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ORIGINAL ARTICLE

Phosphodiesterase 10A: a novel target for selective inhibition of colon tumor cell growth and β-catenin-dependent TCF transcriptional activity

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The cyclic nucleotide phosphodiesterase 10A (PDE10) has been mostly studied as a therapeutic target for certain psychiatric and neurological conditions, although a potential role in tumorigenesis has not been reported. Here we show that PDE10 is elevated in human colon tumor cell lines compared with normal colonocytes, as well as in colon tumors from human clinical specimens and intestinal tumors from *Apc*$^{Min/+}$ mice compared with normal intestinal mucosa, respectively. An isozyme and tumor-selective role of PDE10 were evident by the ability of small-molecule inhibitors and small interfering RNA knockdown to suppress colon tumor cell growth with reduced sensitivity of normal colonocytes. Stable knockdown of PDE10 by short hairpin RNA also inhibits colony formation and increases doubling time of colon tumor cells. PDE10 inhibition selectively activates cGMP/cGMP-dependent protein kinase signaling to suppress β-catenin levels and T-cell factor (TCF) transcriptional activity in colon tumor cells. Conversely, ectopic expression of PDE10 in normal and precancerous colonocytes increases proliferation and activates TCF transcriptional activity. These observations suggest a novel role of PDE10 in colon tumorigenesis and that inhibitors may be useful for the treatment or prevention of colorectal cancer.

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INTRODUCTION

Specific phosphodiesterase (PDE) isozymes hydrolyze the second messengers, cyclic adenosine and/or guanosine 3′, 5′-monophosphate (cAMP and/or cGMP) to suppress cyclic nucleotide signaling.[1] The human genome encodes 21 different PDE genes categorized into 11 families with unique substrate specificity, regulatory properties, tissue localization and sensitivity to inhibitors.[2] Studies have implicated the involvement of specific PDE isozymes in tumorigenesis. For example, PDE inhibitors that target PDE1, 4, 5, 7 or 9 have been reported to induce apoptosis or cell cycle arrest in various tumor cell lines,[3–7] while various PDE isozymes have been reported to be over-expressed in various cancer types.[3,6–9] Despite the well-known regulatory influence of cyclic nucleotides over tumor cell proliferation and survival,[10] no PDE inhibitor has received FDA approval for cancer.

cGMP has an important role in the colon to regulate chloride secretion and water transport.[11] Aside from its physiological function, studies suggest that elevation of intracellular cGMP levels can selectively inhibit proliferation and induce apoptosis of human colon tumor cells.[12,13] In addition, ligands for guanylyl cyclase C such as uroguanylin are lost during colon tumorigenesis, while treatment with such peptides can suppress colon tumor growth in the *Apc*$^{Min/+}$ mouse model of intestinal tumorigenesis.[13–15] The cGMP-dependent protein kinase (PKG) is considered to mediate the anti-proliferative and proapoptotic activity from elevation of intracellular cGMP levels.[16] For example, PKG levels have been reported to be decreased in colon tumor cells compared with normal colonocytes,[17] while ectopic expression of PKG induces apoptosis.[18] However, the upstream cGMP PDE isozymes that are expressed in colon tumor cells and pathways downstream from PKG that regulate tumor cell growth have not been well defined. A potentially important target of cGMP/PKG signaling involves the inhibition of transcriptional activity associated with β-catenin-dependent T-cell factor (TCF) to suppress proteins such as cyclin D and survivin that regulate tumor cell proliferation and survival.[18,19]

PDE10 was independently identified by three groups in 1999.[20–22] Compared with other PDE isozymes, PDE10 has the most limited tissue distribution with high levels in the brain striatum, but low levels in peripheral tissues except testes.[23,24] Studies have suggested that PDE10 has an important role in the central nervous system by regulating striatal output to reduce the sensitivity of medium spiny neurons to dopaminergic and glutamatergic excitation.[25] Preclinical studies of PDE10 inhibitors in rodent models as well as genetic knockout experiments suggest that PDE10 may impact cognition, behavior and motor function. Several inhibitors are in clinical trials for treatment of schizophrenia and Huntington's disease.[26] However, a role of

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PDE10 in tumorigenesis has not been reported in the scientific literature, nor have inhibitors been described to have anti-cancer activity.

Here we provide evidence that PDE10 is involved in colon tumorigenesis from studies showing that this enzyme is overexpressed in colon tumor cells compared with colonocytes. PDE10 was found to be essential for colon tumor cell growth as evident by experiments showing anti-proliferative and proapoptotic effects from small-molecule inhibitors and genetic silencing. Consistent with the differential expression of PDE10 in colon tumor cells compared with colonocytes, the growth of inhibitory effects resulting from PDE10 inhibition were only apparent in tumor cells. An oncogenic function of PDE10 was also evident by experiments demonstrating increased mitogenesis resulting from ectopic expression of PDE10 in normal or precancerous colonocytes. We also describe a novel pathway by which PDE10 inhibition and activation of cGMP/PKG signaling can inhibit colon tumor cell growth by attenuating β-catenin-dependent TCF transcriptional activity.

RESULTS

PDE10 levels are elevated in human colon cancer

Initial experiments using human colon cell lines demonstrated that PDE10 protein levels are elevated in HT29, HCT116, SW480 and Caco2 adenocarcinoma cell lines compared with normal colonocytes (NCM460) as determined by western blotting (Figure 1a). PDE10 is also elevated in the human colon LT97 adenoma line[27] compared with NCM460 colonocytes. Quantitative real-time RT–PCR demonstrated that PDE10 expression is upregulated at the messenger RNA (mRNA) level in colon tumor cell lines compared with colonocytes (Supplementary Figure 1). Levels of the cGMP-specific PDE isozyme, PDE5, were also elevated, which we and others have previously reported in colon and other cancer types.[4,9,12,28–31] However, other cGMP-degrading isozymes (PDE1, 2, 3, 9 and 11) were either not expressed (PDE1), displayed limited expression (PDE2, 3, 11), or had reduced expression (PDE9) in colon tumor cell lines as shown in Supplementary Figure 2.

To assess the clinical relevance of these observations, PDE10 mRNA levels were measured in human colon tumor specimens using real-time PCR-based tissue arrays. Among 40 colon tumors with disease stages ranging from Stage I–IV, 29 exhibited a significant increase in PDE10 mRNA levels, compared with eight samples from normal colonic mucosa (Figure 1b). As shown in Figure 1c, the magnitude of this increase was progressive between normal colonic mucosa and Stage II colon cancer ($P < 0.05$). A trend showing a correlation between PDE10 levels with more advanced colon cancer (Stage III and IV) was also apparent, but not statistically significant due to samples with extremely high levels of PDE10 mRNA.

