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GPR56 is a GPCR that is overexpressed in gliomas and functions in tumor cell adhesion

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GPR56 (also known as TM7XN1) is a newly discovered orphan G-protein-coupled receptor (GPCR) of the secretin family that has a role in the development of neural progenitor cells and has been linked to developmental malformations of the human brain. GPR56 diverges from other secretin-like family members in that it has an extremely large N-terminal extracellular region (381 amino acids) and contains a novel feature among this new subclass, consisting of four cysteine residues that define a GPCR proteolytic site (GPS motif) located just before the first transmembrane spanning domain. The rest of the amino-terminal domain contains a large number of possible N- and O-linked glycosylation sites similar to mucin-like proteins. These features suggest a role in cell–cell, or cell–matrix interactions. Here, we demonstrate upregulation of GPR56 in glioblastoma multiforme tumors using functional genomics. Immunohistochemistry studies confirmed the expression of GPR56 protein in a majority of glioblastoma/astrocytoma tumor samples with undetectable levels of expression in normal adult brain tissue. Immunofluorescence analysis of human glioma cells using anti-GPR56 antibodies demonstrate that GPR56 is expressed on the leading edge of membrane filopodia and colocalizes with α-actinin. Purified recombinant GPR56 extracellular domain protein inhibits glioma cell adhesion and causes abnormal cytoskeletal morphology and cell rounding. These results indicate that the extracellular domain may compete for unidentified ligand(s), and block the normal function of GPR56 in cell attachment. In reporter assays, overexpression of GPR56 activates the NF-κB, PAI-1 and TCF transcriptional response elements. These pathways have been implicated in cytoskeletal signaling, adhesion and tumor biology. The above results indicate that GPR56 serves as an adhesion GPCR and is involved in adhesion signaling.
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Introduction

Malignant gliomas are a devastating form of cancer that requires novel approaches for study and therapeutic intervention. Currently, the most effective treatment for most forms of cancer is cytoreductive surgery followed by some form of chemotherapy. Brain tumors remain exceptionally difficult to treat, due to the vital and complex function of the brain, the blood–brain barrier and resistance to radiation and chemotherapy. Glioblastoma multiforme (GBM/Astrocytoma IV) is the most frequent brain tumor representing 50–60% of all astrocytic tumors and 20% of all intracranial tumors (Berger and Wilson, 1999). It has a peak incidence at age 45–60 years and accounts for approximately 15 000–20 000 cases per year in the US and the mean survival time after diagnosis is less than a year. Glioblastomas are the most malignant astrocytic tumors, composed of poorly differentiated neoplastic cells. Glioblastomas show a strong regional heterogeneity that poses a serious challenge for the analysis of these tumors. The wide dissemination of glioblastoma in the brain typically follows anatomical structures of the white matter. Glioblastoma cell migration utilizes ligands in the matrix and is associated with changes in a variety of receptors, including extracellular matrix (ECM), homotypic cell receptors and developmentally associated matrix proteins. Glioblastomas are one of the most vascularized tumors in humans, making them extremely fast growing and invasive. Understanding tumor cell invasion is of considerable significance in glioblastoma and other cancers. Alterations in several classes of adhesion molecules have been implicated in the progression of various forms of cancers. An essential step in tumor progression is the interaction of tumor cells with ECM leading to its destruction and the tumor cells' invasive behavior (Belkin *et al*., 2001). Tumor invasion is dependent upon a delicate balance between cell adhesion and cell detachment. Cell adhesion receptors and their ligands provide traction, repulsion and stimulus for tumor cell migration. The modulation of therapeutic targets involved in tumor cell invasion and adhesion has great potential for the treatment of cancer (Haier *et al*., 2002).

One such class of targets includes the newly defined adhesion G-protein-coupled receptors (GPCRs). GPCRs constitute a vast family of proteins that

encompass a wide range of functions including various autocrine, paracrine and endocrine processes. They show considerable diversity at the sequence level, on the basis of which they can be separated into distinct groups. Based on certain key sequences, GPCRs can be divided into three major subfamilies: class A containing the rhodopsin-like receptors, class B related to the secretin-like receptors and class C containing the metabotropic glutamate-like receptors. Despite a similar 3D framework to other GPCRs, the secretin-like receptors have their own unique '7TM' signature. Within the secretin-like family, a subgroup of GPCRs form their own phylogenetic cluster, comprised of the so-called 'adhesion GPCRs' (Krasnoperov *et al*., 1999; Hayflick, 2000).

GPR56 (TM7XN1) is an orphan adhesion GPCR with homology to the better characterized HE6 (human epididymis-specific protein 6). Like HE6, GPR56 possesses both an exceptionally long extracellular region, characteristic of cell adhesion proteins, and an intracellular region reminiscent of other GPCRs (Kierszenbaum, 2003; Obermann *et al*., 2003). The cloning of GPR56 and identity of conserved sequence motifs are detailed in two publications (Liu *et al*., 1999; Zendman *et al*., 1999). The potential function of GPR56 had been largely speculative based on these studies. For instance, Zendman identified GPR56 from a human melanoma metastasis model, wherein, Northern blot analysis showed expression in the poorly and intermediately metastasizing cell lines and a marked downregulation in the highly metastatic cell lines. A role for GPR56 in metastasis was thus suggested. GPR56 has other characteristics suggestive of its dual role in adhesion and signaling. GPR56 has an N-terminal extracellular region comprised of 381 amino acids, which contains a novel feature of four cysteine residues (GPS motif) located just before the first transmembrane spanning domain (Fredriksson *et al*., 2002, 2003). The rest of the amino-terminal domain contains a large number of possible N- and O-linked glycosylation sites similar to mucin-like proteins. In addition, the short C-terminus contains some possible phosphorylation sites and a putative cAMP-binding domain (aa 675–686), which, together with a potential tyrosine kinase phosphorylation site (aa 546) between TM4 and TM5, may be indicative for interaction with signaling components. GPR56 and heterotrimeric G protein subunits, Gαq/11 and Gβ, associate with each other as part of a larger complex with tetraspanins (Little *et al*., 2004). The nature and function of this tetraspanin–GPCR complex is largely unknown. Members of the tetraspanin family of cell surface proteins act as molecular scaffolds with known adhesion proteins such as integrins to facilitate their function.

