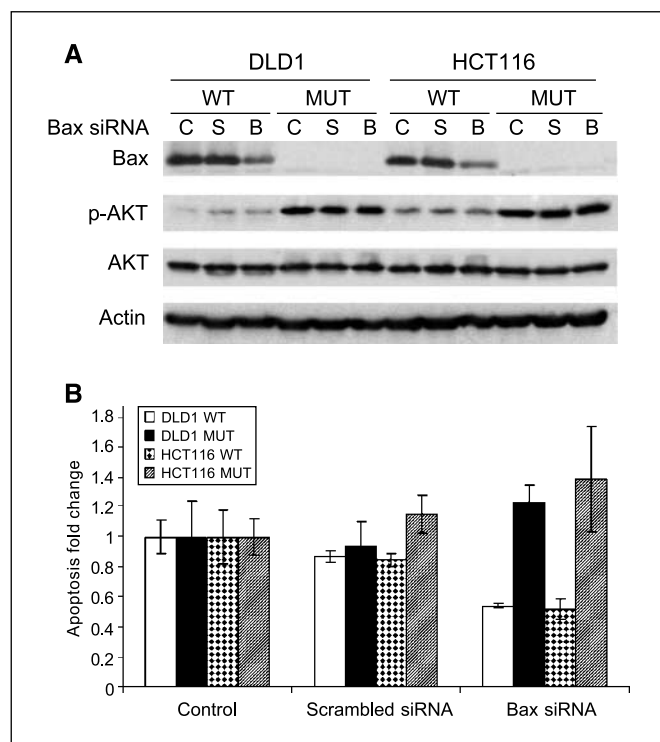


Figure 4. The effects of Bax down-regulation on apoptosis. *A*, the WT and MUT cells were transfected with Bax siRNA (*B*) or scrambled siRNA (*S*) as described in Fig. 3*A*. Nontransfected cells were used as a control (*C*). Western blots were done to analyze Bax expression and phosphorylation of AKT. *B*, the cells were seeded in 96-well culture plates and transfected with Bax siRNA or scrambled siRNA. The cells were then subjected to growth factor and serum deprivation for 72 h, and DNA fragmentation assays were done as described in Materials and Methods.

Because the MUT cells had undetectable Bax expression, we would expect that Bax siRNA should not affect their apoptosis. DNA fragmentation assays were used to detect apoptosis. The apoptosis of untransfected control WT and MUT cells was normalized to 1.0 and that of scrambled or Bax siRNA-transfected cells was calculated as fold change relative to the untransfected control. As expected, DNA fragmentation assays showed that Bax siRNA reduced GFDS-induced apoptosis by ~50% in the WT cells compared with the scrambled siRNA, whereas it had little effect in the MUT cells (Fig. 4*B*). These results were confirmed by TUNEL assays. Although there were similar numbers of apoptotic cells in the WT and MUT cells when transfected with Bax siRNA, there were more apoptotic cells in the WT cells than in the MUT cells when untransfected or transfected with the scrambled siRNA (Fig. 3*C*). Consequently, there was a greater degree of decrease in the apoptotic rate of the WT cells than that of the MUT cells. This indicated that reduction of Bax made the WT cells more resistant to GFDS-induced apoptosis. This result suggested that the higher Bax expression of the WT cells at least partially contributed to GFDS-induced apoptosis in these cells.

The MUT cells are more metastatic than the WT cells in an orthotopic model of colon cancer. Recently, *in vivo* and *in vitro* observations indicated that the metastatic potential of tumors is associated with an increased resistance to apoptosis (39–41). Because MUT cells have more robust cell survival signaling than WT cells, we determined whether the MUT cells display enhanced metastatic potential in an *in vivo* orthotopic model of colon cancer.

GFP-labeled MUT and WT cells were s.c. injected into BALB/c nude mice. Once xenografts were established, pieces of xenograft (1 mm³) were subserosally implanted into other BALB/c nude mice.

As shown in Table 1, all animals showed growth of the primary tumor with invasion of the bowel 35 days after implantation. DLD1 MUT cells were weakly metastatic (7% and 10% for liver and lung metastasis, respectively), whereas DLD1 WT cells showed no metastases in the orthotopic model. Although animals bearing HCT116 MUT tumors showed higher incidence of distant colonization to both liver and lungs (72% and 76%, respectively) than those with HCT116 WT tumors, there was considerable metastasis in mice bearing HCT116 WT tumors. However, on GFP imaging, HCT116 MUT-bearing animals showed a much higher tumor burden of lung metastases compared with the WT-bearing animals (Fig. 5*A*).

