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Original Article

RNAi targeting GPR4 influences HMEC-1 gene expression by microarray analysis

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Abstract: G-protein coupled receptor 4 (GPR4) belongs to a protein family comprised of 3 closely related G protein-coupled receptors. Recent studies have shown that GPR4 plays important roles in angiogenesis, proton sensing, and regulating tumor cells as an oncogenic gene. How GPR4 conducts its functions? Rare has been known. In order to detect the genes related to GPR4, microarray technology was employed. GPR4 is highly expressed in human vascular endothelial cell HMEC-1. Small interfering RNA against GPR4 was used to knockdown GPR4 expression in HMEC-1. Then RNA from the GPR4 knockdown cells and control cells were analyzed through genome microarray. Microarray results shown that among the whole genes and expressed sequence tags, 447 differentially expressed genes were identified, containing 318 up-regulated genes and 129 down-regulated genes. These genes whose expression dramatically changed may be involved in the GPR4 functions. These genes were related to cell apoptosis, cytoskeleton and signal transduction, cell proliferation, differentiation and cell-cycle regulation, gene transcription and translation and cell material and energy metabolism.

Keywords: GPR4, RNAi, microarray

Introduction

G-protein coupled receptor 4 (GPR4) belongs to a protein family comprised of 3 closely related G protein-coupled receptors (GPCRs): GPR4, OGR1/GPR68 and TDAG8/GPR65 [1, 2]. GPR4 is highly conserved during evolution, with more than 90% homology in the amino acid sequences among mammalian orthologs, and 70% homology between human and zebrafish. GPR4 is expressed in many tissues including ovary, liver, heart, kidney, placenta and skeletal muscle [2-4]. G protein coupled receptor 4 (GPR4) is previously identified as a receptor for sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC). The Kd values for SPC and LPC were estimated to be 36 and 159 nM, respectively, in the binding experiments of GPR4-expressing cells [5]. GPR4 is response to SPC and LPC, resulting in the stimulation of DNA synthesis and migration of Swiss 3T3 cells [5]. But later on, this ligand-receptor relationship has not been always confirmed [2]. Later studies have further showed that GPR4 function as a proton sensor. GPR4 senses extracellular protons and responds to acidic pH changes, leading to increased intracellular levels of cyclic AMP [3, 4]. GPR4 is fully activated in the acidic pH 6.8. and partially activated in the physiological range (pH 7.4 to pH 6.8) [6-9]. The overexpression of GPR4 has been shown to stimulate serum response element (SRE)-, nuclear factor of activated T-cell (NFAT), and cAMP response element (CRE)-driven transcription [6, 10].

Our previous studies and some other studies suggest that GPR4 may participate in the angiogenesis and the endothelial barrier function [11-14]. Our previous study indicated that GPR4 is capable of mediating the tube formation of blood vessels by regulating the function of endothelial cells [11]. We found GPR4 is highly expressed in vascular endothelial cells of both

arteries and veins [11, 13, 15]. When GPR4 was knocked down in endothelial cells, the growth, migration, and tube formation of endothelial cells were significantly inhibited. In vivo studies have provided more evidence in support of the GPR4 functions in angiogenesis [16]. Higher perinatal mortality rate was found in GPR4 null neonates [3, 16]. Dilated and tortuous subcutaneous blood vessels, spontaneous hemorrhages, and defective vascular smooth muscle cell coverage were found in a certain percentage of GPR4-null embryos and neonates. These observations indicated that GPR4 is required for the normal vascular development of multiple tissues/organs.

Besides, other available data suggested GPR4 may be oncogenic GPCRs with increased expression levels in different types of malignancies. GPR4 overexpression has been found in kidney tumors, ovarian tumors, colon tumors, breast tumors, and liver tumors [10]. Our previous study also suggested that GPR4 may play roles in the development of EOC, and its overexpression might be required for the angiogenesis of EOC [17]. Our study also shown that, after GPR4 was knockdown in ovarian cancer cells, cell colony formation, migration, invasion, and survivals were significantly inhibited, which suggested GPR4 behaves as an oncogenic gene.

