Cell Reports

Sildenafil Potentiates a cGMP-Dependent Pathway to Promote Melanoma Growth

Graphical Abstract

Highlights

- Melanoma cells express a cGMP signaling pathway involving PDE5

- The cGMP pathway promotes MAPK signaling and melanoma cell growth and migration

- PDE5 degrades cGMP and thus acts as a brake on the growth-promoting cGMP pathway

- The PDE5 blocker sildenafil releases the PDE5 brake, leading to increased tumor growth
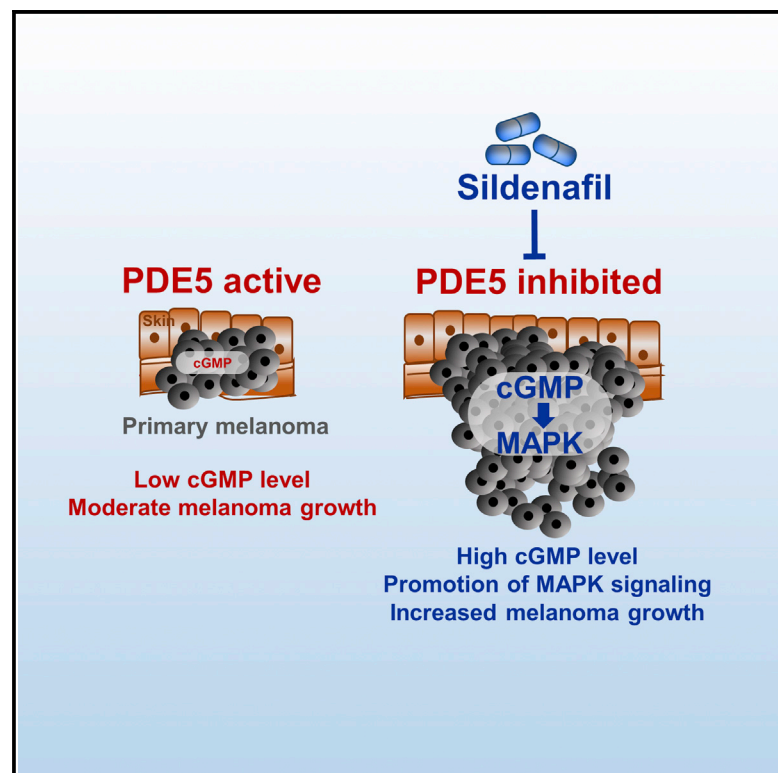
Authors

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In Brief

Use of the phosphodiesterase 5 inhibitor sildenafil (Viagra) has been linked to an increased risk of melanoma. Dhayade et al. explore the underlying mechanism and identify a growth-promoting cGMP-MAPK pathway in melanoma cells that is potentiated by sildenafil treatment.

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Sildenafil Potentiates a cGMP-Dependent Pathway to Promote Melanoma Growth

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SUMMARY

Sildenafil, an inhibitor of the cGMP-degrading phosphodiesterase 5 that is used to treat erectile dysfunction, has been linked to an increased risk of melanoma. Here, we have examined the potential connection between cGMP-dependent signaling cascades and melanoma growth. Using a combination of biochemical assays and real-time monitoring of melanoma cells, we report a cGMP-dependent growth-promoting pathway in murine and human melanoma cells. We document that C-type natriuretic peptide (CNP), a ligand of the membrane-bound guanylate cyclase B, enhances the activity of cGMP-dependent protein kinase I (cGKI) in melanoma cells by increasing the intracellular levels of cGMP. Activation of this cGMP pathway promotes melanoma cell growth and migration in a p44/42 MAPK-dependent manner. Sildenafil treatment further increases intracellular cGMP concentrations, potentiating activation of this pathway. Collectively, our data identify this cGMP-cGKI pathway as the link between sildenafil usage and increased melanoma risk.

INTRODUCTION

Malignant melanoma is one of the most aggressive cancers. It arises from melanocytes and accounts for the majority of deaths related to skin cancer. In the Western world, its incidence almost doubles each decade. If melanoma is diagnosed early, it can be cured by surgical resection. However, due to its distinct tendency to metastasize, in about 20% of patients it progresses to an invasive disease that is refractory to therapy and has a poor prognosis (Gray-Schopfer et al., 2007; Miller and Mihm, 2006). At the molecular level, the mitogen-activated protein kinase (MAPK) pathway is hyperactivated in the majority of melanomas, and this typically occurs through somatic gain-of-function mutations in either NRAS (15%–20% cutaneous melanomas) or BRAF (40%–

50%). These mutations can be identified in benign melanocytic proliferation and all stages of invasive and metastatic melanoma (Lo and Fisher, 2014; Omholt et al., 2003; Shtivelman et al., 2014; Sullivan and Flaherty, 2013). Interestingly, oncogenic BRAF signaling and invasiveness of melanoma cells in mice are associated with an increased level of cyclic guanosine-3′, 5′-monophosphate (cGMP) due to downregulation of PDE5A gene expression, which encodes the cGMP-degrading phosphodiesterase 5 (PDE5) (Arozarena et al., 2011). In this study, melanoma cell invasion could be induced in vitro by the PDE5 inhibitor sildenafil, which is clinically used to treat erectile dysfunction and pulmonary hypertension. Moreover, a recent prospective cohort study in men in the United States indicated that sildenafil use may be linked to an increased risk of developing melanoma (Li et al., 2014). Together, these findings point to a crosstalk between cGMP and MAPK signaling that might be relevant to the pathophysiology and therapy of melanoma in mouse and human. However, the molecular players of cGMP signaling, for example, the cGMP generators and effectors, in melanoma cells are not well characterized.

cGMP is an intracellular signaling molecule that transmits the effects of NO and various peptides and regulates diverse cellular functions in eukaryotes (Beavo and Brunton, 2002; Kemp-Harper and Feil, 2008; Kots et al., 2009). It can be generated from GTP by two classes of guanylate cyclases, NO-sensitive soluble guanylate cyclases (sGCs) (Mergia et al., 2009) or peptide-activated membrane-bound guanylate cyclases (Kuhn, 2009; Potter et al., 2006), such as the atrial natriuretic peptide (ANP)-responsive GC-A or the C-type natriuretic peptide (CNP)-responsive GC-B. The amplitude and duration of cGMP signals are regulated through a dynamic balance between its rate of synthesis by guanylate cyclases and degradation by phosphodiesterases (Francis et al., 2009). In many cell types, the major downstream effector of cGMP is the cGMP-dependent protein kinase I (cGKI, also known as protein kinase G or PKG), which belongs to the serine/threonine family of protein kinases (Hofmann et al., 2006). The mammalian *prkg1* gene encodes two isoforms of cGKI, cGKIα and cGKIβ, which differ in their N-terminal region of approximately 100 amino acids. cGKI activity in intact cells can be monitored by immunodetection of
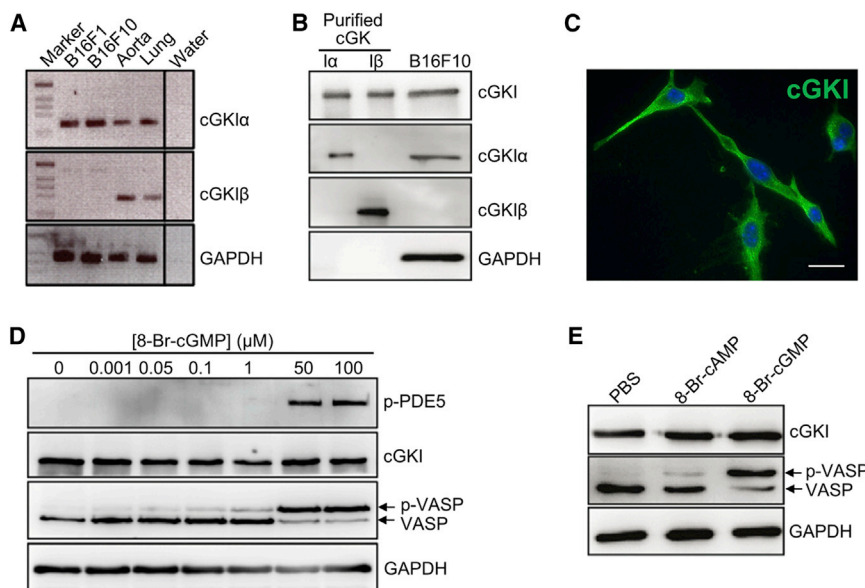
Figure 1. B16 Melanoma Cells Express Functional cGKI

(A) Expression of cGKIα and cGKIβ mRNA in B16 melanoma cells as detected by RT-PCR. RNA from mouse lung and aorta served as positive controls and water as negative a control. GAPDH was used as a loading control. A DNA size marker was loaded in the left lane.

