Microenvironment and Immunology

GPR56 Inhibits Melanoma Growth by Internalizing and Degrading Its Ligand TG2

Liquan Yang, Scott Friedland, Nancy Corson, and Lei Xu

Abstract

Excessive accumulation of extracellular matrix (ECM) is a hallmark of tumor microenvironment and plays active roles during tumor progression. How this process is regulated and whether it is reversible for cancer treatment are outstanding questions. The adhesion G protein–coupled receptor GPR56 inhibits melanoma growth and binds to tissue transglutaminase (TG2), a major crosslinking enzyme in ECM. To understand the function of TG2 in GPR56-mediated melanoma inhibition, we performed xenograft studies in immunodeficient Tg2−/− mice. Our results revealed an antagonistic relationship between GPR56 and TG2 in melanoma, although TG2 and its crosslinking activity promote melanoma growth. GPR56 antagonizes this effect by internalizing and degrading it. The negative regulation of TG2 by GPR56 associates with the decreased deposition of a major ECM protein, fibronectin, and impaired accumulation of focal adhesion kinase, indicating that the GPR56–TG2 interaction regulates ECM deposition and cell–ECM adhesion. Taken together, our findings establish the roles of TG2 in GPR56-mediated melanoma inhibition. The uncovered antagonistic relationship between GPR56 and TG2 proposes a mechanism by which ECM accumulation/crosslinking in tumors may be reversed, and thus could have therapeutic potential for cancer control and treatment. Cancer Res; 74(4); 1022–31. ©2013 AACR.

Introduction

Cancer cells are in constant communication with their microenvironment and these interactions are essential for their survival, proliferation, and malignancy (1). One critical component of tumor microenvironment is the extracellular matrix (ECM; refs. 2, 3), which forms a scaffold to support tissue structures and actively regulates many aspects of cell behavior (4–6). The assembled ECM undergoes regulated turnover and removal, via protease-mediated degradation and receptor-mediated internalization (7). The former process is mostly accomplished by the matrix metalloproteinases (MMP). The latter was shown to occur through endocytosis of adhesion receptors and lysosome-mediated degradation (8–10).

Increased density, elasticity, and crosslinking of ECM were correlated with cancer malignancy (11, 12). In animal models, these ECM changes were shown to promote cancer progression, via stimulation of integrins and assembly of focal adhesion complexes (13, 14). Elevated ECM density/rigidity in tumors has been mostly attributed to increased ECM production complexes (13, 14). Elevated ECM density/rigidity in tumors has been shown to promote cancer progression (14, 16). The function of crosslinking by transglutaminases in cancer, however, is less clear (17). Transglutaminases consist of eight members (18). Tissue transglutaminase, TG2, is the most ubiquitously expressed. It has been implicated extensively in cancer and, in most cases, has been shown to be tumor promoting (17, 19–23).

Increased density, elasticity, and crosslinking of ECM were correlated with cancer malignancy (11, 12). In animal models, these ECM changes were shown to promote cancer progression, via stimulation of integrins and assembly of focal adhesion complexes (13, 14). Elevated ECM density/rigidity in tumors has been mostly attributed to increased ECM production and crosslinking (15). Two families of crosslinking enzymes have been characterized in ECM. One is formed by transglutaminases and the other by lysyl oxidases (LOX). Increased activities of both have been detected in cancer samples, and the crosslinking by LOXs was reported to actively promote tumor progression (14, 16). The function of crosslinking by transglutaminases in cancer, however, is less clear (17). Transglutaminases consist of eight members (18). Tissue transglutaminase, TG2, is the most ubiquitously expressed. It has been implicated extensively in cancer and, in most cases, has been shown to be tumor promoting (17, 19–23).