But how GPR4 conducts its functions including proton sensor, angiogenic activity, and as an oncogene? Rare has been known. In order to detect the genes related to GPR4, microarray technology was employed. GPR4 is highly expressed in human vascular endothelial cell HMEC-1. Small interfering RNA against GPR4 was used to knockdown GPR4 expression in HMEC-1. Then RNA from the GPR4 knockdown cells and control cells were analyzed through genome microarray.

Expression profile microarray technology should provide a useful experimental strategy to define cellular target genes by identifying transcriptionally altered genes upon silencing of endogenous gene expression. Combining microarray with bioinformatics technology to analyze the gene expression patterns of various tumors is an important method in functional genomic studies. It surpasses the sole gene research pattern obviously and may illuminate the gene expression and regulation network of tumor cells in the whole.

Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20-25 base pairs in length. siRNA plays many roles, but its most notable is in the RNA interference (RNAi) pathway, where it interferes with the expression of specific genes with complementary nucleotide sequence [18]. siRNA also acts in RNAi-related pathways, e.g., as an antiviral mechanism or in shaping the chromatin structure of a genome.

A small hairpin RNA or short hairpin RNA (shRNA) is a sequence of RNA that makes a tight hairpin turn that can be used to silence target gene expression via RNA interference (RNAi) [19]. Expression of shRNA in cells is typically accomplished by delivery of plasmids or through viral or bacterial vectors. The promoter choice is essential to achieve robust shRNA expression. At first, polymerase III promoters such as U6 and H1 were used; however, these promoters lack spatial and temporal control. As such, there has been a shift to using polymerase II promoters to regulate expression of shRNA. shRNA is an advantageous mediator of RNAi in that it has a relatively low rate of degradation and turnover.

In order to detect the genes related to GPR4, microarray technology was employed. GPR4 is highly expressed in human vascular endothelial cell HMEC-1. Small interfering RNA against GPR4 was used to knockdown GPR4 expression in HMEC-1. Then RNA from the GPR4 knockdown cells and control cells were analyzed through genome microarray. Microarray results shown that among the whole genes and expressed sequence tags, 447 differentially expressed genes were identified, containing 318 up-regulated genes and 129 down-regulated genes. These genes whose expression dramatically changed may be involved in the GPR4 functions. These genes were related to cell apoptosis, cytoskeleton and signal transduction, cell proliferation, differentiation and cell-cycle regulation, gene transcription and translation and cell material and energy metabolism.

Materials and methods

Cell culture

The HMEC-1 cell line was from the Centers for Disease Control and Prevention (Atlanta, GA)

and were maintained in RPMI 1640 Medium (Invitrogen) supplemented with 5 mM glucose, 10% foetal bovine serum (FBS; HyClone, Australia) and 0.48% (w/v) HEPES, pH 7.4, in a CO_2 humid incubation chamber at 37°C.

Lentivirus vectors for GPR4 RNAi

The GPR4-specific target sequences were designed using the GPR4 reference sequence (Gene Bank Accession No NM_005282). Double-stranded DNA were synthesized according to the structure of a GV115 viral vector (GeneChem) and then inserted into a linearized vector. The shuttle vector and viral packaging system were cotransfected into HEK293T cells to replicate competent lentivirus. The lentivirus containing the GPR4 shRNA (short hairpin RNA) expressing cassette was used for lentivirus production and denoted as LV-shGPR4 in the next experiments. The GV115 mock vector was also packaged and used as a negative control, denoted as LV-eGFP, which has no significant homology to gene sequences. The titers averaged 1×10^8 TU/mL.

The sequences for the hairpin structures were chosen using the "RNAi oligo retriever" program (www.cshl.org/public/SCEINCE/hannon.html) provided by Dr. Greg Hannon. The U6 promoter followed by a sequence encoding a 29 nt short hairpin RNA (shRNA) targeting LPA3 or GPR4 was amplified by PCR with the primers AGATCTGATTTAGGTGACACTATAG and AAAAAAGAGACAATTCCAGCCCAGCGTAGGAACCGCAAGCTTCCGGTCCCCACACTGGGCTGGAATTGCCTCGGTGTTTCGTCCTTTCCACAA (for LPA3-RNAi) or AAAAAAGTGCTGGCGACAGCACCTTCAACTACACCCAAGCTTCGGTGCAGCTGAAGATGCTGCCGCCAGCACGGTGTTTCGTCCTTTCCACAA (for GPR4-RNAi).