(B) Detection of cGKIα and cGKIβ protein in B16 melanoma cell lysates (10 μg) by western blot with antibodies that recognize both cGKI isoforms (cGKI) or selectively cGKIα or cGKIβ. Purified cGKI isozymes (5 ng) served as controls.

(C) Immunofluorescence staining of B16F10 cells with the cGKI antibody (green); nuclei (blue); scale bar, 25 μm.

(D) B16F10 cells were incubated for 10 min in PBS ("0") or with the indicated concentrations of 8-Br-cGMP. Then, protein lysates (10 μg) were analyzed by western blot with antibodies detecting phospho-PDE5 (p-PDE5), cGKI, or phospho-VASP (p-VASP) and VASP.

(E) B16F10 cells were incubated for 10 min under control conditions (PBS) or in the presence of 100 μM 8-Br-cAMP or 100 μM 8-Br-cGMP. Then, protein lysates (10 μg) were analyzed by western blot with antibodies against cGKI or p-VASP/VASP.

For the western blots shown in (B), (D), and (E), GAPDH was used as a loading control. Representative data from one of three independent experiments are shown.

phosphorylated cGKI substrate proteins such as vasodilator-stimulated phosphoprotein (VASP) (Lohmann and Walter, 2005) or PDE5 (Rybalkin et al., 2002).

It is well established that activation of the cGMP-cGKI axis exerts acute cardiovascular effects such as relaxation of vascular smooth muscle and modulation of platelet aggregation. Additionally, cGMP has been implicated in the regulation of growth and survival in multiple cell types including tumor cells (Fajardo et al., 2014; Feil et al., 2003, 2005). The role of cGMP in cancer appears to be complex and dependent upon the type of tumor and model system under investigation (Barsoum et al., 2014; Fajardo et al., 2014; Ying and Hofseth, 2007; Zhang et al., 2014). Both pro- and anti-cancer effects of cGMP have been reported. The variable effects of cGMP on tumor growth are likely due to the fact that different tumor cells express different cGMP generators and effectors and that cGMP signaling also affects various processes in the tumor microenvironment, such as blood flow, angiogenesis, inflammation, and immune response.

The aim of the present study was to characterize the expression and functional role of components of the cGMP signaling system in melanoma cells of murine and human origin. We have identified a cGMP pathway that promotes MAPK signaling and melanoma growth in vitro and in vivo. Importantly, it was found that the growth-promoting cGMP pathway could be potentiated pharmacologically by treatment of melanoma cells or mice with sildenafil.

RESULTS

Identification of a CNP-cGMP-cGKI Pathway in B16 Melanoma Cells

Initially, we analyzed the expression and function of cGMP signaling pathway components in murine B16 melanoma cells.

At the mRNA level, we detected the expression of the cGKIα isoform, but not the cGKIβ isoform, in B16F1 as well as B16F10 cells (Figure 1A). The selective expression of the cGKIα isozyme was confirmed at the protein level by western blot analysis with isoform-specific antibodies (Figure 1B) and immunofluorescence staining of cells (Figure 1C). Unless otherwise stated, subsequent experiments were performed with B16F10 cells. To monitor cGKI enzymatic activity in intact cells, we analyzed phosphorylation of the cGKI substrates, PDE5 and VASP. Phospho-PDE5 was detected with a phospho-specific antiserum and phospho-VASP was detected with an antiserum that recognizes both dephospho-VASP and phospho-VASP (phosphorylated at Ser157). Phospho-VASP (Ser157) migrates at a discernible higher molecular weight compared to dephospho-VASP. VASP can be phosphorylated on Ser 157 by multiple kinases including cGKIα, cGKIβ, and cAMP-dependent protein kinase (cAK) (Lohmann and Walter, 2005; Weber et al., 2007). B16 cells expressed both PDE5 and VASP, and treatment of intact cells with the membrane-permeable cGMP analog and cGKI activator, 8-Br-cGMP, stimulated the phosphorylation of both PDE5 and VASP in a dose-dependent manner (Figure 1D). To test for the presence of a functional cAMP-cAK pathway, B16 cells were incubated with the cAK activator, 8-Br-cAMP, and then phosphorylation of VASP was monitored. However, the level of phospho-VASP induced by 8-Br-cAMP (100 μM) was much smaller than with an equimolar concentration of 8-Br-cGMP (Figure 1E). Taken together, these findings showed that B16 melanoma cells express functional cGKI as well as its substrates VASP and PDE5, and that the cGMP-cGKI pathway might be more prominent than the cAMP-cAK pathway in these cells. While PDE5 could be involved in cGMP-degradation in B16 cells, it was not clear how cGMP is generated in these cells.
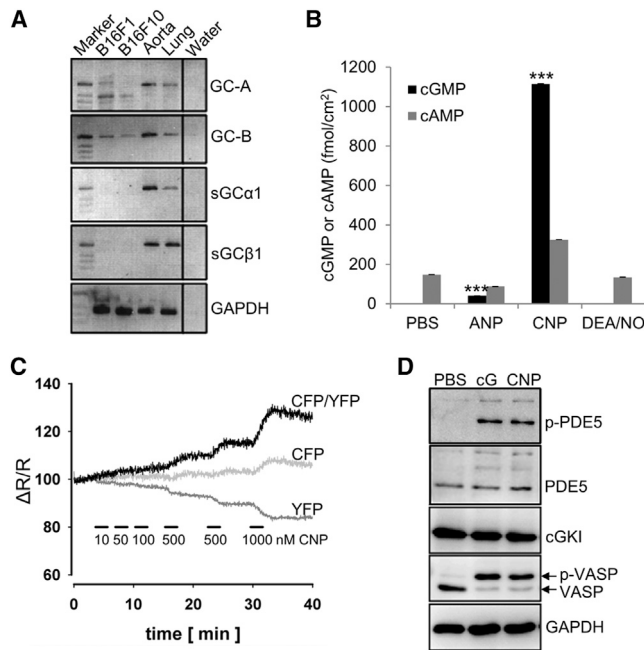
Figure 2. B16 Melanoma Cells Express a CNP-cGMP-cGKI Pathway

(A) Expression of mRNA encoding the membrane-bound guanylate cyclases, GC-A and GC-B, or the soluble guanylate cyclase subunits, $sGC\alpha_1$ and $sGC\beta_1$, in B16 melanoma cells as detected by RT-PCR. RNA from mouse lung and aorta served as positive controls and water as a negative control. GAPDH was used as a loading control. A DNA size marker was loaded in the left lane.

(B) Cyclic nucleotide levels in B16F10 cells. Cells were incubated for 10 min under control conditions (PBS) or with 1 μM ANP, 1 μM CNP, or 100 μM DEA/NO. Then, cells were lysed and cGMP (black bars) or cAMP (gray bars) levels were determined in cell extracts by enzyme immunoassays. Data are presented as mean ± SEM (n = 3); ***p < 0.001 compared to PBS. Representative data from one of two independent experiments are shown.

(C) Real-time imaging of cGMP signals in living B16F10 cells by FRET microscopy. Cells were transfected with the fluorescent FRET-based cGMP sensor, cGi500. To trigger cGMP elevations, cells were superfused with increasing concentrations of CNP (horizontal bars). CFP and YFP emissions were simultaneously recorded (light gray and gray traces, respectively). The CFP/YFP ratio (black trace) reflects the intracellular cGMP concentration. The diagram shows the response of an individual B16 cell that was representative of ten cells measured overall.

(D) B16F10 cells were incubated for 10 min under control conditions (PBS) or in the presence of 100 μM 8-Br-cGMP (cG) or 1 μM CNP. Then, protein lysates (10 μg) were analyzed by western blot with the indicated antibodies. GAPDH was used as a loading control. Representative data from one of three independent experiments are shown.

See also Figure S1.

