Gene chip analysis of the GPR56 silenced cells

Affymetrix GeneChip™ array analysis

Affymetrix GeneChip™ array analysis was described previously (1). Briefly, total cell RNA was isolated with TRIzol® (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and further purified with RNeasy columns (Qiagen, Valencia, CA). Two independent RNA isolations were used for each cell line. Ten micrograms of RNA were used for each sample to generate cDNA with the SuperScript Choice System (Invitrogen), and the cDNA was in vitro transcribed to generate biotinylated cRNA (BioArray HighYield RNA Transcript Labeling Kit, Affymetrix, Santa Clara, CA). The cRNA was fragmented and hybridized to U133A probe arrays during a 16 hour incubation at 45°C. The probe arrays were washed and stained with streptavidin phycoerythrin in a GeneChip® Fluidics Station 400 (Affymetrix). Finally, the probe arrays were scanned with a GeneChip 2500A GeneArray scanner (Affymetrix).

The data were analyzed with Microarray Suite 5.0 to generate probeset intensity signals and Comparison Analyses (.CHP files), and Data Mining Tool 4.0 to query the comparisons (Affymetrix). Additionally, ArrayAssist 4.0 (Stratagene) was utilized to compute probeset signals using the RMA method, as well as the significance of differential gene expression between GPR56-silenced cells and controls using standard T-tests. A number of criteria were utilized to mine the data for genes of interest. The Comparison Analysis algorithm was applied to the probeset summaries between all GPR56-silenced and control replicates, and probesets were ranked by the percentage of
Increase or Decrease calls. Additionally, t-test analyses were run between GPR56-silenced and controls, and probesets were ranked by p-value following a False Discovery Rate (FDR) estimation to 5% or less (Benjamini-Hochberg). Average Signal Log Raio (SLR) between GPR56-silenced and controls provided a quantitative metric for the level of change. Genes of interest were filtered by applying thresholds to the various metrics, or looking at intersecting gene lists from different tests; resulting genes were considered for functional and pathways analysis. Stringency was adjusted to generate lists of different sizes; for Increase and Decrease calls, an agreement between all comparisons of 80 - 100% was used as a threshold. For statistical analysis (t-test) p-values as stringent as < 0.00001 were used with FDR <5%. Additional stringency could also be achieved by intersecting significant probesets from two separate replications of the entire experiment. A subset of genes mined from the data were analyzed with qPCR to further verify their change.

**Gene Network and Pathways Analysis**

Functional analyses of significant changes were conducted in Ingenuity Pathways Analysis® (IPA) by preparing tab-delimited text files with simple lists of Affymetrix probeset IDs with the corresponding mean Signal Log Ratios and t-test p-values. Following file upload and processing, a variety of gene networks and canonical pathway images were created providing a functional view of the changes. The number and exact composition of significant gene networks and pathways depended on the number of genes used in the query, which in turn depended on the stringency used. Analyses conducted with the top few hundred changing probesets (in the range of 400 – 800) were often
found most useful in terms of identifying significant impact on pathways and cell functions.

*Genes involved in integrin pathway*

Genes involved in integrin signaling pathway by GPR56 silencing in A2058 cells are shown in supplement table 1. The integrin signaling pathway was displayed in supplement figure 1.

**Supplement Table 1. Genes involved in integrin signaling pathway are regulated by GPR56 silencing in A2058 cells**

| gene name | Description | Probe set* | Log ratio$ |
|-----------|-------------|------------|------------|
| ACTB      | actin, β    | 229119_s_at| -0.672     |
| ACTG2     | actin, gamma 2, smooth muscle, enteric | 202274_at| -1.318     |
| ARF3      | ADP-ribosylation factor 3 | 211622_s_at| 0.457      |
| ARF6      | ADP-ribosylation factor 6 | 224788_at| 0.437      |
| ARPC4     | actin related protein 2/3 complex, subunit 4, 20kDa | 211672_s_at| 0.67       |
| ARPC1B    | actin related protein 2/3 complex, subunit 1B, 41kDa | 201954_at| -0.468     |
| FYN       | FYN oncogene related to SRC, FGR, YES | 212486_s_at| -1.59      |
| GRB2      | growth factor receptor-bound protein 2 | 215075_s_at| 0.309      |
| ITGA2     | integrin, α4 | 227314_at| 0.972      |
| ITGA4     | integrin, α4 (CD49D, α4 subunit of VLA-4 receptor) | 213416_at| -0.596     |
| ITGA6     | integrin, α6 | 215177_s_at| -0.541     |
| ITGB3     | integrin, β3 (platelet glycoprotein IIIa, antigen CD61) | 204628_s_at| -0.46      |
| ITGB4     | integrin, β4 | 204990_s_at| 0.273      |
| ITGB5     | integrin, β5 | 201125_s_at| -0.424     |
| LAMA4     | laminin, α4 | 202202_s_at| -0.634     |
| LIMS1       | LIM and senescent cell antigen-like domains 1 | 207198_s_at | 0.344 |
|------------|---------------------------------------------|-------------|-------|
| PARVB      | parvin, β                                   | 37965_at    | 0.322 |
| PIK3CB     | phosphoinositide-3-kinase, catalytic, β polypeptide | 212688_at   | 0.603 |
| PIK3CD     | phosphoinositide-3-kinase, catalytic, δ polypeptide  | 203879_at   | -0.318 |
| PPP1R12B   | protein phosphatase 1, regulatory subunit 12B | 201957_at   | 0.569 |
| PTK2       | PTK2 protein tyrosine kinase 2              | 208820_at   | -0.47 |
|            | ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2) | 207419_s_at | -0.5 |
| RAC2       | v-raf-1 murine leukemia viral oncogene homolog 1 | 201244_s_at | 0.494 |
| RAF1       | ras homolog gene family, member J           | 238905_at   | -0.371 |
| RHOJ       | ras homolog gene family, member J           | 212117_at   | -0.526 |
| RHOQ       | ras homolog gene family, member Q           | 209263_x_at | -0.167 |

