GPR56 Regulates VEGF Production and Angiogenesis during Melanoma Progression

Liquan Yang1, Guangchun Chen1, Sonali Mohanty1, Glynis Scott2, Fabeha Fazal3, Arshad Rahman3, Shahinoor Begum4, Richard O. Hynes4, and Lei Xu1,2

Abstract

Angiogenesis is a critical step during cancer progression. The VEGF is a major stimulator for angiogenesis and is predominantly contributed by cancer cells in tumors. Inhibition of the VEGF signaling pathway has shown promising therapeutic benefits for cancer patients, but adaptive tumor responses are often observed, indicating the need for further understanding of VEGF regulation. We report that a novel G protein–coupled receptor, GPR56, inhibits VEGF production from the melanoma cell lines and impedes melanoma angiogenesis and growth, through the serine threonine proline-rich segment in its N-terminus and a signaling pathway involving protein kinase Cα. We also present evidence that the two fragments of GPR56, which are generated by autocatalyzed cleavage, played distinct roles in regulating VEGF production and melanoma progression. Finally, consistent with its suppressive roles in melanoma progression, the expression levels of GPR56 are inversely correlated with the malignancy of melanomas in human subjects. We propose that components of the GPR56-mediated signaling pathway may serve as new targets for antiangiogenic treatment of melanoma.

Cancer Res; 71(16); 5558–68. ©2011 AACR.

Introduction

Angiogenesis is a process of nascent blood vessel formation (1) and is critical for tumor growth and metastasis (2). When tumors reach approximately 1 mm in diameter, hypoxia develops and induces the secretion of vascular growth factor A (VEGFA, or VEGF) from cancer cells (3). VEGF recruits endothelial cells and stimulates new blood vessel formation (4) to ensure sufficient oxygen and nutrient supply for the proliferation of cancer cells. Inhibiting this process reduces tumor sizes and has been proposed as a nonconventional therapy for cancer treatment (5, 6). Various angiogenesis inhibitors have been developed and many of them show promising tumor inhibitory effects (7). However, adaptive responses to single antiangiogenic therapy, manifested as increased invasion and metastasis of cancer cells, have been observed both experimentally and clinically (8–10). The mechanisms for this adaptive response are not clear, but its occurrence strongly argues for combinations of antiangiogenic regimens to effectively treat cancer.

VEGF is a potent growth factor for angiogenesis and is a common target for antiangiogenesis inhibition (11). It binds to the VEGF receptors (VEGFR) on endothelial cells and promotes their proliferation and migration during angiogenesis. The main source of VEGF in tumors is cancer cells and its expression is tightly regulated at both transcriptional and posttranscriptional levels (12). Under hypoxic conditions, VEGF mRNA is induced by the hypoxia-inducible factor alpha and subsequently regulated by alternative splicing. Four main isoforms of VEGF have been identified: VEGF121, VEGF165, VEGF189, and VEGF206, with VEGF165 being the most abundantly expressed. All VEGF isoforms except VEGF121 could associate with extracellular matrix (ECM). The ECM-bound VEGF can be released and activated by heparinases or matrix metalloproteinases, providing an additional layer of regulation for VEGF activity in vivo (13). A less well-characterized mechanism of VEGF regulation is secretion. VEGF contains a signal peptide (14) and presumably is secreted through conventional vesicle trafficking process, from ER to Golgi, and to plasma membrane. In mast cells (15) and neutrophils (16), for example, VEGF is stored in secretory granules, and its release is stimulated by the activation of protein kinase C (PKC).

We report here that VEGF production from melanoma cells is regulated by an atypical G protein–coupled receptor (GPCR), GPR56. GPR56 belongs to the family of adhesion GPCRs, a newly identified family of class B GPCRs implicated in both cell adhesion and G protein–coupled signaling (17). The adhesion GPCRs are highly conserved and their importance in development and diseases has been increasingly...
recognized (17–22), but their regulatory mechanisms remain poorly understood. Adhesion GPCRs share a GPCR proteolytic site (GPS), through which the extracellular stalks are separated from the transmembrane domains by autocatalytic cleavage. The cleaved fragments could still associate with each other noncovalently and form a heterodimeric complex. This cleavage in adhesion GPCRs is required for their proper functions, because mutations in GPS motifs resulted in failure of the receptors to localize on cell surface (23, 24).

