Gene Expression Analysis of \(G^{\alpha 13-}\) Knockout Mouse Embryos Reveals Perturbations in \(G^{\alpha 13}\) Signaling Related to Angiogenesis and Hypoxia

Ji Hwan Park and Sangdun Choi*

Department of Molecular Science and Technology, Ajou University, Suwon 443-749, Korea

Abstract

Angiogenesis is regulated by a large number of molecules and complex signaling mechanisms. The G protein \(G^{\alpha 13}\) is a part of this signaling mechanism as an endothelial cell movement regulator. Gene expression analysis of \(G^{\alpha 13}\) knockout mouse embryos was carried out to identify the role of \(G^{\alpha 13}\) in angiogenesis signaling during embryonic development. Hypoxia-inducible response factors including those acting as regulators of angiogenesis were over expressed, while genes related to the cell cycle, DNA replication, protein modification and cell-cell dissociation were under expressed. Functional annotation and network analysis indicate that \(G^{\alpha 13-}\) embryonic mice were exposed to hypoxic conditions. The present analysis of the time course highlighted the significantly high levels of disorder in the development of the cardiovascular system. The data suggested that hypoxia-inducible factors including those associated with angiogenesis and abnormalities related to endothelial cell division contributed to the developmental failure of \(G^{\alpha 13}\) knockout mouse embryos.

Keywords: angiogenesis, gene expression, G protein \(G^{\alpha 13}\), G protein signaling, hypoxia

Introduction

The heterotrimeric (\(\alpha \beta \gamma\)) G proteins (guanine nucleotide binding proteins) are molecular switches that turn on intracellular signaling cascades in response to the activation of GPCRs (G protein coupled receptor) by extracellular stimuli (Oldham and Hamm, 2008). These G proteins are classified into four different families (\(G^{\alpha n}\), \(G^{\alpha (16)}\), \(G^{\alpha (17)}\) and \(G^{\alpha (12/13)}\)) based on the nature of the \(G^{\alpha}\) subunit present in the heterotrimer (Simon et al., 1991). \(G^{\alpha 13}\) belongs to the well studied, albeit poorly understood, \(G^{\alpha 12}\) family (Ribbo and Manning, 2005). Initially, \(G^{\alpha 12}\) and \(G^{\alpha 13}\) were shown to induce neo-plastic transformations in Rat-1 fibroblasts when activated constitutively (Voyno-Yasenetskaya et al., 1994). It was only later that the true function of \(G^{\alpha 13}\) was determined when it was shown that \(G^{\alpha 13}\), but not \(G^{\alpha 12}\), was involved in the LPA (lysophosphatidic acid)-induced activation of Rho (Gohla et al., 1998).

Subsequent studies identified p115 RhoGEF (Rho guanine nucleotide exchange factor 1 or also known as ARHGEP1) as the guanine nucleotide exchange factor involved in the \(G^{\alpha 13}\)-mediated Rho activation (Hart et al., 1998; Kozasa et al., 1998), \(G^{\alpha 12}/G^{\alpha 13}\) proteins also activate several RhoGEFs such as PDZ-RhoGEF (Fukuhara et al., 1999), Lbc-RhoGEF (Dutt et al., 2004) and LARG (leukemia-associated RhoGEF; RhoGEF12) (Suzuki et al., 2003). Once activated by these factors, Rho triggers a cascade of cellular signals mediated by Rho-dependent kinase (ROCK) (Ribbo and Manning, 2005). \(G^{\alpha 12}\) and \(G^{\alpha 13}\) not only regulate the activity of low molecular weight G proteins of the Rho family, but also the Na\(^+\)/H\(^+\) exchanger (Dhanasekaran et al., 1994), phospholipase D (PLD) (Plonk et al., 1998) and inducible nitric oxide synthase (iNOS) (Kitamura et al., 1996). Recently, it was shown that \(G^{\alpha 12}/G^{\alpha 13}\) basally regulated p53 by serine phosphorylation via mdm4 and this phosphorylation event was found to be distinct from p53 phosphorylation elicited by genotoxic agents (Kim et al., 2007).

\(G^{\alpha 13}\) is necessary for formation of actin stress fibers (Gohla et al., 1999), focal adhesion (Buhl et al., 1995), increasing Na\(^+\)/H\(^+\) exchange (Voyno-Yasenetskaya et al., 1994), aggregation of platelets (Huang et al., 2007), differentiation of embryonic cells (Jho and Malbon, 1997) and apoptosis (Berestetskaya et al., 1998). In the growth factor-induced cell migration, \(G^{\alpha 13}\) promotes the receptor tyrosine kinase (RTK) signaling which is independent of GPCR (Shan et al., 2006), \(G^{\alpha 13}\) and Rac-dependent cell migration is mediated by the formation of lamellipodia (Dhanasekaran, 2006) and the polymerization of actin to the leading edge of the cell (Radhika et al., 2004).

\(G^{\alpha 13}\) is also involved in patterning of blood vessels by controlling cell movement, shape and cell-cell/cell-matrix interactions of the endothelial cells (Ruppel et al., 2004).
2005). Endothelial cells of Gα13 knockout mice lose the ability to form an organized vascular system, resulting in intrauterine death (Offermanns et al., 1997). Moreover, the head mesenchyme of E8.5–E9.5 (embryo stage 8.5–9.5 day) Gα13 homozygous knockout mouse embryos exhibited enlarged blood vessels in comparison to the small vessels found in the wild-type or heterozygous embryos. This suggested that Gα13 activated physiological angiogenesis rather than vasculogenesis (Offermanns et al., 1997).