Real-time PCR

Real-time PCR analysis was performed in a total volume of 20 μL containing template DNA, 100 nM of sense and antisense primers, 10 μL of SYBR® Green master mix (TaKaRa). After denatured at 95°C for 2 min, the mixtures were subjected to 40 amplification cycles (10 seconds at 98°C for denaturation and 20 sec for annealing and extension at 60°C). Incorporation of SYBR® Green dye into PCR products was monitored in real time by using a Light Cycler480 Detection System (Roche) and allowing determination of the threshold cycle (CT) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with one peak, indicating the specificity of the amplification. A CT value was obtained from each amplification curve by using the software provided by the manufacturer (Roche).

Western blot

Cell lysates of lentivirus infected HMEC-1 cells and controls were harvested and subjected to SDS-PAGE in 10% polyacrylamide gel. Bands were probed for GPR4 protein expression using mouse monoclonal antibody and secondary goat anti-mouse antibody (Santa Cruz). GAPDH detection was performed using monoclonal anti-GAPDH (Santa Cruz). Western blot products on images captured by the Fusion FX6 system (Vilber Lourmat).

Gene expression analysis

LV-eGFP and LV-shGPR4 infected HMEC-1 cells were harvested and total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA quantity and purity were assessed by measurement of OD260/280 using a NanoDrop® ND-1000 spectrophotometer. The quality was verified by the integrity of 28S and 18S rRNA using the Agilent Total RNA Nano chip assay on a Model 2100 Bioanalyzer (Agilent Technologies). The intensity of the 28S band should be twice the intensity of the 18S band. Gene expression profiling was conducted using the Human OneArray™ (Phalanx Biotech) containing over 30,000 sixty-mer polynucleotide probes with each probe mapped to the latest draft of the human genome (GoldenPath) printed on standard 1-in. x 3-in. glass slides. Analysis was performed according to the manufacturer's recommendations. After hybridization, arrays were scanned using GenePix 4000B (Axon Instruments) and analyzed with GenePix Pro 6.0 (Axon Instruments) to obtain gene expression ratios. Transformed data were normalized using the Lowes procedure [1]. The normalized data were used for clustering analysis. Clustering analysis was performed using Genespring GX 10.0 software (Agilent Technologies) to provide a graphical display of the expression patterns.