To identify the cGMP-generating mechanism(s) in B16 cells, the mRNA expression of guanylate cyclases was analyzed in B16F1 and B16F10 cells. We detected very weak expression of the ANP receptor, GC-A, relatively strong expression of the CNP receptor, GC-B, and no expression of the $\alpha 1$ and $\beta 1$ subunits of the NO receptor, sGC (Figure 2A). To test the ability of the melanoma cells to generate endogenous cGMP, intact cells were incubated with physiological stimulators of the respective guanylate cyclases, and then cGMP was measured in cell extracts by enzyme immunoassay. In line with their expression pro-

file for guanylate cyclases, treatment of B16 cells with ANP (1 μM), CNP (1 μM), or the NO-releasing compound DEA/NO (100 μM) resulted in a weak increase, strong increase, or no significant change of the intracellular cGMP concentration, respectively, as compared to control conditions (PBS) (Figure 2B, black bars). None of the compounds significantly altered the level of cAMP in B16 cells compared to control conditions (PBS) (Figure 2B, gray bars). The dynamic change of cGMP signals upon CNP stimulation could also be visualized in real time in living melanoma cells that expressed a cGMP biosensor. B16 cells were transfected with an expression plasmid encoding the fluorescent cGMP sensor, cGi500 (Russwurm et al., 2007; Thunemann et al., 2013b). This sensor contains the cGMP-binding domain of the cGKI flanked by CFP and YFP. Binding of cGMP alters the sensor's conformation and the efficiency of fluorescence resonance energy transfer (FRET) from CFP to YFP. Thus, the FRET-based cGi500 sensor reports changes of the cGMP concentration via a change in its CFP/YFP emission ratio. Application of CNP increased the intracellular cGMP concentration dose-dependently as indicated by the CFP/YFP ratio (Figure 2C, black trace). Moreover, the physiological GC-B ligand, CNP (1 μM), was as effective as the synthetic cGMP analog, 8-Br-cGMP (100 μM), in stimulating phosphorylation of the cGKI substrates, PDE5 and VASP, in intact B16 cells (Figure 2D). Thus, we concluded that B16 melanoma cells express a functional CNP-cGMP-cGKI pathway.

cGMP Promotes B16 Melanoma Cell MAPK Signaling, Growth, and Migration

To determine the downstream mechanisms and functions of cGMP in melanoma cells, we tested the effects of pharmacological and physiological stimulation of cGMP signaling with 8-Br-cGMP and CNP, respectively, on MAPK pathway activity and on B16 cell growth and migration. Exposure of B16F10 melanoma cells for 10 min to 8-Br-cGMP (100 μM) or CNP (1 μM) caused phosphorylation of PDE5 and VASP and a dramatic increase in the phosphorylation of p44/42 MAPK (Figure 3A). Similar results were obtained with B16F1 melanoma cells (Figure S1A). The p44/42 MAPK is normally activated by phosphorylation via mitogen and extracellular signal-regulated protein kinase kinase (MEK). Co-incubation of the cells with the MEK inhibitor, U0126 (10 μM), abolished CNP/cGMP-stimulated phosphorylation of p44/42 MAPK (Figure 3A). The cGMP-induced MAPK phosphorylation was also suppressed by lower concentrations of U0126 (1 μM, 0.1 μM) as well as by two alternative MEK inhibitors, PD98059 (50 μM, 10 μM) and trametinib (1 μM, 0.1 μM) (Figure S1B). These results indicated that cGMP acts upstream of MEK to promote p44/42 MAPK signaling. Incubation with 8-Br-cGMP for 10 min and MEK inhibition did not affect the expression level of PDE5 (Figure S1B), and MEK inhibition did not alter the CNP/cGMP-induced phosphorylation of PDE5 (Figure 3A) and VASP (Figure 3A; Figure S1B). Thus, MAPKs did not appear to regulate the activity of cGMP generators and effectors under the conditions tested.

In line with their stimulating effects on MAPK activity, both 8-Br-cGMP (1 mM) and CNP (1 μM) provoked increases in melanoma cell growth as measured by a conventional endpoint assay (Figure 3B) as well as by monitoring living cells with an
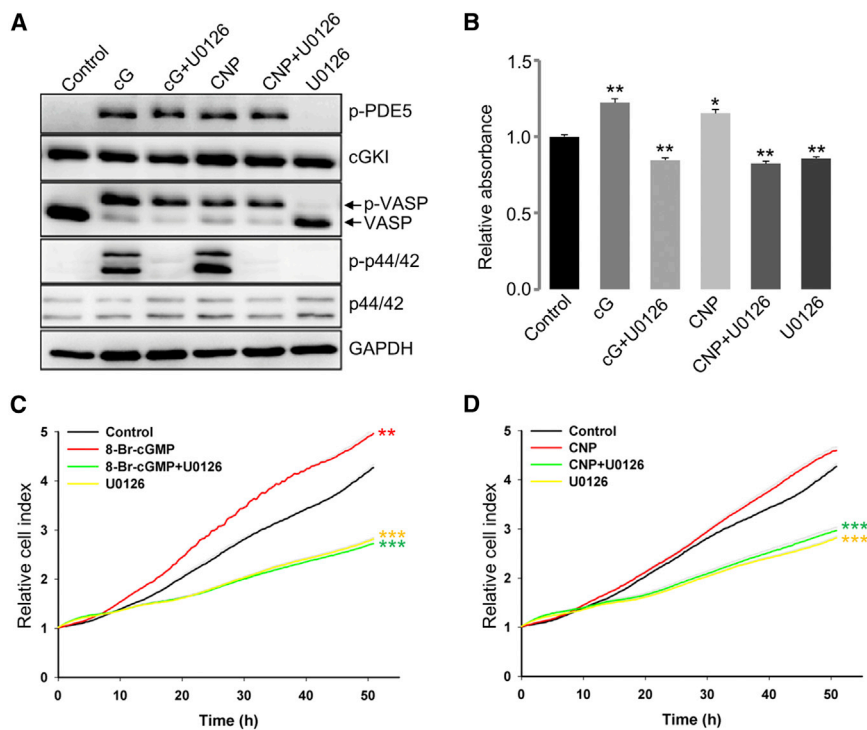
Figure 3. Activation of the cGMP Pathway in B16F10 Melanoma Cells Promotes MAPK Signaling and Melanoma Cell Growth In Vitro

(A) B16 cells were incubated for 10 min in PBS (control) or in PBS in the presence of 100 μM 8-Br-cGMP (cG), 1 μM CNP, 10 μM U0126, or 8-Br-cGMP plus U0126, or CNP plus U0126. Protein lysates (10 μg) were analyzed by western blot with the indicated antibodies. GAPDH was used as a loading control.

(B) B16 cells were grown for 48 hr under control conditions or in the presence of 1 mM 8-Br-cGMP (cG), 1 μM CNP, 10 μM U0126, or 8-Br-cGMP plus U0126, or CNP plus U0126. Then, cell growth was determined by the MTS assay. Data are normalized to growth under control conditions, which was set to 1, and presented as mean $\pm$ SEM (n = 4); *p < 0.05 and **p < 0.01 compared to control.