Recently, mutations in GPR56 were linked to a human brain cortical malformation called bilateral frontoparietal polymicrogyria (BFPP) (Piao *et al*., 2004). The work suggests a role for GPR56 in early development involving cell and tissue migration and folding during regional development and patterning of human cerebral cortex. The GPR56 transcript was also identified for its selective expression in hematopoietic and neuronal stem cells using cDNA microarray techniques (Terskikh *et al*., 2001). Together, these features suggest that GPR56 is an adhesion GPCR, normally involved in development, but when improperly expressed likely plays a role in tumor cell biology. We describe the increased expression of GPR56 in astrocytic tumors, its subcellular localization and its role in glioma cell adhesion.

Results

GPR56 expression profile

A cDNA library was created using high-quality brain tumor samples that provided a disease-specific clone collection ($\sim$25 000 clones) for array analysis. Differentially regulated clones were first identified from the entire library of cDNA clones, and probes were made from normal and tumor samples. All regulated clones were sequenced and automatically compared to public and private databases for sequence homology, protein function and predicted domain structure. The regulated clones were then reprinted onto a new set of filters for the direct comparison of multiple tumors. Gene expression was analysed using a combination of bioinformatics techniques including principle component analysis, pattern recognition and natural language processing. Clones that behaved within stringent statistical parameters in the 14 tumor and six normal brain samples were selected and subjected to sequence analysis and annotation. We determined that clones corresponding to the GPR56 gene were significantly upregulated in the panel of 14 GBM tumor samples. Specifically, GPR56 clones were upregulated by a factor of 1.905-fold with a *P*-value of 0.0001635. The expression profile of GPR56 in normal human tissue was examined using multiple tissue Northern blot analysis (Figure 1). These studies indicate low expression of a 4.7 kb GPR56 mRNA transcript in normal brain and significant GPR56 mRNA levels in placenta, kidney and pancreas. Other tissue lacked detectable GPR56 mRNA. A short 1 kb mRNA is also apparent in some tissue but its significance is unknown. The relative intensities of the 4.6 kb GPR56 mRNA transcript are shown in Figure 1b. In order to confirm and expand upon our functional genomics studies, GPR56 protein was evaluated for its expression profile in tissues and cell lines.

Since mRNA measurements and cDNA arrays do not necessarily reflect the magnitude of protein expression, we endeavored to study this target by additional means. We generated rabbit polyclonal antibodies against a peptide sequence mapping to the extracellular N-terminus of GPR56. These affinity-purified antibodies were selected for their specificity to the extracellular domain of GPR56 and utility in a number of detection methods (Figure 2a). GPR56 protein expression was examined by immunoblot analysis of a collection of human glioblastoma-derived cell lines, as well as lung, liver, brain and glioblastoma tumor tissue sample

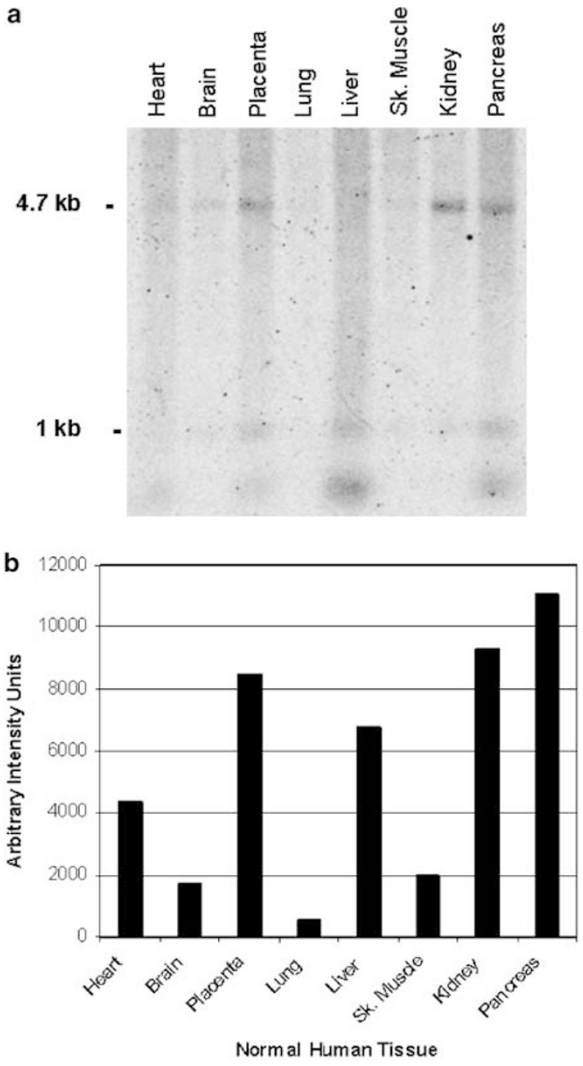


Figure 1 (**a**) Northern blot analysis of GPR56 mRNA expression in multiple human tissue. Multiple tissue Northern blots were probed with labeled GPR56 cDNA and a 4.6 kb band detected in placenta, kidney and pancreas. A 1 kb band of unknown significance was also detected in these tissues. (**b**) The intensity of signal from the 4.6 kb band was quantitated and represented graphically