Immunohistochemistry studies were also performed to analyze PDE10 expression in human clinical specimens from patients with colorectal cancer or non-malignant disease. As shown in Figure 1d, PDE10 labeling was essentially undetectable in normal colonic mucosa from patients with non-malignant disease (panels i and ii), but readily apparent in specimens from a patient with colorectal cancer containing either adenocarcinoma or uninvolved colonic mucosa as shown in panels iii and iv, respectively. Similar labeling of another specimen from a patient with colon adenocarcinoma is shown in panels v and vi, in which high magnification demonstrates that PDE10 is uniformly distributed in the cytoplasm, although there is evidence of localization in the perinuclear, nuclear and cell membrane regions of the cell. PDE10 was also expressed in additional adenocarcinoma specimens analyzed, as well as precancerous colonic adenomas and metastatic lesions in the liver as shown in panels vii and viii, respectively.

Additional evidence that PDE10 is induced during tumorigenesis was obtained from analyzing PDE10 mRNA levels in specimens from the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis. Consistent with human clinical specimens, PDE10 mRNA levels were elevated in intestinal tumors from $Apc^{Min/+}$ mice compared with normal intestinal mucosa from wild-type *Apc* mice (Figure 1e), as well as in paired tumor and normal mucosa samples from $Apc^{Min/+}$ mice (Figure 1f). PDE10 levels were also elevated in normal intestinal mucosa from $Apc^{Min/+}$ mice compared with normal intestinal mucosa from wild-type mice.

PDE10 inhibitors selectively suppress tumor cell growth

Three chemically distinct PDE10 selective inhibitors, papaverine, PQ-10 and Pf-2545920, as shown in Figure 2a, were evaluated for inhibitory effects on the growth of human colonocytes and colon tumor cell lines. The PDE10 inhibitors suppressed the growth of human HT29, SW480 and HCT116 colon tumor cell lines by greater than 90% with IC_{50} values ranging from 0.1–28 μmol/l (Figures 2b and d). Consistent with low levels of PDE10 in colonocytes, NCM460 cells were appreciably less sensitive to treatment in which IC_{50} values ranging from of 8–118 μmol/l were measured. We also determined the effect of Pf-2545920 on proliferation and apoptosis of HT29 colon tumor cells. As shown in Figures 2e and f, Pf-2545920 increased the activity of caspase-3 and 7 and inhibited DNA synthesis in a dose-dependent manner within the same concentration range as its IC_{50} value for inhibiting colon tumor cell growth. In addition, Pf-2545920 treatment led to a dose-dependent increase in the percentage of cells in the G_0/G_1 phase of the cell cycle (Figure 2g), indicating G_1 cell cycle arrest.

Small interfering RNA knockdown of PDE10 selectively inhibits colon tumor cell growth

To further study the requirement of PDE10 for colon tumor cell proliferation and survival, small interfering RNA (siRNA) knockdown studies were performed using human HCT116 and HT29 tumor cells and compared with colonocytes. Two sequences of PDE10 siRNA, designated as siPDE10-1 and siPDE10-2, were used to avoid potential off-target effects of siRNA. Knockdown efficiency was confirmed by western blotting, which showed substantial reductions of PDE10 levels in all three cell lines, including colonocytes that express appreciably lower levels of PDE10 (Figure 3a).

PDE10 siRNA suppressed the growth of colon tumor cells within 3 days of transfection by ~40% compared with scrambled control cells. Consistent with the selective growth inhibitory activity of PDE10 inhibitors, PDE10 knockdown by siRNA did not significantly affect the growth of colonocytes (Figure 3b). Knockdown of PDE10 also induced apoptosis of colon tumor cells as measured by increased activity of caspase-3 and 7, while caspase activity in colonocytes was not significantly affected (Figure 3c). In addition, a significant inhibition of DNA synthesis was measured in colon tumor cells transfected with PDE10 siRNA in comparison with cells transfected with scrambled control siRNA, which also occurred in a tumor cell-specific manner (Figure 3d). The effect of PDE10 knockdown on cell cycle distribution was also determined. As shown in Figure 3e, a significant increase in the percentage of cells in G_0/G_1 with a corresponding decrease in the S phase was measured in HT29 colon tumor cells transfected with PDE10 siRNA compared with cells transfected with scrambled control siRNA. Observations from PDE10 inhibitors and siRNA knockdown studies indicate that the tumor cell growth inhibitory activity caused by PDE10 inhibition involves the inhibition of tumor cell proliferation resulting from G_1 cell cycle arrest and apoptosis induction.

Stable PDE10 knockdown inhibits colon tumor cell growth

Stable PDE10 knockdown cell lines were also developed using short hairpin RNA (shRNA). For establishing stable clones, two