In contrast to the effects of ECM accumulation/crosslinking on tumor progression, the effects of ECM removal via endocytosis of adhesion receptors are largely unexplored. Adhesion receptors bind to ECM proteins and translate their signals into cellular changes (6). They have been shown to induce endocytosis of ECM proteins (10), but whether this effect influences cancer progression is not known. A newly described family of adhesion receptors are adhesion G protein–coupled receptors (GPCR; ref. 24). These receptors contain adhesion motifs in their extracellular stalks upstream of the seven transmembrane domains (24, 25), and are predicted to regulate cell adhesion through G protein–coupled signaling. Interestingly, the extracellular stalk and the seven transmembrane domains of an adhesion GPCR are separated through an autocatalyzed cleavage at the GPCR proteolytic site (GPS; ref. 26). The cleaved fragments can stay associated with each other to form a heterodimeric complex (27, 28).

We reported previously that the adhesion GPCR, GPR56, inhibits melanoma growth (28) and its N-terminus binds to the tissue transglutaminase (TG2), leading to the prediction that GPR56 influences melanoma progression via TG2-mediated…

1022 Cancer Res; 74(4) February 15, 2014

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

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doi: 10.1158/0008-5472.CAN-13-1268

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ECM remodeling. To test this prediction, we generated immunodeficient Tg2 knockout mice and analyzed the function of GPR56–TG2 interaction in melanoma growth. Our results show that TG2 and its crosslinking activity promote melanoma growth, but this tumor-promoting function is antagonized by GPR56, via receptor-mediated internalization and degradation. Furthermore, TG2 is tightly associated with a major ECM protein, fibronectin, and the downregulation of TG2 by GPR56 led to a reduced fibronectin deposition. These findings shed light on the function of GPR56–TG2 interaction in melanoma progression, and revealed a cellular mechanism by which the accumulation/crosslinking of ECM and its tumor-promoting function may be reversed.

Materials and Methods

Mice

The Tg2−/− mice were provided by Dr. Gerry Melino (University of Rome, Italy; ref. 29). They were crossed with the Rag2−/− mice to obtain the Rag2−/− Tg2−/− strain. The NOD scid gamma (NSG) mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/Sjcl) were purchased from the Jackson Laboratory. All mice were housed in the animal facility at the University of Rochester Medical Center (Rochester, NY), in accordance to the animal care guidelines from the Division of Laboratory Animal Medicine at University of Rochester Medical Center. 

Extraction of ECM proteins

Cells were lysed in lysis buffer containing 0.5% sodium deoxycholate and 1% Triton X-100. After centrifugation at 13,000 rpm for 15 minutes, pellets were collected and washed with lysis buffer, and then dissolved in SDS sample buffer.

In situ TG2 activity assay

The in situ TG2 activities in MC-1(GPR56-TG2KD) cells expressing wild-type TG2, TG2(C277S), TG2(W241A), or empty vector (EV), were measured based on the published protocol (30), with some modifications. Briefly, serum-starved cells were incubated with 0.1 mmol/L 5-(biotinamido)pentylamine (BAP; Pierce) for 3 hours. The level of BAP incorporated onto cell surface was used as an indicator of TG2 activity and was detected by horseradish peroxidase using the Substrate (Pierce) for 3 hours. The level of BAP incorporated onto cell surface was used as an indicator of TG2 activity and was detected by horseradish peroxidase using the Substrate (Pierce) for 3 hours. The level of BAP incorporated onto cell surface was used as an indicator of TG2 activity and was detected by horseradish peroxidase using the Substrate (Pierce) for 3 hours. The level of BAP incorporated onto cell surface was used as an indicator of TG2 activity and was detected by horseradish peroxidase using the Substrate (Pierce) for 3 hours. The level of BAP incorporated onto cell surface was used as an indicator of TG2 activity and was detected by horseradish peroxidase using the Substrate (Pierce) for 3 hours.

