

# Chemoresistant KM12C Colon Cancer Cells Are Addicted to Low Cyclic AMP Levels in a Phosphodiesterase 4–Regulated Compartment via Effects on Phosphoinositide 3-Kinase

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## Abstract

One of the major problems in treating colon cancer is chemoresistance to cytotoxic chemotherapeutic agents. There is therefore a need to devise new strategies to inhibit colon cancer cell growth and survival. Here, we show that a combination of low doses of the adenylyl cyclase activator forskolin together with the specific cyclic AMP (cAMP) phosphodiesterase-4 (PDE4) inhibitor rolipram, but not the cAMP phosphodiesterase-3 (PDE3) inhibitor cilostamide, causes profound growth arrest of chemoresistant KM12C colon cancer cells. Low-dose forskolin causes KM12C cells to exit the cell cycle in G<sub>1</sub> by inducing p27<sup>Kip1</sup> and primes cells for apoptosis on addition of rolipram. The effect of the low-dose forskolin/rolipram combination is mediated by displacement of the phosphatidylinositol 3,4,5-trisphosphate/phosphoinositide 3-kinase signaling module from the plasma membrane and suppression of the Akt/protein kinase-B oncogene pathway, to which KM12C cells are addicted for growth. The cAMP and phosphoinositide 3-kinase pathways form a critical intersection in this response, and reexpression of the tumor suppressor lipid phosphatase, phosphatase and tensin homologue, which is commonly lost or mutated in colon cancer, sensitizes KM12C cells to growth inhibition by challenge with low-dose forskolin. Certain chemoresistant colon cancer cells are therefore exquisitely sensitive to subtle elevation of cAMP by a synergistic low-dose adenylyl cyclase activator/PDE4 inhibitor combination. Indeed, these cells are addicted to maintenance of low cAMP concentrations in a compartment that is regulated by PDE4. Well-tolerated doses of PDE4 inhibitors that are already in clinical development for other therapeutic indications may provide an exciting new strategy for the treatment of colon cancer. [Cancer Res 2007;67(11):5248–57]

## Introduction

Colorectal cancer is the third commonest cancer in the United Kingdom, which can, at present, only be cured by complete resection of the primary tumor and isolated metastasis. In reality, the majority of metastatic tumors are not resectable, and

chemotherapy is the first-line treatment for a large number of patients. Currently, chemotherapy, which is usually 5-fluorouracil (5-FU), or capecitabine, which is processed to generate 5-FU in tumor cells, folinic acid, or newer agents such as irinotecan or oxaliplatin, improves survival in only a proportion of cases (reviewed in ref. 1). Although chemotherapy can also give a modest improvement in time to tumor progression and overall survival in more advanced disease, there remains an urgent need for new treatments to improve survival. Here, we have used cancer cells of various origins, including those from the Fidler model of colorectal metastasis (2), to examine whether modulators of cAMP may successfully intervene in chemoresistant cancers, and to identify both mechanism and circumstances in which this might be useful.

Cyclic AMP (cAMP) acts as a second messenger that controls a diverse range of cellular processes (3), usually through activation of either or both protein kinase A (PKA; ref. 4) and the cAMP-GTP exchange factor Epac (5). cAMP signaling is regulated in both spatial and temporal manner by cAMP phosphodiesterases (PDE; ref. 6), which provide the sole route for degradation of cAMP in cells (3, 7). Whereas a large and complex enzyme family provide for cAMP phosphodiesterase activity within cells, invariably the majority of cAMP-hydrolyzing activity is provided by members of the phosphodiesterase 3 (PDE3) and phosphodiesterase 4 (PDE4) families (7, 8). However, enzymes of the PDE4 family have attracted much recent interest because they play a key role in underpinning compartmentalized cAMP signaling in many cell types (9) and because PDE4-specific inhibitors seem to have therapeutic potential as anti-inflammatory agents for treating chronic obstructive pulmonary disease and as cognitive enhancers and antidepressants (7, 10).

In the cancer context, there are some reports suggesting that modulating intracellular cAMP levels may have effects on the behavior of cancer cells (11). For example, the archetypal PDE3-selective inhibitor cilostazol (12) and the archetypal PDE4-selective inhibitor rolipram (7) both suppress colon cancer cell motility (13), whereas inhibition PDE4 by rolipram can negatively affect chronic lymphocytic leukemia (14). Interestingly, rolipram can also induce expression of cyclin-dependent kinase (CDK) inhibitors, leading to growth inhibition and differentiation of glioma cells (15), although a high concentration of rolipram was required for these effects. The cAMP-elevating agent forskolin (16), when used at high doses, has been reported to inhibit DNA replication in lymphocytes via PKA-mediated effects on p21<sup>CIP1</sup>, leading to dephosphorylation of the retinoblastoma protein (pRb) and disrupted tethering of proliferating cell nuclear antigen to DNA (17). Taken together, these reports tantalizingly suggest that modulating intracellular cAMP,

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-07-0097

perhaps in a localized manner by targeting particular PDE types, may affect the proliferation of cancer cells.

In the present study, we set out to test the hypothesis that some chemoresistant epithelial cancer cells are "addicted" to oncogenic growth-regulatory pathways that may be influenced by cAMP modulation. This would provide a novel, and much needed, way to inhibit such cancer cells, particularly if it could be achieved by well-tolerated synergistic low doses of cAMP modulators. We found that a combination of relatively low doses of forskolin and rolipram (but interestingly not cilostamide) can work together to cause growth arrest and apoptosis via sustained inhibition of the phosphoinositide 3-kinase (PI3K)/Akt pathway and effects on regulators of G<sub>1</sub> progression. Reexpression of the phosphatase and tensin homologue (PTEN) lipid phosphatase, which negatively regulates PI3K and is commonly lost or mutated in many human malignancies (18, 19), slows the growth of KM12C cells at low density and renders them more sensitive to growth inhibition by the low-dose forskolin/rolipram treatment. Therefore, KM12C colon cancer cells, which are resistant to cytotoxic agent-induced cell death, can be effectively growth inhibited and killed by particular modulators of cAMP degradation and synthesis; in this case, specifically by a mechanism that ablates signaling through the PI3K/Akt pathway, to which these cells are addicted for growth and survival. In a survey of 11 cancer cell lines (including 7 colon cancer cell lines), we found that up to 8 of these are sensitive to the forskolin/rolipram combination to a greater or lesser extent, implying that this may have more general applicability as a way of inhibiting cancer cells that are otherwise extremely difficult to kill.

## Materials and Methods

**Cell culture and cell lines.** KM12C cells were provided by Prof. I. Fidler (Department of Cancer Biology, M.D. Anderson Cancer Center, Houston, TX) and KM12/2C4 cells were derived as previously reported (20). MCF7, HT29, A431, WiDr, RKO, A375, H630, Du145, SW480, and SW620 were obtained from the American Type Culture Collection. MCF7 and KM12C cells were cultured in Eagle's MEM with Earle's salts supplemented with MEM vitamins, nonessential amino acids, L-glutamine (2 mmol/L), and sodium pyruvate (1 mmol/L; all from Life Technologies) in the presence of 10% fetal bovine serum (Autogen Bioclear). HT29, A431, WiDr, RKO, A375, and Du145 cell lines were cultured in DMEM supplemented with L-glutamine (2 mmol/L; Life Technologies) and 10% fetal bovine serum (Autogen Bioclear). SW480, SW620, and H630 cells were cultured in RPMI (Invitrogen) supplemented with L-glutamine (2 mmol/L; Life Technologies) and 10% fetal bovine serum (Autogen Bioclear). All cells were routinely maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub> and subcultured before reaching confluence. Cells expressing PTEN-green fluorescent protein (GFP; NH<sub>2</sub>-terminal tag) were generated by retroviral infection of KM12C/2C4 cells with PTEN-GFP in pWZL vector, and single-cell clones were selected in growth media containing 400 µg/mL hygromycin B (Calbiochem).

**Modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay.** Cell proliferation and viability was assayed indirectly by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the enzymatic reduction of MTT (Sigma) to formazan crystal by mitochondria and cellular dehydrogenase enzymes (21). Briefly, 50 µL of cell suspension containing 1,000 cells were dispensed into 96-well flat-bottomed microplates. Dilutions of pharmacologic agents in growth media were done in four replicate rows per cell type and per dilution. Plates were then incubated in a humidified incubator in 5% CO<sub>2</sub> at 37°C. At the time points indicated, 50 µL of MTT solution (5 mg/mL MTT in PBS) were added to a total volume of 100 µL and incubated in 5% CO<sub>2</sub> at 37°C for 4 h. Formazan crystals were dissolved with 100-µL DMSO and absorbance at 570 nm was determined with a plate reader.

**Immunoblotting.** Cells were treated with DMSO (vehicle), forskolin, rolipram (all obtained from Sigma-Aldrich), or LY294002 (obtained from Calbiochem) at the concentrations and times indicated before generation of cell extracts. Cell extracts were prepared in lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100, 10% glycerol pH 7.4) from subconfluent cell cultures and clarified by centrifugation at 4°C. Total protein was measured using microBCA reagent (Pierce). Proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and blocked before probing with indicated specific antibodies and detection by horseradish peroxidase (HRP)-conjugated secondary antibodies (antimouse and anti-rabbit HRP, Cell Signaling). Antibodies used in this study include anti-p27<sup>KIP1</sup> (Becton Dickinson Transduction Laboratories); anti-vinculin (Sigma-Aldrich); anti-p21<sup>CIP1</sup>, anti-cyclin A, anti-cyclin E, and anti-CDK1 (Cdc2 p34; Santa Cruz Biotechnology); and anti-phospho-Akt (Ser<sup>473</sup> and Thr<sup>308</sup>) and total Akt (Cell Signaling Technologies). pRb was resolved by 7.5% SDS-PAGE (29.74% acrylamide/0.24% bis-acrylamide) gels before transfer and probed with anti-total pRb antibody (Becton Dickinson Transduction Laboratories). For immunoblotting, 50 to 100 µg of cellular proteins were resolved as above.

**Cell cycle analysis and apoptosis detection.** Cells were fixed in 70% ethanol in PBS overnight. For DNA content analysis, cells were pelleted and resuspended in PBS containing 1 µg/mL RNase (Qiagen Ltd.) and 10 µg/mL propidium iodide, incubated at room temperature for 30 min, then analyzed using a Becton Dickinson (Oxford, United Kingdom) FACScan flow cytometer. To monitor bromodeoxyuridine (BrdUrd) incorporation, cells were incubated with 20 µmol/L BrdUrd for the final hour of treatment, fixed, and incubated with an anti-BrdUrd antibody (Dako) followed by FITC-conjugated secondary antibody. Apoptosis was quantified using an Annexin V-FITC detection kit (Becton Dickinson) and staining was carried out per manufacturers' instructions. Briefly, KM12C cells were set up at low density and treated for 24, 48, or 72 h with the treatments indicated. At each time point, cells were washed with cold PBS, trypsinized, and resuspended in binding buffer (100 mmol/L HEPES, 1.4 mol/L NaCl, 25 mmol/L CaCl<sub>2</sub>, pH 7.4) at a concentration of 1 × 10<sup>6</sup>/mL and 100 µL of resuspended cells were incubated with Annexin V-FITC and propidium iodide.

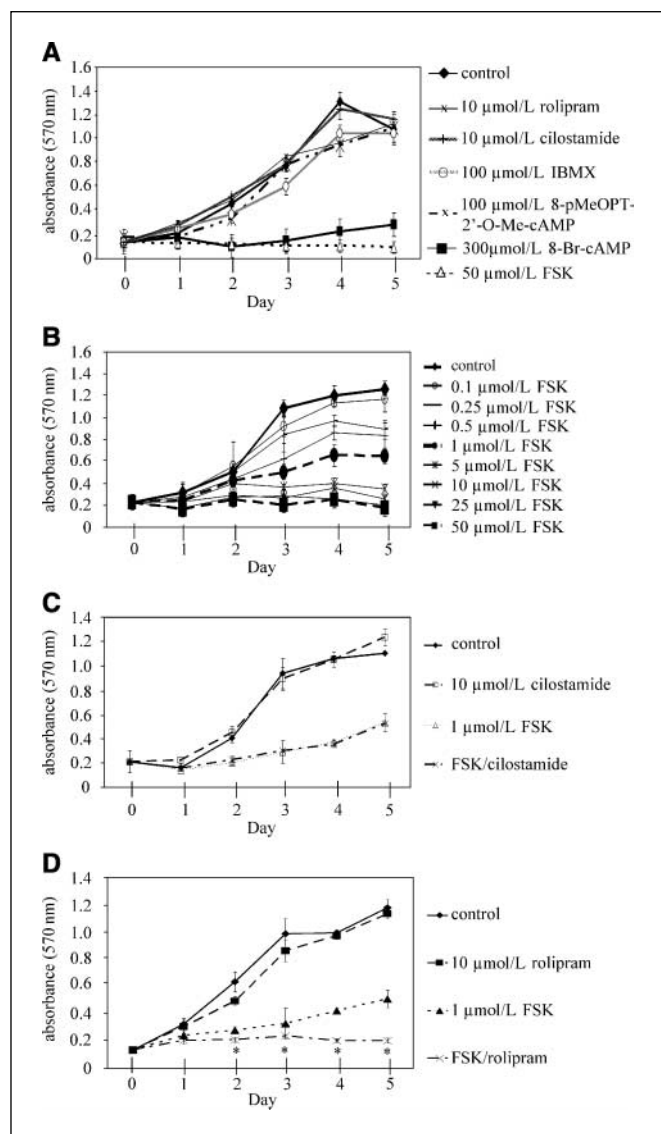
**Microscopy and immunofluorescence.** Cells were plated at a density of 1.5 × 10<sup>5</sup> per glass coverslip for transfection and 7 × 10<sup>4</sup> per glass coverslip for all other imagings. KM12C cells were transiently transfected with 1.5 µg of GFP fused to the plektstrin homology (PH) domain of Akt (GFP-PH; ref. 22) construct for 4 h using Polyfect (Qiagen) and left in fresh media overnight. Cells were treated with pharmacologic agents and then fixed using 4% paraformaldehyde for 15 min at room temperature. GFP was visualized with a confocal microscope (Leica).

**Statistical analysis.** Statistical analysis was done using the nonparametric Mann-Whitney test and *P* < 0.05 was considered significant.

**Phosphodiesterase assay.** PDE assays were done by a modification (23) of the two-step method by Thomson and Appleman (24). In brief, cells were lysed in KHEM buffer [50 mmol/L KCl, 50 mmol/L HEPES (pH 7.2), 10 mmol/L EGTA, 1.92 mmol/L MgCl<sub>2</sub>] containing protease inhibitors (Roche Molecular Biochemicals). Cells were then subjected to 14,000 × *g* for 15 min at 4°C and the resulting supernatants were assayed for total PDE activity using 1 µmol/L cAMP and [<sup>3</sup>H]cAMP as a substrate. To determine the contribution of various PDE family members to the total PDE activity, family specific inhibitors were used at a final concentration that completely inhibited their activities. PDE3 and PDE4 activities were determined using 10 µmol/L cilostamide (PDE3; ref. 12) or 10 µmol/L rolipram (PDE4; refs. 7, 10).