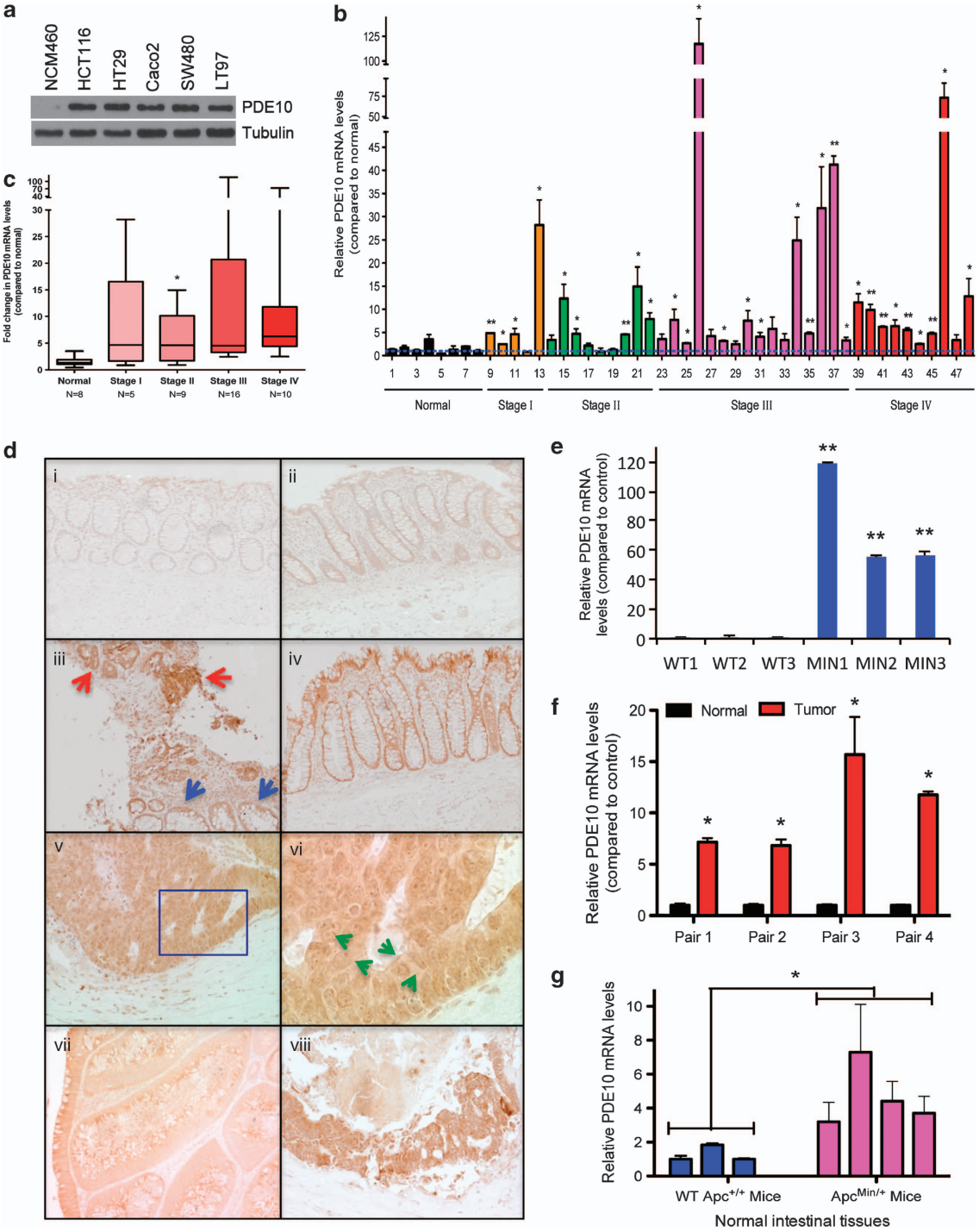

Figure 1. PDE10 expression is elevated in human colon cancer and intestinal tumors from $Apc^{Min/+}$ mice. (**a**) PDE10 protein levels in human normal colonocytes (NCM460) and colon tumor cell lines derived from adenocarcinomas (HCT116, HT29, Caco2, SW480) or adenomas (LT97) as determined by western blotting. The antibody recognizes a protein band with a MW of ~90 kDa, which corresponds to the predicted MW of PDE10. (**b**) PDE10 mRNA expression in human normal colon and adenocarcinomas as determined by quantitative real-time PCR. (**c**) Data for induction fold for each cancer stage in panel (**b**) are represented as box-and-whisker plots with minimum and maximum. The boxed area represents 50% of samples (from the 25th to the 75th percentile) and the band inside the box represents the median. (**d**) Expression of PDE10 in normal colonic mucosa (i, ii), adenocarcinoma and matched uninvolved mucosa distal to tumor (iii, iv), another adenocarcinoma (v, vi), adenoma (vii) and metastasis to liver capsule of colorectal cancer (viii) as determined by immunohistochemistry. Tissues were labeled either with a PDE10-specific antibody from Novus (i–vi) or a polyclonal PDE10 antibody from GeneTex (vii, viii), while controls with no antibody showed no labeling. Red arrows show the border of a colon tumor adjacent to uninvolved colorectal glands (blue arrows). The area within the blue squares is enlarged and presented to the right of image. Green arrows indicate the localization of PDE10 in perinuclear, nuclear, cytoplasmic and cell memebrane regions of the cell. The final magnification ofi–iv and vii is × 100. The final magnification of v and viii is × 200. The final magnification of vi is × 630. (**e**) PDE10 mRNA levels were increased in intestinal tumors from $Apc^{Min/+}$ mice compared with normal intestinal mucosa from wild-type mice. (**f**) PDE10 mRNA expression in paired normal intestine and tumors from $Apc^{Min/+}$ mice. (**g**) PDE10 mRNA levels in normal intestinal mucosa from $Apc^{Min/+}$ mice and wild-type mice. All data in panel (**e**–**g**) are represented as mean ± s.e.m. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

