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GPR56 drives colorectal tumor growth and promotes drug resistance through upregulation of MDR1 expression via a RhoA-mediated mechanism

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Abstract

Drug resistance continues to be a major obstacle of effective therapy for colorectal cancer, leading to tumor relapse or treatment failure. Cancer stem cells (CSCs) or tumor-initiating cells are a subpopulation of tumor cells which retain the capacity for self-renewal and are suggested to be implicated in drug resistance. LGR5 is highly expressed in colorectal cancer and marks CSCs that drive tumor growth and metastasis. LGR5($^+$) CSCs cells were shown to interconvert with more drug resistant LGR5($^-$) cancer cells and treatment with LGR5-targeted antibody-drug conjugates (ADCs) eliminated LGR5($^+$) tumors, yet a fraction of LGR5($^-$) tumors eventually recurred. Therefore, it is important to identify mechanisms associated with CSC plasticity and drug resistance in order to develop curative therapies. Here we show that loss of LGR5 in colon cancer cells enhanced resistance to irinotecan and 5-fluorouracil and increased expression of adhesion G-protein coupled receptor, GPR56. *GPR56* expression was significantly higher in primary colon tumors versus matched normal tissues and correlated with poor survival outcome. GPR56 enhanced drug resistance through upregulation of MDR1 levels via a RhoA-mediated signaling mechanism. Loss of GPR56 led to suppression of tumor growth and increased sensitivity of cancer cells to chemotherapy and MMAE-linked anti-LGR5 ADCs, by reducing MDR1 levels. These findings suggest that upregulation of GPR56 may be a mechanism associated with CSC plasticity by which LGR5($^-$) cancer cells acquire a more drug resistant phenotype.

Implications—Our findings suggest that targeting GPR56 may provide a new strategy for the treatment of colorectal cancer and combatting drug resistance.

Keywords

GPR56; LGR5; MDR1; antibody-drug conjugate; colorectal cancer

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Introduction

Resistance to chemotherapy continues to be a major obstacle in the treatment of colorectal cancer (CRC), leading to relapse or failure of treatment. To develop more effective therapies, it is important to identify the underlying mechanisms that drive resistance. Cancer stem cells (CSCs) are subpopulation of tumor-initiating cells within the bulk tumor that retain the capacity for self-renewal, promote metastasis, and are relatively more resistant to systemic chemotherapies (1,2). A key mechanism of drug resistance in cancer cells and CSCs is increased expression of membrane proteins belonging to the ATP-binding cassette (ABC) transporter family of efflux pumps that decrease the cellular accumulation of anticancer drugs (3). ABC transporters, in particular P-glycoprotein/multidrug resistance protein 1 (P-gp/MDR1), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP), have been reported to be up-regulated in colorectal tumors cells and CSCs (4–7). Thus, elimination of CSCs could be an effective strategy to combat drug resistance.

Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) is highly expressed in CRC (8,9). LGR5 has been authenticated as marker of normal intestinal crypt stem cells (10) and CSCs that fuel tumor growth and metastasis in CRC (11–13). We and others showed that monomethyl auristatin E (MMAE)-linked anti-LGR5 antibody-drug conjugates could eliminate colon tumors. However, a fraction of the tumors eventually recurred subsequent to treatment termination, likely due to LGR5 downregulation or resistance to MMAE (8,9). Selective ablation of LGR5($^+$) colon CSCs showed that LGR5($^-$) cancer cells can sustain tumors with the capacity to transition back to LGR5($^+$) colon CSCs, resulting in more aggressive tumor growth and metastasis (11,13). LGR5($^+$) CSCs were also shown to interconvert with LGR5($^-$) cancer cells and LGR5($^-$) were shown to be more drug and radio-resistant (14,15). Therefore, cure of CRC tumors will require the eradication of both LGR5($^+$) and LGR5($^-$) cancer cells. While LGR5($^+$) cells can be effectively eliminated by anti-LGR5 ADCs, targets and associated mechanisms that are upregulated in LGR5($^-$) cancer cells and involved in mediating CSC plasticity remain poorly understood.

GPR56, or ADGRG1, is a member of the adhesion G-protein coupled receptor (GPCR) subfamily and is comprised of a large N-terminal extracellular domain (ECD), a GPS domain, and a seven transmembrane domain typical of the secretin family of GPCRs (16). The receptor has been shown to couple to the $G_{\alpha 12/13}$ class of heterotrimeric G proteins to promote RhoA activation (17–19). GPR56 is reported to be highly expressed in cancers of the breast, lung, ovary, pancreas, colon, and in glioblastomas (20–22). Recently, GPR56 has been shown to be expressed in intestinal crypt stem cells (23) and identified as a marker of a subgroup of acute myeloid leukemia (AML) CSCs associated with high risk genetic lesions and poor outcome (24,25). Still the function and signaling mechanism of GPR56 in CRC and colon CSCs remains to be elucidated.

Here we set out to identify potential targets that are upregulated with LGR5 ablation and associated with drug resistance. We show that loss of LGR5 in LoVo colon cancer cells resulted in increased proliferation and resistance to chemotherapy with concomitant upregulation of the adhesion receptor GPR56. Knockdown of GPR56 (KD) in multiple colon cancer cell lines led to suppression of tumor growth and decreased drug resistance.

GPR56 was found to regulate MDR1 levels and associated drug resistance via a RhoA-mediated signaling mechanism. Furthermore, loss of GPR56 or direct inhibition of MDR1 sensitized cancer cells to anti-LGR5-MMAE ADC treatment. This study demonstrates a new role for GPR56 in the regulation of drug resistance.

Materials and Methods

Plasmids and cloning

The sequence encoding hGPR56 (amino acids 26–693) was subcloned from pCAG-hGPR56-IRES-GFP and fused with sequences encoding a Myc tag at the N terminus, and cloned downstream of a sequence encoding the CD8 signal peptide (MALPVTALLLPLALLLHAA) in the vector pIRESpuro3 (Clontech). pCAG-hGPR56-IRES-GFP was from Christopher A Walsh (Addgene, 52297) (26). Similarly, myc-mGPR56 was subcloned from mouse *adgrg1* cDNA (Clone ID:3709247, Dharmacon). The pRK5-myc-RhoA-T19N was from Gary Bokoch (Addgene, 12963).

Anti-LGR5-MMAE ADC, cytotoxic drugs, and inhibitors

The cleavable anti-LGR5-mc-vc-PAB-MMAE (anti-LGR5-MMAE) ADC with drug-to-antibody ratio of 4 was generated as previously described (8). MMAE was purchased from ALB Technology. Irinotecan and 5-fluorouracil were purchased from Biotang and Acros Organics, respectively. Tariquidar and Y27632 were from Selleck Chemical. The cell permeable C3 transferase-based Rho inhibitor I was purchased from Cytoskeleton.

Cell culture, transfection, and stable cell line generation

DLD-1, HT-29, and LS180 cells were purchased from ATCC. LoVo cells were obtained from Dr. Shao-Cong Sun (M.D. Anderson Cancer Center). Cell lines were authenticated utilizing short tandem repeat profiling, routinely tested for mycoplasma, and cultured in RPMI medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C with 95% humidity and 5% CO_2. Transient transections were performed using Dharmafect Duo (Dharmacon) or jetPRIME (Polypus Transfection). Stable pLKO.1 (control), LGR5, and GPR56 shRNA KD cells were generated by lentiviral infection as previously reported (8,18). The shRNAs used were, TRCN0000011586 (shLGR5-1), TRCN0000011589 (shLGR5-2), TRCN0000011618 (shGPR56-1), and TRCN0000011619 (shGPR56-2) from GE Dharmacon. Stable DLD-1 cells over-expressing hGPR56 and vector cells were generated as previously described (27).