(C and D) B16 cell growth was continuously monitored by impedance-based RTCA. Cells were grown under control conditions and (C) in the presence of 1 mM 8-Br-cGMP, 10 μM U0126, or 8-Br-cGMP plus U0126, or they were grown (D) in the presence of 1 μM CNP, 10 μM U0126, or CNP plus U0126. Data shown are mean $\pm$ SEM (n = 3); **p < 0.01 and ***p < 0.001 compared to control. In (A)–(D), representative data from one of three independent experiments are shown. See also Figure S1.

impedance-based real-time cell analyzer (RTCA) (Figures 3C and 3D). U0126 (10 μM) reduced basal and CNP/cGMP-stimulated growth of B16 cells (Figures 3B–3D). Next, the effect of cGMP on B16 cell migratory potential was tested in two assays. In the scratch assay, 8-Br-cGMP (1 mM) increased the number of cells in the wounded region after 24 hr compared to control (Figures 4A and 4B). In an alternative migration assay performed with the RTCA, the migration of B16 cells through a membrane (8-μm pore size) was significantly enhanced by 8-Br-cGMP (1 mM) at 6 hr, 12 hr, and 18 hr in comparison to control conditions (Figure 4C). Together, these findings indicated that activation of the cGMP pathway is associated with increased B16 melanoma cell growth and migration, presumably via a crosstalk with MAPK signaling.

cGKI Overexpression or Sildenafil Treatment Enhances B16 Melanoma Growth in Mice

To determine the consequences of increased cGMP signaling for tumor growth under in vivo conditions, we analyzed the effects of overexpressing cGKIα or sildenafil administration on B16 melanoma growth in mice. B16 cells infected with an adenovirus encoding cGKIα (Ad-cGKIα) expressed much higher amounts of the protein kinase than cells infected with a control virus (Ad-EGFP) (Figures 5A and 5B). Both the basal level of VASP phosphorylation in the presence of PBS as well as VASP phosphorylation induced by 8-Br-cGMP (10 μM) or CNP (5 nM) were higher in Ad-cGKIα-infected cells than in Ad-EGFP-infected control cells (Figure 5B). Mice that received intracutaneous injections of cGKIα-overexpressing B16 melanoma cells developed significantly bigger tumors than mice injected with Ad-EGFP-infected

control cells. Fourteen days after implantation, tumors derived from cGKIα-overexpressing B16 cells were $\sim$2-fold bigger in volume and area than control tumors (Figures 5C and 5D).

Since B16 cells express PDE5 (Figures 1D, 2D, and 3A), we asked whether the clinically used PDE5 inhibitor, sildenafil, would enhance the level of cGMP and the associated downstream signaling events in these cells. Indeed, cGMP imaging in living B16 melanoma cells expressing the cGMP biosensor, cGi500, revealed that sildenafil (100 μM) strongly increased CNP-triggered cGMP signals as compared to stimulations with CNP alone (Figure 6A). Moreover, sildenafil potentiated CNP-induced phosphorylation of PDE5, VASP, and p44/42 MAPK (Figure 6B). In line with these biochemical data, sildenafil (100 μM) enhanced CNP-stimulated growth of the melanoma cells as determined by both an endpoint assay (Figure 6C) as well as by impedance-based real-time monitoring of cells (Figure 6D). To determine whether PDE5 inhibition affects melanoma cell growth in vivo, B16 cells were injected intracutaneously into mice that received sildenafil (200 mg kg^{-1}day^{-1}) in their drinking water or water without the drug. Compared to control animals, sildenafil-treated mice showed increased cardiac cGMP levels (Figure 6E) and tumor growth (Figure 6F). Collectively, these data showed that stimulation of cGMP signaling in B16 cells by overexpression of cGKIα or pharmacologically via treatment with sildenafil promotes melanoma growth both in vitro and in vivo in mice.

The Growth-Promoting cGMP Pathway Is Also Present in Human Melanoma Cells

Since our previous studies were performed with a mouse model, it was important to evaluate the relevance of cGMP signaling in
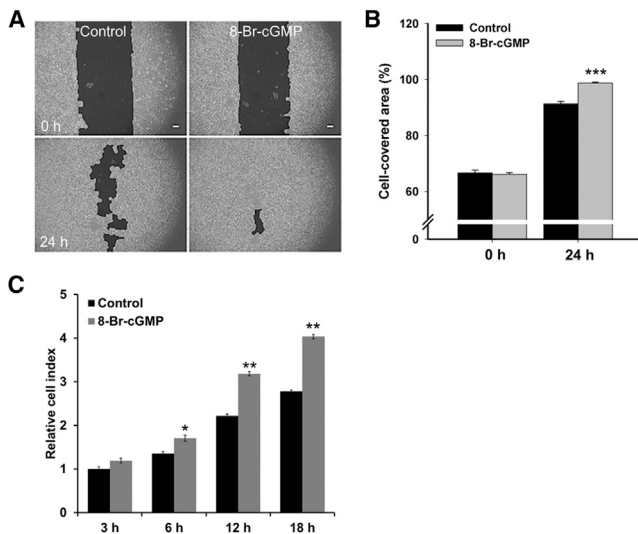
Figure 4. Activation of the cGMP Pathway Promotes B16F10 Melanoma Cell Migration In Vitro

(A and B) Analysis of B16 cell migration by a scratch assay. A confluent monolayer of cells was wounded (0 hr) and then incubated for 24 hr under control conditions or in the presence of 1 mM 8-Br-cGMP. (A) shows representative photomicrographs of the cell monolayer (scale bars, 25 μm), and (B) shows the quantitative evaluation of cell migration reflected by percentage of cell-covered area. Data are presented as mean ± SEM (n = 3); ***p < 0.001 compared to untreated control at 24 hr.

(C) B16 cells were plated on membranes (8-μm pore size) and migration under control conditions or in the presence of 1 mM 8-Br-cGMP was monitored 3, 6, 12, and 18 hr later by impedance-based RTCA. Data are presented relative to control migration at 3 hr, which has been normalized to 1 and expressed as mean ± SEM (n = 3); *p < 0.05, **p < 0.01 compared to the untreated control at the respective time point.

human melanoma. Indeed, expression of cGKIα and cGKIβ mRNA could be detected by qRT-PCR in several human primary/non-metastatic and metastatic melanoma cell lines (Figure 7A). Western blot analysis (Figure S2A) confirmed the expression of cGKI in primary cell lines (WM35, WM3211) as well as in metastatic cell lines (1205Lu, WM852, and SKMel147). PDE5 protein was also detected in these cell lines except in WM852 cells. The cell lines 1205Lu, WM852, and SKMel147 responded to treatment with both 8-Br-cGMP (100 μM) and CNP (1 μM) with strongly increased phosphorylation of the cGKI substrate VASP (Figure S2A). Interestingly, WM35 and WM3211 cells showed relatively weak to undetectable VASP phosphorylation in response to 8-Br-cGMP or CNP (Figure S2A). This could be due to differences in the components of cGMP signaling in these cells as compared to the other cGKI/PDE5-positive cell lines. RT-PCR analysis of PDE expression in these human cell lines correlated well with the western blot results for PDE5. Furthermore, the data indicated that human melanoma cells express varying levels of other cGMP-PDEs including PDE1C, PDE3B, PDE9A, PDE10A, and PDE11A (Figure S2B). Thus, we cannot exclude that in addition to PDE5 other PDEs might be involved in cGMP signaling in human melanoma cells. The cGKI protein was also detected in melanomas from human patients. Immunohistochemical staining of cGKI was positive in

tumor cells of primary melanomas from nine of the 17 patients (Figure 7B, left; data not shown) and in tumor cells of metastases from one of the 11 patients analyzed (data not shown). Interestingly, cGKI protein was not detectable in tumor cells of most metastases tested, including a matched metastasis derived from a patient with a cGKI-positive primary tumor (Figure 7B, right). Together, the results obtained with human melanoma cell lines and tumor tissues indicated that the cGMP-cGKI pathway is expressed in many but not all human melanomas. Importantly, analysis of 418 melanoma patients from The Cancer Genome Atlas (TCGA) (Weinstein et al., 2013; Cheng et al., 2015) revealed that the median survival time of patients with high cGKI expression in their tumors is dramatically reduced compared to patients with low cGKI expression (Figure S3).

For further functional studies, we selected the non-metastatic human melanoma cell line WM3211 (BRAF-wild-type), and the metastatic cell line 1205Lu (BRAF-mutated). Both cell lines expressed cGKI and PDE5 (Figure 7A; Figure S2). Both WM3211 and 1205Lu cells showed a strong increase in their intracellular cGMP concentration upon treatment with CNP (1 μM) and this increase was further augmented by co-treatment with sildenafil (100 μM) as measured in cell extracts by a cGMP immunoassay (Figure 7C). Dynamic cGMP measurements in living 1205Lu cells confirmed these results and showed that in addition to sildenafil, cGMP signals were also enhanced by two other clinically used PDE5 inhibitors, tadalafil and vardenafil (Figure S4).