## Results

**cAMP modulators cause growth suppression.** Treatment of KM12C cells with the adenylyl cyclase activator forskolin (at 50 µmol/L) completely inhibits their growth (Fig. 1A), an effect that is mediated by cAMP as it is mimicked by challenging cells with the cell-permeant cAMP analogue 8-bromo-cyclic AMP (8-Br-cAMP; 300 µmol/L; Fig. 1A). However, treatment of KM12C cells



**Figure 1.** Specific cAMP elevation inhibits KM12C proliferation. Proliferation of KM12C cells was monitored over a 5-d period using a MTT dye-based assay, during which the cells were treated with modulators of cAMP. **A**, cells were treated with vehicle only (*control*; DMSO), 50  $\mu\text{mol/L}$  forskolin (FSK; a adenylyl cyclase activator), 300  $\mu\text{mol/L}$  8-Br-cAMP (a nonhydrolyzable cAMP analogue), 100  $\mu\text{mol/L}$  8-pMeOPT-2'-O-Me-cAMP (a Epac-specific activator), 100  $\mu\text{mol/L}$  IBMX (a nonspecific PDE inhibitor), 10  $\mu\text{mol/L}$  cilostamide (a PDE3-specific inhibitor), and 10  $\mu\text{mol/L}$  rolipram (a PDE4-specific inhibitor). **B**, a concentration range of forskolin (0.1–50  $\mu\text{mol/L}$ ) was carried out to establish which concentration (1  $\mu\text{mol/L}$ ) gave an  $\sim 50\%$  inhibition of proliferation. **C**, stimulation of KM12C cells with low-dose forskolin (1  $\mu\text{mol/L}$ ) in combination with a PDE3 inhibitor (10  $\mu\text{mol/L}$  cilostamide) indicated that PDE3 enzymes do not control the cAMP pool that regulates proliferation on stimulation with forskolin. **D**, PDE4 inhibition (10  $\mu\text{mol/L}$  rolipram) in combination with a low forskolin concentration (1  $\mu\text{mol/L}$ ) completely inhibited the proliferation of KM12C cells, whereas neither agent alone (at these concentrations) was able to do this. *Points*, mean of three independent experiments; *bars*, SD. \*,  $P < 0.03$ , compared with 1  $\mu\text{mol/L}$  forskolin alone.

with the cell-permeant cAMP analogue 8-pMeOPT-2'-O-Me-cAMP, which selectively activates Epac rather than PKA, did not result in growth suppression (Fig. 1A). Interestingly, treatment with the nonselective PDE inhibitor I-methyl-3-isobutylxanthine (IBMX; 100  $\mu\text{mol/L}$ ), the specific PDE3 inhibitor cilostamide (10  $\mu\text{mol/L}$ ), or the specific PDE4 inhibitor rolipram (10  $\mu\text{mol/L}$ ), at concen-

trations known to induce selective PDE inhibition (7, 9, 12), did not cause growth cessation (Fig. 1A).

We next titrated the action of forskolin and found that 1  $\mu\text{mol/L}$  forskolin gave rise to  $\sim 50\%$  inhibition of KM12C cell growth (Fig. 1B). We therefore used this "low dose" of forskolin to look for potential synergistic action with inhibitors specific for the PDE3 and PDE4 families because these are collectively responsible for  $\sim 35\%$  of cAMP-hydrolyzing activity in KM12C cells (Supplementary Table S1). We found that the PDE3-selective inhibitor cilostamide, when used at a dose known to maximally inhibit PDE3 (10  $\mu\text{mol/L}$ ; ref. 12), did not potentiate low-dose (1  $\mu\text{mol/L}$ ) forskolin-induced growth suppression (Fig. 1C). In marked contrast to this, addition of rolipram at a dose (10  $\mu\text{mol/L}$ ) known to maximally inhibit PDE4 (7, 10) enhanced the growth suppression induced by 1  $\mu\text{mol/L}$  forskolin (Fig. 1D); rolipram plus low-dose forskolin caused complete growth cessation of KM12C cells, despite rolipram having no effect on its own. Statistical analysis of the data indicated that there was no significant difference between control and rolipram ( $P > 0.09$ ) for all time points; however, for control versus forskolin ( $P < 0.03$ ), forskolin versus forskolin/rolipram ( $P < 0.02$ ), and control versus forskolin/rolipram ( $P < 0.02$ ), the data were deemed statistically significant from day 2 onward. Thus, under conditions of submaximal adenylyl cyclase activity, inhibition of specific cAMP-hydrolyzing PDE4 can suppress growth regulatory pathways in KM12C cells. The profound growth arrest is intriguing because these cancer cells are resistant to cytotoxic agents and to inhibitors of the major oncogenic Src and Ras pathways.<sup>4</sup> We may therefore have uncovered an apparent "Achilles heel" for these chemoresistant cancer cells.

#### Effects of forskolin and rolipram on cell cycle regulators.

Next, we addressed known regulators of the  $G_1$ -S transition in response to five conditions: (a) DMSO vehicle control; (b) high-dose (50  $\mu\text{mol/L}$ ) forskolin, which alone blocks KM12C cell proliferation; (c) low-dose (1  $\mu\text{mol/L}$ ) forskolin, which only suppresses proliferation by  $\sim 50\%$ ; (d) low-dose (10  $\mu\text{mol/L}$ ) rolipram, which does not affect proliferation; and (e) the combined low doses of both forskolin (1  $\mu\text{mol/L}$ ) and rolipram (10  $\mu\text{mol/L}$ ; forskolin/rolipram), which causes complete growth cessation (Fig. 1). These treatments were used throughout of the remainder of this study to investigate mechanism of action.

We found no consistent difference in p21<sup>CIP1</sup> expression induced by forskolin or rolipram (Fig. 2A, top). However, p27<sup>KIP1</sup> expression was increased by treatments that blocked proliferation, particularly by high-dose forskolin and the synergistic low-dose combination of forskolin/rolipram (Fig. 2A, middle, lanes 2 and 5). In addition, Skp2, an oncogenic F-box protein component of the SCF ubiquitin ligase complex, which is known to target p27<sup>KIP1</sup> for proteosomal degradation (25), is regulated in a reciprocal manner to p27<sup>KIP1</sup> (Fig. 2B, top, lanes 2 and 5); Skp2 protein expression is reduced when p27<sup>KIP1</sup> is enhanced after treatment with high-dose forskolin or the low-dose forskolin/rolipram combination (Fig. 2B, top, lanes 2 and 5). This suggests that the mechanism by which p27<sup>KIP1</sup> accumulates during cell cycle withdrawal may be due, at least in part, to loss of Skp2-mediated degradation. As expected, the induction of p27<sup>KIP1</sup> was paralleled by loss of phosphorylated pRb (and reduced pRb expression) as well as decreased expression of the pRb/E2F-regulated cyclins A, B1, and E, together with their

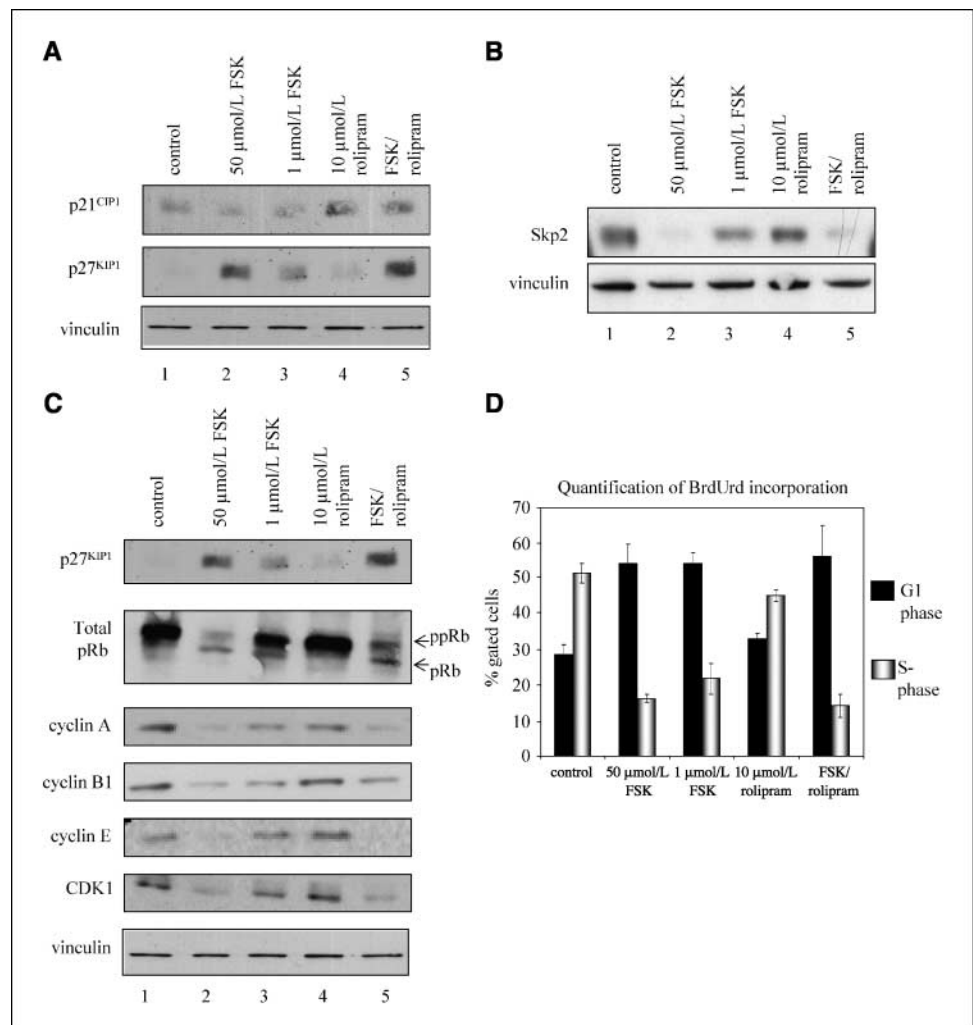
<sup>4</sup> Our unpublished data.