Endocytosis analyses

MC-1(GPR56), MC-1(GPR56KD), and respective control cells were surface-labeled with a cell-impermeable but SH-electrocative sulfo-NHS-Ss-biotin (Pierce) at 4°C. The cells were then incubated at 37°C for various lengths of time to allow internalization of biotinylated proteins. The remaining biotin on cell surface was removed by a cell-impermeable reducing reagent MESNA (2-mercaptoethanesulfonic acid sodium salt). The cells were then lyzed in radioimmunoprecipitation assay (RIPA) buffer and biotinylated proteins were pulled down by streptavidin agarose beads, separated on an SDS–polyacrylamide gel, and detected by Western blot analyses, using the mouse anti-TG2 antibody (Abcam) or sheep anti-GPR56 antibody (R&D).

To directly visualize the internalization of TG2, purified guinea pig liver TG2 (Sigma) was labeled with Alexa 488 in vitro using the Alexa Fluor 488 ProteinLabeling Kit (Life Technologies), following the instruction from the manufacturer. The labeled TG2 protein was added onto MC-1(EV) or MC-1 (GPR56) cells at approximately 10 μg/mL at 4°C. The cells were then shifted to 37°C for various lengths of time, fixed and visualized under microscope. To detect colocalization of N-terminus of GPR56 (GPRN) and TG2 during endocytosis, MC-1(GPR56) cells were surface-labeled with the sheep anti-GPR56 antibody (R&D) before the addition of Alexa 488-labeled TG2 protein. After internalization, cells were fixed, permeabilized, and stained with the Alexa 594-conjugated anti-sheep secondary antibody. Images were acquired under the ×20 objective lens by an Axio Imager.M2m microscope (Zeiss), captured by the Axiocam camera (Zeiss), and processed by the Axiovision and Adobephotoshop softwares.

Results

GPR56 antagonizes the tumor-promoting roles of TG2 in melanoma

Before our analyses, it was reported that human GPR56 may not bind to human TG2, but bind to mouse TG2 (31), questioning the impact of TG2 on GPR56-regulated human cancer progression. To resolve this issue, we confirmed the binding between human GPR56 and human TG2 by coimmunoprecipitation and glutathione S-transferase (GST) pull-down analyses. The results showed that GPR56 coprecipitated with TG2, by either the anti-TG2 antibody or the anti-GPR56 antibody, from lysates overexpressing GPR56 (Supplementary Fig. S1A and S1B), demonstrating that human TG2 associates with human GPR56. To determine whether this association was a result of direct binding, a GST–human TG2 fusion protein (GST-huTG2) was mixed with the fusion protein between the N-terminus of human GPR56 and the Fc fragment of human immunoglobulin G (FcGPRN). The GST-huTG2 protein, but not the GST protein alone, was pulled down with FcGPRN (Supplementary Fig. S1C), arguing strongly that human TG2 directly binds to human GPR56. The GST fusion protein of the C-terminus of mouse TG2 (GST-mTG2Ct) was used as a positive control and, as expected, was pulled down with FcGPRN (Supplementary Fig. S1C).