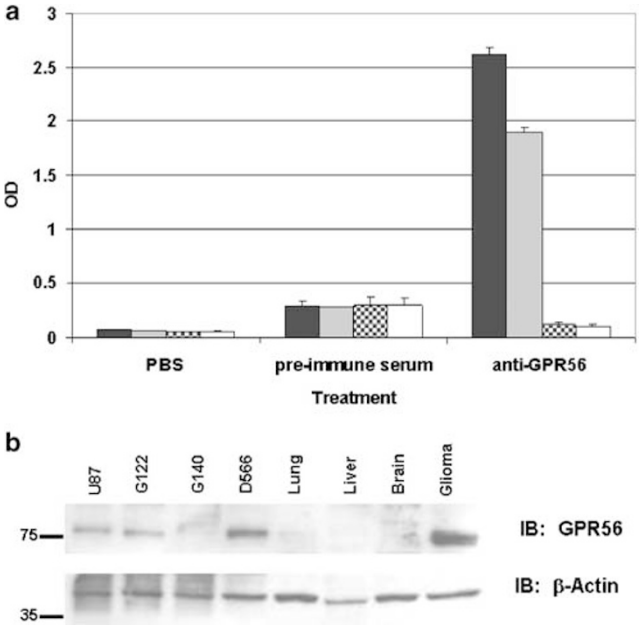


Figure 2 (**a**) The affinity-purified anti-GPR56 rabbit polyclonal antibodies were evaluated by ELISA for specificity and recognition of both the peptide immunogen and recombinant human GPR56 extracellular domain protein. The anti-GPR56 antibodies specifically recognized the peptide immunogen (black bars) and GPR56-ECD (gray bars) and not an unrelated peptide (checkered bars) or unrelated protein (white bars). The preimmune serum did not recognize any of the peptides or protein (error bars, $\pm$s.d.). (**b**) Immunoblot analysis of human glioblastoma-derived cell lines, glioma tumor and other tissue. Protein lysates were immunoblotted using anti-GPR56 antibodies, and subsequently reprobed with β-actin as a loading control. The $\sim$75 kDa GPR56 protein is expressed in several glioblastoma cell lines (U87, G122 and D566) and in the glioblastoma tumor sample shown here. Normal lung, liver and brain tissues do not express significant levels of GPR56 protein

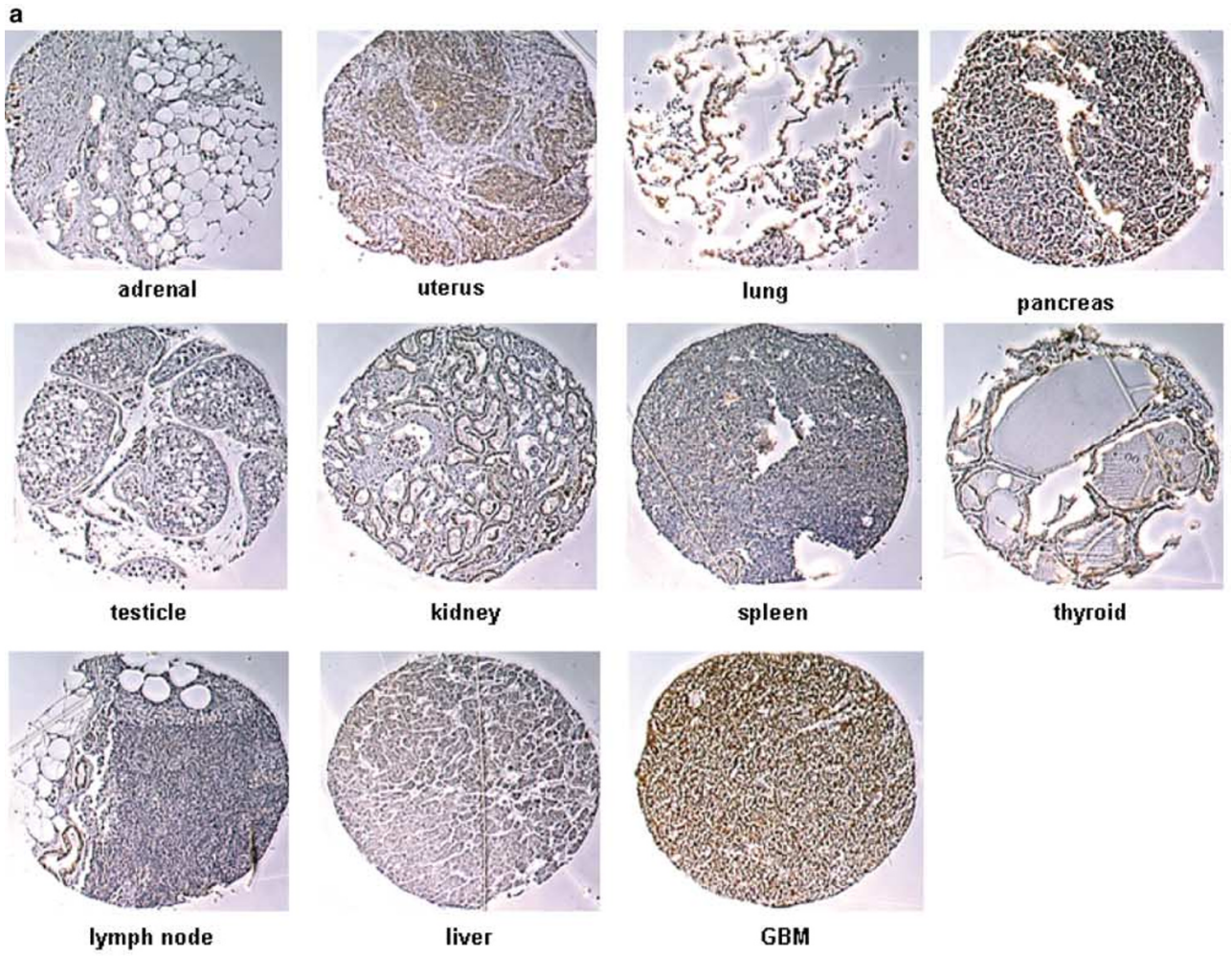
(Figure 2b). GPR56 protein runs at about 75 kDa and was upregulated in glioblastoma tumors and cell lines. Very little or no GPR56 protein is expressed in lung, liver and normal brain. Understanding how the differential regulation of GPR56 impacts tumor cell biology was the next challenge undertaken.