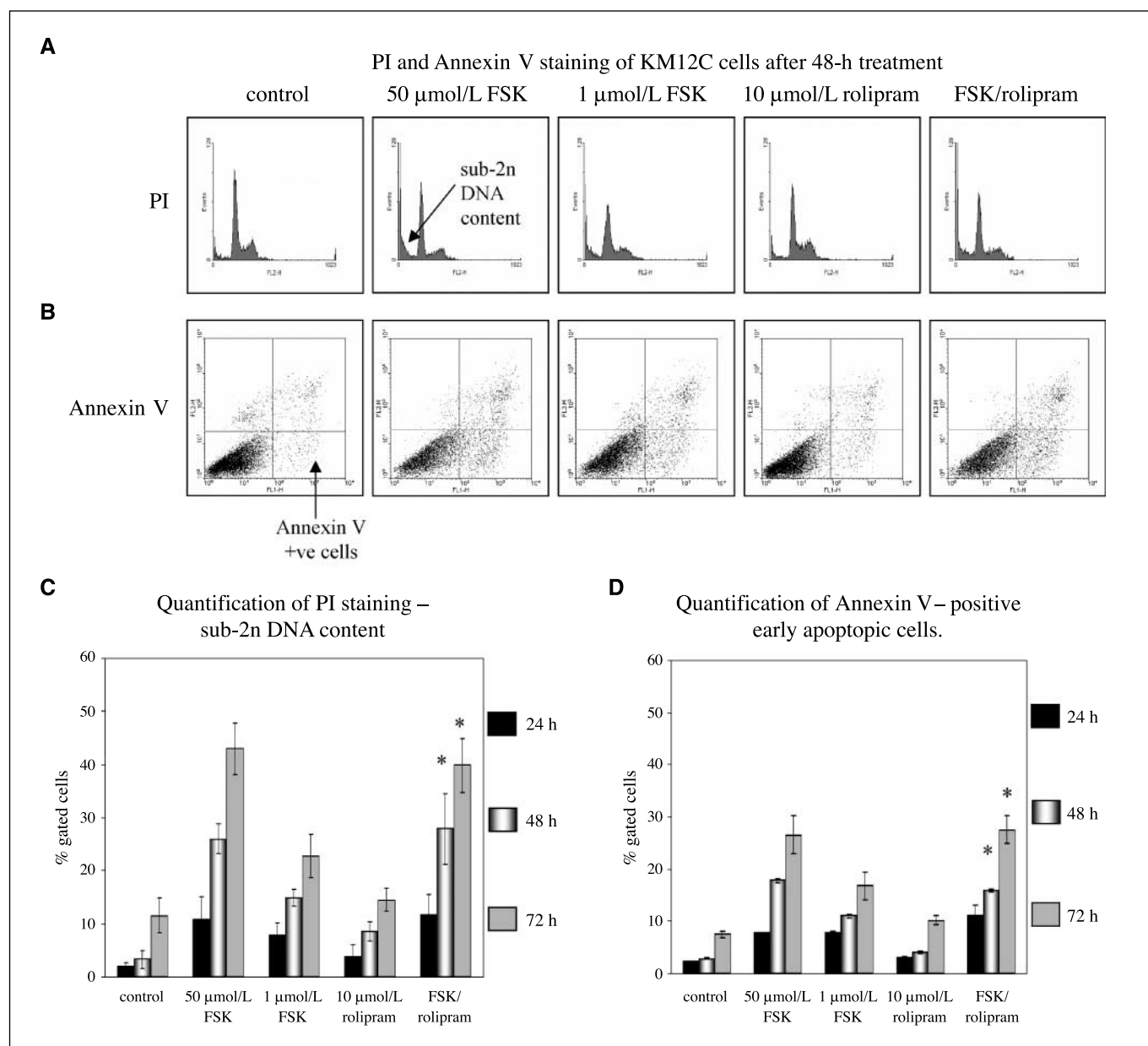
kinase partner CDK1 (Fig. 2C, lanes 2 and 5). These results imply that high-dose forskolin and the low-dose forskolin/rolipram combination induce cell cycle arrest via inhibition of the pRb/cyclin/CDK pathway, which normally controls progression through G<sub>1</sub>-S phase of the cell cycle, and that this is via stabilization of p27<sup>KIP1</sup>.

**Low-dose forskolin causes cell cycle arrest and primes KM12C cells for rolipram-induced apoptosis.** To complement the results of the proliferation assays (Fig. 1) and analysis of cell cycle regulators (Fig. 2), we next cultured cells in the combination of cAMP-modulating agents for 24 h and then pulsed with BrdUrd during the final hour. Cells were stained with propidium iodide and analyzed by flow cytometry to determine BrdUrd incorporation into the DNA at various cell cycle stages. In keeping with the antiproliferative effects observed by MTT assays, quantification of BrdUrd incorporation showed that high-dose forskolin and the low-dose combination of forskolin/rolipram (forskolin/rolipram) caused a partial G<sub>1</sub> arrest, with ~20% of cells still in S phase (Fig. 2D). Surprisingly, low-dose (1 μmol/L) forskolin caused a similar G<sub>1</sub> arrest although these cultures were still able to grow to ~50% of control cells in proliferation assays (Fig. 1). To investigate the reason why high-dose forskolin and low-dose forskolin/rolipram cause complete growth cessation whereas low-dose forskolin-treated cultures can still proliferate, albeit more slowly,

we examined cell viability. Cells were treated with the cAMP-modulating agent combinations for 24, 48, or 72 h, and the cells were fixed and stained with propidium iodide (Fig. 3A) or an Annexin V-FITC conjugate (Fig. 3B) and analyzed by flow cytometry to detect apoptotic cells. Quantification and statistical analysis of sub-2n DNA by propidium iodide (Fig. 3C) and Annexin V staining (Fig. 3D) showed that whereas both high-dose (50 μmol/L) and low-dose (1 μmol/L) forskolin caused G<sub>1</sub> arrest (Fig. 2D), only high-dose forskolin-treated cells were apoptotic. In contrast to low-dose forskolin alone or rolipram alone, the low-dose forskolin/rolipram combination caused both G<sub>1</sub> arrest and apoptosis that was statistically significant from 48 h onward ( $P < 0.05$ ) when compared with 1 μmol/L forskolin alone (Fig. 2D, quantified in Fig. 3C and D). This correlates with the data in Fig. 1D, in which a statistically significant difference in the proliferation between 1 μmol/L forskolin alone versus forskolin/rolipram combination is observed from day 2 onward and reflects the increase in apoptosis observed in forskolin/rolipram-treated cells (Fig. 3C and D). These data indicate that low-dose forskolin not only causes G<sub>1</sub> arrest but also primes KM12C cells to die, presumably from the G<sub>1</sub> arrested population, on addition of the PDE4 inhibitor rolipram (Fig. 3). Challenge with rolipram alone did not cause apoptosis (Fig. 3). This suggests that the combination of low-dose forskolin/rolipram can arrest and kill chemoresistant KM12C colon cancer cells. This