We subsequently examined the effects of TG2 on GPR56-mediated melanoma growth using Tg2−/− mice, which were bred into the immunodeficient Rag2−/− background to allow the growth of human melanoma cell lines. Melanomas grown in these mice lack mouse TG2 in stroma, but still carry TG2 protein from the human cells. To achieve the maximum depletion of TG2, we knocked down TG2 by short hairpin RNAs (shRNA) in the human melanoma cell line, MC-1, that was already expressing GPR56 cDNA or GPR56 shRNA (Fig. 1A and C). These cells were injected subcutaneously into Tg2−/− Rag2−/− mice. The derived tumors were devoid of...
TG2 from either cancer cells or stroma and were denoted as "-TG2" in the remainder of the article. Meanwhile, control cells expressing normal levels of human TG2 were injected subcutaneously into Tg2+/+Rag2−/− mice. The derived tumors contain normal levels of TG2 and were denoted as "+TG2" in the remainder of the article. Similar to what was reported previously in NSG mice (28, 32), expression of GPR56 inhibited subcutaneous growth of MC-1 cells in the Tg2+/+Rag2−/− mice (+TG2), and this inhibition was only observed in vivo but not in vitro (Supplementary Fig. S2). In the absence of TG2, however, the tumor-inhibitory roles of GPR56 were abolished (Fig. 1B and D, -TG2), indicating that TG2 mediates the inhibitory role of GPR56 on melanoma growth. TG2 itself, however, seemed to promote melanoma growth, because the depletion of TG2 in MC-1 cells led to a reduction in tumor weight (Fig. 1B and D). Immunohistochemical analyses using the anti-Ki67 antibody or by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining showed that the tumor-promoting role of TG2 was associated with an increase of cell proliferation but not a reduction in apoptosis (Supplementary Fig. S3). TG2 was reported previously to promote metastasis. To examine whether this is the case for melanoma, MC-1 cells expressing TG2 shRNA or control shRNA were injected intravenously into Tg2+/+Rag2−/− or Tg2+/+Rag2+/− mice, and lung metastases were counted. A dramatic reduction in the number of macroscopic metastases was observed upon depletion of TG2 (Supplementary Fig. S4, -TG2), suggesting that TG2 promotes melanoma metastasis.

Based on the above observations, we predict that GPR56 inhibits melanoma progression by antagonizing the tumor-promoting role of TG2 (Fig. 1E). Interestingly, melanoma growth of MC-1 cells was almost completely abolished when both TG2 and GPR56 were depleted (Fig. 1D). Perhaps, in the absence of TG2, GPR56 also exhibits some tumor-promoting functions.

The effects of the GPR56–TG2 interaction on melanoma progression were also analyzed in WM115 cells, a human melanoma cell line that is unrelated to MC-1. Similar antagonistic functions of GPR56 and TG2 were observed: expression of GPR56 suppressed melanoma growth in both NSG mice (Fig. 2A and B) and Tg2+/+Rag2−/− mice (Fig. 2C, +TG2), and the depletion of TG2 diminished these effects and grossly inhibited melanoma growth (Fig. 2C, -TG2).
The tumor-promoting function of TG2 requires its crosslinking activity

TG2 is a major crosslinking enzyme in ECM and its activity is frequently elevated during tumor progression. However, TG2 also exhibits crosslinking-independent functions and regulates cell adhesion (33). It thus remains unclear whether the tumor-promoting effects of TG2 require its crosslinking activity. To test this, wild-type human TG2 or TG2 mutants (C277S or W241A) that are enzymatically inactive (34) were expressed in MC-1 cells, in which both GPR56 and TG2 were knocked down [i.e., MC-1 (GPRKD-TG2KD) cells; Fig. 3A]. In situ transamidation activity assays were performed to measure the TG2 activities in the derived cell lines (see Materials and Methods). As expected, cells expressing wild-type TG2, but not the mutants, exhibited increased crosslinking activity relative to the vector control (Fig. 3B), although the proteins were expressed at equivalent levels (Fig. 3A). Furthermore, tumor growth from the cells expressing wild-type TG2 was significantly enhanced relative to the vector control, but no difference in tumor growth was observed from cells expressing the mutants (Fig. 3C), demonstrating that the crosslinking activity of TG2 is required for its tumor-promoting roles in melanoma.