Note: A list of genes in the integrin signaling pathway is shown whose expression is affected in A2058-siGPR56 cells as compared to A2058-siCNTL cells. * represents affymatrix probe set, and $ indicates expression changes represented as log ratio.

**Supplement Figure 1 legend.**

Genes involved in integrin signaling pathway are shown. Color indicates gene expression changes in shGPR56 silenced cells compared to controls, with blue representing down-regulation, red representing up-regulation, and red-green indicates a node with a mixture of up-regulated and downregulated gene products. The regulated gene list is also shown in supplemental Table 1.
Involvement of Fyn in cell transformation downstream of GPR56 in A2058 cells

Fyn is a protein tyrosine kinase and a well-known member of the Src oncogene family. It is involved in transmembrane signaling, but is not known to bind with high stoichiometry to any surface receptor protein. Fyn has been suggested to be involved in cell adhesion (2) and the metastatic potential of cancer cells (3). Our expression profiling data indicated that FYN expression is associated with GPR56 expression, and a correlated down-regulation of FYN expression in GPR56 silenced cells is further confirmed by Real-time RT-PCR (Supplemental figure 2A). Similar down-regulation of FYN expression, along with GPR56 down-regulation, was also observed in HeLaHF cells, as compared to HeLa cells (4). We then asked whether Fyn was relevant to GPR56 mediated cell transformation in the A2058 cell system. We silenced Fyn using the pSD31-shFyn vector (90% down-regulation at the mRNA level, Supplement figure 2B), in a similar manner as in the GPR56 silencing experiment (data not shown). Interestingly, Fyn silencing also caused a similar phenotype as that seen for GPR56 silencing, namely reduced anchorage-independent growth (Supplement figure 2B,C), suggesting a role of Fyn in the GPR56 mediated cell transformation pathway. Similar reduced growth phenotype was also observed in HeLa cells when Fyn expression is down-regulated, further confirming our observations (4). Fyn-silenced cells did not display reduced GPR56 expression as monitored by mRNA levels (data not shown), implying that Fyn functions downstream of GPR56 in the A2058 transformation pathway.

Supplement Figure 2 legend.
Fyn silencing reduced cell survival. A) GPR56 and Fyn mRNAs in the A2058 cells containing shCNTL, shGPR56A, or shGPR56B were determined and normalized to those of shCNTL cells. B) Fyn mRNA was determined for the cells with either shCNTL or shFyn, and expressed as fold of shCNTL. C) A2058 cells with either shCNTL or shFyn were assayed for soft agar culture growth. Mean value of triplicate samples is shown; error bar stands for standard deviation and p < 0.01 is shown as *. 

Down-regulation of Erk in GPR56 silenced cells.

PI3K-ERK signaling pathways have been implicated in anchorage dependent cell cycle progression mediated by growth factor receptor and/or integrin receptor signaling. It is also involved in anchorage-independent proliferation and anoikis mediated by the cell adhesion responses of integrin receptor signaling (5). If GPR56 is involved in the cell adhesion related growth response, it may also impact Erk activity. We tested this hypothesis by assessing whether Erk phosphorylation would be affected by GPR56 silencing in A2058 cells. The results showed that Erk was partially dephosphorylated (inactivated) in GPR56 silenced cells (Supplement Table 2), supporting the assumption that GPR56 is also involved in Erk activation. The lower Erk activity is further confirmed by the lower activity of its downstream target Elk activity as assayed by the Elk-responsive reporter assay (data not shown).

If GPR56 is indeed involved in ERKs or PI3K-ERK pathway physiologically, the inhibition of this pathway by the inhibitors of MEK in A2058 cells should yield the same phenotypes as observed for GPR56 silencing. Indeed when inhibitors of MEK (PD98059) were used to treat A2058 cells, reductions of Erk phosphorylation and cell
proliferation (Supplement table 2), as well as reduced cell adhesion to ECM (data not shown), were observed. These data support a role of GPR56 in the integrin mediated cell adhesion pathway and A2058 cell transformation.

**Supplement Table 2. Effects of GPR56 silencing or MEK inhibitor on ERK activation and cell growth**

| Agents     | *Reduction of Erk phosphorylation | $Reduction of cell growth |
|------------|----------------------------------|--------------------------|
| shGPR56    | 30%                              | 30%                      |
| PD98059    | 80%                              | 60%                      |

Note: PD98059 is MEK inhibitor. *Reduction of Erk phosphorylation is conducted by Western blotting of cell lysates with p-Erk specific antibody and actin antibody. The ratio between p-ERK and actin band intensities was normalized to that of the control samples (non-treated for PD98059; shCNTL for shGPR56). $ Cell growth is determined by AlamarBlue staining and reduction of cell growth is shown.

**References:**

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