To further investigate and quantitate the tumor burdens in the lungs of the animals orthotopically implanted with xenografts formed by HCT116 WT or MUT cells, RNA was extracted from one lung of each mouse and semiquantitative RT-PCR was done using a human-specific GAPDH primer. The level of human GAPDH mRNA expression in each sample represents the amount of human RNA, which is a reflection of tumor burden in the lungs of the mice. RT-PCR results showed that the human-specific GAPDH mRNA level was almost undetectable in the WT group except for one sample, whereas the levels were quite high in most of the samples in the MUT group (Fig. 5*B*), which confirmed that the tumor burdens of the MUT group were much larger than the WT group. Taken together, these results indicated that the MUT cells had greater capacity than the WT cells to colonize and survive in the distant organs, such as lungs. These results suggested that MUT PI3K might play an important role in metastasis of colon cancer and can be used as a target for colon cancer treatment.

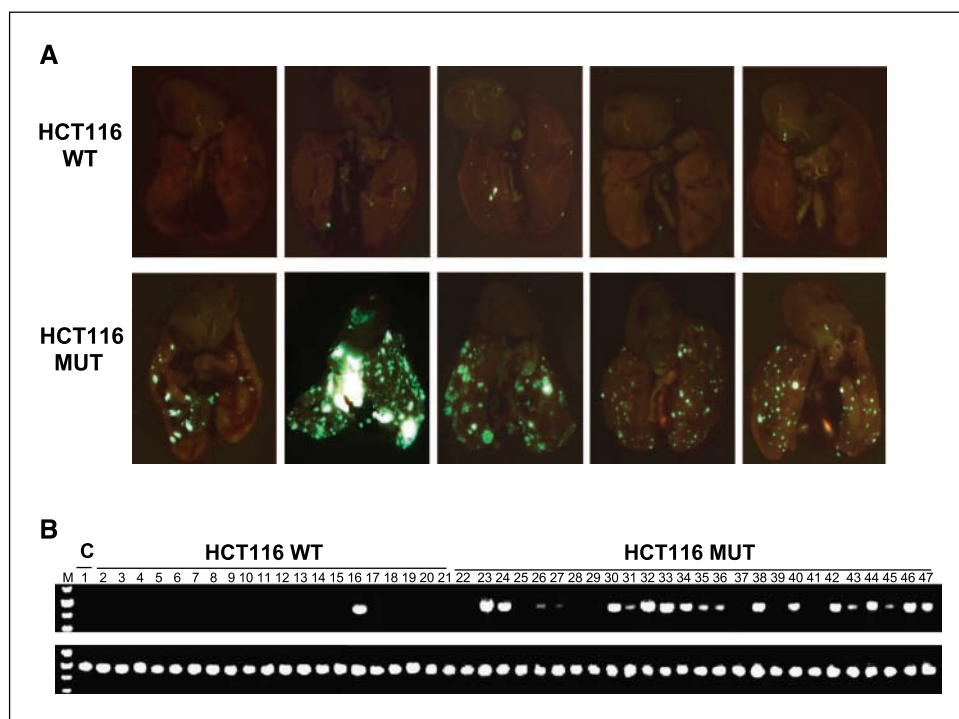
Discussion

Gain-of-function *PIK3CA* mutations have been identified in a variety of human cancers, including colon cancer. Recently, Samuels et al. asymmetrically knocked out the WT or MUT *PIK3CA* allele in DLD1 and HCT116 colon cancer cells (30). They showed that the cells bearing only MUT *PIK3CA* allele showed constitutive PI3K activity, resulting in attenuation of apoptosis and enhanced tumor formation (30). Consistent with their study, we showed here that MUT *PIK3CA*-bearing colon cancer cells displayed resistance to GFDS-induced apoptosis through constitutive activation of PI3K pathway. In addition, our results indicate that low Bax expression in the MUT cells contributes to increased survival capacity under stress. More importantly, we showed for the first time in an orthotopic model that MUT PI3K increased the metastatic potential of colon cancer cells. Recent studies have

Table 1. Summary of liver and lung metastasis of WT and MUT cells

Cell line	Primary invasion	Liver metastasis	Lung metastasis
HCT116 MUT	29/29 (100%)	21/29 (72%)	22/29 (76%)
HCT116 WT	28/28 (100%)	14/28 (50%)	12/28 (46%)
DLD1 MUT	30/30 (100%)	2/30 (7%)	3/30 (10%)
DLD1 WT	27/27 (100%)	0/27 (0%)	0/27 (0%)

Figure 5. *PIK3CA* mutations promote tumor metastasis. **A**, HCT116 WT and MUT cells were labeled with GFP and injected s.c. into athymic nude mice. Tumor xenografts were implanted as described above. Lung metastases were detected at autopsy by GFP imaging. Photographs show representative examples of GFP imaging results. **B**, one of the lungs from each mouse was harvested on day 35 and RNAs were isolated. *Top*, RT-PCR assays were done to detect the mRNA level of human-specific GAPDH; *bottom*, nonspecific GAPDH was used as loading control. RNA from normal mouse lungs was used as negative control.



indicated that apoptosis is an important process regulating metastasis and that resistance to apoptosis favors the metastatic process (39–41). Therefore, our studies suggest that mutational activation of PI3K provides cancer cells with survival advantage, which allows them to survive an environment deficient in blood supply and nutrients due to inadequate vascularization. The ability of malignant cells to withstand these stresses is considered a key factor in tumor development and progression to metastasis.