**Figure 2.** Combined low-dose forskolin/rolipram induces p27<sup>KIP1</sup>, loss of Skp2, pRb phosphorylation, and cyclin/CDK components. Subconfluent KM12C cells were cultured for 24 h under normal conditions and in the presence of DMSO (control; lane 1), 50 μmol/L forskolin (lane 2), 1 μmol/L forskolin (lane 3), 10 μmol/L rolipram (lane 4), or 1 μmol/L forskolin + 10 μmol/L rolipram (FSK/rolipram; lane 5), and the protein levels of various cell cycle regulators were analyzed via immunoblotting with specific antibodies as probes. A, high forskolin and forskolin/rolipram combination treatment increases p27<sup>KIP1</sup> (middle, lanes 2 and 5) but does not affect p21<sup>CIP1</sup> levels (top). B, forskolin and forskolin/rolipram treatment causes a decrease in levels of the SCF ubiquitin ligase adapter protein Skp2 (top, lanes 2 and 5). C, high forskolin (lanes 2) and low-dose forskolin/rolipram treatment (lanes 5) causes loss of hyperphosphorylated (ppRb) and total pRb and deregulation of the pRb/E2F regulated cell cycle control proteins cyclin A, cyclin B1, cyclin E, and CDK1. Vinculin immunoblotting was used as a loading control in all of the above. D, quantification of BrdUrd pulse-labeled KM12C cells. Cell cycle distribution is presented as percentage of gated cells. Columns, mean of three independent experiments; bars, SD.





**Figure 3.** Forskolin/rolipram induces apoptosis of KM12C cells. Effects of treatments on cell viability. KM12C cells were cultured for 24, 48, or 72 h in the presence of DMSO, 50  $\mu\text{mol/L}$  forskolin, 1  $\mu\text{mol/L}$  forskolin, 10  $\mu\text{mol/L}$  rolipram, or 1  $\mu\text{mol/L}$  forskolin + 10  $\mu\text{mol/L}$  rolipram, and then washed, trypsinized, and incubated with either propidium iodide (PI; A) or anti-Annexin V-FITC conjugate and propidium iodide (B) and analyzed by fluorescence-activated cell sorting for the detection of apoptotic cells. Results shown are for 48 h. Quantification of sub-2n DNA regions of the histograms (C) and lower right quadrants of the dot plots for the detection of Annexin V-positive early apoptotic cells (D) were used to calculate percentages of gated cells (columns, mean of three independent experiments; bars, SD). \*,  $P < 0.05$ , compared with 1  $\mu\text{mol/L}$  forskolin alone.

raises the exciting possibility that such combinations of relatively low doses of cAMP-elevating agents may provide a means of inhibiting the growth of some advanced cancer cells, which are otherwise extremely difficult to kill. This also indicates that KM12C cell viability and growth requires maintenance of cAMP at low levels, at least in the compartments that are regulated by PDE4.

**Low-dose forskolin/rolipram works by suppressing PI3K signaling.** Because the PI3K pathway plays a major role in regulating cell growth and survival (26), we examined whether it was important for continued proliferation of KM12C cells and whether it impinged on the novel, cAMP-induced, inhibitory effects on survival. Intriguingly, we found that GFP-PH [used as a reporter

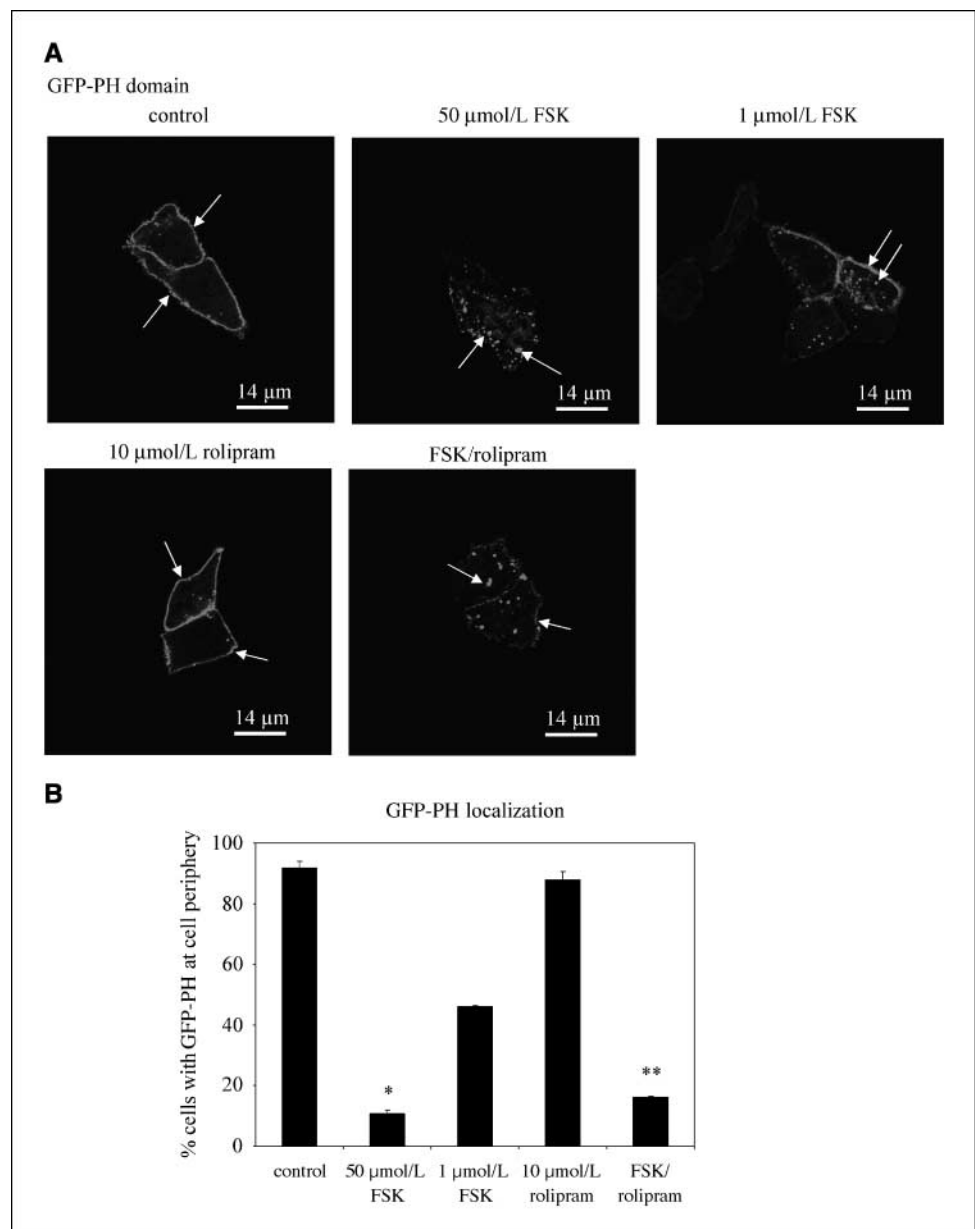
of phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) localization; ref. 22] was displaced from its normal membrane localization in cells treated with either high-dose forskolin ( $P < 0.05$ , compared with control) or the low-dose forskolin/rolipram combination ( $P < 0.05$ , compared with 1  $\mu\text{mol/L}$  forskolin alone), but not with rolipram alone ( $P > 0.09$ ; Fig. 4A, quantified in Fig. 4B). We saw similar displacement of the membrane-proximal location of the PI3K regulatory subunit p85 $\alpha$  on treatment with forskolin or the lower-dose forskolin/rolipram combination (data not shown). These data indicate that under growth inhibitory cAMP-elevating conditions, there was loss of membrane-associated PI3K and PIP<sub>3</sub>. In addition, low-dose forskolin/rolipram combination treatment