Next, we asked whether stimulation of the cGMP pathway in human melanoma cells would also promote MAPK signaling as in murine melanoma cells. Note that, as expected, BRAF-mutated 1205Lu cells exhibited a higher basal level of p44/42 MAPK phosphorylation than BRAF-wild-type WM3211 cells (Figure 7D, PBS). Compared to control (PBS), 8-Br-cGMP (100 μM) or CNP (1 μM) strongly stimulated phosphorylation of p44/42 MAPK in WM3211 cells and, to a lesser extent, also in 1205Lu cells (Figure 7D). The latter finding was of particular interest because it suggested that cGMP might even be able to stimulate the growth of BRAF-mutated melanoma cells, such as 1205Lu cells, which already exhibit hyperactive MAPK signaling. Indeed, 8-Br-cGMP (100 μM) or CNP (1 μM) significantly increased the growth of both WM3211 and 1205Lu cells, and sildenafil (100 μM) further augmented the pro-growth effect of CNP in both melanoma cell lines (Figure 7E). We conclude that, similar to murine melanoma cells, human melanoma cells can express a growth-promoting cGMP pathway that is also responsive to pharmacological stimulation with sildenafil.

DISCUSSION

Previous work has indicated a role of cGMP signaling in melanoma, but the underlying mechanism(s) remained largely unknown (Arozarena et al., 2011; Li et al., 2014). In the present study, we have identified a cGMP pathway in murine and human melanoma cells that promotes p44/42 MAPK signaling and melanoma growth in vitro and in vivo. Moreover, the clinically used PDE5 inhibitor, sildenafil, potentiated the biochemical activity of the cGMP signaling cascade in melanoma cells and enhanced tumor growth. With the discovery of a CNP-cGMP-cGKI-MAPK pathway in melanoma cells, our study provides the cGMP
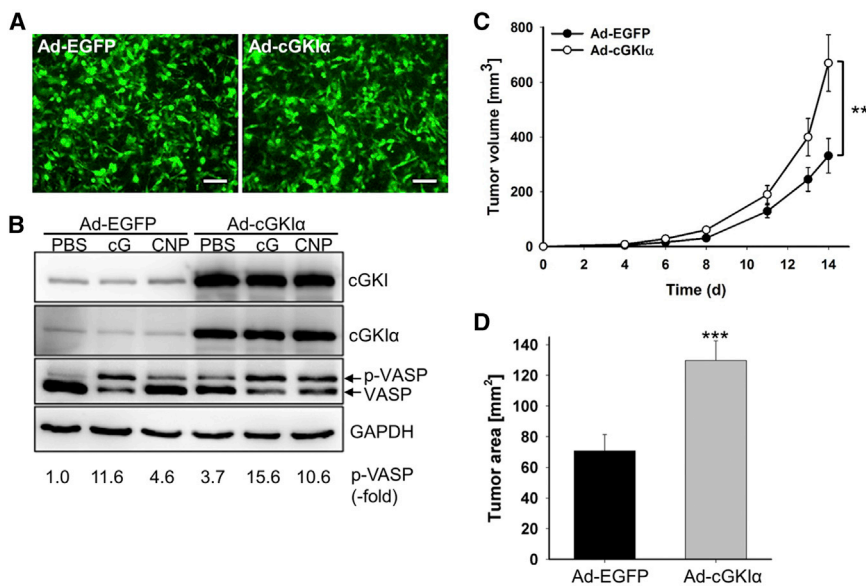
Figure 5. Overexpression of cGKIα Augments cGMP Signaling in B16F10 Melanoma Cells In Vitro and Tumor Growth In Vivo

(A) B16 cells were infected with a control adenovirus expressing EGFP (Ad-EGFP) or with an adenovirus encoding EGFP and cGKIα (Ad-cGKIα) and analyzed 48 hr post-infection; scale bars, 50 μm.

(B) Melanoma cells infected with Ad-EGFP or Ad-cGKIα were incubated for 10 min in PBS or in PBS in the presence of 10 μM 8-Br-cGMP (cG) or 5 nM CNP. Protein lysates (10 μg) were analyzed by western blot with the indicated antibodies. GAPDH was used as a loading control. Values below the panels represent fold increase in VASP phosphorylation as determined by densitometric analysis of the p-VASP to VASP ratio and normalization to control conditions (Ad-EGFP-infected cells incubated with PBS). Representative data from one of two independent experiments are shown.

(C and D) B16 melanoma cells infected with Ad-EGFP or Ad-cGKIα were injected intracutaneously (i.c.) into C57BL/6 mice (2 × 75,000 cells/mouse, n = 18 injections with Ad-EGFP cells and n = 16 injections with Ad-cGKIα cells). (C) Tumor growth curves were determined by measuring the tumor volume over time. Data are presented as mean ± SEM. Differences between groups were assessed by repeated-measures ANOVA followed by a Bonferroni t test; $**p < 0.01$. (D) Mice were sacrificed on day 14, and tumor area was determined. Data are presented as mean ± SEM; $***p < 0.001$ compared to tumors from Ad-EGFP-infected cells.

generator and effector mechanisms that are potentially involved in the pro-melanoma effects of sildenafil in men.

This report describes a role for CNP and its receptor GC-B in melanoma. We are aware of few studies that have linked CNP signaling to tumorigenesis. One study (Lelièvre et al., 2001) showed that subnanomolar concentrations of CNP stimulated the proliferation of human and rodent neuroblastoma cell lines, presumably via activation of cGMP-dependent protein kinase. In another study (Schönrath et al., 2011), the authors observed increased cGMP levels in murine skin squamous cell carcinoma cells following CNP stimulation, but the functional relevance of CNP-induced cGMP in tumor cell growth was not investigated. Interestingly, however, the high cGMP levels in response to CNP appeared to correlate with the aggressiveness/invasiveness of the analyzed tumor cell lines (Schönrath et al., 2011). In contrast to CNP, ANP and its receptor GC-A (Zhang et al., 2014) as well as NO-derived cGMP (Barsoum et al., 2014; Ying and Hofseth, 2007) have been previously implicated in various cancers including melanoma. For instance, genetic GC-A deficiency protected mice from grafted lung, skin, and ovarian cancer, possibly because GC-A positive cells in the tumor stroma are involved in the regulation of local inflammation and tumor angiogenesis (Kong et al., 2008). More recently, Nojiri et al. (2015) reported that ANP via GC-A on vascular endothelial cells prevented metastasis of B16 melanoma cells to the lung by inhibiting their adhesion to the inflamed endothelium. The roles and mechanisms of cGMP signaling in melanoma cells themselves are, however, not well understood.

We have identified the cGMP effector cGKI as downstream component of CNP/GC-B signaling in melanoma cells and, as expected from previous reports, its substrates VASP (Kim et al., 2011) and PDE5 (Arozarena et al., 2011; Drees et al.,

1993). While the functional significance of VASP in melanoma cells is not clear (Kim et al., 2011), our pharmacological experiments with PDE5 inhibitors (sildenafil, tadalafil, and vardenafil) demonstrated that PDE5 contributes to the degradation of cGMP and is, thus, an important "brake" for cGMP signaling in melanoma cells. Stimulation of melanoma cells with the physiological agonist CNP resulted in increased growth as determined by conventional endpoint assays as well as by impedance-based real-time monitoring of living cells. Mechanistically, activation of the cGMP-cGKI cascade was associated with increased MAPK signaling as shown by detection of phospho-p44/42 MAPK. Experiments with MEK inhibitors (U0126, PD98059, and trametinib) demonstrated that the cGMP pathway interacts with MAPK signaling upstream of MEK and that this crosstalk is required for the growth-promoting effect of cGMP in melanoma cells.