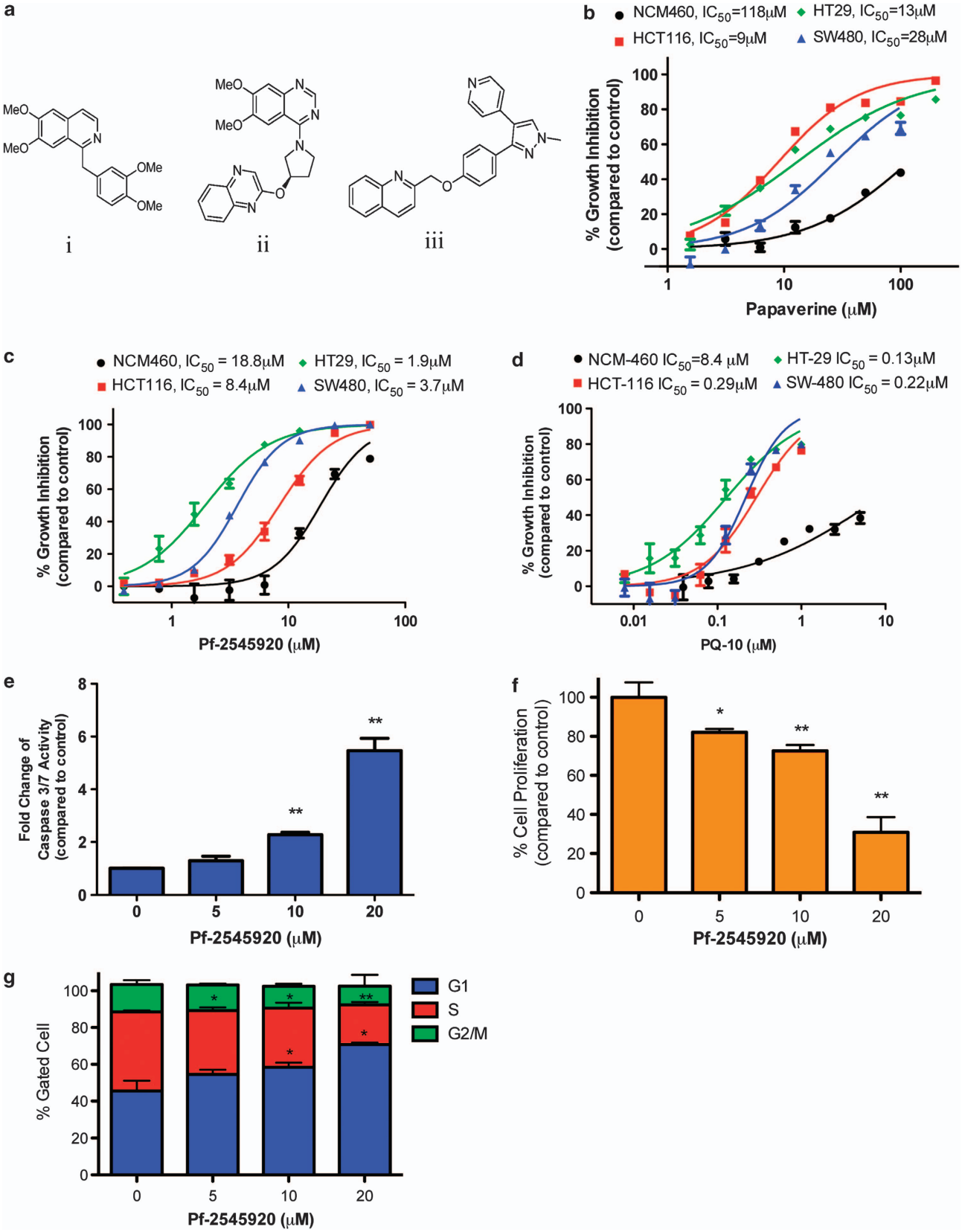

Figure 2. PDE10 inhibitors selectively inhibited colon tumor cell growth. (**a**) chemical structures of papaverine (i), PQ-10 (ii) and Pf-2545920 (iii). (**b**–**d**) Dose-dependent cell growth inhibitory activity of PDE10 inhibitors papaverine (**b**), PQ-10 (**c**) and Pf-2545920 (**d**) as measured by luciferase-based ATP assay after 72 h of treatment. (**e**) Apoptosis induction of HT29 cells by Pf-2545920 after 24 h of treatment. (**f**) Inhibition of proliferation by Pf-2545920 treatment after 24 h in HT29 colon tumor cells. (**g**) Induction of G1 cell cycle arrest in HT29 cells after 48 h of treatment with Pf-2545920. All data are represented as mean ± s.e.m. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

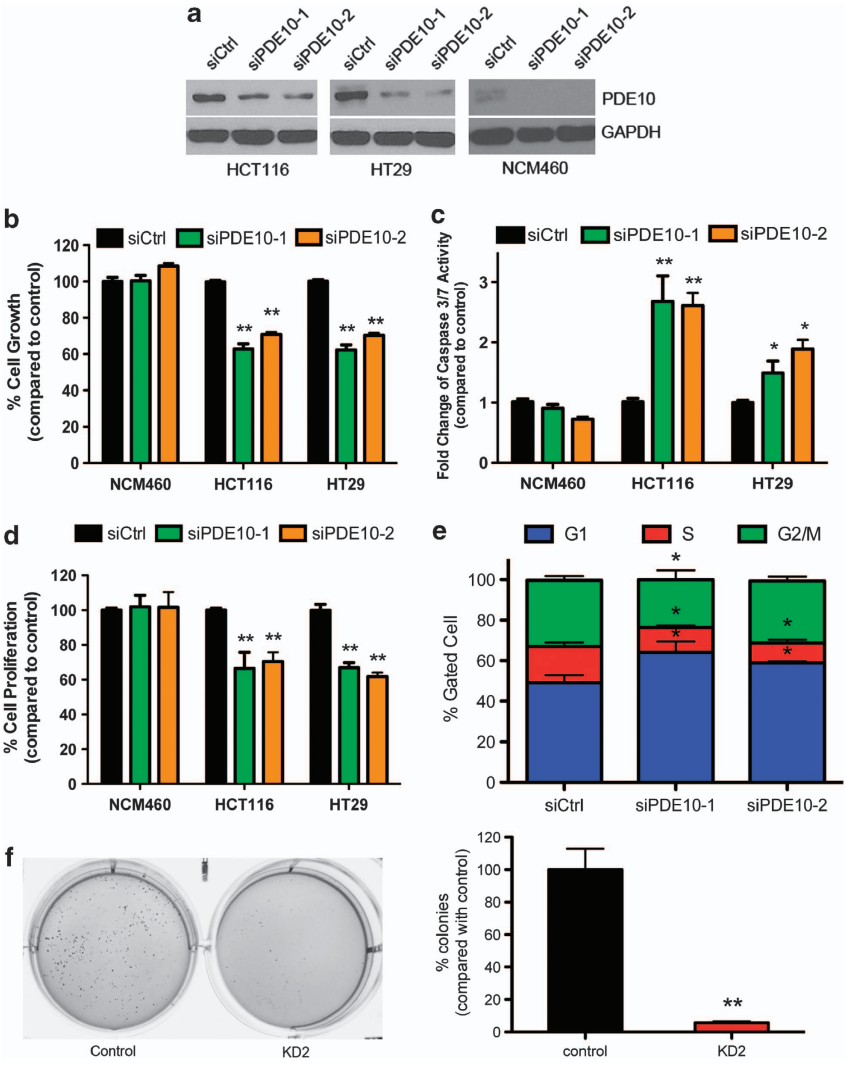

Figure 3. Knockdown of PDE10 exhibits potent anti-cancer activity. (**a**) siRNA-mediated knockdown of PDE10 expression as determined by western blotting. (**b**) PDE10 knockdown by siRNA selectively inhibited tumor cell growth. (**c**) Tumor cell-specific induction of apoptosis by PDE10 siRNA. (**d**) Selective inhibition of tumor cell proliferation by PDE10 siRNA. (**e**) siRNA-mediated PDE10 knockdown arrested HT29 colon tumor cells in the G_1 phase of the cell cycle. (**f**) Stable PDE10 knockdown leads to a decrease of anchorage-independent colony formation in HT29 cells. Cells were grown in soft agar for 21 days then stained and counted. Images from a representative experiment are shown on the left, and statistical analysis is shown on the right. All data are represented as mean ± s.e.m. of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$.

different colon tumor cell lines, HT29 and SW620, were used to eliminate the potential for cell line-specific effects. Two stable PDE10 knockdown clones were selected from the HT29 cell line, and one from the SW620 cell line. Knockdown of PDE10 was confirmed by western blotting (Supplementary Figures 3a and b). As shown in Supplementary Figures 3c and d, a 50–75% reduction of growth was observed in stable PDE10 knockdown cells compared with vector control cells after 6 days of growth. The effect of PDE10 knockdown on growth was especially evident by confocal microscopy (Supplementary Figure 3e). Stable PDE10 knockdown also increased doubling time of the cell population from 21 h in vector control HT29 cells to 45 h in the knockdown cells (Supplementary Figure 3f). In addition, increased apoptosis following PDE10 knockdown was observed by measuring cleaved caspase-3 levels using western blotting (Supplementary Figure 3a).

Colony formation assays were also conducted using stable PDE10 knockdown cells. In these experiments, PDE10 knockdown significantly interfered with the ability of HT29 and SW620 cells to form colonies by as much as 90% within 14 days of monolayer culture as compared with vector control cells (Supplementary Figures 4a, b). In addition, soft agar growth assays were performed to evaluate the effect of stable PDE10 knockdown on colony formation under conditions requiring anchorage-independent cell growth. As shown in Figure 3f, PDE10 knockdown substantially impaired the ability of the cells to form colonies in comparison with vector control cells. These results indicate that PDE10 is necessary for anchorage-dependent and -independent growth of HT29 colon tumor cells.