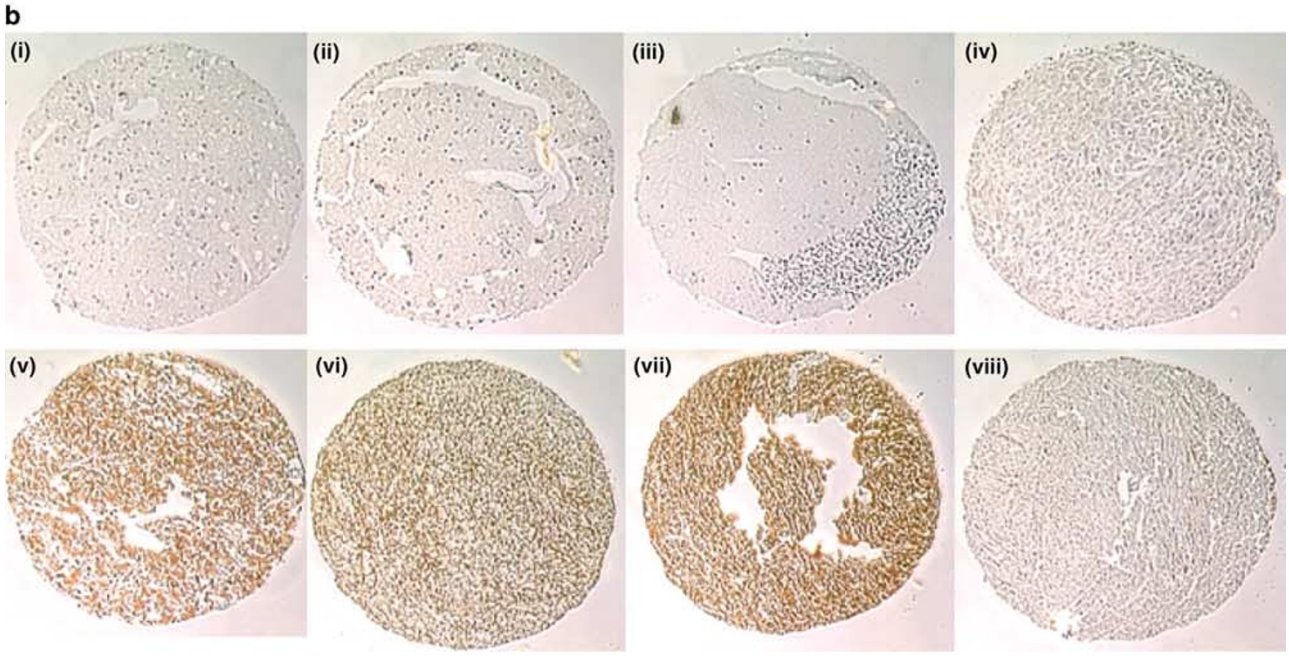
We analysed the expression of GPR56 protein by immunohistochemistry (IHC) on paraffin sections of normal tissue to determine its expression in peripheral tissues (Figure 3a). Using this technique, GPR56 expression was seen in uterus, pancreas and a subset of kidney cells. Very little or no GPR56 was detected in adrenal, lung, testicle, spleen, thyroid, lymph node or liver. A GPR56-positive GBM tissue section was included for comparison in this study. The immunoblot and IHC data is largely consistent with the multiple tissue Northern blot and array analysis. In order to expand on the study of GPR56 protein expression in brain tumors, we examined a panel of normal brain tissue and tumor specimens (Figure 3b and Table 1). In this study, 19 out of 24 (87%) astrocytoma tumors stained positive for GPR56 (v–viii). Normal brain tissue sections (i–iii) did not have detectable levels of signal, nor did certain other brain cancer samples including meningioma (iv). These results were consistent with the cDNA array data, and demonstrate the specific upregulation of GPR56 in human astrocytic tumors. GPR56 does not seem to be expressed in meningiomas or schwannomas (Table 1), cancers with nonastrocytic lineages. This is significant because GPR56 was identified as a neural stem cell marker (Terskikh *et al*., 2001) and suggests a specific role in the development of astrocytic tumors.

Role of GPR56 in glioblastoma cell adhesion

A hallmark feature of adhesion proteins is the localization to membrane substructures such as filopodia and lamellopodia (Honda *et al*., 1998; Belkin *et al*., 2001; Woods *et al*., 2002). We therefore tested for localization of GPR56 to such structures in human glioblastoma

cells and observed GPR56 expression on the leading edge of extending cell membranes (Figure 4). GPR56-specific antibodies detect the expression of this protein as indicated by the green fluorescent stain (panel a). α-Actinin is a protein known to play a role in cell adhesion and migration as part of focal adhesion





complexes; therefore, we stained for its expression (red fluorescence – panel b). The expression of GPR56 is coincident with α-actinin and is localized to the leading edge of the extending membrane structure (see arrows, magnified image and panel c). The nuclei stain (Hoechst) is blue in these images. These results support the notion that GPR56 likely plays a role in cell adhesion and migration due to its colocalization with a protein that has a demonstrated role in these processes.

The extracellular domain of GPR56 is uncharacteristically large for a GPCR and contains a mucin-like domain, features suggestive of a role in cell–cell or cell–matrix interactions. In order to test the functional role of GPR56 in adhesion, we explored the possibility that its N-terminal extracellular domain could act to modulate glioblastoma cell adhesion. To this end, we expressed and purified the extracellular domain of GPR56 (GPR56-ECD) protein using a baculovirus system. The baculovirus-produced GPR56-ECD protein is secreted, glycosylated and runs with a molecular weight of about 51 kDa, significantly larger then the predicted size of 44 kDa (Figure 5a). We tested if purified GPR56-ECD could act as a ligand, competitor or dominant-interfering agent during cell attachment. Consistent with its role in cell adhesion, glioblastoma cells did not adhere effectively to tissue culture plates that had been coated with GPR56-ECD, in contrast, cells attached normally to dishes coated with the control protein, BSA (Figure 5b). GPR56-ECD behaved similarly to NgCAM, a component of the extracellular matrix that acts as a repulsive substrate to this cell type. In contrast, cells attached vigorously to plates coated with Fibronectin. Glioma cell lines expressing high or low levels of GPR56 are differentially repulsed by GPR56-ECD, but no obvious correlation was observed, indicating that soluble GPR56-ECD is not a ligand for endogenous GPR56 (data not shown). Cells plated onto GPR56-ECD rounded up and displayed abnormal cell morphology. The difference in morphology of cells plated onto GPR56-ECD-coated plates, when compared to those plated onto BSA-coated plates was visualized by staining with Phalloidin (Figure 5c). Fluorescent-conjugated Phalloidin specifically stains F-actin and thereby provides visual evidence of the disorganized cytoskeleton and cell rounding caused by GPR56-ECD coating. This inhibition of cell adhesion by GPR56-ECD indicates that GPR56 may play a role in tumor adhesion and invasion.