Our previous work showed that GPR56 inhibited melanoma growth and metastasis in a xenograft model (25). We present evidence here that, depending on the presence or absence of a serine threonine proline-rich (STP) segment in its N-terminus, GPR56 differentially regulates VEGF production, angiogenesis, and melanoma growth, via a signaling pathway involving PKCα. We propose that GPR56 exists in different activation states in melanoma cells, which are modulated by its STP segment and induce opposing outcomes on angiogenesis and melanoma progression. Specific targeting of tumor-promoting activity of GPR56 may thus serve as a new strategy for angiogenesis inhibition and melanoma treatment.

Materials and Methods

Cell lines
The MC-1, SM, and MA-1 cells were derived from the human melanoma cell line, A375 (ATCC #CRL-1619; refs. 25, 26) and maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS and 4 mmol/L glutamine. WM266-4 cells were obtained from American Type Culture Collection (ATCC; CRL-1676) and maintained in Minimal Essential Medium with Earle’s salt, 10% FBS, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate, and 2 mmol/L glutamine. YURIF cells were purchased from the Yale University Cell Core Facility (27) and maintained in Opi-MEM (Invitrogen) with 5% FBS. HEK293 cells were maintained in DMEM with 10% FBS and 2 mmol/L glutamine. All the cell lines were passaged in our laboratory for less than 6 months after receipt.

Mice
Nonobese diabetic/severe combined immune-deficient (NOD-SCID) mice (NOD.CB17-Prkd<sup>−/−</sup>/J) and NSG mice (NOD.Cg-Prkd<sup>−/−</sup>Il2rg<sup>−/−</sup>/SzJ) were purchased from the Jackson Laboratory. Gpr56<sup>−/−</sup> mice (Genentech Inc.) were maintained through crossbreedings between Gpr56<sup>−/−</sup> mice. All the mice were housed in the animal facility at the University of Rochester Medical Center or Massachusetts Institute of Technology, in accordance to the animal care guidelines from the Division of Laboratory Animal Medicine at University of Rochester Medical Center or MIT.

ELISA for VEGF quantification
Cells were seeded at a density of 1.0 × 10<sup>5</sup> cells per well in 24-well plates and cultured in serum-free medium for 3 days. Media were collected every 24 hours for VEGF-specific ELISA analyses, following the instructions from the manufacturer (R&D Systems).

To test the effects of PKC on VEGF production, MC-1 cells were incubated with the PKC inhibitors, chelerythrine chloride and Ro-31-8425 (EMD Biosciences), or the PKC activator, phorbol myristate acetate (PMA; Sigma), for 48 hours in serum-free medium. The media of the treated cells were subjected to ELISA. To examine the effects of PKCα on VEGF production, MC-1 (ΔSTP-GPR56) and MC-1 (GPRC) cells were transiently transfected with the construct expressing a dominant negative (DN) mutant of PKCα, or vector control (Addgene). Media were collected 48 hours after transfection for ELISA analyses. To assess whether the DN-PKCα sequencers PMA-induced VEGF production, MC-1 (GPR56) cells transiently expressing DN-PKCα or vector control were subjected to PMA (10 μg/mL) stimulation. Media were collected 48 hours after stimulation for ELISA analyses.

Analyses of activation of PKC isoforms
Cell fractionation to assess activated PKC isoforms was done as previously reported (28). Briefly, cells were lysed in isotonic buffer (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 10 mmol/L EDTA, 5 mmol/L EGTA, 20 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, 1 mmol/L sodium vanadate, 1 μmol/L okadaic acid, and a cocktail of protease inhibitor). Membrane fractions were collected after 2 rounds of centrifugations and were subjected to Western blot analyses by using antibodies against PKCα, PKCβ1, PKCβ2, PKCy, PKCδ, or PKCε (1:1,000; Santa Cruz Biotechnology). The rabbit anti-LRP6 antibody (1:500; Cell Signaling) was used as a loading control. The intensity of each PKC band was measured over that in MC-1 (EV) cells (which was arbitrarily set to 1) was defined as the "Relative Intensity" score.