RNA isolation and quantitative RT-PCR

Patient colorectal cancer tumor and adjacent normal tissues were obtained from the MD Anderson's Institutional Tissue Bank. RNA from cell lines or tissues was isolated using TRIzol (Invitrogen), purified using an RNeasy kit (Qiagen), and treated with DNase I digestion. RNA quality was verified using a bioanalyzer (Agilent Technologies) and RNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific). Quantitative RT-PCR of was performed by the Quantitative Genomic & Microarray Core Lab (University of Texas Health Science Center, Houston, TX). Briefly, a total of 100 ng RNA was run in triplicate per assay (along with no-template and nonamplifying controls) using the following Taqman primer/probes: ADGRG1 (GPR56); forward GATTACAGGTGGTGACTTCCAA, reverse ACCAGGAAGAGCAGACTCA, probe FAM-TGCTGCAGACGACACTGTTCCTG-BHQ1 and 18S rRNA; forward CGGCTTAATTTGACTCAACAC, reverse ATCAATCTGTCAATCCTGTCC, probe FAM-AAACCTCACCCGGCCCG-BHQ1. Quantified expression levels of GPR56 were determined from an ssDNA standard curve and expression was normalized to levels of 18S rRNA.

Microarray analysis

Total RNA was purified from LoVo cells (n = 2/cell line). Microarrays and data analysis were performed at the UT Health Quantitative Genomic & Microarray Core Lab. Gene expression profiles were performed using Illumina HumanHT-12 v4 bead array chips and data were preprocessed with BeadStudio (Illumina) using quantile normalization with background subtracted, and expressed genes were identified using a detection threshold of $P < 0.01$. The P-values were determined by two-tailed t-test. Differential expression was assessed with Bonferroni correction and a false discovery rate of 0.05. Microarray data have been deposited in the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo) under accession number GSE135749.

Western blot and RhoA pulldown assays

RhoA-GTP pulldown activation assay (Cytoskeleton, #BK036) was carried out according to protocol. For western blots, protein extraction was performed using RIPA buffer (Sigma) supplemented with protease/phosphatase inhibitors. Cell lysates were incubated at 37°C for 1 hour in 2xSDS buffer prior to loading on SDS-PAGE. Commercial antibodies were used in accordance to manufacturer's guidelines: anti-LGR5 (Abcam, ab75732), anti-GPR56 (Abnova, H00009289-B01P), anti-MDR1 (Abcam, ab170904 or Cell Signaling, 13342S), anti-MRP1 (Novus, NB400-156 or Cell Signaling, 72202S), anti-ABCG2 (Cell Signaling, 42078S), anti-myc (Cell Signaling, 2276S), and anti-β-actin (Cell Signaling, 4970). HRP-labeled secondary antibodies were utilized for detection with the standard ECL protocol. Quantification was performed using ImageJ.

In vitro cytotoxicity and proliferation assays

Cells were plated at 1000 cells/well in 96 half-well plates. Serial dilutions of drugs or anti-LGR5 ADC were added and allowed to incubate at 37°C for 3–4 days as indicated. For experiments using Rho inhibitor I or tariquidar, cells were pretreated for approximately 24 hours and 1 hour prior to drug or ADC treatment, respectively. Cell cytotoxicity was measured using CellTiter-Glo (Promega) according to manufacturer's protocol. Luminescence was measured using EnVision mulitlabel plate reader (PerkinElmer). For proliferation studies, measurements were acquired once a day for 4–5 days (n =3–4 experiments). Each condition was tested in at least triplicates. Cytotoxicity data is shown as a single experiment representative of 3–4 independent experiments.

Multi-drug resistance calcein-AM assay

Cell were plated in a 96-well plate at 10,000 cells/well and the next day treated with PBS vehicle or 50 nM tariquidar for 1 hour at 37°C. Calcein-AM (BD Biosciences) was added at a final concentration of 0.5 μM for 15 min at 37°C. Cells were washed three times with PBS and fluorescence intensity was quantified at 494/517 nm using Tecan Infinite M1000 plate reader. Cell numbers for different cell lines were normalized using alamarBlue (ThermoFisher) according to protocol.

In vivo tumor growth

Animal studies were carried out in strict accordance with the recommendations of the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Houston (AWC-17-0148). Female 6–8 week old nu/nu mice (Charles River Laboratories) were subcutaneously inoculated with or 1×10^6 HT-29 cells or 2×10^6 DLD-1 cells in 1:1 mixture of PBS:matrigel (BD Biosciences, San Jose, CA) into lower right flank. Tumor volumes were measured bi-weekly and estimated by the formula: Tumor volume= length x width^2/2. Mice were euthanized when tumor volume reached ~1000 mm^3.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Data are expressed as mean +/− SEM. The Cancer Genome Atlas (TCGA) datasets were partitioned into low and high expression values based on overall distribution range of each cohort. Normal and tumor samples were compared and analyzed using paired t-test. IC_{50} values were determines using logistic nonlinear regression model. For in vitro proliferation and in vivo tumor studies, differences between groups were analyzed by two-way ANOVA. Other multiple comparisons used one-way ANOVA and Tukey's post-hoc analysis unless otherwise specified. P-values ≤ 0.05 were considered statistically significant.

Results

LGR5 knockdown enhances proliferation and drug resistance in LoVo cells

To characterize the functional effects of LGR5, we measured changes in cell proliferation and drug resistance in response to LGR5 KD (shLGR5) in LoVo colon cancer cells. LoVo cells were selected since they are the colon cancer cell line with the highest level of LGR5 based on gene expression data extracted from the Cancer Cell Line Encyclopedia (CCLE) and by protein expression, as we previously reported (8). LGR5 KD using 2 independent shRNA constructs was confirmed by western analysis (Fig. 1A). Using the CellTiter-Glo assay we showed that loss of LGR5 expression resulted in increased cell proliferation (Fig. 1B) compared control KD (shCTL) and parental cells. To test LGR5-mediated effects on drug resistance, cells were treated with increasing concentrations of irinotecan or 5-fluorouracil (5-FU) for 3 days. Interestingly, loss of LGR5 lead to enhanced resistance to both drugs (Fig. 1C–D). The IC_{50} values for LGR5 KD cells were approximately 6–8 fold higher for irinotecan and 5-fold higher for 5-FU compared to shCTL cells (Table 1). LoVo parental and shCTL cells exhibited similar IC_{50}s for each drug.

GPR56 is upregulated with loss of LGR5 expression

To identify genes that may be involved in mediating the proliferative and drug resistant phenotype of LGR5 KD cells, we performed genome-wide microarray analysis of LoVo cells with and without KD of LGR5. One of the most highly upregulated genes in LGR5 KD cells was *ADGRG1* or *GPR56* (Fig 1E). Interestingly, LoVo cells have little to no endogenous *GPR56* expression, however mRNA levels were markedly induced by ~25 and 100-fold in shLGR5-1 and shLGR5-2 cells, respectively. Western blot analysis verified that GPR56 protein levels were also induced in response to LGR5 KD (Fig. 1A). Interestingly, when we transfected increasing amounts of GPR56 into LoVo cells we observed a concomitant decrease in LGR5 expression, suggesting that GPR56 and LGR5 cannot be co-expressed at high levels in this cell line (Fig. 1F). Of note, GPR56 expression can appear as a broad band, depending on the cell line, due to posttranslational modifications and proteolytic cleavage of the extracellular domain (ECD), typical of adhesion GPCRs (17,28). Major bands between ~55–60 and 70–75 kDa are more obvious with recombinant expression and represent the ECD and full-length, respectively. Increased GPR56 expression was also observed in LS180 colon cancer cells in response to CRISPR/Cas9 knockout (KO) of LGR5 (Supplementary Fig. S1A). Similar to LoVo cells, loss of LGR5 expression resulted in increased cell proliferation and enhanced resistance to irinotecan (Supplementary Fig. S1B–C and Table 1). LGR5 KO did not have a significant effect on LS180 resistance to 5-FU (Supplementary Fig. S1D). On the other hand, no significant change in GPR56 levels or proliferation was observed with LGR5 KD in DLD-1 cells (Supplementary Fig. S1E–F), indicating that LGR5-mediated regulation of GPR56 expression may be cell line dependent. These finding suggest that GPR56 may play a role in enhancing cell proliferation and drug resistance in response to LGR5 KD in LoVo cells.

