Supplementary Methods - Experimental protocol

RNA preparation (BMSCs, human tissue)
The BMSC lines were derived as previously described \(^1\) from two different female donors who were 45 years and 9 years old, (BMSC 1 and 2 respectively). BMSC3 was prepared from marrow obtained from a 19 year old male. Use of human subjects was approved under NIH protocol 94-D-0188.

Fragments of trabecular bone were obtained in the course of surgical procedures and marrow was scraped off the bone with a steel blade into \(\alpha\)-modified Minimum Essential Medium (\(\alpha\)MEM). The marrow samples were pipetted up and down repeatedly to release individual cells and passed consecutively through 16 and 20 gauge needles to break up cell aggregates. Finally, the cell suspensions were filtered through a Falcon 2350 nylon cell strainer to remove remaining cell aggregates.

To generate BMSC strains, single cell-suspensions were plated at \(1.0 \times 10^7\) nucleated cells per 75cm\(^2\) flask. Growth medium (\(\alpha\)MEM, 2 mM glutamine, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin sulfate, and 20% fetal bovine serum, (growth medium for BMSC3 contained dexamethasone and L-ascorbic acid phosphate magnesium salt n-hydrate in addition at \(10^{-8}\) M and \(10^{-4}\) M, respectively) was replaced on day 6 or 7. The cultures were first passaged when they approached confluence, usually on day 11 to 14, following two consecutive applications of 1x trypsin-EDTA for 5-10 min at room temperature. The cells were replated at \(4.5-7 \times 10^6\) per 75 cm\(^2\) flask, harvested at 80-90% confluency, and lysed in buffer RLT (RNeasy kit, Qiagen). RNA was extracted from the BMSCs and frozen tissues (liver, kidney, cerebral cortex, ovary, and hippocampus) using RNeasy (Qiagen, Valencia CA) according to the manufacture’s instructions.

We prepared RNA from BMSC1 and 2 at passage 2 and from BMSC3 at passage 4.

DNase treatment
The volume of the RNA solution was adjusted to 60\(\mu\)l. 20\(\mu\)l RDD buffer and 20\(\mu\)l DNase were added and incubated at room temperature for 45 minutes. RNA was purified using an RNeasy kit (Qiagen). Two washes with buffer RW1 (350\(\mu\)l added, 1 minute
Selection of primers and oligonucleotides
Starting with the human GPCRs found at NCBI EntrezGene, we assembled a list of RefSeq id's for representatives of each receptor. We included splice variant transcripts if they were potentially different enough from one another to identify using specific primers/probes. Examples of receptors with such variant forms are CCR2 and GPR74. The two variants of CCR2 contain two different exons, and the variant forms can be distinguished from one another using two pairs of primers specific for these exons. GPR74 represents a less ideal case. One of the variants of GPR74 has and the other lacks a specific exon. Two primers were designed in this case, one that recognizes both transcripts and the other that only recognizes the transcript with the extra exon. If the mRNA without this exon is the only one expressed, or if the second transcript is expressed at low levels, only the primer common to both forms will generate a detectable product. If the transcript with the extra exon is present, both primers will give products. The exon-plus transcript could either be found alone in an extract or be made along with the exon-minus product. These possibilities cannot be distinguished from one another.

We created a blast-formatted database containing all human mRNA RefSeq sequences at NCBI (28,424 transcript sequences at the time the analysis was done). We then used the 'dublastn' part of the ROSO package, (http://pbil.univ-lyon1.fr/roso/Home.php) to compare our set of GPCR sequences to this database. dublastn conveniently sends a list of sequences to a local installation of NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast), and it allowed us to tabulate GPCR sequences that were similar to other sequences in the database at or above a predetermined level. We ran dublastn twice to create two tables. The first was a list of regions of GPCR transcripts with similarities ≥ 60% to human RefSeq sequences; the second was a list of regions with similarities ≥80%. Using these two lists, we defined areas of each GPCR mRNA from which we did not want primers to be selected—i.e., areas similar to at least one other RefSeq. This allowed us to mask portions of each GPCR transcript at two levels of stringency prior to primer selection.
The GPCR sequences, with specific regions masked out, were submitted to a local installation of “Primer3” (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for primer generation. First, we attempted to select PCR primers for each sequence after masking regions of 60% or greater identity. The reaction products were designed to be 150-200 bases long, and have a melting temperature of 60°C (57-63°C) and GC content of 50% (20-80%). Primer 3 was programmed to use the human mispriming library (http://frodo.wi.mit.edu/cgi-bin/primer3/cat_humrep_and_simple.cgi)--a collection of interspersed repeats and microsatellites that should be avoided when selecting primers from human sequence data--to identify and prevent selection of primers within known repeat regions.