To evaluate the potential clinical relevance of the cGMP-cGKI pathway in melanoma, we have analyzed human melanoma cells and the effect of the PDE5 inhibitor sildenafil on melanoma growth. Indeed, we could demonstrate the growth-promoting cGMP cascade not only in murine B16 cells but also in human melanoma cells, although this pathway is probably not universally conserved in all human melanomas. Importantly, treatment with sildenafil increased the biochemical activity of the cGMP-cGKI pathway as well as melanoma cell growth in vitro, and it significantly enhanced melanoma growth in mice in vivo. Our results with sildenafil are in agreement with a recent study that indicated a link between PDE5 and cGMP signaling in melanoma. Arozarena et al. (2011) reported that in melanoma cells oncogenic BRAF, acting through MEK and the transcription factor BRN2, downregulates PDE5 expression. PDE5 downregulation was associated with a relatively modest increase in cGMP and
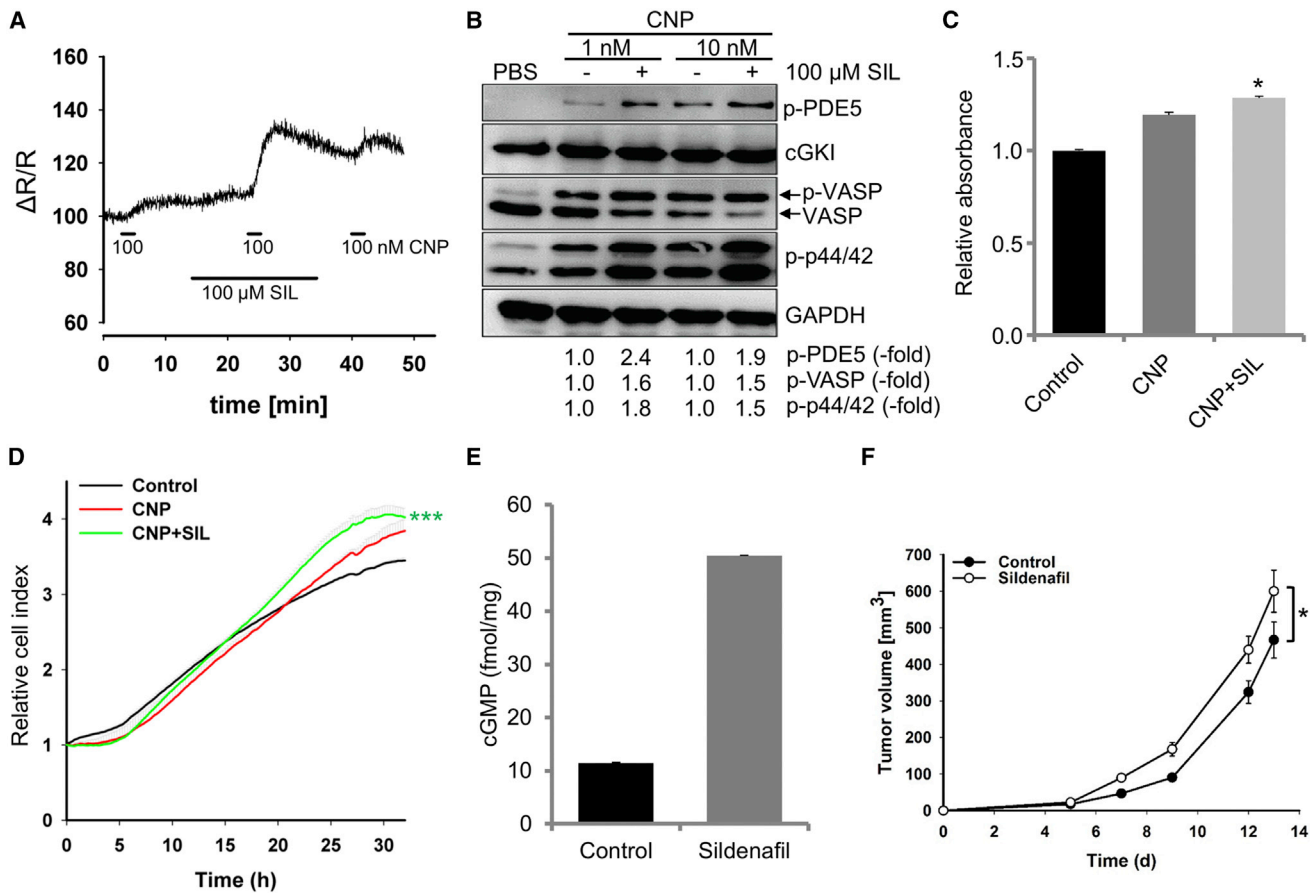
Figure 6. Sildenafil Augments cGMP Signaling in B16F10 Melanoma Cells In Vitro and Tumor Growth In Vivo

(A) Real-time imaging of cGMP signals in living B16 cells by FRET microscopy. Cells were transfected with the fluorescent FRET-based cGMP sensor, cGi500. Elevations of cGMP were triggered by superfusion of the cells with 100 nM CNP (short horizontal bars) in the absence and presence of 100 μM sildenafil (SIL, long horizontal bar). The black trace denotes the CFP/YFP ratio signal, which reports the intracellular cGMP concentration. The diagram shows the response of an individual B16 cell that was representative of ten cells measured overall.

(B) B16 cells were incubated for 10 min in PBS or in PBS with CNP (1 nM, 10 nM) in the absence (–) or presence (+) of 100 μM sildenafil (SIL). Protein lysates (10 μg) were analyzed by western blot with the indicated antibodies. GAPDH was used as a loading control. Values below the panels represent fold increase in phosphorylation of PDE5, VASP, and p44/42 as determined by densitometric analysis of the ratios of p-PDE5 to GAPDH, p-VASP to VASP, and p44/42 to GAPDH, respectively. Values were normalized to the respective control conditions (1 nM CNP or 10 nM CNP in the absence of SIL). Representative data from one of three independent experiments are shown.

(C) B16 cells were grown for 48 hr under control conditions or in the presence of 1 μM CNP or 1 μM CNP plus 100 μM sildenafil. Then, cell growth was determined by the MTS assay. Data were normalized to growth under control conditions, which was set to 1, and presented as mean ± SEM (n = 4); *p < 0.05 compared to control.

(D) B16 cell growth was continuously monitored by impedance-based RTCA. Cells were grown under control conditions or in the presence of 1 μM CNP or 1 μM CNP plus 100 μM sildenafil. Data are shown as mean ± SEM (n = 3); ***p < 0.001 compared to control.

(E and F) B16 melanoma cells were injected intracutaneously (i.c.) into C57BL/6 mice (2 × 75,000 cells/mouse). A subgroup of the experimental animals received sildenafil (200 mg kg^{-1}day^{-1}) in their drinking water, while the remaining mice had water without the drug. Administration of sildenafil was started 2 days before injection of the melanoma cells and continued throughout the study. (E) At the end of the study (day 13 after injection of melanoma cells), the cGMP levels in the hearts of control and sildenafil-treated mice were determined by enzyme immunoassay. Data are presented as mean values (n = 2 mice per group). (F) Tumor growth curves were determined by measuring the tumor volume over time. Data are shown as mean ± SEM (n = 20 tumors from control mice and n = 18 tumors from sildenafil-treated mice). Differences between groups were assessed by repeated-measures ANOVA followed by a Bonferroni t test; *p < 0.05.

cytosolic Ca^{2+} and promoted the invasiveness of the melanoma cells. However, the effect of sildenafil on the growth of primary tumors was not determined in this study, and the potential cGMP generators and effectors were not identified. Thus, while Arozarena et al. describe an influence of MAPK on cGMP signaling, in that enhanced MAPK activity results in increased cGMP levels via downregulation of PDE5, our study reports