Table 1 Immunohistochemistry of brain tumor tissue

Tumor type	*Incidence*	*Average area stained (%)*	*Average intensity (0–3)*
Normal brain	0/4 (0%)	0	0
Glioblastoma	19/24 (87%)	63	2
Astrocytoma III	3/3 (100%)	95	2
Astrocytoma II	4/4 (100%)	80	2.5
Meningioma IV	0/2 (0%)	0	0
Meningioma I	0/8 (0%)	0	0
Schwannoma I	0/4 (0%)	0	0

Signaling by the GPR56 adhesion GPCR

We carried out reporter assays in order to demonstrate functional expression of GPR56 in cDNA-transfected cells and determine its ability to activate known signaling pathways. No ligand has been identified for GPR56. HEK 293 cells were transiently cotransfected

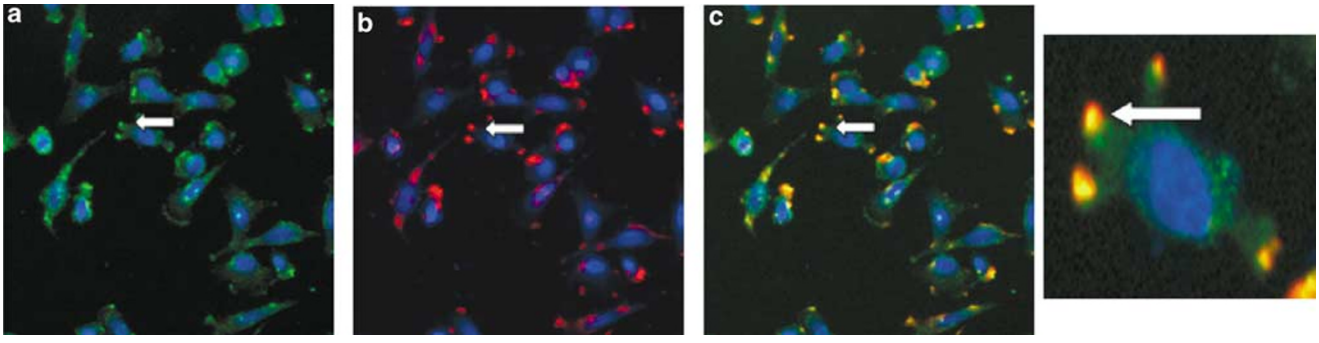


Figure 4 Localization of GPR56 in the leading edge of human glioblastoma cells. Human U87 cells were fixed and stained for protein expression and localization. Representative immunofluorescent images are shown here. Panel a demonstrates GPR56 staining (green) on the cells. Panel b shows α-Actinin staining (red) localized to focal adhesions. Panel c demonstrates coincident staining of GPR56 with α-actinin (yellow), nuclei stain blue (Hoechst) in all of the above images. Arrows indicate representative coincident GPR56-α-actinin staining, observed on the extended membranes of the cells and in focal adhesions in particular. A cell from panel c is shown as a magnified image

Figure 3 (**a**) Anti-GPR56 rabbit polyclonal antibodies were used to stain tissue microarrays. The brown chromagen (DAB) indicates GPR56 expression and the blue (Meyers' hemotoxylin) provides a counter stain. GPR56 was detected at significant levels in uterus, pancreas and a subset of kidney tissue and at low or undetectable levels in adrenal, lung, testicle, spleen, thyroid, lymph node and liver. A stained glioblastoma (GBM) tissue section was also included. (**b**) Immunohistochemical analysis of normal brain and brain tumor tissue demonstrates astrocytic tumor-specific expression of GPR56. Representative images of a normal brain cortex and cerebellum tissue are negative for GPR56 expression (i–iii). Glioblastoma tumor samples are positive for GPR56 expression (v–vii). Section (viii) is a matched benign specimen for comparison to (vii). Section (iv) is a grade 4 meningioma. The data is also represented in Table 1