PDE10 knockdown selectively inhibits cGMP hydrolysis and activates the PKG pathway

To determine the effect of the PDE10 knockdown by siRNA on cyclic nucleotide hydrolysis, whole-cell lysates from transfected cells were assayed for PDE activity using cGMP or cAMP as substrates. In comparison with lysates obtained from cells

transfected with scrambled siRNA, transfection with PDE10 siRNA reduced cGMP hydrolysis by ~35% in HCT116 and HT29 colon tumor cell lines, but did not affect cGMP hydrolysis in normal colonocytes (Figure 4a). PDE10 siRNA also did not significantly affect cAMP degradation in cell lysates from the three PDE10 knockdown cell lines compared with scrambled control cell lysates (Figure 4b). The selective effect of PDE10 knockdown on cGMP signaling was substantiated by measuring the activation of the PKG and PKA pathways in intact cells using phospho-specific antibodies against vasodilator-stimulated phosphoprotein, which is known to be phosphorylated at the serine 239 residue by PKG[32] or the serine 157 residue by protein kinase A.[33] As shown in Figure 4c, PDE10 knockdown by siRNA increased PKG-mediated phosphorylation of vasodilator-stimulated phosphoprotein compared with scrambled control cells, but did not affect PKA-mediated phosphorylation. These results are consistent with the lack of effect of PDE10 knockdown on cAMP hydrolysis in whole-cell lysates and suggest that PDE10 inhibition can selectively activate the cGMP/PKG pathway in intact cells.

Tumor cell growth inhibitory activity from PDE10 inhibition is associated with suppression of β-catenin and TCF transcriptional activity

Previous studies have reported that PKG activation can inhibit the Wnt/β-catenin pathway to suppress the TCF transcription factor.[18,34] To determine if PDE10 knockdown can affect this oncogenic pathway, β-catenin levels were measured along with the expression of survivin, which is regulated by TCF transcriptional activity.[35] As shown in Figure 4d, β-catenin and survivin levels were appreciably lower in PDE10 knockdown HCT116 colon tumor cells compared with scrambled control cells. In addition, PDE10 knockdown resulted in reduced TCF transcriptional activity in HCT116 and HT29 colon tumor cells as measured using a

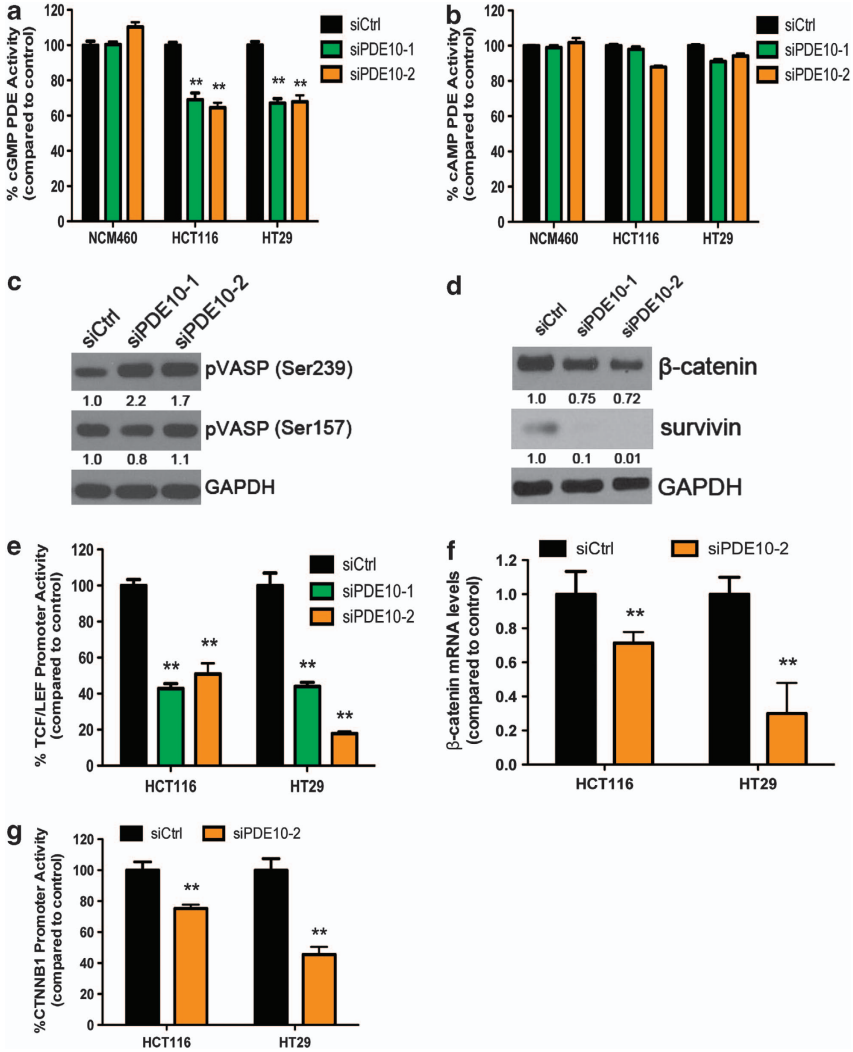

Figure 4. Knockdown of PDE10 by siRNA activates the cGMP/PKG pathway and inhibits Wnt/β-catenin signaling. (**a**) Selective suppression of cGMP hydrolysis by PDE10 siRNA in HCT116 and HT29 colon tumor cells, but not NCM460 colonocytes. (**b**) No effect of PDE10 siRNA on cAMP hydrolysis in HCT116, HT29 and NCM460 cells. (**c**), siRNA-mediated PDE10 knockdown activated PKG, but not PKA, as determined by increased phosphorylation of vasodilator-stimulated phosphoprotein at the serine 239 residue, but not the serine 157 residue, in HCT116 colon tumor cells. (**d**) PDE10 siRNA suppressed the expression of β-catenin and survivin in HCT116 colon tumor cells. The numbers indicated below the blots (panel **c** and **d**) derive from band quantification normalized to GAPDH and are expressed as ratio of scrambled control siRNA treatment. (**e**) PDE10 knockdown by siRNA inhibited β-catenin-dependent-TCF transcriptional activity. (**f**), PDE10 knockdown decreased β-catenin mRNA levels in colon tumor cells at 72 h post transfection. β-catenin mRNA expression was normalized to GAPDH expression. (**g**) suppression af *CTNNB1* (β-catenin gene) transcriptional activity by PDE10 siRNA after 72 h transfection in HCT116 and HT29 colon tumor cells. All data are represented as mean ± s.e.m. of three independent experiments. $^{**}P < 0.01$.

luciferase reporter gene assay (Figure 4e). To determine the mechanism by which PDE10 siRNA suppresses β-catenin signaling, the effect of PDE10 knockdown on steady-state levels of β-catenin mRNA in colon tumor cells was measured. As shown in Figure 4f, a marked decrease in β-catenin mRNA levels was observed in PDE10 knockdown colon tumor cells compared with scrambled control cells. Knockdown of PDE10 also significantly inhibited the transcription of β-catenin as measured using a reporter construct that encodes for the promoter regions of β-catenin gene (*CTNNB1*) (Figure 4g). In addition, levels of phosphorylated β-catenin were measured by western blotting using a phospho-specific antibody with specificity for Ser33/37/Thr41 residues known to target β-catenin for proteasomal degradation, but we did not observe an increase of β-catenin phosphorylation in response to PDE10 knockdown (Supplementary Figure 5), suggesting that suppression of PDE10 can inhibit β-catenin expression at the transcriptional level rather than by increasing proteasomal degradation. These findings were confirmed by observations of reduced expression of β-catenin, cyclin D1 and survivin along with inhibition of TCF transcriptional activity in stable PDE10 knockdown cell lines (Supplementary Figure 6).