Loss of GPR56 in LGR5 knockdown cells partially rescues drug sensitivity

To determine if aberrant GPR56 expression is implicated in mediating the functional effects observed in LoVo LGR5 KD cells, we generated a LGR5/GPR56 double KD cell line. Knockdown of both LGR5 and GPR56 was confirmed by western blot and immunocytochemistry (Fig. 1G and Supplementary Fig. S1G). Cell proliferation of LGR5/GPR56 KD cells was significantly decreased compared to LGR5 KD (Fig. 1H). Furthermore, relative to LGR5 KD cells, LGR5/GPR56 KD cells showed increased sensitivity to both irinotecan and 5-FU (Fig. 1I–J). IC_{50} values for double KD cells were approximately 2- and 7-fold lower for irinotecan and 5-FU, respectively, compared to LGR5 KD cells (Table 1). These results suggest that loss of GPR56 can at least partially reverse the LGR5 KD-mediated effects on proliferation and drug resistance in LoVo cells. However, since GPR56 KD rescue was incomplete, it is likely other mechanisms are also involved.

GPR56 is highly expressed in colorectal cancer and correlates with poor survival

To further investigate the importance of *GPR56* in colon cancer we evaluated expression levels in patient samples and colon cancer cell lines. Quantitative RT-PCR analysis showed that *GPR56* mRNA expression was significantly higher in primary colon tumors versus matched normal adjacent tissue obtained from MD Anderson Cancer Center, with 80% of samples showing an increase of at least 2-fold (Fig. 2A). Similarly, analysis of whole transcriptome sequencing from TCGA colorectal adenocarcinoma (COADREAD) dataset showed that *GPR56* is highly upregulated in tumors when compared to matched normal tissue based on values of RSEM (RNA-Seq by Expectation-Maximization) (Fig. 2B) (29). Implementation of a fold change cut-off of 2 revealed that 66% of the patient population had high *GPR56* tumor expression. Importantly, partitioning of data from the COADREAD cohort showed that high *GPR56* expression strongly correlated with poor disease-free (Fig. 2C, median value = 26 vs. 109 months) and overall survival (Fig. 2D, median value = 47 vs. 100 months). Furthermore, examination of CCLE microarray datasets revealed that *GPR56* is abundantly expressed in approximately 90% of colon cancer cell lines (Log2 Robust Multi-array Average (RMA)-normalized ≥ 6) (Fig. 3A) (30).

Loss of GPR56 suppresses colon tumor growth

To further demonstrate a role for GPR56 in promoting colon cancer cell growth, independent of LGR5 expression, we performed GPR56 KD in two colon cancer cell lines that express high levels of endogenous GPR56 and different levels of LGR5 based on CCLE analysis, HT-29 and DLD-1 (Fig. 3A). DLD-1 cells expressed relatively high LGR5 protein levels, whereas in HT-29 cells LGR5 was undetectable (Fig. 3B). Western blot analysis confirmed significant KD of protein levels in both cell lines using two distinct GPR56-targeted shRNA constructs (Fig. 3B). Using the CellTiter-Glo assay, we found proliferation of GPR56 KD cell lines was significantly decreased in both HT-29 and DLD-1 cells (Fig. 3C–D). To evaluate in vivo tumor growth, HT-29 and DLD-1 (shCTL and shGPR56) cells were implanted into nude mice. After approximately 4 weeks a significant reduction in tumor growth was observed in both HT-29 and DLD-1 cells (Fig 3E–F). To test if overexpression of GPR56 could increase proliferation and tumor growth, we generated DLD-1 cells stably overexpressing vector (control) or recombinant myc-tagged human GPR56 (hGPR56). GPR56 overexpression was verified by western blot (Fig. 3G). Though we did not observe a significant change in proliferation in vitro (Supplementary Fig. S2A), GPR56 overexpression did significantly increase tumor growth (Fig. 3H). The difference in vitro versus in vivo may be attributed to the tumor microenvironment and potential increased accessibility to endogenous ligands in vivo. Together, these finding indicate that GPR56 may have a significant role in driving colon tumor initiation and growth.

GPR56 knockdown sensitizes cancer cells to chemotherapy

Next, we investigated how loss of endogenous GPR56 expression effects the sensitivity of colon cancer cells to chemotherapeutic agents. HT-29 and DLD-1 parental, shCTL, and GPR56 KD cells were treated with increasing concentrations of irinotecan or 5-FU (Fig. 4A–B and Supplementary Fig. S2B–C) for 3 days. Loss of GPR56 resulted in a significant increase sensitivity of HT-29 to 5-FU and DLD-1 to both drugs. Compared to HT-29 shCTL cells, average IC_{50} values for both drugs decreased by 2-fold for HT-29 shGPR56-2 cells, which exhibited a more complete knockdown than HT-29 shGPR56-1 cells (Table 1). To test if overexpression of GPR56 could enhance drug resistance, DLD-1-vector and DLD-1-hGPR56 cells were treated with increasing doses of irinotecan or 5-FU. Results showed that overexpression of GPR56 enhanced resistance with a 2- and 4-fold increase in IC_{50}, respectively (Fig. 4C–D and Table 1). Furthermore, transient overexpression of myc-tagged mouse GPR56 (mGPR56) DLD-1 GPR56 KD cells demonstrated that mGPR56 rescued the KD phenotype with respect to irinotecan sensitivity (Fig. 4E–F). Similar to hGPR56, mGPR56 also increased irinotecan resistance in DLD-1 shCTL cells. Taken together, these results suggest that GPR56 functions to regulate drug resistance in colon cancer cells.

GPR56 modulates expression and function of ABC transporter proteins

To explore mechanisms underlying GPR56-mediated drug resistance, we mined the CCLE datasets for expression of ABC transporters commonly implicated in driving drug efflux in colon cancer (i.e. *ABCB1*, *ABCC1, ABCG2*) (31). The findings showed that LoVo cells express relatively high levels of all three transporters, DLD-1 cells express high levels of *ABCB1* and *ABCC1*, HT-29 cells express high levels of *ABCC1,* moderate levels of *ABCG2,* and low to undetectable levels of *ABCB1*, and LS180 cells express high levels of *ABCB1* and *ABCC1* and low to undetectable levels of *ABCG2* (Supplementary Fig. S2D). We then performed western blot analysis to confirm protein expression levels and measure changes in response to altered GPR56 expression. As shown in the left panel of Fig. 5A, LoVo LGR5 KD cells, which express high levels of GPR56 showed an increase in expression of a smaller variant form of the MDR1 (*ABCB1*) transporter. There was also a noticeable increase in MRP1 (*ABCC1*) and a reduction in BCRP (*ABCG2*) expression. Of note, LS180 LGR5 KO cells, which have high levels of GPR56, also exhibited an increase in MDR1, but no change in MRP1 and BCRP was undetectable (Supplementary Fig. S2E). Loss of GPR56 in LoVo LGR5 KD cells resulted in a dramatic decrease in both MDR1 and MRP1 levels. GPR56 knockdown in DLD-1 cells showed a decrease in MDR1 expression with no change in MRP1 levels (Fig. 5A, right panel). HT-29 cells do not express MDR1, however there was a notable decrease in MRP1 expression with GPR56 KD with no change in BCRP (Supplementary Fig. S2F).