led to a rapid dephosphorylation of the PI3K/PIP<sub>3</sub>-regulated protein kinase Akt/protein kinase B (PKB) at Ser<sup>473</sup> (Fig. 5A). Moreover, although the biological effects of cAMP-elevating agents were long term and sustained, suppression of Akt/PKB phosphorylation was evident between 5 and 10 min after drug addition (Fig. 5A). Decreased phosphorylation of Akt/PKB, at both Ser<sup>473</sup> and Thr<sup>308</sup>, which are known to regulate Akt/PKB activity (27), correlated with cell death induced by high-dose forskolin or the low-dose forskolin/rolipram combination (Fig. 5B). We confirmed the implied necessity for the PI3K/Akt pathway for continued proliferation and survival of KM12C cells by showing that the PI3K inhibitory drug LY294002 recapitulated the growth inhibitory effects induced by forskolin or the low-dose forskolin/rolipram combination (Fig. 5C). Indeed, more detailed analysis indicated that 20 μmol/L LY294002 caused G<sub>1</sub> arrest, with <20% of cells still incorporating BrdUrd (Supplementary Fig. S1A, quantified in Fig. 5D), and also resulted in

accumulation of cells with sub-2n DNA content when compared with DMSO-treated controls (Supplementary Fig. S1B). In keeping with a similar mechanism of action, LY294002 also induced p27<sup>KIP1</sup>, inhibited pRb phosphorylation, and reduced the expression of cyclins A, B1, and E and CDK1, effects that were similar to the forskolin/rolipram combination (Supplementary Fig. S2). These data show that PI3K membrane localization and phosphorylation of Akt/PKB were strongly inhibited by the low-dose forskolin/rolipram combination, and this is almost certainly how these agents induce growth arrest and cell death.

**PTEN reexpression suppresses growth at low density and sensitizes KM12C cells to forskolin.** To determine whether known oncogenic or tumor suppressor regulators of the PI3K pathway influenced KM12C cell growth, we examined cells in which either PTEN or Src had been modulated by exogenous expression. Src, which positively regulates PI3K (28), is commonly

**Figure 4.** Forskolin/rolipram perturbs PIP<sub>3</sub> localization. **A**, a GFP-PH-expressing plasmid was transiently transfected into KM12C cells to monitor PIP<sub>3</sub> distribution. Its localization after 3-h treatment with DMSO, 50 μmol/L forskolin, 1 μmol/L forskolin, 10 μmol/L rolipram, or 1 μmol/L forskolin + 10 μmol/L rolipram was visualized by confocal microscopy. *Arrows*, distribution of the PIP<sub>3</sub> reporter. **B**, quantification of membrane-localized GFP-PH (Akt PH domain) reporter of PIP<sub>3</sub> after treatment with cAMP modulators or DMSO control was carried out by counting 100 transfected cells under each condition. *Columns*, mean number of cells (in percentage) from three independent experiments; *bars*, SD. \*, *P* < 0.05, compared with control; \*\*, *P* < 0.05, compared with 1 μmol/L forskolin alone.



activated, or overexpressed, in late-stage colon cancer cells (29, 30), whereas PTEN, which acts as a PIP<sub>3</sub> lipid phosphatase to down-regulate the PI3K pathway (19), is frequently lost or mutated (26). We found that overexpressing a constitutively active Src-Y527F mutant did not alter the growth properties of KM12C colon cancer cells (specifically in KM12C/2C4 cells described in ref. 20; data not shown). However, reexpression of PTEN, the expression of which is lost in these cells, slows down growth rate of KM12C/2C4, particularly evident at lower cell densities (Fig. 6B), with accumulation of cells in G<sub>1</sub> (Fig. 6C). Reexpression of PTEN resulted in reduced Akt/PKB phosphorylation to levels found in colon cancer cells that have retained PTEN expression (shown for HT29 cells in Fig. 6A). However, although PTEN-mediated control of the PI3K/Akt pathway was restored, this did not result in complete growth cessation or cell death induced by complete loss of phospho-AKT caused by the PI3K inhibitor LY294002. Together, these data provide support for a critical role for the PI3K pathway in KM12C proliferation, presumably mediated, at least in part, by PTEN loss (Fig. 6A and B). Interestingly, we found that reexpression of PTEN resulted in a 4-fold greater inhibition of cell proliferation in the presence of 1 μmol/L forskolin (or 0.5 μmol/L forskolin; Fig. 6D).

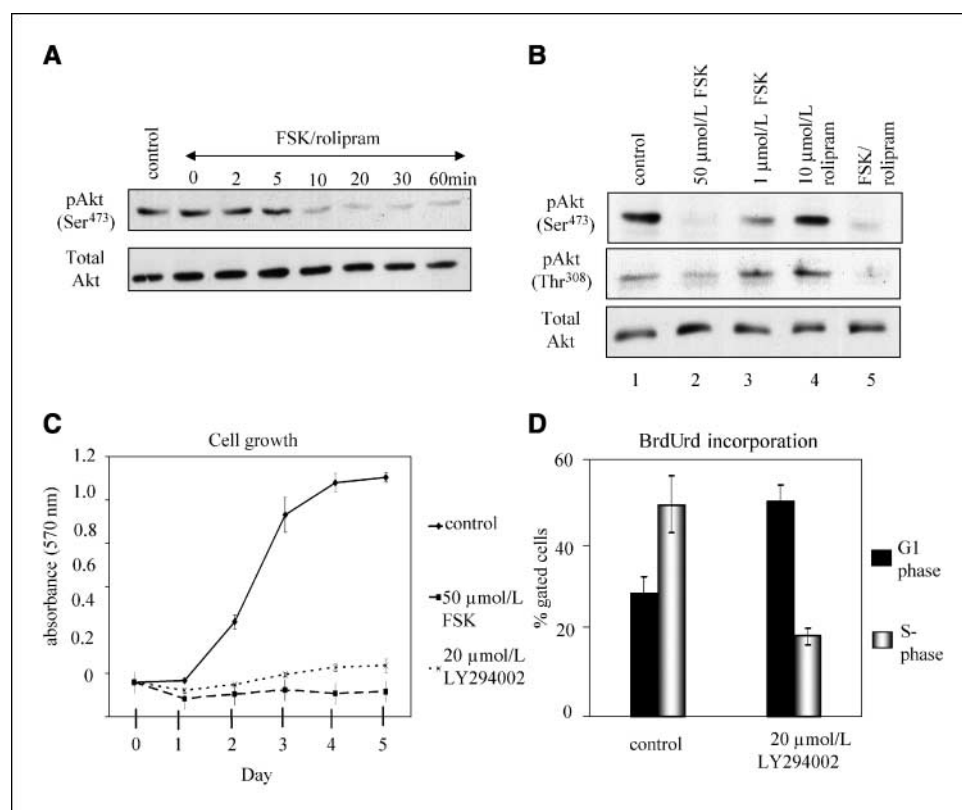
**Sensitivity to growth inhibition by cAMP modulation is not restricted to KM12C cells.** In considering the potential therapeutic benefit of any new strategy (e.g., the potentiating low-dose combination of cAMP modulators), it is important to test whether the observed effects are not particular to one cell line, in this case KM12C colon cancer cells. We therefore examined a number for their ability to be growth inhibited by the low-dose forskolin/rolipram combination. Of the 11 cancer cell lines tested, 3 of these (KM12C, MCF7, and HT29) were extremely sensitive to forskolin/

rolipram, displaying ~80% inhibition of proliferation (Supplementary Fig. S3A). Another five cell lines (A431, WiDr, RKO, A375, and H630) were partially sensitive, displaying between 40% and 60% inhibition (Supplementary Fig. S3B), whereas three cell lines (Du145, SW480, and SW620) were all insensitive to forskolin/rolipram-induced growth inhibition (Supplementary Fig. S3C). Thus, there is a subset of cancer cells that respond to a greater or lesser extent to the forskolin/rolipram combination (8 of 11 in our study), implying that a significant proportion of cancer cells may be sensitive to this type of growth modulation.