vice versa that cGMP also impacts MAPK signaling, in that increased levels of cGMP enhance the activity of MAPK via cGKI. Arozarena and colleagues could not detect cGKI expression in seven BRAF mutant melanoma cell lines analyzed in their study (Arozarena et al., 2011). Our expression analysis of a panel of human melanoma cell lines and tumor sections of human melanoma patients also indicated that cGKI is probably not the only
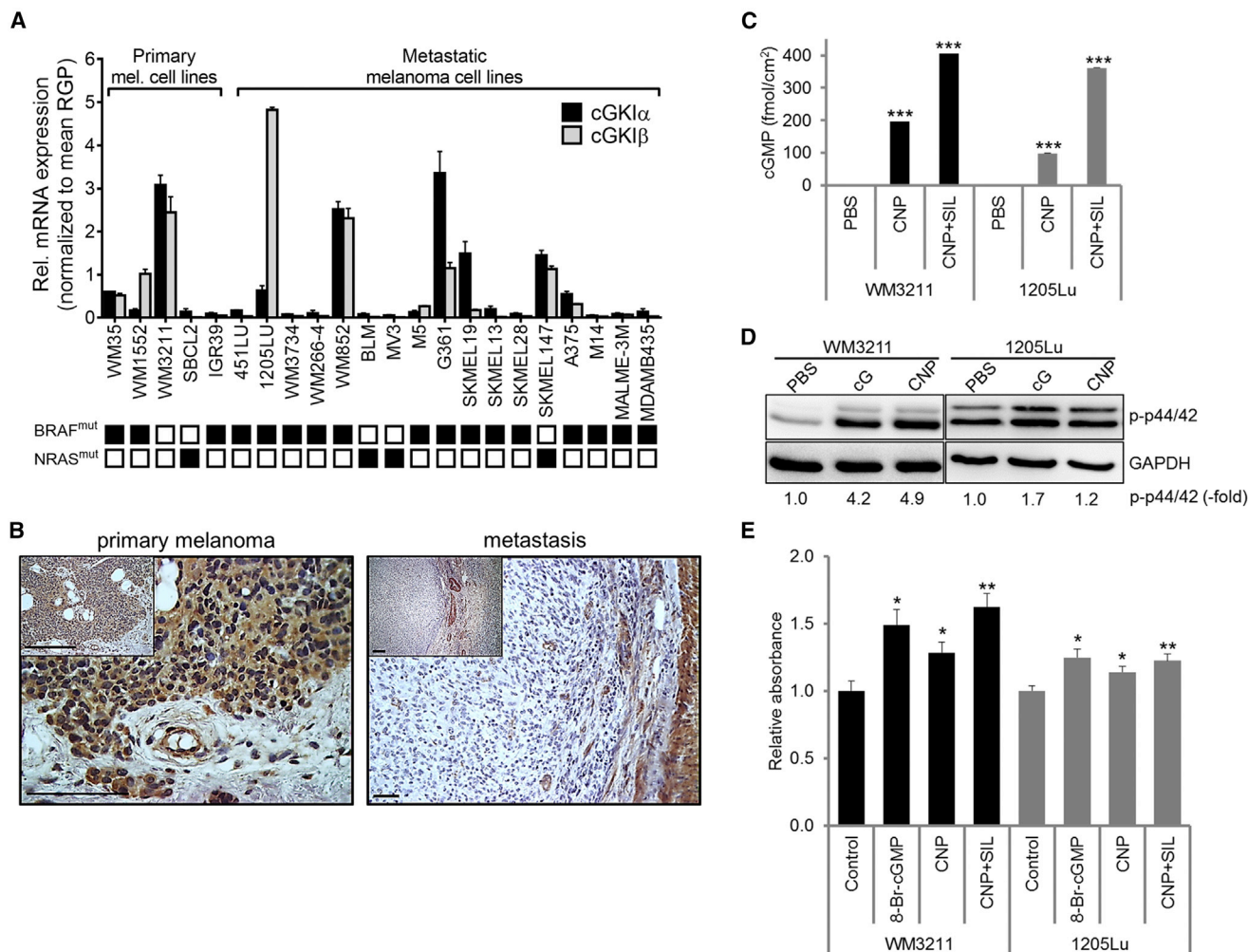
Figure 7. Human Melanoma Cells Also Express a Growth-Promoting cGMP Pathway

(A) Analysis of cGKIα and cGKIβ mRNA expression in human melanoma cell lines by qRT-PCR. Data normalization was carried out against 18S rRNA, and values were referenced to the mean expression level of non-metastatic, radial growth phase (RGP) melanoma cell lines (WM35, WM1552, WM3211, SBCL2). Primary and metastatic melanoma cell lines are indicated by the horizontal brackets. The mutation status of BRAF and NRAS for each cell line is given below the diagram (open squares and filled squares denote wild-type and mutated, respectively). More information about the cell lines is given in Table S1. Data were analyzed in triplicates and are presented as mean ± SD (n = 3).

(B) Immunohistochemical detection of cGKI (brown) in human melanoma sections. Representative photomicrographs of a cGKI-positive primary tumor (left panel) and a cGKI-negative metastasis (right panel) of the same patient; scale bars, 100 μm. The insets show overviews of the respective sections; scale bars, 250 μm. Note that the well-known expression of cGKI in blood vessels is also detected, which serves as a positive control for successful immunostaining of the sections.

(C) cGMP levels in WM3211 (black bars) and 1205Lu (gray bars) human melanoma cells. Cells were incubated for 10 min in PBS or in PBS with 1 μM CNP or 1 μM CNP plus 100 μM sildenafil (SIL). Then, cells were lysed, and cGMP levels were determined in cell extracts by an enzyme immunoassay. Data are presented as mean ± SEM (n = 3); ***p < 0.001 compared to PBS.

(D) WM3211 or 1205Lu human melanoma cells were incubated for 10 min under control condition (PBS) or in the presence of 100 μM 8-Br-cGMP (cG) or 1 μM CNP. Then, cell lysates (10 μg) were analyzed by western blot with the indicated antibodies. GAPDH was used as a loading control. Values below the panels represent fold increase in p44/42 phosphorylation as determined by densitometric analysis of the p-p44/42 to GAPDH ratio and normalization to control conditions (cells incubated with PBS). Representative data from one of two independent experiments are shown.

(E) WM3211 (black bars) and 1205Lu (gray bars) cells were grown for 48 hr under control conditions or in the presence of 1 mM 8-Br-cGMP, 1 μM CNP, or 1 μM CNP plus 100 μM sildenafil (SIL). Then, cell growth was determined by the MTS assay. Data were normalized to growth under control conditions, which was set to 1, and presented as mean ± SEM (n = 4); *p < 0.05 and **p < 0.01 compared to control.

See also Figures S2, S3, S4, and S5.

effector of cGMP in melanoma cells. Taken together, the results from the present study and from Arozarena et al. (2011) may reflect the well-known heterogeneity of melanoma cells and suggest that the functional outcome of increased cGMP in melanoma cells may in fact depend on the availability of cGKI or alternative cGMP effectors, which have yet to be identified. Indeed,

the survival of melanoma patients correlates with the expression level of cGKI in their tumors, with a dramatically shorter median survival time for patients with high cGKI expression (Figure S3).

Based on the present study and previous findings (Arozarena et al., 2011; Schönrath et al., 2011), we suggest a model in which a bidirectional crosstalk of cGMP and MAPK signaling promotes the switch of non-metastatic cells in primary melanomas to invasive/metastatic cells. In this model (Figure S5), CNP via GC-B stimulates cGMP generation in melanoma cells of primary tumors resulting in activation of cGKIα and p44/42 MAPK, resulting in cells with increased potential for growth, migration, and invasiveness to develop. Degradation of cGMP via PDE5 acts as a brake in this switching process. However, a persistent increase in MAPK signaling, for instance, by sustained activity of the cGMP-cGKI cascade and/or by somatic gain-of-function mutation of BRAF, results in downregulation of PDE5. This releases the PDE5 brake, thus establishing a feedforward, self-reinforcing loop that further enhances the aggressiveness of the melanoma cells. Importantly, the PDE5 brake can also be released pharmacologically by sildenafil, thus explaining the pro-melanoma effects of this drug (Figure S5). Since primary melanomas appear to express substantially higher levels of cGKI (Figure 7B; data not shown) and PDE5 (Arozarena et al., 2011) than metastases, it is likely that CNP and sildenafil act primarily on cells of primary tumors and promote their metastatic potential. Cancer metastasis requires multiple steps including resistance to anoikis, which is defined as induction of apoptosis due to detachment from the extracellular matrix (Buchheit et al., 2014). Indeed, we have shown previously that in vascular smooth muscle cells activation of cGKI promotes cell adhesion and survival (Weinmeister et al., 2008; Wolfsgruber et al., 2003) leading us to propose that cGMP-cGKI signaling can protect cells from anoikis (Weinmeister et al., 2008). A similar mechanism may contribute to cGMP-mediated growth of melanoma cells and tumor progression. Finally, it is important to note that cGMP generation in melanoma cells is initially triggered by CNP and that a basal CNP tonus would also be required for an increase in cGMP after PDE5 downregulation/inhibition. It is well known that CNP can be secreted by endothelial cells, particularly in the presence of cytokines (Potter et al., 2006). Thus, it is likely that the pro-melanomagenic CNP is released by the tumor vasculature, particularly under inflammatory conditions, also providing a link between inflammation and melanoma (Figure S5).