Expression of GPR56 causes changes in TG2 distribution in melanoma

Our above data indicate that GPR56 antagonizes the tumor-promoting function of TG2 in melanoma. One potential mechanism is that GPR56 regulates the distribution/expression of TG2 in ECM. To test this, sections from MC-1 cell–derived tumors overexpressing GPR56 [MC-1(GPR56)] or empty vector [MC-1(EV)] were stained with the anti-GPR56 and anti-TG2 antibodies. A dramatic change of TG2 distribution was observed in response to GPR56 expression (Fig. 4A). In MC-1(EV) tumors, TG2 was deposited on most of the individual melanoma cells, or in some cases at the periphery of small clusters of melanoma cells (Fig. 4A, top). In MC-1(GPR56) tumors, however, TG2 was not detectable on individual melanoma cells, but mostly deposited at the periphery of large clusters of melanoma cells, exhibiting a "fishnet" pattern of distribution (Fig. 4A, bottom). These observations suggest that GPR56 negatively regulates the deposition of TG2 in melanoma ECM. Consistent with this, TG2 was more ubiquitously
In Fig. 5A, increasing amounts of biotinylated TG2 were used to investigate this (see Materials and Methods for details). As shown in Fig. 5A, increasing amounts of biotinylated TG2 were efficiently secreted into the media when expressed. GPRN fragments ending at three different positions were expressed in MC-1 cells expressing GPRN or GPR56 shRNA, and the level of TG2 was measured by Western blot analyses. As predicted, TG2 was significantly reduced in the ECM from MC-1(GPR56) cells (Fig. 4C), but did not change in their cytosolic fractions (Supplementary Fig. S7). MC-1 cells expressing the Nf fragment exhibited the highest level of TG2-expressing cells. Five mice were used in each category.

**Internalization and degradation of TG2 by GPR56 in melanoma cells**

GPRCRs are typically internalized upon activation by their ligands, and then are either recycled back to cell surface or degraded in lysosomes (S3). We postulated that TG2 might act as such a ligand for GPR56 and be internalized and subsequently degraded in melanoma cells after binding with GPR56. A biotin-based endocytosis analysis was performed to investigate this (see Materials and Methods for details). As shown in Fig. 5A, increasing amounts of biotinylated TG2 were observed over a 60-minute time course of endocytosis. Notably, this increment was more pronounced in MC-1(GPR56) cells than in MC-1(EV) cells, suggesting that GPR56 facilitates the internalization of TG2. Similar regulation of TG2 internalization by GPR56 was observed in WM115 cells (Supplementary Fig. S6).

To directly visualize the GPR56-dependent internalization of TG2, fluorescently labeled TG2 protein was added onto MC-1(EV) or MC-1(GPR56) cells and its internalization was tracked over a period of 60 minutes. The fluorescence signals were readily detectable on the surface of MC-1(GPR56) cells 5 minutes after addition of the labeled protein and seemed to be intracellular vesicles at the 30-minute time point (Fig. 5B). Internalization of TG2 was more prominent in MC-1(GPR56) cells than in MC-1(EV) cells (Fig. 5B), and an almost complete colocalization of GPRN and TG2 was observed throughout the endocytosis process (Fig. 5C), further supporting that GPR56 is a mediator of TG2 internalization.

Both the biotin-based and fluorescence-based endocytosis analyses showed a decline of endocytosed TG2 at a later time point (60 minutes; Fig. 5A and B), suggesting that TG2 is degraded after internalization. To determine whether this degradation occurred in lysosomes, MC-1 cells expressing GPR56 or GPR56 shRNAs were treated with the lysosome inhibitors, NH4Cl or chloroquine. The GPR56-dependent degradation of TG2 was blocked after the treatments (Fig. 5D and E), consistent with its degradation in lysosomes.