Since GPR56 overexpression in DLD-1 cells led to an increase in drug resistance, we next examined these cells for changes in ABC transporter expression and function. As expected, overexpression of GPR56 led to a significant 12-fold increase in MDR1 levels with no change in MRP1 (Fig. 5B and Supplementary Fig. S3A). To test if changes in ABC transporter expression levels were consistent with changes in function, we performed the multidrug resistance assay which measures accumulation of calcein AM, a substrate for both MDR1 and MRP1. DLD-1 hGPR56 cells showed a significant reduction in calcein AM accumulation compared to parental and vector cells, which was rescued by pretreatment with the MDR1 specific inhibitor, tariquidar (Fig. 5C). GPR56 KD cells showed a significant increase in calcein AM retention, likely attributed to decreased MDR1 expression, and tariquidar pretreatment had little effect (Fig 5C). DLD-1 cell uptake of Hoechst 33342, which is a substrate for MDR1 and BCRP, showed a similar pattern of dye retention (Supplementary Fig. S3B). For LoVo LGR5 KD cells, the calcein AM retention was significantly decreased compared to shCTL cells potentially due to the increase in MRP1 and a variant form of MDR1 (Supplementary Fig. S3C). Correspondingly, double LGR5/GPR56 KD cells showed a significant increase in calcein AM retention compared to LGR5 KD and control cells, consistent with the reduction in MDR1 and MRP1 expression and associated efflux. LS180 LGR5 KO cells, which have higher MDR1 levels, showed decreased calcein AM retention compared to control and increased uptake in the presence of tariquidar. (Supplementary Fig. S3D). We then tested if inhibition of MDR1 could rescue drug sensitivity of DLD-1 cells. As shown in Fig. 5D, cells pretreated with tariquidar exhibited an increase in irinotecan sensitivity with an average 2- and 4-fold decrease in IC_{50} for vector and hGPR56 cells, respectively. Tariquidar also sensitized vector and hGPR56 cells to 5-FU (Supplementary Fig. S4A). Together, these findings suggest that GPR56 modulates ABC transporter expression of colon cancer cells and potentially promotes drug resistance through changes in transporter function, as shown by tariquidar-inhibition of MDR1.

GPR56 regulates MDR1 expression through RhoA

GPR56 has been shown to induce activation of the small GTPase RhoA (17–19). Therefore, we examined whether GPR56 regulation of MDR1 expression is mediated by a RhoA-dependent signaling pathway. Using a GTPase pulldown assay which employs the Rho binding domain of the Rho effector protein, Rhotekin, we showed that GPR56 overexpression increased levels of active GTP bound RhoA (RhoA-GTP) in DLD-1 cells independent of exogenous ligands (Fig. 5E). GPR56 KD considerably decreased RhoA-GTP levels (Fig. 5F). Next, we tested if abrogating RhoA activity would alter MDR1 levels. Transfection of dominant-negative RhoA T19N (RhoA-DN) in DLD-1 cells reduced levels of MDR1 after 3 days (Fig. 5G). Next, DLD-1 vector and hGPR56 cells were treated with Rho inhibitor I, a cell permeable C3 transferase which inhibits Rho, or Y27632 which inhibits Rho-associated protein kinase (ROCK), a kinase activated by RhoA. As shown in Fig. 5H and Supplementary Fig. S4B, western analysis was performed 3 days post-treatment and showed that both Rho and ROCK inhibitors reduced levels of MDR1 in vector (2.5-fold) and hGPR56 cells (2 and 1.3-fold, respectively). To test if Rho inhibition could rescue drug sensitivity of GPR56 cells, vector and GPR56 cells were pretreated with Rho inhibitor I for 24 hours then treated with different concentrations of irinotecan for 4 days. Intriguingly, treatment with Rho inhibitor restored sensitivity of GPR56 cells, reducing the IC_{50} of irinotecan to a value analogous to that of vector cells (Fig. 5I). Rho inhibitor I also sensitized DLD-1 GPR56 cells to 5-FU (Supplementary Fig S4C–D). Notably, treatment with Rho Inhibitor I alone did not affect cell survival of vector or GPR56 cells (Supplementary Fig. S4E). These findings suggest that GPR56-mediated induction of MDR1 expression and associated drug resistance is regulated by a RhoA-dependent signaling mechanism.

Loss of GPR56 enhances cancer cell sensitivity to anti-LGR5-MMAE ADCs

Previously we reported that MMAE-linked anti-LGR5 ADCs could target and eradicate LGR5(+) colon cancer cells and LoVo xenograft tumors (8). However, certain colon cancer cell lines, such as DLD-1, are more resistant than LoVo cells to ADC treatment despite expressing high levels of LGR5, due to MMAE resistance (IC_{50} 10-fold greater for DLD-1 cells, Fig. 6A and Table 1). Reports have shown that resistance to MMAE can be mediated by high expression of MDR1 (32,33). Since, GPR56 KD sensitized DLD-1 cells to chemotherapy via downregulation of MDR1, we tested if these cells were also more sensitive to MMAE and anti-LGR5-MMAE ADC treatment. To evaluate in vitro cytotoxicity to free MMAE, DLD-1 shCTL, GPR56 KD, vector, and hGPR56-overexpressing cells were incubated with increasing concentrations of MMAE and cell viability was measured after 4 days (Fig. 6B). Similar to our findings with irinotecan, GPR56 KD cells were more sensitive to MMAE, whereas hGPR56 cells were more resistant with a ~4.5-fold difference in IC_{50}s compared to their respective controls (Table 1). Furthermore, anti-LGR5-MMAE ADC treatment was in fact more cytotoxic to shGPR56 cells compared to shCTL cells (Fig. 6C). Of note, no significant change in LGR5 expression was detected in response to GPR56 KD (Supplementary Fig. 3B), demonstrating this finding was not a target-based effect. Co-treatment of vector and hGPR56 cells with tariquidar, to block MDR1 function, enhanced the cell killing effect of anti-LGR5-MMAE ADC (Fig. 6D). These findings show that loss of GPR56 can potentially overcome resistance to MMAE-based ADCs through downregulation of MDR1.

Discussion

Recent studies have demonstrated the role of LGR5($^+$) CSCs and their plasticity in tumor growth, metastasis, and drug resistance (11,13,15). However, the actual function and mechanisms of LGR5 in these processes remains unclear. LGR5 gene knockdown and overexpression studies have demonstrated that LGR5 can have a growth suppressive effect in colon cancer cells (34–36), whereas others have shown that LGR5 promotes tumor growth (37). In this study, we found that LGR5 KD in LoVo and KO in LS180 cells led to a significant increase in proliferation (Fig. 1B and Supplementary Fig. S1B), but had only a minor impact in DLD-1 cells (Supplementary Fig. S1F), suggesting the extent of effect of LGR5 KD on proliferation may be cancer cell dependent. We also found that loss of LGR5 in LoVo and LS180 cells led to an increase in resistance to irinotecan and 5-FU, two common chemotherapy drugs used for the treatment of colon cancer. Intriguingly, we show that LGR5 KD led to a significant induction of GPR56 expression in LoVo and LS180 cells, but not DLD-1 cells (Fig. 1A, 1E–F and Supplementary Fig. S1A and S1E). Furthermore, GPR56 overexpression in LoVo cells reduced LGR5 levels, suggesting that in this cell line these proteins are inversely regulated and cannot be co-expressed at high levels. Loss of GPR56 in LGR5 KD cells partially rescued the effects on proliferation and drug resistance (Fig. 1G–J). Since the rescue was incomplete, it is possible that additional factors and signaling pathways may be involved in mediating these observed functional effects in response to loss of LGR5. In fact, we have shown that LGR5 KD modulates Wnt signaling and cell adhesion (18), which may contribute to changes in proliferation and drug resistance.