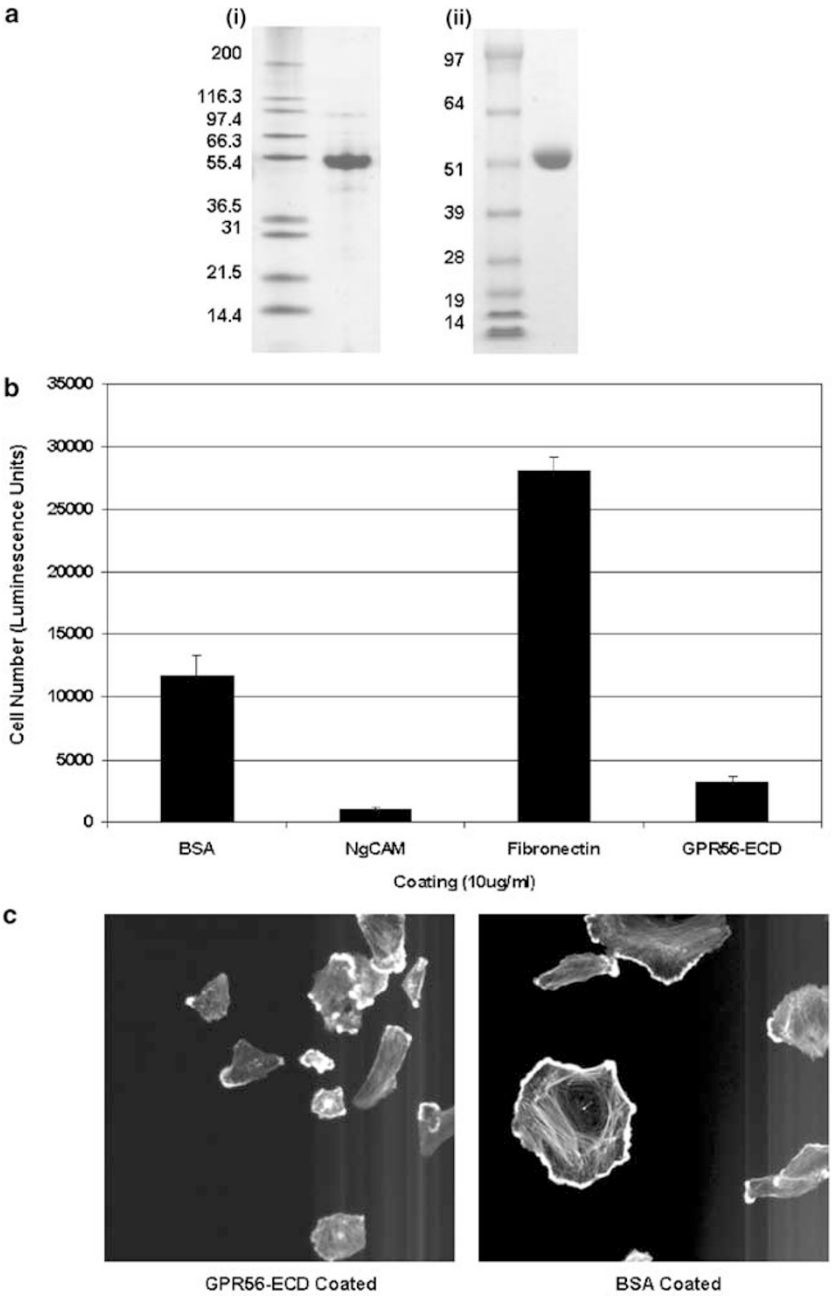


Figure 5 (**a**) GPR56 extracellular domain protein (GPR-56-ECD) expression and purification. The C-terminal 6X Histidine-tagged GPR56-ECD protein is secreted into the media and was purified using a nickel affinity column. (i) SDS–PAGE analysis (1 μg protein loaded) showed the GPR56-ECD band with a molecular weight of about 51 kDa, and a purity of approximately 90%. (ii) N-terminal sequencing of purified GPR56-ECD identified the first residue as Arg_{26}, giving a predicted molecular weight of 44 kDa, but SDS–PAGE analysis and mass spectrometry (data not shown) indicated a larger molecular weight. This difference is likely due to glycosylation, as indicated by a total glycoprotein stain of the purified protein, shown here. (**b**) Effect of GPR56-ECD on cell adhesion. Tissue culture plates (96 well) were coated with BSA (10 μg/ml), NgCAM (10 μg/ml), Fibronectin (10 μg/ml) or GPR56-ECD (10 μg/ml), washed and then the cells were added and allowed to attach for 15 min. The plates were then washed three times with PBS and the number of attached cells quantitated using Cell Titer Glo Luminescence Reagent (error bars, $\pm$s.d.). (**c**) Cells plated onto GPR56-ECD-coated wells did not adhere normally. Tissue culture plates (96-well) were coated with GPR56-ECD (10 μg/ml) or BSA (10 μg/ml), washed and then cells were added and allowed to attach for 30 min. The plates were then washed and the morphology of attached cells was visualized by staining with Phalloidin-594 (Molecular Probes)

with GPR56 cDNA construct, a panel of luciferase reporter constructs and a β-galactosidase transfection control. These reporter assays demonstrate that GPR56 overexpression activated the PAI-1, TCF and to a lesser extent, NF-κB response elements (Figure 6a). Therefore, GPR56 transient overexpression led to the activation of specific signaling cascades culminating in the upregulation of PAI-1 gene promoter and activation of TCF and

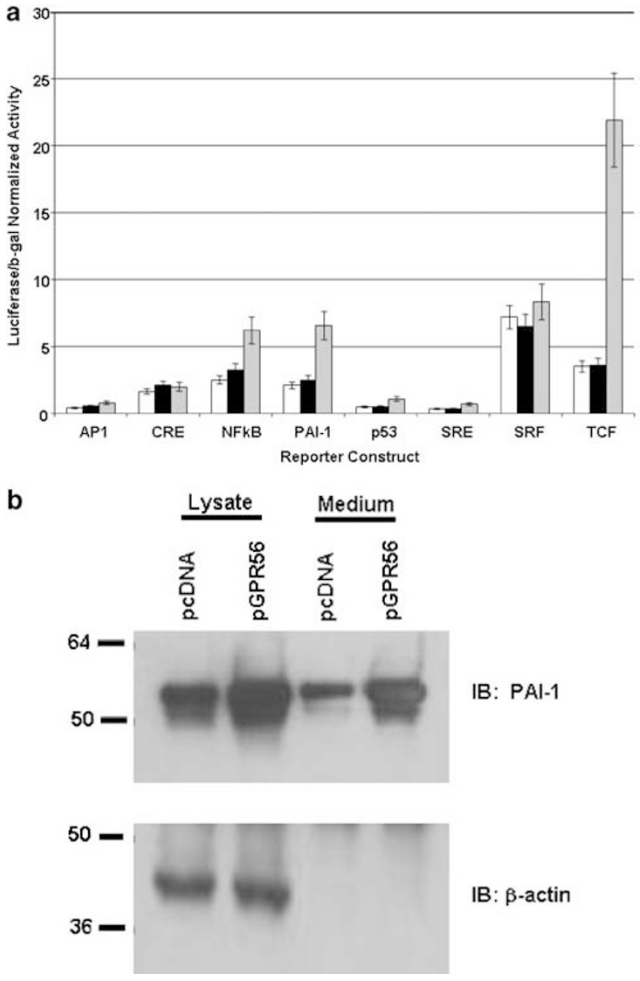


Figure 6 (**a**) Activation of downstream transcription factors by transient overexpression of GPR56. HEK 293 cells were transiently transfected with pcDNA, pcDNA-GPR56 (pGPR56) or with pcDNA followed by GPR56-ECD (10 μg/ml) treatment. A panel of luciferase reporter constructs (AP1-Luc, CRE-Luc, NFκB-Luc, PAI-1-Luc, p53-Luc, SRE-Luc, SRF-Luc and TOPFLASH LEF/TCF) and β-galactosidase were cotransfected in combination with the pcDNA (white bars), pcDNA plus GPR56-ECD (black bars) or pGPR56 (gray bars). Luciferase activity was measured the next day and normalized to β-galactosidase activity (error bars, $\pm$s.d.). pGPR56 activates signaling cascades leading to transcriptional upregulation of the NF-κB, PAI-1 and TCF reporter constructs. (**b**) Upregulation of PAI-1 following transfection with pGPR56. HEK 293 cells were transiently transfected with pcDNA or pGPR56. After 2 days, HEK 293 cell lysate and conditioned media was immunoblotted for PAI-1 protein and subsequently reprobed with β-actin as a loading control

NF-κB response elements. Other commonly investigated signaling pathways such as AP-1, CRE and SRE were not significantly activated. These data indicate that GPR56 is able to activate a specific subset of signaling pathways when transiently overexpressed in HEK 293 cells. In order to confirm the upregulation of PAI-1, HEK 293 cells were transfected with pCDNA or pCDNA-GPR56 (pGPR56) and incubated for 2 days prior to testing for PAI-1 expression. Cell lysates and conditioned media was immunoblotted for the expression of PAI-1 protein (Figure 6b). PAI-1 protein levels were elevated in the pGPR56 transfectants, thus confirming the ability of GPR56 to upregulate PAI-1.