Overexpression of PDE10 promotes growth of colonocytes and adenoma cells

Based on the above loss-of-function experiments, we predicted that increased PDE10 expression in colonocytes could stimulate growth by activating the Wnt/β-catenin pathway. To test this possibility, gain-of-function experiments were performed using colonocytes derived from either normal colonic intestinal mucosa (NCM460) or precancerous adenomas (LT97) that were transiently transfected with a PDE10-expressing vector to determine if ectopic expression of PDE10 is associated with changes in cell growth. Increased expression of PDE10 in both cell lines compared with vector control cells was confirmed by western blotting (Figure 5a). A 30% increase in viable cell number compared with vector control cells was observed in colonocytes, while adenoma-derived cells displayed a 20% increase within 3 days following transfection (Figure 5b). Consistent with experiments described above in which knockdown of PDE10 by siRNA suppressed Wnt/β-catenin signaling, cyclin D1 and survivin expression were induced in colonocytes by overexpressing PDE10 (Figure 5c). These observations suggest that the mitogenic effects from PDE10 overexpression result from an activation in Wnt/β-catenin signaling.

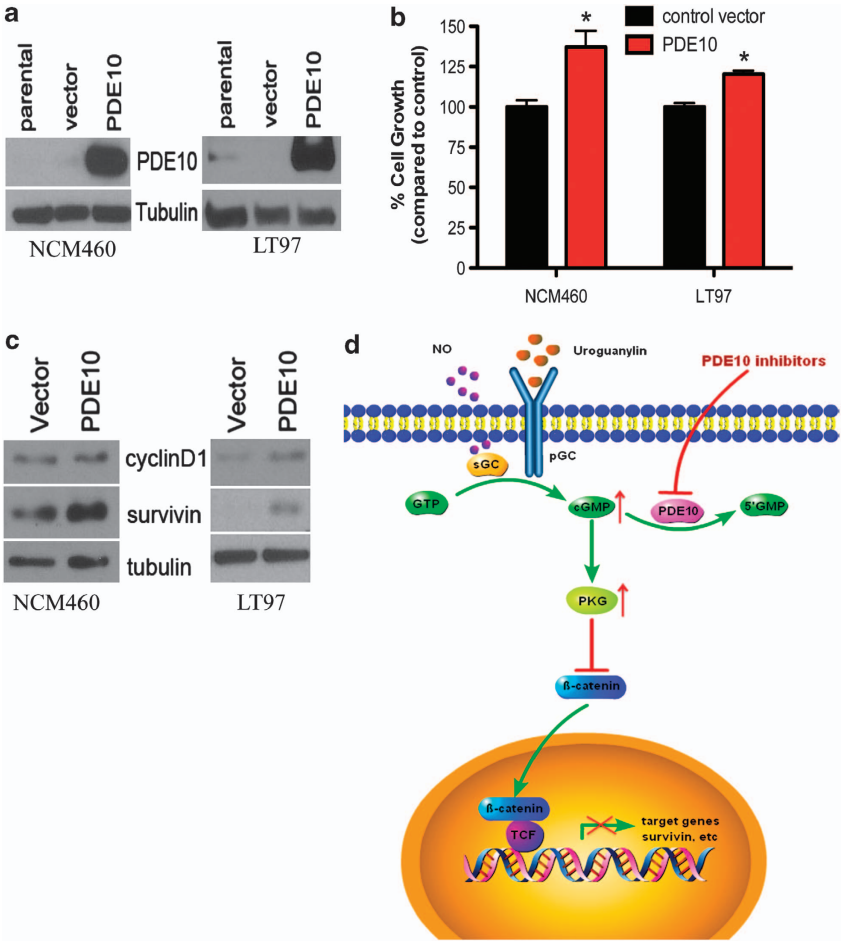

Figure 5. Overexpression of PDE10 promotes colon cell growth. (**a**) Confirmation of ectopic expression of PDE10 in colonocytes (NCM460) and adenoma cells (LT97) by western blotting. (**b**) Ectopic expression of PDE10 promoted growth of colonocytes and adenoma cells after 72 h transfection of PDE10-expressing vector compared with cells transfected with control vector. All data are represented as mean ± s.e.m. of three independent experiments. *$P < 0.05$. (**c**) PDE10 overexpression activated β-catenin signaling pathway as shown by increased expression of cyclin D1 and survivin, two genes that are regulated by β-catenin signaling, in PDE10-experssing vector transfected cells. (**d**) Schematic hypothesis for the mechanism underlying the antitumor activity of PDE10 inhibition in colon tumor cells. Inhibition of PDE10 elevates intracellular cGMP levels and activates PKG to suppress the expression of β-catenin and inhibit TCF transcription of target genes (for example, cyclin D and survivin).

DISCUSSION

We show here *in vitro* and *in vivo* evidence that PDE10 levels are elevated in colon tumors cells compared with normal colonocytes. An isozyme and tumor-selective role of PDE10 was demonstrated by the ability of specific PDE10 inhibitors and siRNA to selectively suppress colon tumor cell growth by inhibiting proliferation and inducing apoptosis. Other experiments showed mitogenic effects resulting from ectopic expression of PDE10 in normal colonocytes. The growth inhibitory activity resulting from PDE10 inhibition appears to be mediated by the cGMP/PKG pathway to inhibit β-catenin-dependent TCF transcriptional activity, while the mitogenic activity from PDE10 overexpression involves the induction of β-catenin-dependent TCF transcriptional activity. These observations suggest an important role of PDE10 in colorectal tumorigenesis and as a novel therapeutic target for developing potentially safe and efficacious drugs for colorectal cancer.

While these studies are limited to colorectal cancer, additional research is necessary to determine if other tumor types involve PDE10 and are sensitive to inhibitors. However, screening of tumor cell lines in the NCI-60 panel revealed broad sensitivity to the growth inhibitory activity of Pf-2545920. Tumor arrays from other cancer types also revealed that PDE10 is expressed in numerous other tumor types.