**Secreted N-terminal fragment of GPR56 blocked internalization of TG2 and promoted melanoma growth**

We next assessed whether the internalization of TG2 by GPR56 is required for the inhibitory effects of GPR56 on melanoma growth. TG2 binds to the GPRN. Ectopic expression of GPRN might then compete with the endogenous receptor and diminish its effects on TG2 internalization and consequently inhibition of melanoma growth. Because the nature of the secretion/shredding of GPRN was not well defined, efforts were first made to identify the portion of GPRN that was necessary for the secretion. Among the three, the Nf fragment exhibited the highest level of secretion into the medium (Fig. 6B) and the least retention in the cell cytosol (Supplementary Fig. S7). Conditioned media were collected and Western blot analyses were performed to assess the efficiency of GPRN secretion. Among the three, the Nf fragment exhibited the highest level of secretion into the medium (Fig. 6B) and the least retention in the cytosol (Supplementary Fig. S7). MC-1 cells expressing the Nf fragment [MC-1(Nf)] were subsequently used to examine the effects of GPRN on TG2 internalization and melanoma growth. As shown in Fig. 6C, the entry of fluorescently labeled TG2 into the MC-1(GPR56) cells was significantly inhibited after cells were treated with conditioned media from MC-1(Nf) cells, demonstrating that the Nf fragment inhibits the internalization of TG2 by GPR56. To understand whether this inhibition of TG2 internalization leads to enhanced melanoma growth, MC-1(Nf) cells were injected subcutaneously into NSG.
mice. Tumor growth was significantly elevated relative to the vector control (Fig. 6D), consistent with the prediction that blocking TG2 internalization by GPR56 leads to increased melanoma growth. Expression of the N or Ntm fragment also enhanced melanoma growth relative to the vector control (Fig. 6D), although the difference was not statistically significant, probably due to their suboptimal efficiency in secretion (Fig. 6B).

The GPR56–TG2 pair regulates fibronectin deposition in melanoma

TG2 has been reported to have pleiotropic functions in ECM stability and deposition (21). It directly binds to a major ECM protein, fibronectin (36), and has been reported to regulate fibronectin deposition/assembly as well as its mediated cell adhesion. Consistent with this, immunostaining analyses showed almost complete colocalization of TG2 and fibronectin in ECM on melanoma sections (Fig. 7A). Like TG2, fibronectin was lost on most individual melanoma cells in GPR56-overexpressing tumors that were grown in either NSG mice or in Tg2+/−Rag2−/− mice (Fig. 7A and Supplementary Fig. S8A and S8C). Conversely, the levels of fibronectin were increased when GPR56 was knocked down by shRNAs (Supplementary Fig. S8B and S8D). These results indicate that GPR56 negatively regulates fibronectin deposition in melanoma. Similar to the effects of GPR56 overexpression and concordant with the reported function of TG2 on fibronectin deposition/assembly, depletion of TG2 resulted in a significant reduction of fibronectin deposition on melanoma sections (Fig. 7B). The above GPR56- and/or TG2-regulated fibronectin deposition was confirmed by Western blot analyses, which showed a decrease of fibronectin level in GPR56-overexpressing and/or TG2-depleted melanoma lysates (Fig. 7C and D). Fibronectin signals through integrins to induce the formation of focal adhesion complexes and activate focal adhesion kinase (FAK; ref. 37). A reduction of fibronectin deposition is expected to result in destabilization of focal adhesion complexes and recruitment of FAK. Indeed, the level of FAK was much reduced in melanomas overexpressing GPR56 and/or depleted of TG2 (Fig. 7C and D).

Discussion

Abnormal accumulation and crosslinking of ECM proteins were shown to have pleiotropic functions in ECM stability and deposition (11, 15). How
this ECM accumulation/crosslinking is controlled and whether it is reversible are poorly understood. We report here that an adhesion GPCR, GPR56, inhibits melanoma growth by internalizing and degrading its ligand, the ECM crosslinking enzyme TG2. These results confirmed the physiologic relevance of TG2 in GPR56-mediated inhibition on melanoma growth, provided the evidence that ECM accumulation/crosslinking in tumors might be reversed through

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Figure 5. TG2 is internalized by GPR56 and degraded in lysosomes. A, biotin-based endocytosis analyses were performed on MC-1 (EV) and MC-1(GPR56) cells. The internalized proteins were probed with anti-TG2 and anti-GPRN antibodies on Western blots. GAPDH was used as a loading control. B, direct visualization of TG2 internalization. Alexa 488-labeled TG2 protein was loaded onto MC-1 (EV) or MC-1(GPR) cells. Cells were fixed at various time points during endocytosis and imaged. Scale bar, 20 μm. C, colocalization of GPRN and TG2 during endocytosis. Scale bar, 20 μm. D and E, downregulation of TG2 by GPR56 is mediated by lysosomes. MC-1(EV) and MC-1 (GPR58) cells or MC-1(control shRNA) and MC-1(GPR56KD) cells were treated with lysosome inhibitors, NH4Cl, and chloroquine. The amount of ECM TG2 was measured on Western blots.