GPR56 is significantly upregulated in colon cancer and high expression correlates with poor overall and disease-free survival (Fig. 2). Analysis of other colon cancer patient datasets have also demonstrated high *GPR56* tumor expression with poor prognosis (22,38). Ablation of endogenous GPR56 in DLD-1 and HT-29 significantly suppressed tumor growth and sensitized cells to chemotherapy, whereas recombinant overexpression had the opposite effect (Fig 3–4 and Supplementary Fig. S2B–C). These GPR56-mediated effects were independent of LGR5 expression status. Consistently, another group recently showed siRNA knockdown of GPR56 could promote apoptosis and suppress colon tumor growth in vivo (38). GPR56−/− mice showed an increase in apoptotic cells in the intestinal crypts compared to wild-type and colonic organoids generated from GPR56−/− mice had reduced survival capacity (23). Lineage tracing demonstrated that GPR56 is expressed in colonic crypt stem cells and RNA-seq data shows that *GPR56* is expressed in both LGR5($^+$) and LGR5($^-$) cells isolated from patient derived organoids from adenomas (23,39). These findings suggests that GPR56 plays an important role in growth and drug resistance of CRC tumors and CSCs.

MDR1, MRP1 and BCRP belong to the family of ABC transporters that decrease the bioavailability of administered drug and enhance drug resistance of tumors and CSCs (4–7). In fact, irinotecan is a reported substrate for MDR1, MRP1, and BCRP (40) and 5-FU has been shown to be a substrate for MDR1 in colon cancer cells (41) in addition to other transporters (42). Here we found that loss of GPR56 in colon cancer cells decreased MRP1 expression and to a greater extent MDR1 (depending on the cell line), whereas increased GPR56 expression in LoVo LGR5 KD, LS180 LGR5 KO, and DLD-1-hGPR56 cells significantly increased MDR1 levels (Fig. 5A–B and Supplementary Figs S2E–F). The changes in ABC transporter expression were consistent with the changes in function based on the multidrug resistance assay and the sensitivity of the different cell lines to irinotecan and 5-FU (Fig. 4–5 and Supplementary Fig. S1C–D, S2B–F, and S3). Enhanced drug resistance of GPR56-overexpresssing cells was rescued by tariquidar treatment, suggesting that irinotecan and 5-FU are MDR1 substrates in DLD-1 cells. Expression of several ABC transporter genes, including the MRP1 gene *ABCC1*, were reported to correlate with GPR56 in AML CSCs (25). Interestingly, we found that LGR5 KD in LoVo cells (GPR56-high) resulted an increase in a variant form of MDR1, which is observed to be present in DLD-1 cells (Fig. 4A). GPR56 KD decreased the level of this variant, suggesting that GPR56 may modulate phosphorylation, glycosylation, or ubiquitination of MDR1. Of note, GPR56 KD did not revert ABC transporter expression back to that of the parental and shCTL lines, suggesting that changes in the expression of both LGR5 and GPR56 in LGR5 KD cells lead to modulation of other mechanisms that alter the cell line and may also affect ABC transporter expression. However, whether GPR56 plays a role in the regulation of transcription, posttranslational modification, and/or degradation of the MDR1 remains to be determined.

Since GPR56 activates RhoA (Fig. 5E–F) (17–19), we evaluated whether RhoA inhibition could suppress GPR56-mediated effects on drug resistance. We showed that blockade of RhoA activity decreased expression of MDR1 and sensitized DLD-1-hGPR56 cells to chemotherapy (Fig. 5G–I and Supplementary Fig. S4B–E). RhoA has been previously shown to regulate MDR1-mediated resistance to irinotecan and doxorubicin in colon cancer cells (43,44). Our findings suggest that GPR56 regulation of drug resistance is RhoA-mediated, yet the cognate ligand and signaling mechanism downstream of RhoA is unclear. Putative ligands for GPR56 have been reported (19,23,45). However, expression of these ligands is low or undetectable in the majority GPR56-expressing colon cancer cell lines (30), suggesting GPR56 has constitutive activity or alternative ligand(s) are likely involved.

We reported that anti-LGR5-MMAE ADC could eradicate LGR5($^{+}$) colon tumors, yet some tumors eventually reappeared due to LGR5 downregulation (8) and potentially resistance to MMAE. ABC transporters have been implicated in resistance to ADCs (46) and MMAE has been reported to be a substrate for MDR1 (32,33). We show that DLD-1 cells are more resistant to free MMAE and anti-LGR5-MMAE than LoVo cells, though both cell lines express relatively high levels of MDR1 (Fig.6A). MMAE resistance of DLD-1 cells may be due to the different variant forms of MDR1, the presence of other ABC transporters that mediate MMAE efflux, or other mechanisms. GPR56 overexpression further enhanced resistance (Fig. 6B–D), whereas GPR56 KD sensitized DLD-1 cells by a greater magnitude than tariquidar to MMAE and anti-LGR5-MMAE treatment (Fig. 6B–C). Thus, GPR56 may regulate other mechanisms involved in MMAE resistance. Of note, since GPR56 KD had a significant impact on DLD-1 tumor growth, the ADC response was not tested in vivo. These findings suggest that inhibition of GPR56 may enhance the potency of MMAE-conjugated ADCs.

In conclusion, our findings suggest that upregulation of GPR56 may be a mechanism associated with colon CSC plasticity by which LGR5(−) cancer cells acquire a more drug resistant phenotype. We show that GPR56 regulation of MDR1 expression is mediated by RhoA. Furthermore, GPR56 is highly expressed in CRC and has a significant effect on tumor growth and patient survival. Thus, targeting GPR56 may provide a new strategy for the treatment of CRC and combatting drug resistant tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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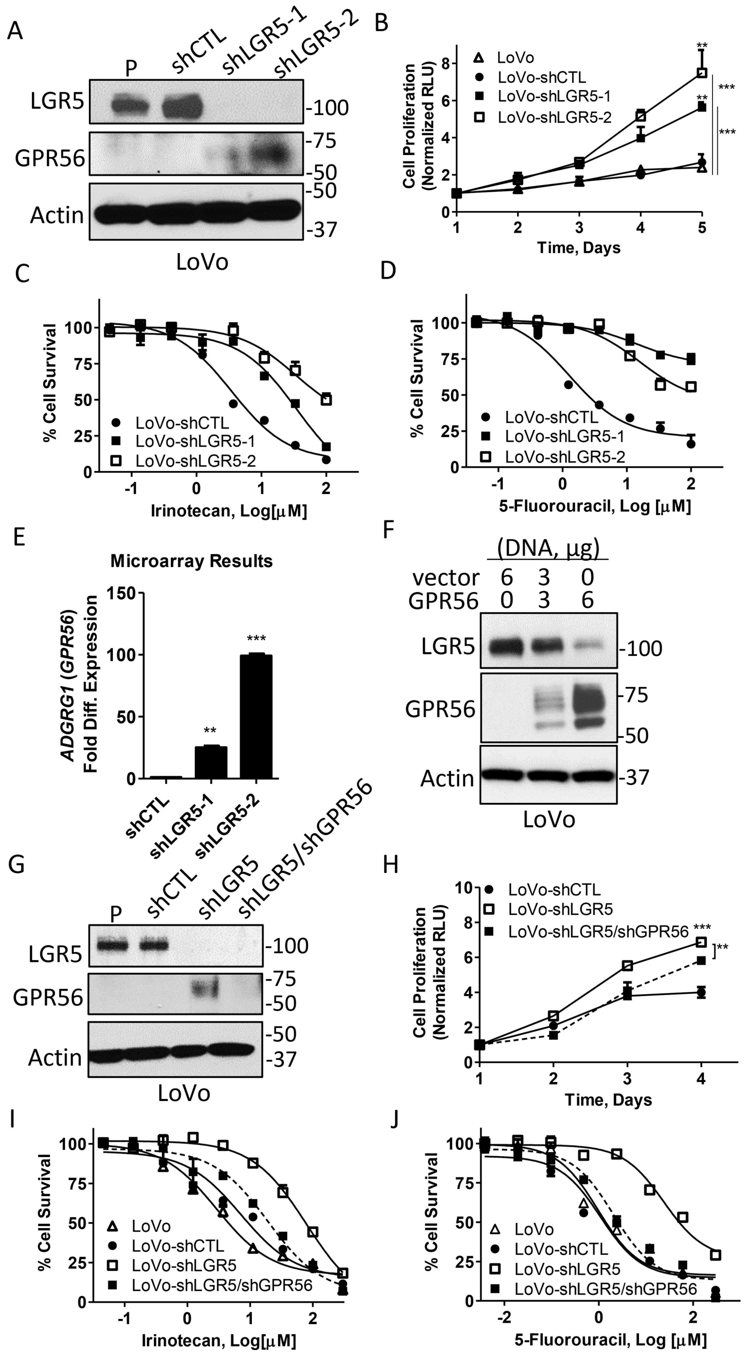

Figure 1. LGR5 knockdown induces GPR56 expression and enhances proliferation and drug resistance in LoVo colon cancer cells.