Western blots
Cells were lysed in radioimmunoprecipitation assay buffer and separated on a 12% SDS-polyacrylamide gel for Western blot analyses by using the goat anti-VEGF antibody (0.2 μg/mL; R&D systems), sheep anti-GPRN (0.4 μg/mL; R&D systems), rabbit anti-GPRC (1:1,000; Santa Cruz Biotechnology), or mouse anti-LRP6 antibody (1:500; Cell Signaling) as a loading control. The intensity of each PKC band was measured over that in MC-1 (EV) cells (which was arbitrarily set to 1) was defined as the "Relative Intensity" score.

Tumor studies
A total of 5 × 10<sup>5</sup> of melanoma cells were injected subcutaneously into NOD-SCID or NSG mice. Tumor were harvested and weighted 5 weeks after injections. To study tumor angiogenesis, frozen sections of tumors were stained with rat anti-PECAM antibody (1:5; BD Pharmingen), or mouse anti-human vimentin antibody (1:50; Leica Microsystems), and rabbit anti-GPRC antibody (1:100) followed by detection with Alexa 488 or Alexa 594 donkey anti-rabbit, anti-mouse, or anti-rat secondary antibodies (1:400; Invitrogen). Images were captured by the SPOT software and processed with Adobe.
Photoshop. The numbers of blood vessels in 2 to 5 randomly chosen fields on each tumor section, or GPR56-positive and GPR56-negative areas on each GPR56-expressing tumor section, were counted.

Results

GPR56 inhibits angiogenesis in melanoma

We previously reported that GPR56 inhibited melanoma growth and metastasis in xenograft models (25). Angiogenesis is a critical process during tumor progression (2). To determine whether GPR56 inhibits melanoma progression by regulating angiogenesis, tumor sections from MC-1 cells expressing GPR56 or vector control were stained with an antibody against the platelet and endothelial cell adhesion molecule-1 (PECAM-1). Significantly fewer blood vessels were observed on sections from MC-1 (GPR) cells than the controls (Fig. 1A and B), suggesting that GPR56 inhibits angiogenesis in melanomas.

We frequently observed loss of GPR56 expression in some areas of tumors from MC-1 (GPR) cells, probably because of the growth advantage of cells that express low levels of ectopic GPR56. We postulated that these areas would contain more blood vessels than the adjacent areas that retain GPR56 expression. To test this, MC-1 (GPR56) tumor sections were costained with the anti-GPR56 antibody (anti-GPRC) and the anti-PECAM antibody, or an antibody against the human-specific vimentin (Fig. 1A and Supplementary Fig. S1). GPR56-low and -high areas that contain tumor cells but not mouse stroma were selected. Quantification of blood vessels in these areas revealed an inverse relationship between expression levels of GPR56 and blood vessel density (Fig. 1A and C), arguing strongly that ectopic expression of GPR56 in MC-1 cells inhibits melanoma angiogenesis.

Expression of the C-terminal fragment of GPR56 induces angiogenesis and melanoma growth

Like many members of the adhesion GPCR family, mature GPR56 receptor is cleaved into 2 fragments at the junction between its N-terminus (GPRN) and C-terminal transmembrane domains (GPRC; ref. 25). The GPRN and GPRC fragments still associate with each other after cleavage, but significant portions of them remain unbound. We hypothesized that the GPRN signals through GPRC to inhibit melanoma growth and angiogenesis and deletion of GPRN in GPR56 should abolish this inhibition. To test this, the GPRC fragment was overexpressed in MC-1 cells. This expression did not alter cell proliferation significantly in vitro (Supplementary Fig. S2).

Figure 1. GPR56 inhibits angiogenesis in melanomas.
A, expression of GPR56 resulted in reduction in angiogenesis. Tumor sections from melanoma cells expressing wild-type GPR56 or vector control were stained with anti-GPRC antibody and anti-PECAM antibody. B, quantification of blood vessel density in GPR56-expressing tumors and vector control. C, quantifications of blood vessel density in areas of the same GPR56-expressing tumors.
but, in vivo, it not only failed to inhibit melanoma growth but dramatically induced melanoma growth and angiogenesis (Fig. 2A–C), in direct contrast with the inhibition observed from full-length GPR56. These results indicated a potential antagonistic relationship between the GPRN and GPRC fragments in MC-1 cells.