We also excluded pairs of primers resulting in amplicons that contained regions masked out prior to primer selection. This gave us confidence that our products would contain no repeat regions found in the mispriming library or regions highly similar to other transcripts.

As noted, we first selected as many primers as possible after masking regions of GPCR mRNAs with 60% identity to other human transcripts, but some sequences in our set did not yield adequate primers at this stage. Consequently, we generated primers as described above after masking sequences that were 80% identical to those in other RefSeqs. In effect, this ‘opened up’ regions previously unavailable for primer selection, and allowed us to select primers to amplify mRNAs from families of similar genes.

At the end of this step, some sequences still lacked suitable primers, so we used Primer 3 one last time without masking any of the data to ensure at least one pair of primers existed per GPCR transcript. 219 primers pairs were generated at step one, 46 at step 2, and 136 at step 3.

As a final analysis of the quality of the primer pairs, we used the AutoDimer program (http://www.cstl.nist.gov/biotech/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm) to check each primer for dimerization against the entire set of candidate primers. We set the cutoff melting temperature to 35°C and the minimum score to 6.
When we discovered a primer-primer interaction, we replaced the offending pair with the next best available one and ran AutoDimer again. We repeated this until no theoretical dimerizations were found. The designed primers have nominal melting temperatures of 60.0°C (58.7-62.6°C) and average GC contents of 52.8% (35.0-67.7%).

Once we settled on a set of primer pairs, we searched the hypothetical PCR products for 55 bp regions suitable for capturing the products on a microarray. We submitted the sequences of the amplicons to our local ROSO installation, and allowed the program to generate up to 4 probes per amplicon. We selected oligonucleotides with nominal melting temperatures of 89.4°C (79.1-98.5°C), GC contents of 50.6% (25.5-72.7%), and no more than 4 identical nucleotides in a row. When more than one good candidate was identified, we arbitrarily chose the 5’-most sequence.

Using the rules and methods described above, we developed a set of 401 primer pairs and 802 oligonucleotide probes (401 sense and 401 antisense probes), which can all be found in Supplementary Tables 4 and 5 online.

Multiplex RT-PCR
For Multiplex RT-PCR, we divided the 401 primer pairs into 8 groups (7x50, 1x51). The assignment of any given primer pair to one of these groups was random. We used the OneStep RT-PCR Kit (Qiagen), driving first strand synthesis with the 3’ primers in each mixture. The 25μl reactions contained 1, 10, or 100ng of total RNA, 2μl of primer solution (with each primer present at 1μM), 1μl of Q-solution, 3μl of enzyme mix for 100ng of template (1μl of enzyme mix for 10ng and 1ng of template), 0.25μl of RNase Inhibitor (RNasin, Promega Mason, WI), and 4 μl of aadNTP mix (dATP, dGTP, dCTP-2.5μM, T-1.625μM (Amersham Pharmacia, Piscataway NJ), 5-[3-aminoallyl]-2'-deoxyuridine 5'triphosphate (aadUTP) 0.875μM (Sigma, St. Louis, MO). The RT-PCR protocol begins with a 30 minute reverse-transcription reaction at 50°C, followed by 15 minutes at 95°C to initiate the PCR reaction. Each amplification cycle consists of 30 seconds at 94°C for denaturation, 30 seconds at 55°C for annealing, and 15 seconds at
72°C for extension. 45 cycles were performed. A final extension at 72°C lasts 15 seconds.

Individual RT PCR:
We used the same protocol and reagents as for multiplex RT PCR, adding 0.2µl of each primer (100µM) per 25µl amplification.

Oligonucleotide array printing and prehybridization
We printed oligonucleotide microarrays on UltraGAPS™ coated slides (Corning, NY 14831). Five nl of a 25 uM solution (7.5x10^10 molecules) were applied to the array to create each element. Prior to hybridization, we exposed the slides to UV light (254nm, 650 Joules/m^2, UV Stratalinker 2400, Stratagene, La Jolla, CA), followed by a background reduction step (Background reduction kit, Corning) according to the manufacture’s instructions and then incubated them in 5xSSC, 0.1% (wt/vol) SDS, 1% (wt/vol) BSA buffer for 1 hour at 42°C, and distilled water for 2 minutes at RT. Finally, we dipped them in isopropanol, and dried them by centrifugation for 2 minutes at 130xg (800rpm; Sorvall Super T-21, Asheville, NC).