A, Western blot analysis of LGR5 and GPR56 expression in parental (P), control (shCTL) and LGR5 shRNA knockdown (shLGR5, KD) cells. **B**, LGR5 KD cells show increased cell proliferation and **C**, drug resistance to irinotecan and **D**, 5-FU using the CellTiter-Glo assay. **E**, Microarray analysis of *ADGRG1* (*GPR56*) gene expression in LoVo control and two LGR5 KD cell lines. **F**, Western blot of GPR56 overexpression effect on LGR5 levels 2-days post-transfection. **G**, Western blot of GPR56 and LGR5 expression in parental, control, LGR5 KD (shLGR5-2), and double KD (shLGR5/shGPR56) cells. Double KD cells show decreased **H**, cell proliferation and **I**, drug resistance to irinotecan and **J**, 5-FU compared to LGR5 KD cells. Cells were treated with chemotherapeutic drugs for 3 days. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus parental or shCTL cells. Error bars indicate SEM.

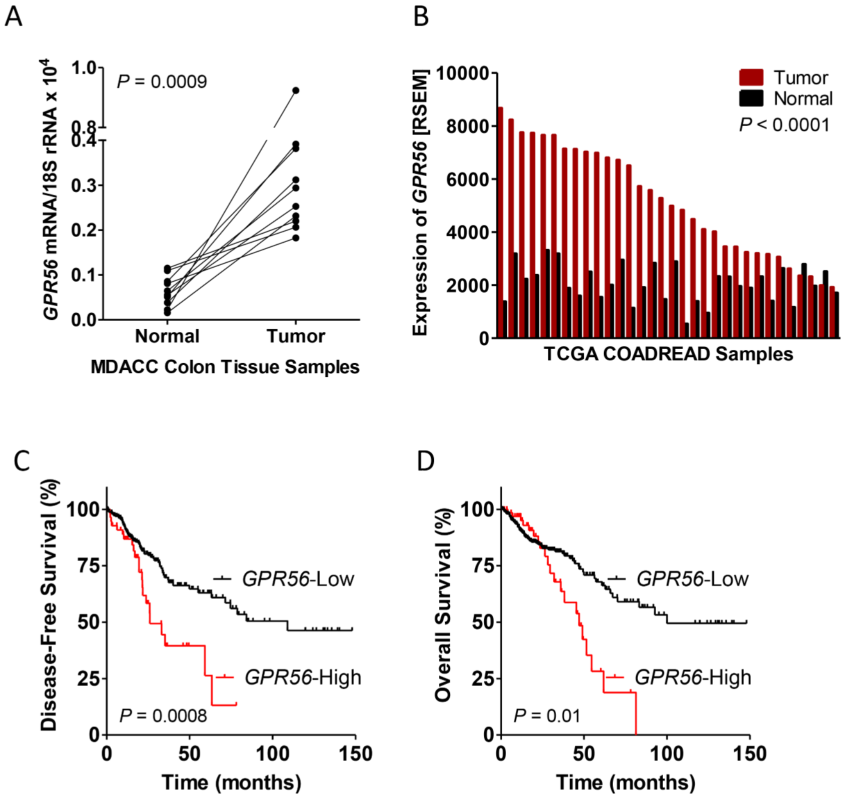

Figure 2. Association of GPR56 overexpression with poor survival outcome in colorectal cancer.

A, qRT-PCR *GPR56* expression data for 10 matched tumor and adjacent normal samples from M. D. Anderson Cancer Center. **B**, GPR56 RNA-Seq RSEM values for 32 matched tumor and adjacent normal from the TCGA colorectal adenocarcinoma (COADREAD) cohort. Statistical analysis was performed using paired *t* test (**A-B**). Kaplan-Meier plots of **C**, disease-free (high, n=58, low, n=271) and **D**, overall survival of patients from the TCGA COADREAD cohort (high, n=67, low, n=307). *P* values were obtained by the log-rank test.

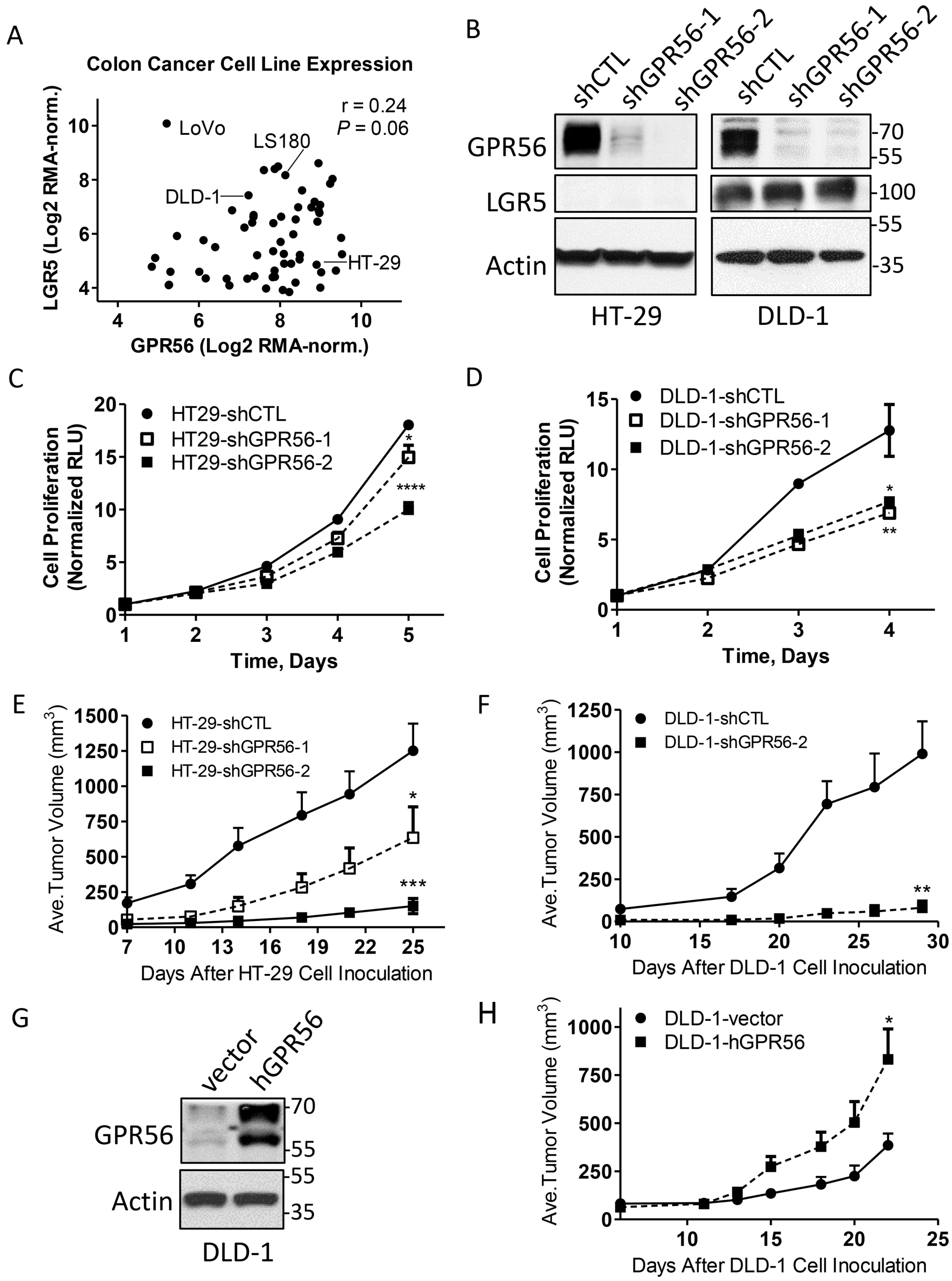

Figure 3. GPR56 expression regulates colon tumor growth.