The STP segment in GPR56 binds to TG2 and its deletion in GPR56 led to enhanced angiogenesis and tumor growth

The antagonistic relationship of GPRN and GPRC fragments implicated from the above finding led us to speculate that a factor might bind to GPRN and modulate the stimulatory or inhibitory effects of GPRC. We previously reported that tissue transglutaminase, TG2, binds to GPRN (25). To test whether TG2 might mediate the inhibitory function of GPRC, we mapped the region in GPR56 that binds to TG2. GPRN fragments of various lengths were expressed and purified as human Fc fusion proteins (Supplementary Fig. S3A and B) and their binding to TG2 was tested on overlay assays (25). A STP segment of around 70 aa was shown to be both necessary and sufficient for binding with TG2 (Supplementary Fig. S3C and D).

We reasoned that, if TG2 mediates the suppressive roles of GPR56 in melanoma progression, deletion of the STP motif in GPR56 would reverse the inhibition by GPR56. To test this hypothesis, ΔSTP-GPR56 was expressed in MC-1 cells (ΔSTP-GPR56), processed into a C-terminal fragment of approximately 25 kDa and an N-terminal fragment of approximately 45 kDa (Fig. 3A), and localized on cell surface as the full-length GPR56 (Supplementary Fig. S4). The expression of ΔSTP-GPR56 did not perturb MC-1 cell proliferation significantly in vitro (Supplementary Fig. S2) but led to increased tumor growth and blood vessel densities in vivo (Fig. 3B–D), similar to GPRC but in contrast to the full-length receptor, indicating that the STP segment is required for the inhibitory effect of GPR56 on melanoma angiogenesis and growth.

ΔSTP-GPR56 induces VEGF production in melanoma cell lines

To examine whether GPR56 affects tumor angiogenesis by modulating VEGF production, the concentrations of VEGF in the media from the MC-1 (EV), MC-1 (GPR), and MC-1 (ΔSTP-GPR56) cells were determined by ELISA. VEGF was lower in the media of MC-1 (GPR) cells than that of MC-1 (EV) cells (Fig. 4A) but dramatically elevated in the media from MC-1 (ΔSTP-GPR56) and MC-1 (GPRC) cells (Fig. 4A and B), suggesting that GPR56 inhibits, but ΔSTP-GPR56 or GPRC promotes, the production of VEGF by MC-1 cells. Consistent with these in vitro observations, the level of VEGF in the circulation of mice bearing MC-1 (ΔSTP-GPR56) cells or MC-1 (GPRC) tumors was higher than controls, and the reverse was observed for MC-1 (GPR56) tumors (Supplementary Fig. S5).
To examine whether GPR56 regulates VEGF production in melanoma cells other than MC-1 cells, D\textsubscript{STP}-GPR56 was expressed in 4 additional human melanoma cell lines, SM, WM266-4, MA-1, and YURIF. Its expression induced VEGF production in all of them (Fig. 4C), suggesting that regulation of VEGF production by GPR56 might be a shared mechanism in malignant melanoma.

The above opposing roles of GPR\textsubscript{C} and full-length GPR56 on VEGF production predicted an antagonistic relationship between GPR\textsubscript{N} and GPR\textsubscript{C} in melanoma cells. To directly test this, purified FcGPRN fusion protein (shown as FcGPRN-1 in Supplementary Fig. S3) was added onto MC-1 (GPR\textsubscript{C}) cells and resulted in a significant reduction of VEGF production relative to controls (Fig. 4D), suggesting strongly that GPR\textsubscript{N} inhibits the activity of GPR\textsubscript{C} during VEGF production of melanoma cells. Because GPR\textsubscript{C} could be detected in both "GPR\textsubscript{N}-bound" and "GPR\textsubscript{N}-free" states in melanoma cell lysates (25), depleting the whole GPR56 molecule would abolish both and, depending on the ratio of the 2 states, may result in no net change or cell-specific changes of VEGF production in melanoma cells. Consistent with this, we did not observe significant changes of VEGF production from MA-1 cells expressing GPR\textsubscript{N}\textsubscript{-}shRNAs (Supplementary Fig. S6).