Treatment of transfected cells with soluble GPR56-ECD for 18 h did not activate any of the reporter constructs. This indicates that soluble GPR56-ECD is unlikely to act as a direct ligand, but it is consistent with our adhesion experiments in which attached cells treated with soluble GPR56-ECD appeared to have normal morphology and adhesive characteristics (data not shown).

Discussion

Owing to their localization, invasiveness and heterogeneity, glioblastomas present a formidable problem of diagnosis and treatment. The median survival time of patients with malignant glioma treated aggressively with surgery, chemotherapy and radiation is 40–50 weeks. The first line of treatment is surgical removal of the tumor mass; however, because glioblastomas are highly invasive, there are few well-defined tumor margins available to guide the clinician during resection. The location of the tumor near important regions of the brain may also preclude complete removal. The recurrence rate of glioblastoma is extremely high (>95%) (Berger and Wilson, 1999). This is due in part to its invasive nature, which contributes to the inability to remove all of the tumor tissue. It has also been suggested that transformed neural stem cells migrate to and replenish the tumor mass (Berger *et al*., 2004). Understanding cell adhesion and migration is of considerable significance in studying invasive, recurrent tumors. Alterations in several classes of adhesion molecules have been implicated in the progression of various forms of cancers, including glioblastoma. An essential step in tumor progression is the interaction of tumor cells with ECM, matrix restructuring and tumor invasion. Tumor invasion is dependent upon a delicate balance between cell adhesion and cell detachment (Belkin *et al*., 2001). Therefore, therapeutic strategies that target cell adhesion receptors have great potential for the treatment of cancer.

Astrocytomas are often heterogeneous, posing a problem for diagnosis and treatment. GPR56 provides a functional biomarker of astrocytoma, in that it is specifically upregulated in a large percentage of these tumors and plays a functional role in tumor biology. The identification of GPR56 as a stem cell marker (Terskikh *et al*., 2001), and recent evidence that gliomas arise from the transformation of normal stem cells (Singh *et al*., 2003; Berger *et al*., 2004), underscore the importance and novelty of these functional studies of GPR56.

Since the clinical course of glioblastoma is so aggressive and invariably recurs, early and accurate assessment of the tumor is essential for proper disease management. In this regard, identification of biologically relevant markers impacts treatment and clinical outcome. For instance, diagnostic markers that accurately predict the invasive capacity of the tumor would

allow clinicians to design a treatment strategy tailored to this characteristic. Therefore, understanding the biology of glioblastoma targets is of critical importance in developing diagnostics and therapeutics based on functional analysis. Functional genomics coupled with detailed molecular and cell-based analysis provides a powerful approach to identifying potential therapeutic targets.

GPR56 is a potential therapeutic target by virtue of its drugability, specific overexpression in astrocytic tumors and its role in tumor cell adhesion. The recent discovery of a subclass of GPCRs with cell adhesion domains presents the likely scenario that cell-to-cell and cell-to-matrix interactions are linked to G-protein-coupled signaling. The human epididymis-specific protein-6 (HE6), CD97, and calcium-independent receptor for α-latrotoxin (CIRL) are three examples of GPCRs with cell adhesion properties (Krasnoperov *et al*., 1999; Obermann *et al*., 2003). Most of the adhesion GPCRs have been discovered and classified based on sequence information and have not been thoroughly tested for functional activity. Among the adhesion GPCRs, only CD97 has a known binding partner, the membrane protein CD55/DAF (Hamann *et al*., 2000). Nevertheless, the adhesion GPCRs have been implicated in diverse cellular functions such as cell polarity, cell activation and secretion. A shared attribute of adhesion GPCRs is the potential cleavage of the extended N-terminus at a consensus site located adjacent to the first transmembrane domain (Fredriksson *et al*., 2002). This GPCR proteolytic site (GPS) contains a cysteine- and tryptophan-rich motif, which is conserved among adhesion GPCR family members. Mutations in this region make CIRL resistant to cleavage and impairs its surface expression (Krasnoperov *et al*., 2002). The functional implications of the processing are unclear. One possibility is that the processing reveals an endogenous agonist, such as demonstrated for the protease-activated receptors. Another possibility is that the intracellular cleavage may regulate trafficking of the adhesion GPCRs. Although GPR56 shares some features with CIRL, including the GPS motif, we did not explore the regulation of GPR56 by intracellular processing. However, we were unable to produce GPR56 stable cell lines, and a carboxy-terminus tagged construct was retained in what appears to be the ER/Golgi (data not shown). This observation may support the notion that the proper control of expression, processing and trafficking are important aspects of GPR56 function.

Endogenous GPR56 is predominantly located on the leading edge of membranes and may play a dynamic role in cell adhesion and detachment, a function which could require tight control between surface expression and signaling. Consistent with this concept, GPR56 activates pathways with demonstrated roles in cell adhesion. The binding of urokinase plasminogen activator (uPA) to its cell surface receptor (uPAR) promotes cell adhesion by increasing the affinity of uPAR for both vitronectin and integrins. The plasminogen activator inhibitor (PAI-1) can detach cells by disrupting uPAR–vitronectin and integrin–vitronectin interactions (Czekay *et al*., 2003). The ability of GPR56 to upregulate PAI-1 may contribute to the regulation of cell adhesion. More broadly, GPR56 overexpression in glioblastoma and other tumors may contribute to PAI-1 overexpression. High PAI-1 levels in cancer patients are associated with a poor prognosis, a feature likely due to its function as a regulator of tumor cell invasion and metastasis (Czekay *et al*., 2003). Similarly, the activation of the TCF reporter by GPR56 implicates the β-catenin pathway in GPR56 signaling. β-catenin dysregulation has been associated with oncogenesis. β-catenin binds and activates TCF, leading to transcriptional regulation of genes involved in adhesion, proliferation and apoptosis (Orford *et al*., 1999). Therefore, the ability of GPR56 to activate the β-catenin/TCF pathway may be involved in the transition from a benign tumor to an invasive, metastatic cancer. Identification of signaling intermediates activated by GPR56 will be a focus of future studies. In summary, the convergence of several observations implicates the adhesion GPCR, GPR56, as an important player in tumor cell biology. Therapies designed to specifically target GPR56 through its unique extracellular domain or by its signaling characteristics could prove beneficial to patients suffering from glioblastoma and other cancers.