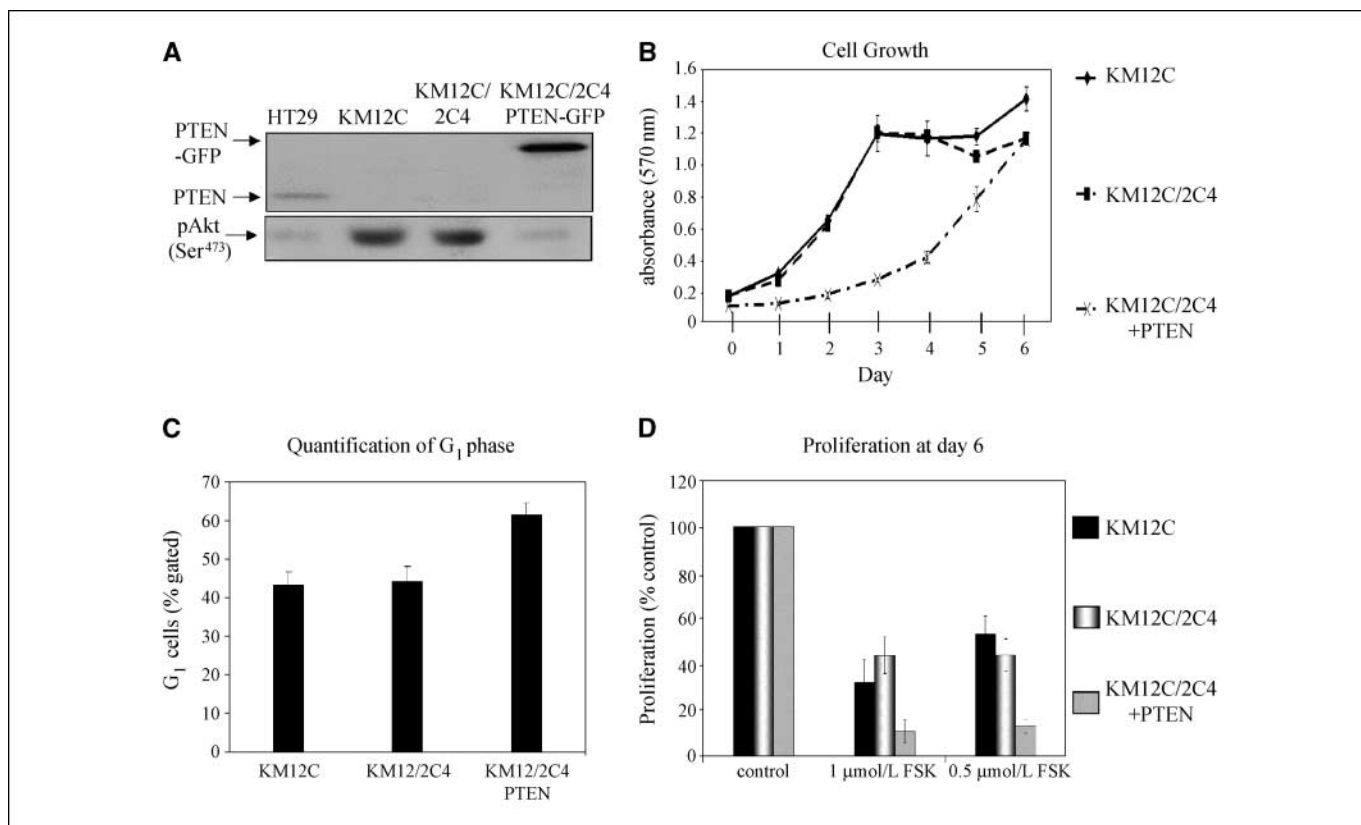
Interestingly, we found that the sensitive cells were also highly sensitive to treatment with LY294002, whereas the forskolin/rolipram-resistant cancer cell lines were relatively insensitive to LY294002 (Supplementary Fig. S3A and C), showing a consistent link between sensitivity to cAMP modulation and PI3K dependence.

## Discussion

Cancer cells, despite having many genetic, epigenetic, and chromosomal abnormalities, are often addicted to one or two oncogenic changes for continued proliferation and survival (31). Major therapeutic advances are likely to come from molecular profiling the oncogenic addictions of individual tumors. This would in turn allow tailored therapy to be more widely applied. There are now a number of spectacular examples of agents that attack critical molecular events having therapeutic benefit. For example, in non-small-cell lung cancer, a subset of patients with activating mutations in the kinase domain of the epidermal growth factor receptor (EGFR) exhibit impressive clinical responses to the EGFR inhibitor gefitinib (32). In this case, oncogene addiction is a result



**Figure 5.** Forskolin/rolipram synergy causes loss of Akt/PKB phosphorylation. **A**, phosphorylated Akt (pAkt) was monitored by Western blot of lysates prepared from cells treated for various times up to 60 min and compared with total Akt. **B**, KM12C cells were treated continuously for 24 h with DMSO, 50 μmol/L forskolin, 1 μmol/L forskolin, 10 μmol/L rolipram, and 1 μmol/L forskolin + 10 μmol/L rolipram. Phospho-Akt (Ser<sup>473</sup>) (top) and phospho-Akt (Thr<sup>308</sup>) (middle) status was monitored by immunoblotting with phospho-specific antibodies and total Akt (bottom) was compared as a loading control. High forskolin and the low forskolin/rolipram combination (lanes 2 and 5, respectively) resulted in loss of phospho-Akt (at both Ser<sup>473</sup> and Thr<sup>308</sup>). **C**, proliferation of KM12C cells was monitored by MTT assay over a 5-d period in the presence of DMSO, 50 μmol/L forskolin, or 20 μmol/L LY294002. Points, mean of three independent experiments; bars, SD. **D**, quantification of BrdUrd incorporation after 24 h of LY294002 treatment resulting in a partial G<sub>1</sub>-S phase block.



**Figure 6.** Exogenous expression of PTEN slows KM12C growth rate and sensitizes to forskolin/rolipram. Cells were plated at low density ( $5 \times 10^5$  in 60-mm<sup>2</sup> dish) for analysis. **A**, KM12C cells do not express PTEN and reintroduction restores lower phospho-Akt (Ser<sup>473</sup>) levels. Expression of endogenous PTEN in HT29 colon cancer cells and PTEN-GFP in KM12C/2C4 (top) correlates with reduced phospho-Akt (*pAkt*; Ser<sup>473</sup>), whereas lack of PTEN in KM12C and KM12C/2C4 cells correlates with increased phospho-Akt. Immunoblots were carried out with the specific PTEN and Akt antibodies as probes. **B**, reintroduction of PTEN affects low-density growth of KM12C cells. Proliferation of KM12C, KM12C/2C4, and KM12C/2C4 PTEN-GFP cells was monitored with an MTT assay over a 6-d period. **C**, PTEN increases percentage of cells in G<sub>1</sub> phase. Propidium iodide analysis of KM12C, KM12C/2C4, and KM12C/2C4-PTEN-GFP was carried out and the percentage of cells in G<sub>1</sub> calculated as described earlier. **D**, PTEN sensitizes cells to low concentrations of forskolin. Proliferation of KM12C, KM12C/2C4, and KM12C/2C4 PTEN-GFP cells in the presence of DMSO and 1 and 0.5 μmol/L forskolin was monitored over a 6-d period and the percentage proliferation of control (DMSO) at day 6 was calculated. Columns, mean of three independent experiments; bars, SD.

of mutation, not simply overexpression or inappropriate cellular activation, and it is thought that addiction may be mediated by constitutive activation of the prosurvival Akt/PKB pathway downstream of activated EGFR (33). Other clear examples of clinical benefit arising from the targeting of critical oncogenes come from treatment of breast cancers, in which the HER2 receptor tyrosine kinase is overexpressed, with the monoclonal antibody trastuzumab (Herceptin; ref. 34) and the use of imatinib (Gleevec) to treat chronic myeloid leukemia and gastrointestinal stromal tumors that are driven by the oncogenic BCR-Abl and c-Kit proteins, respectively (35). It is likely that identification of tumor oncogene addiction will thus provide a key part of delivering effective cancer treatments in the future.