Our data together with the findings of Arozarena et al. (2011) raise concerns that the use of sildenafil (Viagra) or other PDE5 inhibitors like vardenafil (Levitra) or tadalafil (Cialis) could promote melanoma in humans. These drugs are the first-line therapy for most men with erectile dysfunction. Indeed, a recent long-term cohort study that included 14,912 men in the United States indicated that men who used sildenafil for erectile dysfunction had a significantly elevated risk of melanoma (Li et al., 2014). The link between sildenafil and melanoma development is further supported by the finding that PDE5 inhibitors promote melanin synthesis (Zhang et al., 2012), which may exacerbate melanoma development (Noonan et al., 2012). PDE5 inhibitors are also used for the treatment of benign prostate hyperplasia and pulmonary arterial hypertension, and there is a tremendous growth of preclinical and clinical studies exploring new applications of PDE5 inhibitors, such as the management of cardiovascular diseases, diabetes, and even cancer (Das et al., 2015; Ghofrani et al., 2006; Shi et al., 2011). In principle, pharmacological modulation of cGMP signaling can affect both the tumor cells and the tumor microenvironment. For example, sildenafil was reported to have anti-inflammatory effects and to augment endogenous antitumor immunity in several mouse tumor models (Serafini et al., 2006) including melanoma (Meyer et al., 2011). There are also studies indicating that PDE5 inhibition can directly induce apoptosis and growth inhibition of tumor cells in vitro (Sarfati et al., 2003; Tinsley et al., 2009; Zhu et al., 2005). A recent report suggested that activation of the 67-kDa laminin receptor on cancer cells by a green tea polyphenol increases cGMP and induces cancer-selective apoptosis (Kumazoe et al., 2013). In a mouse breast cancer model, PDE5 inhibition by vardenafil potentiated the anticancer effect of the green tea polyphenol. Interestingly, however, when vardenafil was given alone, tumor growth appeared to increase compared to control animals that did not receive drugs (Kumazoe et al., 2013). Thus, the effects of cGMP signaling and its pharmacological stimulation by PDE5 inhibitors on tumorigenesis are complex and might depend on tumor type and context. Although it is not clear whether the sildenafil concentration used in our experiments is also reached in patients, the results of the preclinical melanoma models obtained in the present study and elsewhere (Arozarena et al., 2011; Noonan et al., 2012; Zhang et al., 2012) combined with the recent finding of increased melanoma risk in men using sildenafil (Li et al., 2014) suggest that possible skin adverse effects of PDE5 inhibitors should be considered at least in patients with melanoma.

Taken together, this study has uncovered a previously unknown cGMP-cGKI signaling cascade in murine and human melanoma cells. Activation of this pathway promotes tumor cell growth in vitro and in vivo. This pathway also provides a mechanism for the reported pro-melanoma effects of sildenafil and it could be an interesting therapeutic target in melanoma. Our findings in melanoma cells also support the general notion that signaling via cGMP and cGKI promotes phenotypic modulation, growth, and survival of various cell types when they are exposed to inflammation and/or stress, such as vascular smooth muscle cells (Wolfsgruber et al., 2003), bone marrow progenitor cells (Aicher et al., 2009), cardiomyocytes (Fiedler et al., 2006), erythrocytes (Föller et al., 2008), cochlear hair cells (Jaumann et al., 2012), and bone cells (Marathe et al., 2012).

EXPERIMENTAL PROCEDURES

For detailed procedures and reagents, see Supplemental Experimental Procedures.

Analysis of Gene Expression, Signaling Pathways, Cell Growth, and Cell Migration

Murine B16 melanoma cells were grown in DMEM (Life Technologies) and human melanoma cells were grown in RPMI 1640 medium (Life Technologies). Media were supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were grown at 37°C and 6% CO_2 in a humidified incubator. Further information on the human melanoma cell lines is provided in Table S1.

For RT-PCR analysis, total RNA was isolated from serum-starved (3 hr) cells or from mouse tissues, reverse transcribed, and then amplified using the

primer pairs indicated in Supplemental Experimental Procedures and Table S2. For western blot analysis, serum-starved (3 hr) cells were treated for 10 min with test substances in PBS at 37°C in a 6% CO_2 incubator. Then, cells were lysed in SDS-containing lysis buffer, and protein lysates were subjected to western blot analysis as described (Müller et al., 2012). Primary antibodies are listed in Supplemental Experimental Procedures. For immunofluorescence staining, formalin-fixed cells were incubated with cGKI antibody (1:1,000) (Valtcheva et al., 2009) for 1 hr. For immunohistochemical staining of human tissue sections, paraffin sections were incubated with cGKI antibody (1:500) (Valtcheva et al., 2009) for 12 hr. Archived tissue samples of melanomas between the years 2001 and 2006 from patients of the Department of Dermatology, Inselspital, Bern, Switzerland, were included in this study. The study was approved by the Internal Review Board of the Inselspital.

To measure the levels of cGMP and cAMP, cells were grown in 6-well plates and serum starved for 3 hr. Then, they were incubated with various drugs at 37°C. For cGMP/cAMP immunoassays (Cyclic GMP EIA Kit, Cyclic AMP EIA Kit; Cayman Chemical), cells or tissues were extracted with ice-cold ethanol, and cyclic nucleotide levels were determined as described (Müller et al., 2012). Alternatively, intracellular cGMP signals were monitored in living cells that had been transfected with a plasmid encoding the fluorescence resonance energy transfer (FRET)-based cGMP sensor, cGi500 (Russwurm et al., 2007). FRET/cGMP imaging of the cells was performed 48 hr after transfection as described previously (Thunemann et al., 2013a, 2013b).

To determine cell growth, cells that had been serum starved for 18 hr were grown in the absence or presence of drugs in medium containing 10% FCS. Cell growth was analyzed by an MTS assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega) or, alternatively, by using an xCELLigence Real Time Cell Analyzer Dual Plate (RTCA DP, Roche) with 16-well plates (E-plate, Roche). The RTCA uses a unitless parameter termed cell index (CI) to measure relative changes in electrical impedance and cell status. To monitor cell migration, cells were serum starved for 18 hr. Then, a scratch assay was performed using μ-Dish Culture-Inserts (35 mm, high, Ibidi). Alternatively, cell migration through a membrane with 8 μm-pores was monitored in real time by using the RTCA with modified 16-well plates (CIM-16, Roche).

In Vivo Tumor Growth Model
B16F10 melanoma cells were exposed to adenovirus (MOI = 100) encoding EGFP (Ad-EGFP) or EGFP and bovine cGKIα (Ad-cGKIα) in serum-free medium for 15 min followed by the addition of medium containing 10% FCS for 48 hr. Then, cells were injected intracutaneously (i.c.) into the dorsal skin of anesthetized 5- to 6-week-old female C57BL/6 mice (2× 75,000 cells per mouse). For PDE5 inhibition, sildenafil citrate (Molekula, kindly provided by R. Lukowski, Tübingen) was given orally in the drinking water to a subgroup of mice (200 mg $kg^{-1}day^{-1}$). Administration of sildenafil started 2 days before injection of B16 melanoma cells and was continued for the whole course of the experiment. Tumor size was measured by caliper every 2–3 days, and, on day 13 or 14, animals were sacrificed and tumors were removed by surgery. All animal experiments were approved by the local authority (Regierungspräsidium Tübingen, HT11/13).

Statistics
Data are expressed as mean ± SEM, if not otherwise stated. Independent samples were compared with unpaired Student's t test and differences between groups of mice were analyzed by repeated-measures ANOVA followed by a Bonferroni t test. p values <0.05 were considered to be significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.028.

AUTHOR CONTRIBUTIONS

S.D. performed and analyzed most of the experiments, generated the figures, and drafted part of the initial manuscript. S.K. performed and evaluated the in vivo tumor growth experiments in mice together with S.D. T.S. performed and evaluated RT-PCR experiments and analyzed survival data of melanoma patients. H.D. did several western blot experiments with human melanoma cell lines and analyzed the data. S.P. performed and evaluated cGMP imaging experiments with human melanoma cells. U.N. generated and characterized the adenoviral constructs. H.L. contributed to histological analysis of human melanoma sections. R.E.H. provided human melanoma sections. M.T. helped with cGMP imaging. T.B. contributed to the design of the in vivo tumor growth experiments. B.S. provided melanoma cell lines and contributed to the design of experiments. H.-U.S. contributed to the histological analysis of human melanoma sections. S.F. was involved in the immunostaining of human melanoma sections and contributed to study design and data analysis. R.F. supervised the study and wrote the manuscript.

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