The level of VEGF produced from MC-1 (\textDelta STP-GPR56) cells was increased relative to controls, showing that the STP segment in GPR56 is essential for its inhibitory role on VEGF production. The STP segment binds to TG2; therefore, TG2 might participate in the inhibition of VEGF production by GPR56. To test this, TG2 mRNA was knocked down in MC-1 cells by short hairpin RNAs (shRNA; Supplementary Fig. S7A). Although a minimal increase of VEGF was observed in the MC-1 (TG2-shRNA) cells relative to the controls (Supplementary Fig. S7B), this increase was much smaller than the massive induction of VEGF observed in MC-1 (\textDelta STP-GPR56) cells, indicating that TG2 might not mediate the inhibitory effects of GPR56 on VEGF production.

We next explored the molecular mechanism by which GPR56 might regulate VEGF production in MC-1 cells. We did not observe significant difference in VEGF mRNA levels among the MC-1 (EV), MC-1 (GPR), and MC-1 (GPR\textsubscript{C}) cells; the level of VEGF mRNA in MC-1 (\textDelta STP-GPR56) cells was slightly higher than those in the other 2 cell lines, but the magnitude of increase (~2-fold) could not account for the large increase of VEGF levels in the conditioned medium (>20-fold; Supplementary Fig. S8A). Similarly, no significant difference in intracellular VEGF levels was observed between the
MC-1 (EV) and MC-1 (GPR) cells, and in MC-1 (ΔSTP-GPR56) cells, the amount of intracellular VEGF was reduced relative to the other 2 cell lines (Supplementary Fig. S8B). We also did not observe alterations in alternative splicing or ECM retention of VEGF in MC-1 cells expressing GPR56 or ΔSTP-GPR56 (data not shown). These data collectively imply that GPR56 might not regulate VEGF production at the levels of mRNA or protein synthesis, but instead might inhibit its secretion/release from melanoma cells.

VEGF secreted from MC-1 (EV), MC-1 (GPR56), and MC-1 (ΔSTP-GPR56) cells contribute to angiogenesis and tumor growth

VEGF is a major stimulator of angiogenesis during development and diseases. In tumors, it is mainly secreted by cancer cells (29), and its level correlates with poor prognosis of cancer (30). To test whether VEGF secreted from MC-1 cells contribute to the angiogenesis and tumor progression regulated by GPR56, VEGF mRNA was knocked down in MC-1 (ΔSTP-GPR56) cells by shRNAs (Fig. 4E). These MC-1 (ΔSTP-GPR56 + VEGF-shRNA) cells were injected subcutaneously into mice. Tumor growth from these cells was significantly impaired compared with controls (Fig. 4F), showing that VEGF produced from MC-1 (ΔSTP-GPR56) cells directly contribute to their tumor progression. Consistent with this, conditioned media from MC-1 (ΔSTP-GPR56) cells accelerated the wound closure of endothelial monolayer in a VEGF-dependent manner in scratch assays (Supplementary Fig. S9A and B).

GPR56 regulates VEGF production through protein kinase Cα

PKC activation has been shown to induce VEGF release from specific granules in multiple cell types (16, 31, 32). To test...
whether GPR56 also regulates VEGF secretion in melanoma cells through PKC, PMA, a potent PKC activator, was added to MC-1 (GPR) cells and shown to abrogate the inhibition of VEGF secretion by GPR56 (Fig. 5A). Conversely, administration of PKC inhibitors, chelerythrine chloride and Ro-31-8425, resulted in significant reduction of VEGF secretion in MC-1 (GPR) cells and shown to abrogate the inhibition of VEGF secretion by GPR56. B, inhibition of PKC activities led to a reduction in VEGF secretion from MC-1 (GPR) cells. C, inhibition of PKC activities led to a reduction of VEGF secretion from MC-1 (ΔSTP-GPR56) cells. Serum starved MC-1 (ΔSTP-GPR56) cells were incubated with PKC inhibitors for 4 hours before the media were collected for ELISA analyses. D, PKCα was reduced in the particulate fractions from MC-1 (GPR) cells but increased in those of MC-1 (ΔSTP-GPR) and MC-1 (GPRC) cells, relative to MC-1 (EV) cells. LRP6 was used as a loading control. E, expression of DN PKCα in MC-1 (ΔSTP-GPR) or MC-1 (GPRC) cells led to reduced VEGF production in the medium. F, expression of DN-PKCα inhibited PMA-induced production of VEGF in MC-1 (GPR56) cells.