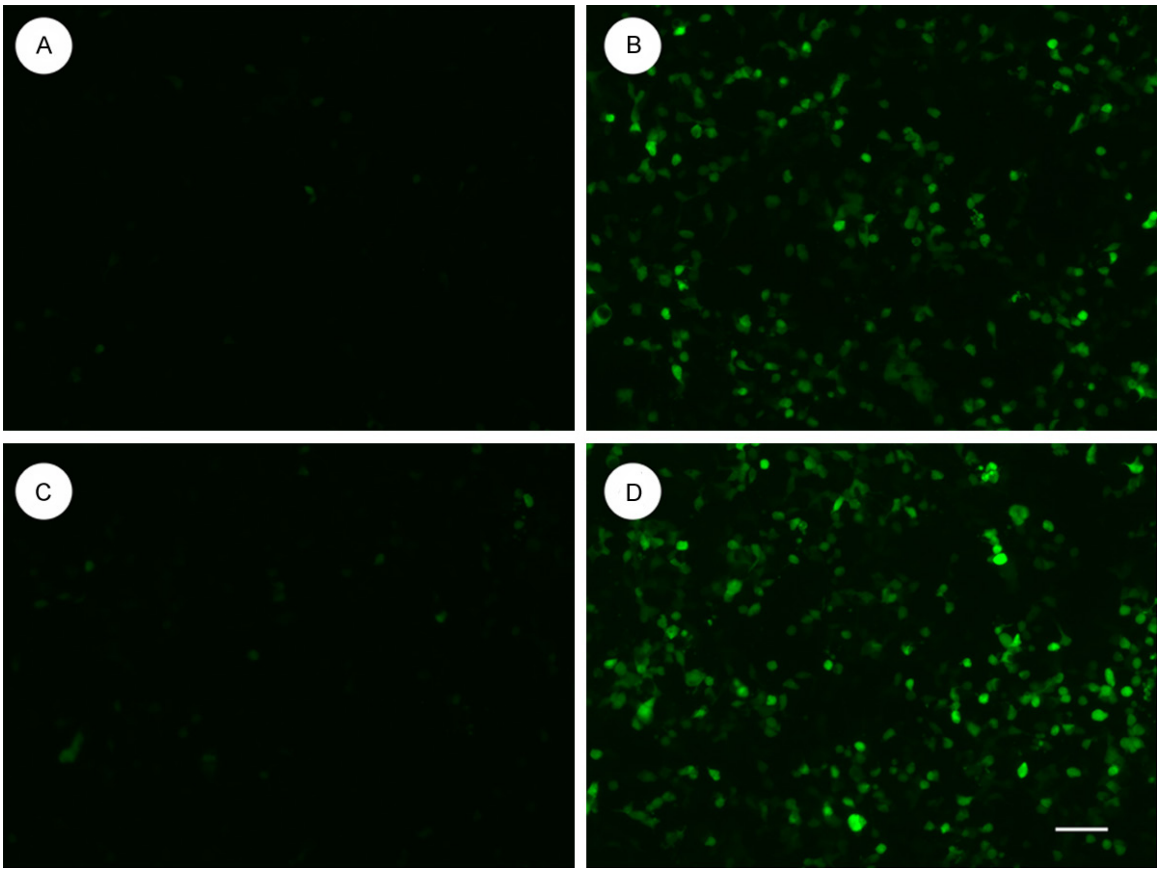

Figure 1. Verification of GPR4 knockdown in HMEC-1 cells by lentiviral-mediated RNA interference. Images of GFP expression showing shRNA delivery efficiency. Bar=50 μM. A: LV-CON-infected HMEC-1 cells for 24 h; B: LV-CON-infected HMEC-1 cells for 48 h; C: LV-shGPR4-infected HMEC-1 cells for 24 h; D: LV-shGPR4-infected HMEC-1 cells for 48 h.