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Figure 6. Ectopic expression of GPRN blocked internalization of TG2 and promoted melanoma growth. A, schematic presentation of GPRN fragments (green) expressed in MC-1 cells. B, the levels of GPRN fragments secreted in the conditioned media were measured on Western blots. Cell lysates were run to control the total expression levels of GPRN fragments. Nf showed the most efficient secretion among the three fragments. C, conditioned medium from MC-1(Nf) cells blocked endocytosis of TG2 in MC-1(GPR56) cells. MC-1(GPR56) cells were incubated with the conditioned medium from MC-1(Nf) cells or that from MC-1(EV) cells (control medium). Alexa 488-labeled TG2 was subsequently added and its endocytosis was imaged at different time points. Scale bar, 20 μm. D, MC-1(EV), MC-1(N), MC-1(Nf), and MC-1(Ntm) cells were injected subcutaneously into NSG mice (n = 5 for each cell line). Tumors were harvested and weighed. *P < 0.05.
receptor-mediated endocytosis, and established, for the first time, the link between this type of ECM turnover and a GPCR.

As one of the major crosslinking enzymes in ECM, TG2 has been extensively implicated in cancer, but conflicting observations have been reported about its in vivo function (17, 21–23). These controversies might be partly due to redundant functions of other transglutaminases, the complicated cellular distributions of TG2, and, in some cases, the incomplete depletion of TG2 in tumors. In our study, a maximum depletion of TG2 in melanomas was achieved by injecting human melanoma cell lines expressing TG2 shRNA into Tg2 knockout mice. The results clearly indicated that TG2 could act as a tumor-promoting factor during melanoma growth. Moreover, this tumor-promoting effect of TG2 requires its crosslinking activity, supporting the positive effects of ECM crosslinking on tumor progression. Because TG2 binds to the N-terminus of GPR56, its effects on GPR56-mediated tumor inhibition are presumably only attributable to the TG2 localized in the extracellular space but not that in the cytosol. This is in line with the recent finding, which showed that ectopic administration of purified TG2 was sufficient to promote cancer progression (20). In addition to GPR56, TG2 has been shown to interact with integrins (38) and the low density lipoprotein receptor-related protein 1 (LRP1; ref. 39), and was internalized by LRP1 in vitro. The regulatory effect of TG2 on ECM is thus likely more complex, but the fact that its activity and turnover directly affect tumor progression and are regulated by a receptor on cancer cells indicates that perturbing it is plausible and may prove effective for cancer control and treatment.

We show that TG2 downregulation by GPR56 resulted in the decrease in fibronectin deposition and FAK expression. Fibronectin is a major ECM component in tumor stroma. Its level is elevated in many types of cancer, including melanoma (40), and thought to play pleiotropic roles at various steps of cancer progression (3, 41, 42). Fibronectin contains a number of protein-binding domains and functions as a scaffold for the assembly of other ECM proteins (43) as well as a repertoire of growth factors (4). Loss of fibronectin in tumors might thus result in broader defects than the impairment of its direct signaling and/or the reduction in FAK expression. The extent of these defects, however, is not clear and awaits further investigations.