A, CCLE microarray expression of GPR56 and LGR5 in colon cancer cell lines. *P*-value evaluated by Spearman's correlation. **B**, Western blot of GPR56 and LGR5 expression in HT-29 and DLD-1 control and KD cell lines. GPR56 KD effects on proliferation of **C**, HT-29 and **D**, DLD-1 cells. Loss of GPR56 causes a significant reduction in tumor growth in both **E**, HT-29 (shCTL and shGPR56-2, n=8; shGPR56-1, n=7) and **F**, DLD-1 xenografts (n=4/group). **G**, Western blot of stable GPR56 overexpression in DLD-1 cells. **H**, Increased GPR56 overexpression significantly increases DLD-1 tumor growth (vector, n=5 and hGPR56, n=7) *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control cells. Error bars indicate SEM.

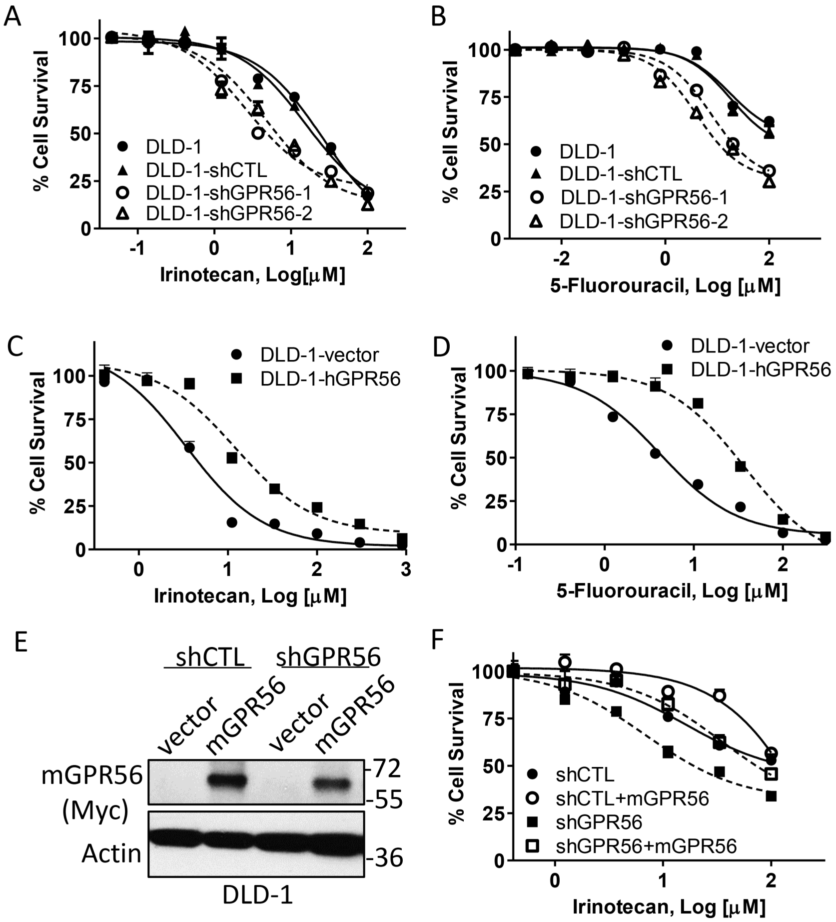

Figure 4. GPR56 promotes drug resistance to chemotherapy.

GPR56 KD enhances drug sensitivity of DLD-1 cells treated with **A**, irinotecan or **B**, 5-FU for 3 days as indicated. Overexpression of hGPR56 increases resistance in DLD-1 cells treated with **C**, irinotecan or **D**, 5-FU for 4 days. **E**, Western blot of mGPR56 overexpression in DLD-1 control and GPR56 KD cell lines. **F**, Effect of mGPR56 overexpression on cytotoxicity of DLD-1 shCTL and GPR56 KD cells after 3 days of irinotecan treatment.

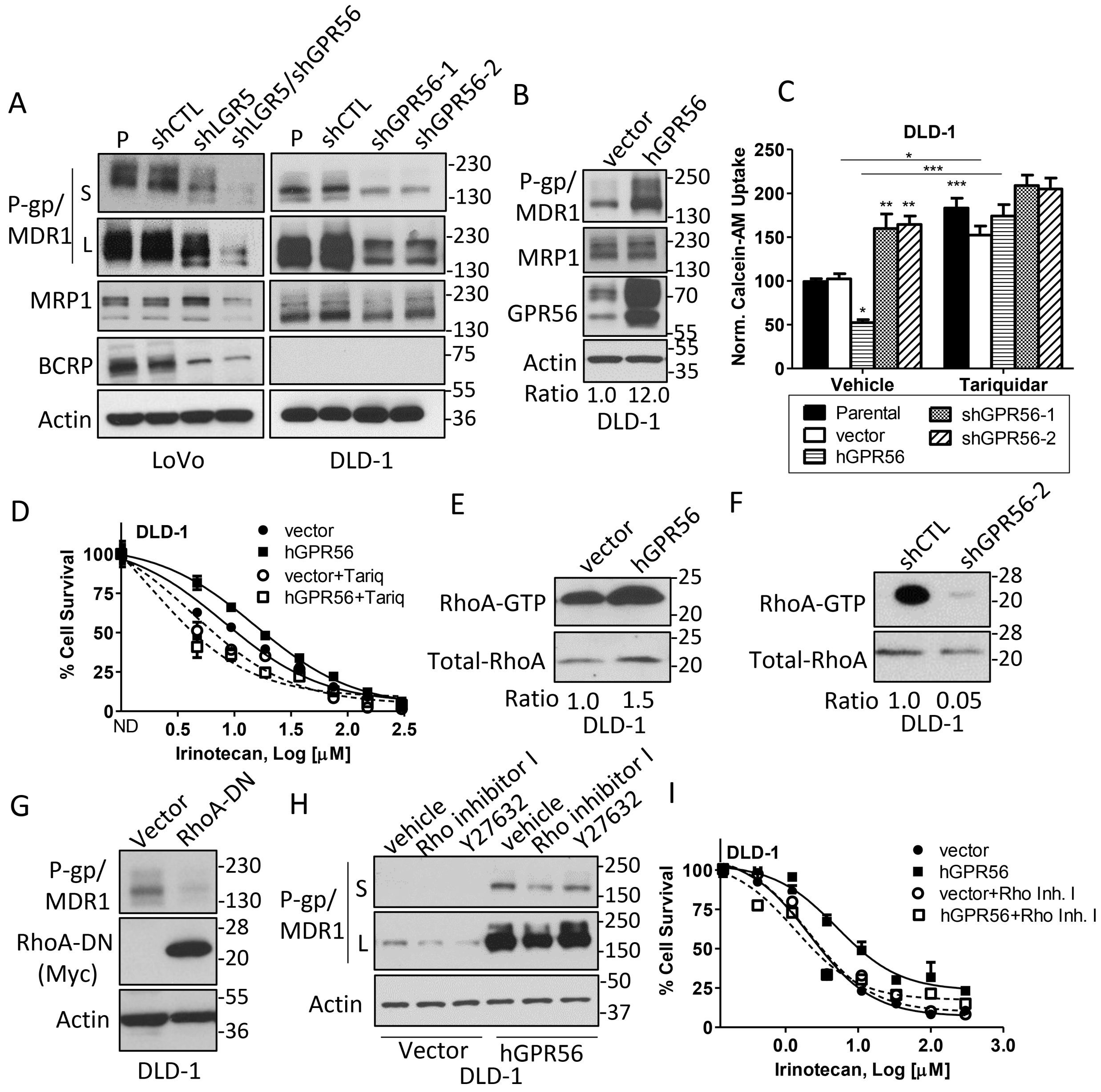

Figure 5. GPR56 regulates MDR1 expression and associated drug resistance via a RhoA-mediated mechanism.