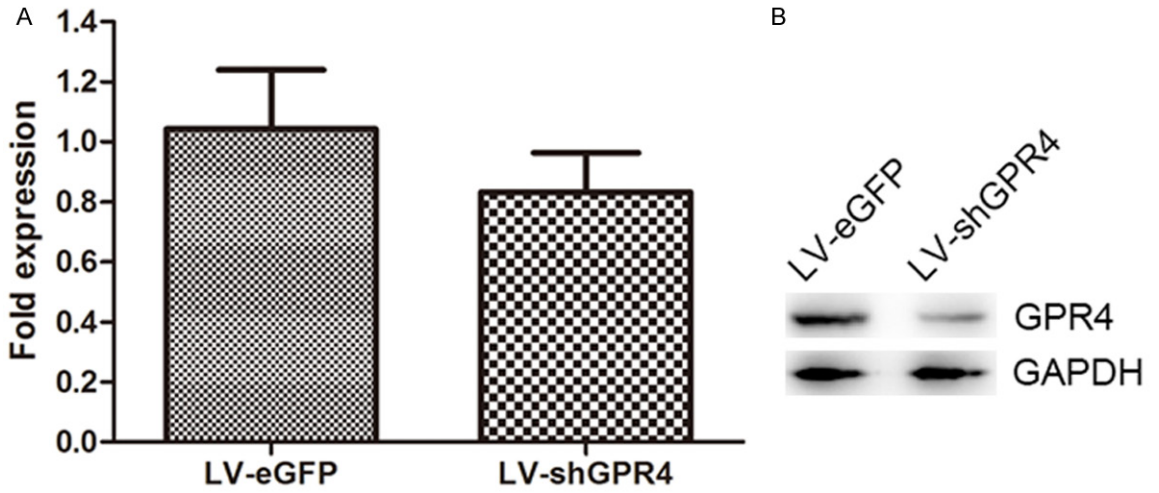

Figure 2. Lentiviral vector-mediated delivery of siRNA targeting GPR4 results in specific knockdown of expression. The indicated lentiviral siRNA expression constructs were cotransfected into HMEC-1 cells with an expression construct for the GPR4 target. A: Real-time PCR analyzed the expression of target gene expression in the RNA level. B: Immunoblotting of whole cell extracts was performed with anti-GPR4 antibody to detect GPR4 (upper panel). An anti-GAPDH antibody was used to confirm equal protein loading (bottom panel).

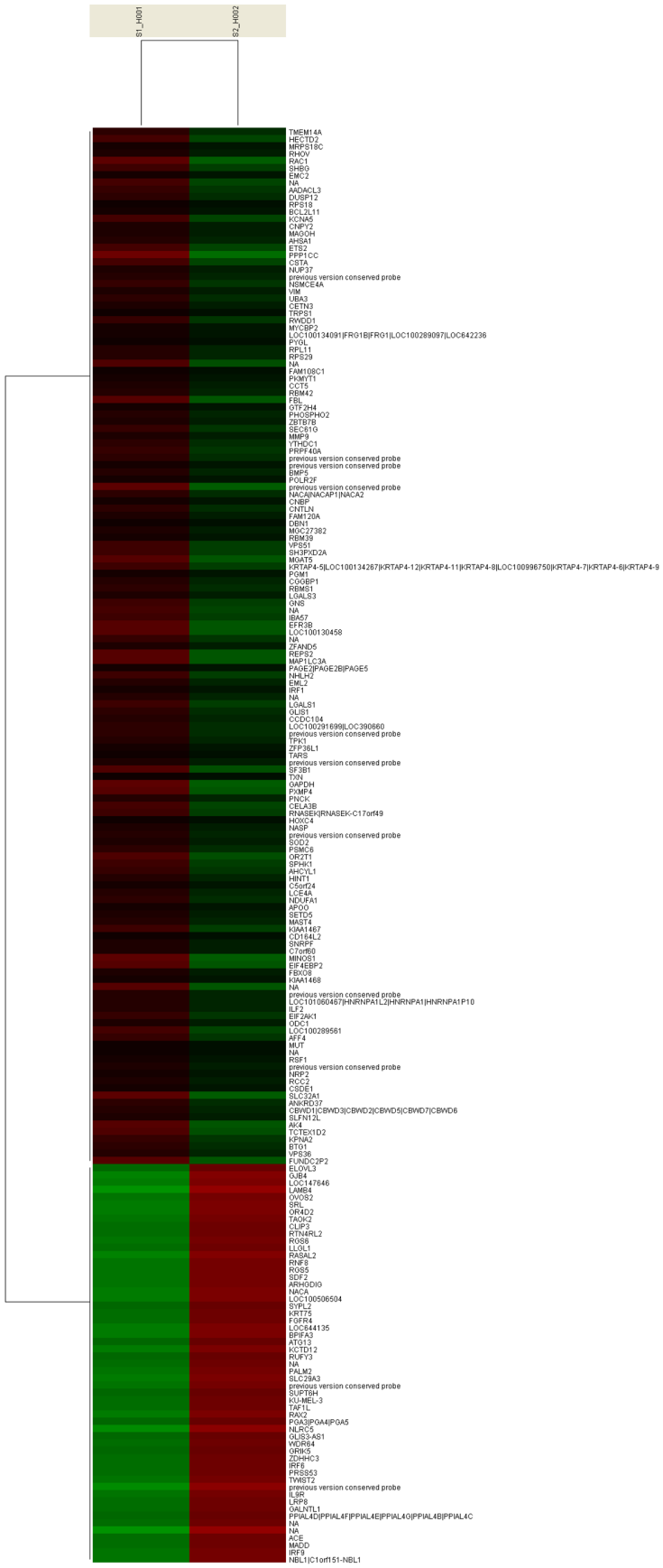

Figure 3. Cluster analysis of the gene expression. Clustering of differentially expressed genes in the HMEC-1 cells infected with LV-Con or LV-shGPR4.