Materials and methods

Cell lines and antibodies

U373 cells (ECACC), U87 cells (ATCC), HEK293 cells (ATCC), D566 cells (kindly provided by Darrell Bigner of Duke University), G140 cells and G122 cells (kindly provided by Manfred Westphal, Frankfurt, Germany). Custom rabbit polyclonal GPR56 antibodies were generated against the N-terminus peptide sequence (aa 35–39) CSQRN QTHRS SLHYK (Zymed). These antibodies were affinity purified and screened by standard ELISA methods. Mouse monoclonal antibodies to α-actinin were obtained from Sigma. Mouse monoclonal anti-β-actin antibodies were obtained from Abcam. Anti-PAI-1 antibody was obtained from Oncogene Sciences. Secondary anti-rabbit-HRP and anti-mouse-HRP conjugates were purchased from Santa Cruz Biotechnology. Phalloidin Alexa-594 dye, anti-rabbit and anti-mouse Alexa-488/594-conjugated secondary antibodies were obtained from Molecular Probes.

Functional genomics

Library construction, hybridization and array analysis were performed as described previously (Mueller *et al*., 2003). Using suppression PCR, subtractive and normalized cDNA libraries were generated and cloned into the pCR2.1 vector using a TA cloning kit (Invitrogen). Four primary high-density arrays, 25 000 individual clones along with standards and empty wells were spotted onto a single filter, and six membrane filters were hybridized per probe. In secondary arrays, 6000 clones were spotted onto a single filter. Each of these filters was hybridized twice with the same probe in two independent hybridization reactions (six data points per probe). The mean and standard deviation from triplicate measurements for each clone was determined to calculate the confidence level (Student's *t*-test)

and ratio of expression between tumor and control tissue. Clones, which were upregulated in the first array analysis at a confidence value greater than 95%, were newly arrayed and spotted in triplicate on one membrane. The array-based screen was repeated twice with the same probes, and clones upregulated in both experiments were submitted for sequencing. Gene identification of clones was performed by an analysis of the sequences using BLAST and Smith–Waterman algorithms with NCBI, Unigene, Swiss-Prot/TrEMBL and Derwent databases with accelerated hardware (TimeLogic).

Northern blot analysis

Multiple tissue Northern Blots from Clontech were used to detect GPR56 transcripts in normal human tissue. GPR56 cDNA probe was radioactively labeled and hybridized according to the manufacturer's instructions (Clontech). The Multiple tissue Northern Blot was exposed and quantitated using phosporimaging (Molecular Dynamics).

Immunohistochemistry

Tissue array slides were obtained from Ambion (Land Mark Tissue MicroArray Low density Brain slides); IHC reagents were obtained as a commercial kit from InnoGenex. All the slides were processed using the protocol provided by the manufacturer and scored using microscopy. The slides were also analysed and scored by USLabs in a blinded outsourced study.

Indirect immunofluorescence

Cells were plated into black-walled, clear bottom 96-well tissue culture plates at a density of 10 000/well, incubated at 37°C overnight and immunostained the following day. Briefly, the cells were fixed with 4% formaldehyde (in PBS) for 10 min at room temperature and permeabilized with 0.1% Triton X-100 (in PBS) for 5 min. BSA (1%) was used for all the wash and blocking steps. Primary antibodies (made up in 1% BSA) were added for 1 h at room temperature followed by staining with Hoechst dye and secondary antibodies tagged with either Alexa-488 or Alexa-594 (Molecular Probes) for 1 h at room temperature. After washing, PBS was added to the wells. Immunofluorescence analysis was performed on an ArrayScan II platform (Cellomics) with a Zeiss fluorescence microscope ($\times 20$ objective).

Immunoblotting

Cells were lysed in RIPA buffer (0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate in PBS) for 30 min on ice. Lysates were then cleared by centrifugation. Protein quantitation was performed using the BCA kit (Pierce). Total cellular proteins were separated on standard Tris-Glycine-SDS–polyacrylamide gels. The fractionated proteins were blotted onto nitrocellulose membranes (VWR). Membranes were then blocked in 5% nonfat milk followed by incubation with antibody. After incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), immunodetection was accomplished using an enhanced chemiluminescent substrate (Amersham Pharmacia), followed by exposure to X-ray film (Kodak). To confirm equal loading, blots were stripped with Western blot stripping buffer (2% SDS, 100 mM β Mercapto ethanol, 62.5 mM Tris) and reprobed with β-actin antibody (Abcam).

GPR56 protein expression and purification

GPR56 extracellular domain (TN7XN1-ECD) residues Met_1-Lys_{403}, including signal peptide was tagged at the C-terminus with six histidines using PCR and expressed in baculovirus and grown in Sf9 host cells. The secreted protein was affinity purified over Ni^{2+}-NTA resin (Qiagen) and estimated to be about 90% pure by Coomassie stain.

Cell adhesion assay

In order to determine the role of GPR56 protein in glioma cell adhesion, 96-well plates were coated overnight at 4°C with different proteins. Appropriate wells were coated overnight with 10 μg/ml BSA (Pierce), NgCAM, Fibronectin (Chemicon) and GPR56-ECD. The plates were washed with PBS, blocked for 1 h at RT with 1% BSA and washed again in PBS. Cells were harvested, suspended into serum-free media and plated onto the coated plates at a density of 30 000 cells/well. After washing the plates three times with PBS, the number of adherent cells were measured using Cell Titer Glo reagent (Promega).

Reporter assay

HEK 293 cells were seeded at 10 000 cells/well on 96-well plates. The next day, quadruplicate wells of HEK 293 cells were cotransfected using Fugene 6 transfection reagent (Roche) with 50 ng of the following luciferase reporter constructs: AP1-Luc, CRE-Luc, NFκB-Luc, PAI-1-Luc, p53-Luc, SRE-Luc, SRF-Luc (Stratagene) and TOPFLASH LEF/TCF reporter plasmid (Upstate), 10 ng of RSV-β-galactosidase, as a normalization control, and either 100 ng of pcDNA3.1 or 100 ng pcDNA3.1-GPR56 cDNA construct encoding the entire coding region of GPR56. After 24 h post-transfection, the cells were lysed and assayed for Luciferase and β-galactosidase activity using the Dual-Lite Reporter Kit (Tropix-Applied Biosystems). All results were normalized to β-galactosidase.

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