PKCs are represented by as many as 10 different isoforms (33). Except for the 2 atypical PKCs, all other 8 isoforms (PKCα, PKCβ1, PKCβ2, PKCγ, PKCδ, PKCe, PKCη, and PKCθ) are sensitive to PMA activation and could be involved in stimulating VEGF secretion. To examine which isofom might be regulated by GPR56, we investigated their activities in MC-1 cells expressing full-length GPR56, ΔSTP-GPR56, GPRC, and vector control. Because activated PKC typically translocates from cytosol to membranes (34), its level in the particulate fraction of cell lysates directly correlates with its activation state. Consequently, the particulate fractions of MC-1 (EV), MC-1 (GPR), MC-1 (ΔSTP-GPR), and MC-1 (GPRC) cells were collected and the levels of PKCα, PKCβ1, PKCβ2, PKCγ, PKCe, PKCδ, and PKCθ in each fraction were determined by Western blotting analyses by using isofom-specific antibodies. We found that the level of PKCα was reduced in the particulate fractions from MC-1 (GPR) cells and increased in those of MC-1 (ΔSTP-GPR) and MC-1 (GPRC) cells (Fig. 5D and Supplementary Fig. S10), suggesting that PKCα is inhibited by full-length GPR56 and activated by ΔSTP-GPR56 and GPRC, and thus may mediate the regulation of VEGF production by these receptors. Consistent with this, expression of a DN mutant of PKCα led to a significant reduction of VEGF secretion in MC-1 (ΔSTP-GPR) and MC-1 (GPRC) cells as well as the PMA-induced VEGF secretion from MC-1 (GPR56) cells (Fig. 5E and F). The level of PKCβ2 was decreased in the particulate fractions of MC-1 (GPR) cells, but did not show any increase in those of MC-1 (ΔSTP-GPR) and MC-1 (GPRC) cells, thus unlikely contribute directly to the VEGF production regulated by GPR56 and its derivatives. None of the remaining PKC isoforms tested showed significant differential regulation by GPR56 (Fig. 5D and Supplementary Fig. S10).
Expression levels of GPR56 are inversely correlated with the progression of human melanomas

To investigate expression of GPR56 in melanocytic lesions, we analyzed the expression pattern of GPR56 on human tissue microarrays (TMA) of nevi, primary melanomas, and metastatic melanomas (35) by immunohistochemistry, using an antibody raised against the STP segment of GPR56. The antibody specifically recognized GPR56 on formalin-fixed sections from mouse tissues and human melanoma xenografts (Fig. 6A and B). Expression intensities of GPR56 on the TMAs were determined by one of us (G.S.) and decreased significantly as melanoma progressed from nevi to primary melanomas, and seemed to follow this trend of decline (although not with statistical significance) in metastatic melanomas (Fig. 6C and D).

Discussion

Malignant melanoma is a devastating disease with significant resistance to current therapies (36). Recently, antiangiogenic inhibitors have shown promising therapeutic potentials in melanoma (7, 37). A common target for antiangiogenic therapy is VEGF. Several VEGF inhibitors have been pursued clinically and some of them have been approved by FDA (38). However, relapse was frequently observed in VEGF inhibitor-treated patients (6, 7), partly attributable to incomplete inhibition of VEGF activity. Most of the VEGF inhibitors block the downstream effects of VEGF, after its secretion and release from cells, either by neutralizing diffusible VEGF or inhibiting the signaling pathway it stimulates. Because the supply of VEGF is not affected, continuous administration of inhibitors is required to sequester its activity and frequently some VEGF escapes the inhibition, by its retention in ECM, for example (39). This problem may be alleviated by reducing production and/or secretion of bioactive VEGF at source, thus increasing the efficacy of antiangiogenic effects of VEGF inhibitors.

G protein–coupled receptors constitute more than 40% of drug targets for treatment of human diseases, but they have not been the main targets for cancer control, mostly because of a lack of understanding of their roles in cancer progression. The involvement of GPCRs on VEGF production in cancer cells has been reported and shown to involve the p38/MAPK pathway (40). We report here that an adhesion GPCR, GPR56, regulates VEGF production and melanoma angiogenesis through a signaling pathway mediated by PKCα (Fig. 7). We also present evidence that the 2 fragments of GPR56, generated by cleavage upon receptor maturation, could play distinct roles in their regulation of melanoma angiogenesis and progression. Although expression of its C-terminal