Western blots of **A**, ABC transporter protein expression with and without GPR56 KD and **B**, MDR1 levels in DLD-1 cells with stable overexpression of GPR56. **C,** Calcein AM retention in DLD-1 cells pre-treated with vehicle or 50 nM MDR1 inhibitor, tariquidar, for 1 hr. $*P<0.05$, $**P<0.01$ and $***P<0.001$ compared to parental and vector unless otherwise indicated. Error bars indicate SEM. **D**, Effect of 100 nM tariquidar on DLD-1-vector and -hGPR56 cell cytotoxicity to irinotecan after 3 days. Western blots of changes in active RhoA (RhoA-GTP) levels in response to **E**, GPR56 overexpression and F, GPR56 KD in DLD-1 cells via active GTPase pulldown assay. Western blots of **G**, MDR1 expression in response to dominant negative RhoA (RhoA-DN) and **H**, effects of 3 day treatment with Rho inhibitor I (0.5 μg/ml) and ROCK inhibitor Y27632 (10μM) on MDR1 levels. S and L indicate short and long exposure, respectively. **I**, Effect of Rho inhibitor I (0.5 μg/ml) on DLD-1-vector and -hGPR56 cell cytotoxicity to irinotecan after 4 days.

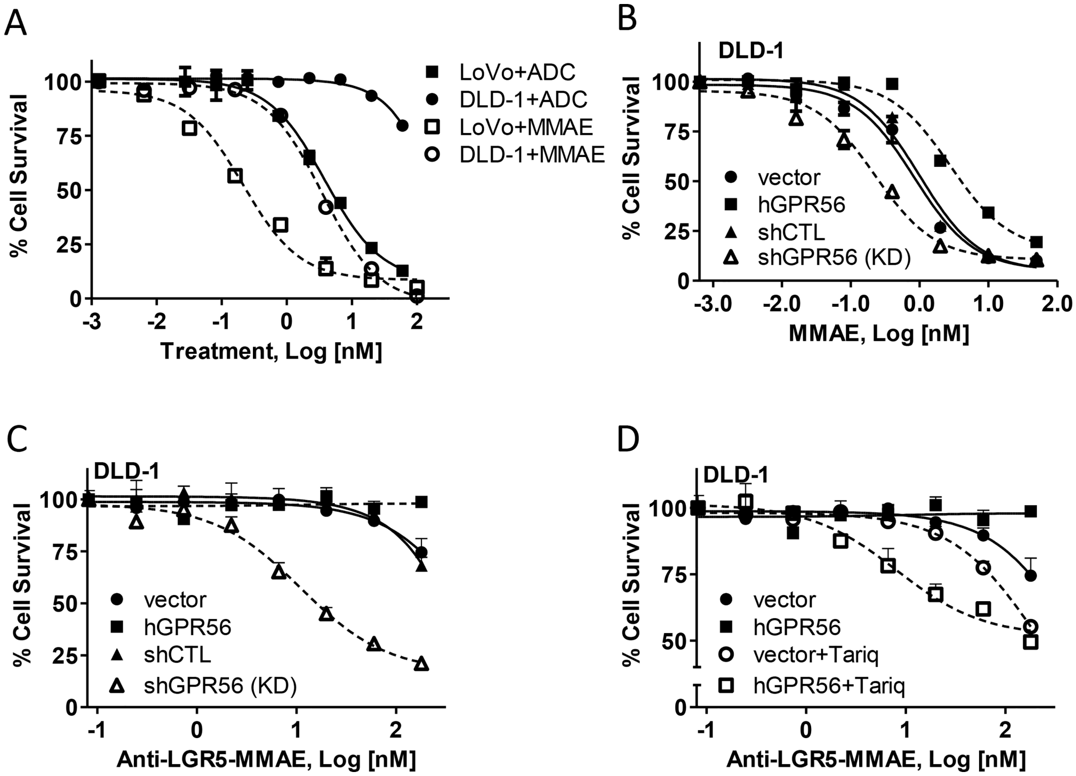

Figure 6. GPR56 knockdown sensitizes cells to anti-LGR5-MMAE ADC.

A, Cytotoxicity of LoVo and DLD-1 cells to free MMAE (nM, drug concentration) after 3 days and anti-LGR5-MMAE ADC (nM, antibody concentration) after 4 days. Viability of DLD-1 cell lines after treatment with **B**, MMAE or **C**, anti-LGR5-MMAE for 4 days. **D**, Co-treatment with 100 nM tariquidar enhances cytotoxicity of DLD-1-vector and -hGPR56 cells to anti-LGR5-MMAE ADC after 4 days.

Table 1.

IC_{50} values for cytotoxic drugs in colon cancer cell lines

Cancer Cell Line	**IC_{50} ± SEM (nM)**		
	Irinotecan	**5-Fluorouracil**	**MMAE**
LoVo-parental	3.52 ± 0.56	3.21 ± 2.26	0.26 ± 0.04
LoVo-shCTL	5.37 ± 1.06.	3.97 ± 1.28	ND
LoVo-shLGR5-1	33.50 ± 1.25*	19.14 ± 5.94**	ND
LoVo-shLGR5-2	42.03 ± 5.41**	20.59 ± 2.30***	ND
LoVo-shLGR5/GPR56	23.65 ± 4.90	3.06 ± 0.96*	ND
DLD-1-parental	26.08 ± 0.63	17.77 ± 0.48	2.37 ± 0.39
DLD-1-shCTL	24.60 ± 2.55	17.96 ± 0.24	1.05 ± 0.03[a]
DLD-1-shGPR56-1	5.00 ± 1.57**	8.09 ± 0.26**	ND
DLD-1-shGPR56-1	8.72 ± 1.33*	4.84 ± 0.80***	0.24 ± 0.02[a]
DLD-1-vector	4.14 ± 0.77[a]	6.62 ± 1.53[a]	0.83 ± 0.11[a]
DLD-1-hGPR56	11.75 ± 1.54*[a]	25.13 ± 5.94*[a]	3.81 ± 1.19*[a]
HT-29-parental	47.57 ± 3.02	5.65 ± 1.07	ND
HT-29-shGPR56-1	60.77 ± 9.68	6.15 ± 0.62	ND
HT-29-shGPR56-2	47.24 ± 0.11	3.18 ± 1.80	ND
LS180-CTL	1.36 ± 0.07	15.03 ± 1.47	ND
LS180-LGR5-KO-1	6.92 ± 0.45*	24.80 ± 3.26	ND
LS180-LGR5-KO-2	5.84 ± 1.66*	20.87 ± 2.19	ND

Values represent an average of at least 3 experiments and were determined 3 days post-treatment unless otherwise indicated.

*$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared to respective control cell lines by one-way analysis of variance

[a] indicates 4 days post-treatment. ND, not determined.