Quantification of target RNA expression

Target gene expression was quantified using qPCR in an Light Cycler480 System (Roche) to confirm the upregulation of expression of target gene that were detected in the human OneArray™ from two cell lines. The expression level of each gene in LV-eGFP or LV-shGPR4 sample was represented relative to the expression of GAPDH, which was used as an internal control. We evaluated the expression levels by calculating the relative expression values in the two cell lines. Analysis of variance (ANOVA) was used for intergroup comparisons, and an appropriate post-hoc test was used to compensate for multiple comparisons (SigmaStat). *P* values, 0.05 were considered significant.

Results

Construction and analysis of the LV-shGPR4

The most efficient shRNA expression cassette was selected and constructed into the lentiviral vector, named LV-shGPR4. To determine the effect of LV-shGPR4 on the expression of GPR4, GFP expression was observed under a fluorescence microscope in the HMEC-1 cells 48 h after infection with LV-shGPR4 and LV-eGFP (**Figure 1**). Next, real-time PCR and Western blot were performed to determine the mRNA and protein levels of GPR4 in the LV-shGPR4 and LV-eGFP cell groups. As shown in **Figure 2A** and **2B**, LV-shGPR4 significantly inhibited expression of GPR4 protein when compared

Table 1. Primers for real-time PC

Primer No.	Sequence of primers (5'-3')	Length (bp)
PPP1CC-F	TTCTGCTGTCATGGAGGTTTATC	124
PPP1CC-R	TATCGGGGTCAGACCACAAAA	
ETS2-F	CTGGGCATTCCAAAGAACCC	85
ETS2-R	CCAGACTGAACTCATTGGTGG	
EIF4EBP2-F	TAGCCCTGGCACCTTAATTGA	91
EIF4EBP2-R	ATCCCCAACTGCATGTTTCCT	
PCDH20-F	AAAATGCACCTGTAAACACCCG	86
PCDH20-R	GCGATAGGTCTGTACCCCATTA	

with the levels in the HMEC-1 cells with LV-CON infection.

Gene array analysis of mrna levels in cells interfered by LV-shGPR4

To identify genes up-regulated or down-regulated by LV-shGPR4, we used human OneArray™ and defined the differential expression genes with a criterion (log ratio, $P \leq 0.01$ and $P \geq 1.5$-fold change in mRNA levels). Among the whole genes and expressed sequence tags, 447 differentially expressed genes were identified, containing 318 up-regulated genes and 129 down-regulated genes (**Figure 3**). These genes were principally classified into several biological process-related functions using the Panther analytical system, including (1) cell cycle; (2) apoptosis; (3) cell proliferation and differentiation; (4) protein biosynthesis, metabolism, and modification; (5) nucleobases, nucleoside, nucleotide, and nucleic acid metabolism; (6) signal transduction; (7) immune and defense; (8) transcription regulation; and so on.

Validation of gene expression changes with real-time PCR

Five genes including PPP1CC, ETS2, EIF4EBP2 and PCDH20 were testified with real-time PCR (**Table 1**). After detecting the expression of GPR4, the target 4 genes were analyzed by real-time PCR. The results of the 4 genes were in concord with microarray, signifying the high reliability of the microarray results (**Figure 4**).

Discussion

Silencing RNA is a highly specific tool for targeted gene knockdown, and it has advantages over the antisense oligo-DNA or ribozyme because it can be introduced into cells with a high efficiency and exerts its gene-silencing effect at a concentration several orders lower. Today, it is generally accepted that RNA interference is an effective, feasible, and stable approach for exploring gene function and identifying and validating new drug targets in functional genomic studies [20].