![Figure 6. The expression levels of GPR56 are inversely correlated with melanoma progression. A and B, immunohistochemical analyses on sections from wild-type or Gpr56+/− testes (A), or tumors from MC-1 cells that express human GPR56 (B). The anti-GPRN antibody specifically recognized GPR56 on these sections. C, immunohistochemical analyses on melanoma tissue microarrays by using the anti-GPRN antibody. D, expression intensity scores of GPR56 on melanoma tissue microarrays. **, P < 0.001; ***, P < 0.0001.](image-url)
levels of GPR56 relative to cancerous tissues (43), and VEGF inhibitors. Normal adult tissues tend to express low levels of GPR56; however, some tumors express higher levels of GPR56. The expression of GPR56 in tumors is associated with increased melanoma progression, especially when combined with other mechanisms and resulted functions of GPR56 may be shared among malignant melanomas. Interestingly, alternatively spliced isoforms of GPR56 were reported recently and 2 of them encode proteins similar to STP-GPR56 (ON) Full-length GPR56 (OFF)

Figure 7. Model of GPR56 function in VEGF production by melanoma cells. We hypothesize that the free GPRC fragment of GPR56 represents a basal/constitutive "on" state to induce the activation of PKCα, VEGF production, angiogenesis, and melanoma progression. The binding of full-length GPRN switches GPRC to an "off" state and inhibits those processes. The binding of ΔSTP-GPRN, however, activates it and promotes melanoma progression.

We reported previously that melanoma development in the Ink4a/Arf<sup>−/−</sup> tyr-HRAS mice did not alter significantly in the absence of GPR56 (43). Perhaps GPR56 plays dual roles in endogenous melanoma progression, as in xenografts, and its complete absence abolishes both and leads to an outcome similar to that in wild-type mice. Alternatively, GPR56 expressed on stromal cells also affect tumor development in endogenous melanomas, which was not assessed in the xenograft model.

The signaling pathways mediated by GPR56, or adhesion GPCRs in general, are poorly understood, despite their increasingly recognized importance in development and diseases (17–22). The results from our study serve as a platform for further investigations in this area. For example, it is not clear whether the inhibition or activation of GPRC imposed by GPRN or ΔSTP-GPRN might involve other interacting proteins. We previously reported that TG2 binds to GPRN (25). However, knockdown of TG2 in MC-1 (GPR56) cells by RNAi did not lead to an increase of VEGF production as observed in MC-1 (ΔSTP-GPR56) cells, suggesting that the rules of TG2 in GPR56-mediated signaling are not straightforward. It is possible that other factor(s) binds to the STP segment in GPRN and mediates the inactivation of GPRC. The expression or stability of this factor(s) may thus determine the activity of GPR56 in cancer cells. Finally, GPR56 was reported to associate with Gtqα (45), consistent with its regulation of PKC activation. Whether this is the case also needs to be investigated.

The apparent separate functions of GPRN and GPRC indicated by our results support the recent proposal that the 2 fragments of adhesion GPCRs might function as distinct entities (46). It was reported that the 2 fragments of latrophilin, another adhesion GPCR, could associate with and potentially signal through fragments from other adhesion GPCRs. The GPCR proteolytic site (GPS) is conserved in all adhesion GPCRs and those tested were shown to be cleaved into 2 fragments (17). Their potential to play distinct functions promises a great complexity of regulation by the adhesion GPCRs in a range of biological processes, including cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgment**

We thank Drs. Hartmut Land and Helene McMurray (University of Rochester) for stimulating discussions.

**Grant Support**

The work was supported by the Ruth L. Kirschstein National Research Service Award (to L. Xu), the NIH (HL67424 to A. Rahman and U54CA126515 to R.O. Hynes), and the Howard Hughes Medical Institute (to R.O. Hynes). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 17, 2010; revised June 8, 2011; accepted June 8, 2011; published OnlineFirst July 1, 2011.
References


GPR56 Regulates VEGF Production and Angiogenesis during Melanoma Progression

Liquan Yang, Guangchun Chen, Sonali Mohanty, et al.

Cancer Res 2011;71:5558-5568. Published OnlineFirst July 1, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-4543

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/07/01/0008-5472.CAN-10-4543.DC1

Cited articles
This article cites 46 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/71/16/5558.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/71/16/5558.full.html#related-urls

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

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

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