Consistent with previous reports concluding that PDE10 has a limited expression in peripheral tissues,[23,24] low levels of PDE10 were apparent in normal colonocytes and intestinal mucosa. By comparison, high PDE10 mRNA and protein levels were evident in human cell lines derived from either adenoma or adenocarcinoma. Elevated levels of PDE10 mRNA were also observed in colon and intestinal tumors obtained from human clinical specimens and the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis, respectively. Immunohistochemistry confirmed that PDE10 protein is expressed in adenomas as well as in metastatic lesions. It was especially interesting that PDE10 levels are also elevated in normal colonic mucosa from patients with colorectal cancer compared with patients with non-malignant disease, as well as in intestinal mucosa from $Apc^{Min/+}$ mice compared with normal intestinal mucosa from wild-type mice. These observations suggest that PDE10 is induced prior to the development of tumors and may have predictive value as a cancer biomarker.

To our knowledge, this is the first report in the scientific literature describing tumor cell growth inhibitory activity of PDE10 inhibitors, including inhibitors in clinical trials for the treatment of schizophrenia and Huntington's disease (for example, Pf-2545920). The PDE10 inhibitors, papaverine, PQ-10 and Pf-2545920, were found to effectively inhibit the growth of human colon tumor cells. By comparison, colonocytes were appreciably less sensitive, which is consistent with low levels of PDE10 in colonocytes. Similar differences in expression and sensitivity to inhibitors were also observed by comparing lung tumor cells and normal airway epithelial cells. The growth inhibitory activity of Pf-2545920 was associated with reduced proliferation, increased apoptosis and G_1 cell cycle arrest.

Additional experiments were performed to further study the role of PDE10 in colon tumor cell growth using an siRNA approach. The human colon tumor cell lines, HCT116 and HT29, along with NCM460 colonocytes were transiently transfected with PDE10 siRNA and effects on cell growth, apoptosis and proliferation were determined. The results from assays measuring viable cell number, caspase activity and the level of DNA synthesis were consistent with anti-proliferative and proapoptotic effects of small-molecule PDE10 inhibitors.

Accompanying reduced protein levels of PDE10 brought about by siRNA in colon tumor cells and colonocytes, reduced levels of cGMP hydrolysis were measured in whole-cell lysates from knockdown cells compared with lysates from scrambled control cells. In agreement with the selectivity by which PDE10 siRNA inhibits colon tumor cell growth, PDE10 siRNA only inhibited cGMP hydrolysis in colon tumor cells, despite comparable reductions of PDE10 in tumor cells and colonocytes.

An unexpected finding was that PDE10 knockdown in colon tumor cells did not affect cAMP hydrolysis, despite the reported dual substrate specificity of this PDE isozyme.[20–22] The selectivity by which PDE10 knockdown induces cGMP signaling, but not cAMP signaling, is supported with experiments showing increased phosphorylation of vasodilator-stimulated phosphoprotein at the serine 239 residue, a PKG substrate,[32] but not at the serine 157 residue, a PKA substrate.[33] The selective effect of PDE10 knockdown on cGMP signaling as observed in intact cells may be attributed to an abundance of cAMP degrading isozymes that compensate for the effects of PDE10 knockdown on cAMP signaling. Alternatively, PDE10 is known to have a higher K_m for binding cGMP (3 μmol/l) compared with cAMP (50 nmol/l) and a fivefold higher V_{max} for hydrolyzing cGMP compared with cAMP. These kinetic properties have led other investigators to conclude that PDE10 may function as a cAMP-inhibited cGMP PDE,[20–22] which would also be supported by our observations.

Colon tumor cell lines with stable knockdown of PDE10 established by lentivirus-mediated transduction of shRNA were found to grow slower and have impaired ability to form colonies in monolayer or soft agar cultures. Since the ability of transformed cells to grow in an anchorage-independent fashion is a predictor of tumorigenicity and metastatic potential, the strong growth inhibitory effect resulting from PDE10 knockdown on the growth and colony formation of colon tumor cells under anchorage-independent conditions provides strong evidence that PDE10 is essential for maintaining their malignant phenotype.

Given the importance of Wnt/β-catenin signaling in colorectal tumorigenesis,[36,37] experiments were conducted to determine if PDE10 inhibition can suppress this oncogenic pathway in colon tumor cells. PDE10 inhibition by isozyme-specific inhibitors or knockdown by siRNA were found to reduce the expression of β-catenin and inhibited TCF transcriptional activity to suppress target genes (for example, cyclin D1 and survivin) that regulate colon tumor cell proliferation and survival. These observations are consistent with previous reports showing that activation of cGMP/PKG signaling can suppress the oncogenic Wnt/β-catenin pathway and implicate a role of PDE10.[9,16,29,34,38]

The mechanism by which PDE10 inhibition suppresses the oncogenic activity of β-catenin appears to be at the transcriptional level given that β-catenin mRNA levels and CTNNB1 promoter activity were reduced in colon tumor cells following transient transfection with PDE10 siRNA. Moreover, the ability of PDE10 siRNA to suppress β-catenin transcription paralleled its ability to inhibit of TCF transcriptional activity in colon tumor cells. Previous studies have reported binding sites of numerous transcription factors within CTNNB1 promoter, including AP-1, TCF/LEF, E2F1, NF-κB, MEF1, Pax5, ISRE2, Smad3/4, GATA and ZIC.[39] In addition, the translocation of PKG to the nucleus was reported,[40] suggesting a broader role of PKG in the regulation of gene transcription. It is possible that PKG can negatively regulate a transcriptional factor, thereby inhibiting transcription of β-catenin, although further experiments are necessary to study this possibility.

In summary, PDE10 was found to be strongly overexpressed in colon cancer cells and is essential for their proliferation and survival. As depicted in Figure 5d, the antineoplastic activity resulting from PDE10 inhibition involves the selective activation of the cGMP/PKG pathway in colon tumor cells, leading to the suppression of β-catenin and inhibition of TCF transcriptional activity, whereby the synthesis of important cell cycle proteins such as cyclin D and survivin are reduced. These observations suggest a novel role of PDE10 in colon tumorigenesis and as a biomarker and therapeutic target for colorectal cancer.