The downregulation of TG2 and fibronectin by GPR56 in melanoma, as reported here, proposes a mechanism by which accumulation and crosslinking of ECM in tumors might be

Figure 7. Expression of GPR56 and/or depletion of TG2 led to reduction in fibronectin deposition and FAK expression in melanoma. A, TG2 and fibronectin colocalize on melanoma sections. Cryosections from MC-1(EV) or MC-1(GPR56) tumors were stained with the mouse anti-TG2 (red) or rabbit anti-fibronectin antibody (green). Scale bar, 40 μm. B, loss of fibronectin in melanomas depleted of TG2. Tumor sections from MC-1(control) cells with or without depletion of TG2 (Fig. 2D) were stained with the mouse anti-fibronectin antibody (green). Blue, 4',6-diamidino-2-phenylindole (DAPI), for nuclei. Scale bar, 80 μm. C, reduced fibronectin (FN) and FAK were observed in tumors overexpressing GPR56 and/or depleted of TG2, relative to the controls (lanes 3–8 vs. lanes 1–2). Tumor lysates were probed with mouse anti-fibronectin antibody and mouse anti-FAK antibody on Western blots. Two tumors in each category were analyzed. γ-Tubulin was used as a loading control. D, quantitation of band intensity of FAK and fibronectin in C. The intensity of FAK and fibronectin bands was measured using the ImageJ software and was normalized against the intensity of γ-tubulin from the same sample. The fold change of each band intensity over that in lane 1 is shown.
reversed. This mechanism differs from ECM degradation by MMPs (44). Fragments of ECM proteins remain after their cleavage by MMPs and could exert multiple physiologic effects, including cell adhesion, immune responses, and fibrosis (44). These multiplex effects may explain the elevated activities of MMPs frequently associated with cancer malignancy (45). In contrast, ECM internalization via adhesion receptors likely results in the removal of ECM molecules (46), as we observed in GPR56-overexpressing tumors. In addition, the process acts through receptors on cell surface, which are more accessible for pharmacologic perturbations. It should be noted, nonetheless, the endocytosis process presumably heavily relies on the availability of receptors on cell surface and may occur concomitantly with their activation or inactivation, which could have pleotropic effects. These limitations need to be taken into consideration when its potential as a cancer target is explored.

Finally, our findings contribute to the understanding of adhesion GPCR biology in general. Mutations in adhesion GPCRs have been associated with a variety of human diseases, such as mental retardation (47) and usher syndrome (48), and more recently, cancer (49). Two of the adhesion GPCRs, LPN3 and BA13, were found frequently mutated in cancer (49), suggesting that they may act as tumor suppressors. However, little is known about how adhesion GPCRs exert their functions, how they receive signals, and how they translate signals into changes in cell behaviors that affect development and diseases. We show for the first time, to our knowledge, the internalization of a ligand and its physiologic relevance for an adhesion GPCR. Interestingly, the cancer-associated mutations in LPN3 and BA13 were found to localize on the surface of their extracellular stalks, named the GPCR autoproteolysis-inducing (GAIN) domains, and were predicted to mediate ligand binding (24). Whether they also regulate the internalization of their ligands, like GPR56, will be an interesting area for future study.

Disclosure of Potential Conflicts of Interest
L. Xu is a consultant with Gerson Lehrman Group. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: L. Yang, S. Friedland, L. Xu
Development of methodology: L. Yang, L. Xu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Yang, S. Friedland, N. Corson, L. Xu
Analysis and interpretation of data (e.g., statistical analysis, biosistatistics, computational analysis): L. Yang, L. Xu
Writing, review, and/or revision of the manuscript: L. Yang, L. Xu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Yang, S. Friedland, N. Corson, L. Xu
Study supervision: L. Xu

Acknowledgments
The authors thank Dr. Gail Johnson (University of Rochester Medical Center, Rochester, NY) for providing the constructs and bacteria strain to express TG2 and various mutants, and Drs. Hartmut Land and Dirk Bohmann (University of Rochester Medical Center) for reading the article.

Grant Support
This work was supported by Postdoctoral Fellowship from James Wilmot Cancer Center at the University of Rochester Medical Center (L. Yang) and R01GM098591 from the National Institute of General Medical Sciences (L. Xu).

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Received May 6, 2013; revised October 31, 2013; accepted November 29, 2013; published OnlineFirst December 19, 2013.

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Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-13-1268

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