Similar to the sensitivity of the gain-of-function EGFR MUTs in lung cancers to EGFR antagonism (29), our studies indicate that the MUT *PIK3CA*-bearing cells are hypersensitive to PI3K inhibition and more dependent on aberrant PI3K signaling, therefore indicating that *PIK3CA* mutations are associated with “oncogenic addiction.” These results imply an important role for gain-of-function MUT PI3K in carcinogenesis and suggest that identification of the subset of patients harboring *PIK3CA* abnormalities and developing adequate drugs for selectively targeting the MUT PI3K forms might provide a novel way to treat this subset of cancers.

We showed that the MUT *PIK3CA*-bearing cells expressed low levels of Bax. In contrast, the WT cells with low PI3K/AKT activation expressed much higher levels of Bax. Inhibition of expression of Bax by siRNA in the WT cells conferred resistance of the cells to GFDS-induced apoptosis. These results suggest that Bax expression is negatively correlated with MUT PI3K/AKT activity and that Bax is an important effector in GFDS-induced apoptosis. Bax is a proapoptotic protein that translocates into mitochondria after a death signal, which then leads to release of cytochrome *c* and apoptosis. It has been reported that the hormone glucose-dependent insulinotropic polypeptide (GIP) potently activated the PI3K/AKT/FKHR signaling pathway and down-regulated Bax expression in pancreatic β -cells (42). GIP decreased Bax promoter activity that reduced Bax at the mRNA level. In our study, despite the striking difference in Bax protein expression, Bax mRNA expression was very similar between the WT and MUT cells (data

not shown). Therefore, different mechanisms may be involved in the regulation of Bax expression in pancreatic β -cells and colon cancer cells. An earlier study showed that AKT, activated by nicotine, colocalized with Bax in the cytoplasm and phosphorylated Bax at Ser¹⁸⁴ in the COOH-terminal hydrophobic tail (43). Phosphorylation inactivated Bax apoptotic activity by blocking translocation of Bax from the cytosol into mitochondria. Bax phosphorylation also reduced the stability of Bax in A549 human lung cancer cells (43). Recently, Bax has been reported to be degraded in an ATP/ubiquitin/proteasome-dependent pathway in cancer cells (44). When we transfected the WT Bax and a MUT Bax (S184A), which cannot be phosphorylated, into the MUT cells, we found that the expression level of the WT Bax was significantly decreased after 72 h, whereas the expression of the MUT Bax remained at a high level (data not shown). Accordingly, the mechanism of Bax repression in the MUT cells seems to be linked to AKT phosphorylation of Bax at S184, thus leading to Bax degradation.

There is a balance between cell survival and apoptosis in the cells, which is maintained by oncogenes and tumor suppressors. Activation of oncogenes and/or inactivation of tumor suppressors lead to aberrant cell survival during tumor development (45). Mutational activation of PI3K confers resistance to GFDS-induced apoptosis, which may be an important mechanism of tumor progression in colon carcinomas given the high frequency with which these mutations are found in this disease.

Metastasis is a complex, multistep process that requires a tumor cell to be able to fulfill the two rate-limiting steps: invasion and distant colony formation. *In vivo* models, such as tail vein injection described in the Samuels article, allow for the study of the ability of a tumor cell to form distant colonies. They do not, however, allow for the study of invasion, which is ultimately necessary before distant colony formation. In the absence of transgenic animal models of metastasis, this orthotopic model of colon cancer allows

for the study of invasion in the bowel wall and of distant colony formation in the liver and the lungs (32). Metastatic spread to the liver and lungs recapitulates the pattern of colorectal cancer metastases in humans and thus shows the potential key role of the MUT *PIK3CA* in colon cancer progression.

Recently, *in vivo* and *in vitro* observations indicated that the metastatic potential of tumors is associated with increased resistance to apoptosis (39–41). We showed that the MUT cells displayed increased survival capacity under stress conditions, which might contribute to increased metastasis in the orthotopic model. However, it has been shown that, relative to their WT counterparts, HCT116 or DLD1 MUT cells had a 6- to 8-fold increased ability to migrate through a porous membrane or to invade through Matrigel (30). Therefore, increased invasion of the

MUT cells might also contribute to increased metastatic potential *in vivo*. More studies will be required to determine whether increased survival, invasion, or both contribute to the metastatic potential of those cells *in vivo*.

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