MATERIALS AND METHODS

Cells and cell culture

Human colon tumor cell lines and HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown under standard cell culture conditions in RPMI1640 medium containing 5% serum at 37 °C in a humidified atmosphere with 5% CO_2. The NCM460 colonocyte line derived from normal human colon mucosa[41] was obtained from INCELL (San Antonio, TX, USA) and grown in INCELL's enriched M3:10 medium with 10% serum as recommended by the supplier. The LT97 adenoma cell line was a generous gift from Dr Brigitte Marian (Medical University Vienna, Austria) and grown as previously reported.[27]

siRNA knockdown of PDE10

One siRNA targeting human PDE10 (siPDE10-1) and scrambled control siRNA were purchased from Qiagen (Valencia, CA, USA). Another human PDE10 siRNA (siPDE10-2) was purchased from Dharmacon (Pittsburgh, PA, USA). The sequences of siRNAs can be found in the Supplementary Table 1. NCM460 and HCT116 cells were transfected with PDE10 siRNAs and scrambled control siRNA using Hiperfect transfection reagent (Qiagen), while HT29 cells were transfected with siRNA duplexes using RNAiMAX reagent (Invitrogen, Grand Island, NY, USA) according to manufacturers' specifications. Cells were then incubated at 37 °C for up to 72 h post transfection.

shRNA-mediated knockdown of PDE10

GIPZ lentiviral shRNA vectors targeting human PDE10, nonsilencing GPIZ lentiviral control vector and transduction kit were purchased from Open Biosystems (Pittsburgh, PA, USA). Lentivirus particles were produced in HEK293T cells according to the manufacturer's instructions. HT29 and SW620 cells were transduced by the lentivirus particles followed by puromycin selection (5 μg/ml) for 2 weeks. Individual cell colonies stably expressing shRNA were selected and isolated in the presence of puromycin, and evaluated by western blotting.

Ectopic expression of PDE10

NCM460 and LT97 cells were transiently transfected with a human full-length PDE10 cDNA (Origene, Rockville, MD, USA) using X-tremeGENE DNA transfection reagent (Roche, Indianapolis, IN, USA) for 72 h. Empty vector PCMV6-XL4 was used as control.

TissueScan colon cancer cDNA array

TissueScan colon cancer cDNA array was purchased from Origene. The panel containing 48 clinical samples covering four disease stages and normal tissues, and were used to evaluate PDE10 expression in human colon cancer according to manufacturer's recommendation. Tissue cDNAs of assay are synthesized from high quality total RNAs of pathologist-verified tissues, normalized and validated with β-actin. The PDE10 primer and RT^2 SYBR Green qPCR Mastermix were purchased from Qiagen. Real-time quantification was performed on the Chromo 4 system purchased from Bio-Rad (Hercules, CA, USA). The results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Quantitative real-time RT–PCR

Tumors were dissected out from $Apc^{Min/+}$ mice, and the paired remaining intestinal epithelium was removed by scraping. Normal and uninvolved intestinal mucosa were also dissected from wild-type and $Apc^{Min/+}$ mice, respectively.

PDE10 mRNA levels in human colon cell lines, wild-type and $Apc^{Min/+}$ mice and β-catenin mRNA levels in PDE10 knockdown and scrambled control cells were evaluated by performing quantitative real-time RT–PCR as described previously.[42] The human *PDE10* and *CTNNB1* real-time PCR primers were purchased from Qiagen. The list of other primers used can be found in the Supplementary Table 1.

Immunohistochemistry

Tissue sections were cut from paraffin-embedded blocks and placed on chrome alum-coated glass slides. Mounted tissue sections were heated at 60 °C for 1 h, deparaffinized with xylene and rehydrated in decreasing concentrations of ethanol. Antigens were retrieved by incubating with Tris-EDTA buffer (pH 9.0) in a scientific pressure cooker for 10 min. Endogenous peroxidase activity was inactivated in 3% hydrogen peroxide solution for 5 min. After blocking with 3% goat serum for 45 min, sections were incubated with either a PDE10 antibody (Novus, Littleton, CO, USA) that the specificity has been demonstrated in previous reports[43] or a PDE10 antibody (GeneTex, Irvine, CA, USA) for 1 h at room temperature. After rinsing with Tris buffer, sections were incubated at room temperature for 30min with horseradish peroxidase-conjugated anti-rabbit antibody. 3,3′-diaminobenzidine (DAB) reactions were performed following three washes in Tris buffer. Sections were counterstained with hematoxylin for 1 min, dehydrated and mounted with permount mounting medium.

Growth assay

The effect on cell growth caused by treatment was determined after 72 h of treatment using the Cell Titer Glo Assay (Promega, Madison, WI, USA) as described previously.[12] Papaverine was purchased from Sigma-Aldrich (St Louis, MO, USA). Pf-2545920 was purchased from Selleck Chemicals (Houston, TX, USA), while PQ-10 was synthesized as described previously.[44]

Caspase, cell proliferation and luciferase reporter assays

Caspase, cell proliferation and luciferase reporter assays were performed as previously described.[12]

Cell cycle analysis

Cells were fixed and permeabilized with ice-cold 70% ethanol at 4 °C overnight. After washing with PBS, cells were treated with RNase at 37 °C for 20 min, and stained with propidium iodide (40 μg/ml). Cells were analyzed for DNA content by FACSCalibur flow cytometer (BD, Franklin Lakes, NJ, USA). A total of 10 000 events were counted for each sample. Data were analyzed with CellQuest software to determine the percentage of cells in each phase (G_1, S and G_2/M).

Cell doubling time assay

Stable PDE10 knockdown and vector control HT29 cells were plated in 6-well tissue culture plates in triplicate at a density of 250 000 cells per well. Cells were grown in RPMI1640 media supplemented with 10% serum and counted after 72 h of culture. The trypan blue exclusion test is used to determine the number of viable cells present in a cell suspension. The assay was performed each week for 4 weeks. The doubling time was calculated as follows: t (in hours) = $h \times \ln(2)/\ln(C2/C1)$. $C1$ is the cell number at the beginning of the incubation, and $C2$ is the cell number at the end of the 72 h incubation.

PDE assay and western blotting

The PDE assay and western blotting procedures and quantification methods were performed as described previously.[12] The PDE antibodies were purchased from GeneTex. All other antibodies were purchased from Cell Signaling (Danvers, MA, USA).

Colony formation assay

For colony formation assays, 500 viable cells were plated in 6-well tissue culture plates and were cultured in RPMI1640 medium supplemented with 5% serum. Colonies were counted using ImageQuant LAS 4000 System (GE Healthcare, Pittsburgh, PA, USA) 14 days after staining with crystal violet.

Soft agar assay

The cell suspension (1×10^4 cells in 1.5 ml of RPMI1640 medium supplemented with 5% serum and 0.3% agar) was plated onto 6-well tissue culture plates containing 2.5 ml of RPMI1640 medium with 5% serum and 0.6% agar. Cells were then cultured for 21 days. Colonies were stained with crystal violet, and counted with ImageQuant LAS 4000 System.

Fluorescence microscopy

GFP-expressing PDE10 stable knockdown and vector control cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cell nuclei were counterstained with 4′,6-diamidino-2-phenyl-indole (DAPI; 1 mg/ml). Images were captured by the Operetta confocal microscope (Perkin Elmer, Waltham, MA, USA).

Experimental design and data analysis

Drug effects on cell growth and IC_{50} values were determined as described previously.[28] All experiments were repeated a minimum of three times to determine the reproducibility of the results. All error bars represent s.e.m. Statistical analysis was performed using Student's *t*-test. *A P*-value of < 0.05 was considered statistically significant.

CONFLICT OF INTEREST

MPM has ownership interest (including patents) in INCELL Corporation. The remaining authors declare no conflict of interest.

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