After GPR4 was knockdown in HMEC-1 cell, among the whole genes and expressed sequecnce tags, 447 differentially expressed genes were identified, containing 318 up-regulated genes and 129 down-regulated genes. These genes were principally classified into several biological process-related functions using the Panther analytical system, including 1) cell cycle; 2) apoptosis; 3) cell proliferation and differentiation; 4) protein biosynthesis, metabolism, and modification; 5) nucleobases, nucleoside, nucleotide, and nucleic acid metabolism; 6) signal transduction; 7) immune and defense; 8) transcription regulation and so on. Those genes whose expression dramatically changed may be involved in the GPR4 functions. These genes were related to cell apoptosis, cytoskeleton and signal transduction, cell proliferation, differentiation and cell-cycle regulation, gene transcription and translation and cell material and energy metabolism. This also confirm that GPR4 has a range of functions including 1) proton sensing; 2) regulating the growth, survive, migration, differentiation, tube formation of endothelial cells; 3) stimulating the cell proliferation, migration, invasion, colony formation, and survival of ovarian cancer cells.

In addition, 318 genes were upregulated in HMEC-1 cells treated with LV-shGPR4 compared with those treated with LV-CON, which were mainly involved in apoptosis, p53 signaling, and metastasis suppressor. Subsequently, RT-PCR was performed to confirm the GPR4-dependent expression of 4 genes identified in the microarray analysis. The expression of many genes is known to have important role in tumor progression.

The protein encoded by this gene belongs to the protein phosphatase family, PP1 subfamily. PP1 is an ubiquitous serine/threonine phosphatase that regulates many cellular processes, including cell division [21]. PPP1CC (protein phosphatase 1, catalytic subunit, gamma isozyme) is a protein-coding gene. Diseases asso-

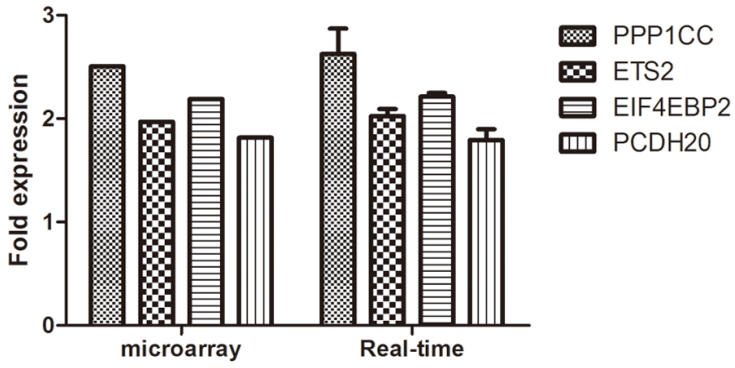

Figure 4. Comparison of 4 differential expression genes between the microarray and RT-PCR analyses.

ciated with PPP1CC include ossifying fibroma, and lipoma, and among its related super-pathways are Regulation of lipid metabolism Insulin signaling-generic cascades and Mitotic Anaphase [22, 23]. ETS2 (v-ets avian erythroblastosis virus E26 oncogene homolog 2) is a protein-coding gene. Diseases associated with ETS2 include frey syndrome, and Horner's syndrome, and among its related super-pathways are Rho Family GTPases and OSM Pathway [24, 25]. EIF4EBP2 (eukaryotic translation initiation factor 4E binding protein 2) encodes a member of the eukaryotic translation initiation factor 4E binding protein family. Diseases associated with EIF4EBP2 include sleep apnea, and Alzheimer's disease, and among its related super-pathways are TGF-Beta Pathway and Erythropoietin Pathway [26]. PCDH20 (protocadherin 20) belongs to the protocadherin gene family, a subfamily of the cadherin superfamily and was associated with the WNT/β-catenin pathway [27, 28]. Epigenetic silencing by hypermethylation of the CpG-rich promoter region of PCDH20 leads to loss of PCDH20 function, which may be a factor in the carcinogenesis of NSCLC [27].

GPR4 mediated an extremely complex and changing process. This process involves a variety of ways and multiple genes. Gene chip technology can provide comprehensive, reliable technical support for the screening of GPR4 related genes. This study may provide new evidence for GPR4 as a promising gene therapeutic target for cancer.

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Disclosure of conflict of interest

None.

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