Here, we establish for the first time that KM12C colon cancer cells, which are resistant to cell death induced by DNA-damaging or other cytotoxic agents commonly used to treat colorectal cancers,<sup>4</sup> are critically dependent on the PI3K pathway for their continued proliferation and survival. The PI3K pathway is frequently deregulated in cancer through a variety of mechanisms, including PTEN loss (reviewed in ref. 36), as in KM12C cells, or activating mutations in PI3K $\alpha$  (37). One consequence of such mutations is activation of downstream effectors, including Akt/PKB and mammalian target of rapamycin (reviewed in ref. 38),

which promote proliferation and cell survival. In cancer cells, such as KM12C, which are addicted to the PI3K pathway, there is an urgent need to devise effective, yet relatively nontoxic, ways to inhibit tumor cell growth and survival. In this regard, inhibitors of PI3K have been developed with against various classes of PI3K (39), although these drugs are not particularly specific (40). Although there is optimism that ongoing efforts will lead to selective isoform-specific PI3K inhibitors as therapeutic agents, these are neither readily available nor at an advanced stage of clinical development (41).

Whereas reexpression of PTEN reduces phosphorylation of Akt/PKB and causes slowed proliferation at low density (Fig. 6), it does not recapitulate complete growth cessation and cell death induced by the PI3K inhibitor LY294002 (Fig. 5C and D and Supplementary Fig. S2). This implies that although loss of PTEN is a contributing factor to the apparent dependence of KM12C cells on PI3K, other mechanisms may also operate. Intriguingly, we show here that KM12C cells can also be efficiently growth arrested and killed by a low-dose combination of the adenylyl cyclase activator forskolin and the PDE4-selective inhibitor rolipram (Figs. 1, 2D, and 3), but not by forskolin and the PDE3-selective inhibitor cilostamide. Such selectivity is consistent with the now well-established notion that cAMP signaling is compartmentalized in cells, with PDE3 and



PDE4 activities contributing to distinct functional compartments (6, 7, 9).

In evaluating the mechanism of action of forskolin/rolipram on these cells, we found induced rapid and sustained inhibition of the PI3K pathway, as judged by displacement of a GFP-PH domain (Akt PH) protein (reporting PIP<sub>3</sub>; Fig. 4), and inhibition of Akt/PKB phosphorylation on both Ser<sup>473</sup> and Thr<sup>308</sup> residues. Although we do not yet know the precise mechanism by which specific cAMP pools are mediating PIP<sub>3</sub> displacement, it is noteworthy that PKA has been shown to phosphorylate p85 $\alpha$  on Ser<sup>83</sup> and that this contributes to PKA-induced growth arrest (42). cAMP can also block the membrane localization of PDK1, an upstream activator of Akt/PKB (43). The forskolin/rolipram-induced inhibition of the PI3K pathway shown here is associated with clear changes in cell cycle regulators, including reduced Skp2, which is linked to induction of p27<sup>KIP1</sup>, together with the dephosphorylation and reduced expression of pRb, cyclins A, B1, and E, and CDK1 (Fig. 2A–C). Such key changes, commonly associated with negative regulation of progression through the G<sub>1</sub> phase of the cell cycle (17, 44), are consistent with the observed accumulation of cells in G<sub>1</sub> (Fig. 2D). In addition, whereas low-dose forskolin (1  $\mu$ mol/L) can, by itself, induce a partial G<sub>1</sub> arrest (Fig. 2D), the coapplication of rolipram (10  $\mu$ mol/L), which has no effect on its own, potentiates the effects of low-dose forskolin to cause growth-arrested cells to undergo apoptosis (Fig. 3). Taken together, these data imply that whereas submaximal stimulation of adenylyl cyclase in KM12C colon cancer cells is sufficient to cause a partial growth arrest, it also primes cells for cell death on further elevation of cAMP in subcellular compartments that are specifically controlled by PDE4 rather than PDE3. This offers a unique opportunity for therapeutic exploitation. Whereas both PDE3 inhibitors and high-dose colforsin daropate, a water-soluble forskolin derivative, exert potent positive inotropic effects on heart, no such actions are evident using PDE4-selective inhibitors (45), which is consistent with PDE3 and PDE4 controlling distinct intracellular compartments also in cardiac myocytes (6, 9). Consistent with this, PDE4-selective inhibitors, which have undergone clinical trials for treating inflammatory lung disease, have shown no inotropic or chronotropic effects on cardiac function (10, 45). Thus, a combination therapy of low-dose forskolin coupled with a PDE4 inhibitor may provide a novel means of treating various colon cancers without associated cardiac toxicity.

We have here discovered a novel way of inhibiting the PI3K pathway by a synergistic combination of relatively low doses of cAMP modulators. Our data suggest that we have found another Achilles heel for these chemoresistant cancer cells: that these cells also critically require PDE4 activity, presumably during the normal adenylyl cyclase/PDE cycle that controls cAMP production and degradation in a localized manner. Thus, PDE4 inhibition, under conditions when adenylyl cyclase activity is stimulated endogenously, results in the death of these colon cancer cells, which display chemoresistance that is extremely hard to overcome. Such

a novel addiction to maintenance of low levels of cAMP in the appropriate subcellular locations, via PDE4 activity, is required to maintain signaling through the PI3K/Akt pathway. This, as we show here, is needed for the proliferation and survival of KM12C cells. It is interesting that by bringing it back under the normal regulatory control exerted by PTEN, the PI3K/Akt pathway acts to sensitize the cells to complete growth cessation and death induced by cAMP modulation, as shown by the enhanced responses to low doses of forskolin that do not normally kill these cells (Fig. 6).

To evaluate the generality of our discovery, we probed a number of cancer cells with forskolin/rolipram combinations. In doing this, we found that 8 of 11 of such cell lines were growth inhibited by the forskolin/rolipram combination to a greater or lesser extent (Supplementary Fig. S3). In particular, three of these cell lines were extremely sensitive to growth inhibition by forskolin/rolipram, suggesting that a significant number of cancer cells may be addicted to the need to maintain low levels of cAMP in the compartment regulated by PDE4. These data raise the exciting possibility that relatively low-dose combinations of (a) pharmacologic agonists that could prime adenylyl cyclase and (b) PDE4 inhibitors, which are undergoing clinical testing in other disease contexts, may have therapeutic benefit in treating advanced colon cancers that are refractory to existing cytotoxic therapies.

In summary, misregulation of signaling proteins occurs in many cancers, leading to distorted circuitry and the establishment of oncogene addiction to one or more signal transduction pathways. It is becoming clear that the identification of such addictions can provide therapeutic opportunities, and so understanding the molecular events driving oncogene addiction, and hence tumor cell proliferation and survival, is becoming increasingly important. Here, we identify for the first time two “addictions” of chemoresistant cancer cells. Both of these addictions are required for maintenance of cell proliferation and survival (i.e., activation of the PI3K pathway and the need to maintain low cAMP levels in compartments regulated by PDE4, which itself mediates effects on the PI3K/Akt pathway). We have therefore identified a key point of cross-regulation of two major second messenger-regulated pathways in these cells, those controlled by cAMP and PIP<sub>3</sub>, which are critical for cancer cell viability. This raises the exciting possibility that the adenylyl cyclase/PDE4 axis may be exploited for therapeutic benefit.

## Acknowledgments

Received 1/15/2007; revised 3/19/2007; accepted 4/5/2007.

**Grant support:** Cancer Research UK studentship (D.G. McEwan), Cancer Research UK Beatson Institute Core Grant (M.C. Frame and V.G. Brunton), and the Medical Research Council (M.D. Houslay and G.S. Baillie).

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We wish to thank David Gillespie for advice on cell cycle analysis and Owen Sansom for advice on statistics.

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## Chemoresistant KM12C Colon Cancer Cells Are Addicted to Low Cyclic AMP Levels in a Phosphodiesterase 4–Regulated Compartment via Effects on Phosphoinositide 3-Kinase

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*Cancer Res* 2007;67:5248-5257.

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