Labeling
We labeled the amplicons as previously reported. We purified the PCR products with a MiniElute kit (Qiagen) pooling as many as four such products (amplicons from 200 primer pairs), combined the pool with 500µl of Buffer PB, added the resulting solution to columns, and centrifuged for 1 minute at 16000xg in this and subsequent steps in an Eppendorf 5415D centrifuge (Westbury, NY). We reapplied the flow through to the columns and centrifuged again, washed the columns twice with of 500µl Buffer PE, emptied the collection tubes to remove residual ethanol, and centrifuged for an additional minute. We then transferred the columns to clean 1.5 ml microcentrifuge tubes. To elute the DNA, we pipetted 10µl of H₂O (pH 8.5) onto the center of the membrane, incubated at room temperature (RT) for 1 minute, and centrifuged for 1 minute. After eluting the membrane a total of three times, we dried down the eluate to 9µl in a vacuum centrifuge (DNA120 SpeedVac, Thermo Savant, Holbrook, NY), added 1µl of sodium bicarbonate
(pH 9.0) and 4.5μl NHS-ester Cy5 (Amersham Pharmacia, Piscataway, NJ, 62.5 μg/μl in dimethyl sulfoxide) to the tube, mixed the resulting solution by pipetting it up and down, wrapped the tube in aluminum foil, and incubated it at RT for 1 hour. To stop the labeling reaction, we added 4.5μl of 4M hydroxylamine hydrochloride, rewrapped the tube in aluminum foil, and incubated it at RT for 30 minutes.

To purify the labeled products, we used Qia-quick PCR purification kits (Qiagen). We added 80μl of H2O and 500μl of Buffer PB to the labeling reaction, applied the solutions to columns, centrifuged for 1 minute, reapplied the flow through to the column, and centrifuged it again. When we amplified 10ng or 1ng of total RNA, we pooled the two labeled products (401 amplicons) from each sample and added 60μl of H2O. We then washed the columns twice with 500μl of Buffer PE, centrifuged them for 1 additional minute to remove ethanol, transferred the columns to clean 1.5 ml microcentrifuge tubes, added 20μl of Buffer EB to the center of the membrane, and centrifuged after a 1 minute incubation at RT. We performed two additional elutions with 20μl of Buffer EB, and dried the eluate down to 12.5μl in a vacuum centrifuge.

Hybridization and washing condition

We added 2.25μl of 20x saline-sodium citrate (SSC) and 0.4μl of 2% (wt/vol) SDS to the labeled product, denatured it for 2 minutes at 98°C, centrifuged for 5 minutes (16000xg), and pipetted 3.5μl of target solution onto each array. After applying coverslips (17x17 mm), we placed the slides in hybridization chambers (Corning) and incubated these in a 65°C water bath for 1 hour, after which we placed the slides in 2xSSC, 0.1% SDS-buffer. We allowed the coverslips to fall off, and then washed the arrays 3 times (0.1xSSC, 0.01% (wt/vol) SDS at 50°C, 0.05xSSC at 50°C and 0.05xSSC at RT, each for 15 minutes) and dried them by centrifugation for 2 minutes at 130xg (Sorvall Super T-21, Asheville, NC).

Array scanning and image analysis
We scanned the arrays with a GenePix 4000A scanner (Axon, Foster City CA) at 10μm resolution and a photomultiplier tube voltage (PMT) of 700, and analyzed the images using IPLab software (Fairfax, VA) as previously described. To capture the non-specific noise possibly present in both fluorescent channels, we measured the signal intensities in both the red and the green channel, extracting the intensity of each spot without background subtraction. Although the Cy3 channel has no input signal, we used signals in this channel to quantify the false positive probability as described below.

In vitro transcription
We inserted GPCR cDNAs (ADCYAP1R1, NM_001118; GLP2R, NM_004246; OPN5, NM_181744; GPR15, NM_005290; GPR141, NM_181791; GPR128, NM_032787) into pcDNA3 or pcDNA3.1 (Invitrogen, Carlsbad, CA), purified plasmid DNA (QIAprep miniprep, Qiagen), linearized the plasmids by cutting them at the 3’ ends of their inserts with appropriate restriction enzymes (enzymes and buffers, New England Biolab, Beverly, MA) at 37°C over night, and transcribed them in vitro using a MEGAscript™High Yield transcription kit (T7) (Ambion, Austin, TX) according to the manufacture’s instructions. To be sure of the quality of the products, we determined their lengths with 1% agarose gel electrophoresis. For spiking experiments we adjusted the concentration of each RNA to 100pg/μl, and added serially diluted samples to 50plex RT-PCR reactions.