The development of blood vessels from differentiation of endothelial cells is called vasculogenesis. On the other hand, the subsequent sprouting of new blood vessels from the organized vascular system is referred to as angiogenesis. Angiogenesis is regulated by a number of stimulators and inhibitors in sophisticated ways. Once activated, epithelial cells begin to degrade the extracellular matrix and loosen the cell-cell adhesion. The adhesion molecules can then help in the sprouting of new blood vessels that are subsequently connected to existing vessels and stabilized with remolded tissues (Pandya et al., 2006).

During embryonic development, Vegf (vascular endothelial growth factor) is a key activator of angiogenesis as well as earlier vasculogenesis. The expression of Vegf is modulated by numerous cytokines such as interleukin 1 beta (IL1β) (Maruyama et al., 1999), CD40 (Tai et al., 2002), tumor necrosis factor alpha (TNFα) (Nabors et al., 2003), transforming growth factor beta (TGFβ) (Wang et al., 2004) and growth factors such as fibroblast growth factor 2 (FGF2) (Seghezzi et al., 1998). Hypoxia-initiated angiogenesis also occurs through the Vegf signaling pathway (Shweiki et al., 1992). Angiogenesis is thus important to both physiological and pathological conditions such as embryonic development, cancer cell metastasis, wound healing, etc.

Microarray analysis of whole genome expression of Gα13 knockout mouse embryos was carried out in order to identify the role of Gα13 in signaling networks during mouse embryonic development. Whole genome expression data highlighted significantly increased levels of disorder in the development of the physiological systems. Considering the importance of angiogenesis in embryonic or tumor development, it should be very crucial to understand the link between angiogenesis and Gα13-mediated signaling.

Methods

Generation of Gα13 knockout mouse embryos

The generation of Gα13 knockout mouse embryos is described elsewhere (Offermanns et al., 1997). Briefly, the first two exons of the Gα13 clone isolated from a 129/Sv mouse genomic λ phage library were replaced with neo gene from the plasmid pMC1neo Poly A (Stratagene, USA). The targeting vector contained a 1.4 kb-upstream sequence as the 5′ arm, with 8 kb of intron sequence and the second exon as the 3′ arm. Gene targeting was carried out in the mouse embryonic stem (ES) cell line CJ7. Correctly targeted ES cell clones were injected into C57BL/6 blastocysts and chimeras were bred with C57BL/6 and 129/Sv mice to generate heterozygous animals (Ramirez-Solis et al., 1993). Heterozygous mice were interbred to generate homozygous-deficient (knockout) embryos in Gα13 or wild-type littermate embryos for the controls. All procedures involving mice were carried out in accordance with the guidelines of the ethical committee of Ajou University.

Genotyping of mutant mouse strains

Total RNA was purified from the embryo or from the yolk sac and used for RT-PCR analysis with Gα13-specific primers. Oligonucleotides used for PCR reaction were 5′-AGC AGC GCA AGT CCA AAG AGA TCG-3′ and 5′-AGG AAC ACT CGA GTC TCC ACC ACC ATC C-3′. The genotypically confirmed knockout embryo sample was directly compared to the wild-type littermate embryo sample.

DNA microarray

Total RNA was extracted from knockout and wild-type littermate embryos using the Trizol method (Invitrogen, USA) and DNA microarray analysis was performed using the Affymetrix in situ synthesized GeneChip as described on the website of the Alliance for Cellular Signaling (AFCS: http://www.signaling-gateway.org/data/cgi-bin/ProtocolFile.cgi?afcs_PP000001T4.pdf?pid=PP000001T4), Briefly, total RNA was prepared from embryos with the use of Trizol (Life Technologies, USA). After the Trizol extraction, cleanup was carried out with a Qiagen RNeasy Mini Kit (Qiagen, USA). Reverse transcription was performed to generate double-stranded cDNAs with the use of SuperScript polymerase (Life Technologies) and an oligo-(dT)24 primer with a T7 RNA polymerase transcription from T7 RNA polymerase promoters. The cDNA prepared from total RNA was used as a template in the presence of a mixture of unlabeled ATP, CTP, GTP, and UTP and biotinylated CTP and UTP. The biotinylated cRNA was hybridized for 16 h at 40°C to a set of oligonucleotide arrays in the GeneChip Fluidics
### Table 1. Functions of the genes in cluster A of Fig. 1B

| Symbol | Gene name                                      | Function                                      | 9.5 day | 9.75 day | 10 day | 10.5 day | References                  |
|--------|-----------------------------------------------|-----------------------------------------------|---------|----------|---------|----------|-----------------------------|
| Adm    | Adrenomedullin                                | Migration, proliferation                      | 3.2     | 4.2      | 5.3     | 3.5      | Cormier-Regard et al., 1998 |
| Anxa2  | Annexin A2                                    | Growth                                        | 0.5     | 1.1      | 1.1     | 1.5      | Hoang et al., 2001          |
| Bhhb2  | Basic helix-loop-helix domain containing, class B, 2 | Differentiation                              | 0.9     | 4.5      | 3.4     | 3.1      | Bosco et al., 2006          |
| Bnip3  | BCL2/adenovirus E1B 19KDa interacting         | Apoptosis                                    | 1.5     | 1.4      | 1.8     | 1.8      | Bosco et al., 2006          |
| Ccnb2  | Cyclin G2                                     | Cycle progression                             | 1.3     | 1.4      | 2.1     | 1.6      | Bosco et al., 2006          |
| Cln3   | Chloride channel 3                            | Ion channel                                  | 0.6     | 1.1      | 1.7     | 2.3      | Schwarzer et al., 2005      |
| Ddit4  | DNA-damage-inducible transcript 4             | Differentiation                              | 2.9     | 3.5      | 3.7     | 3.3      | Bosco et al., 2006          |
| Egr1   | EGL nine homolog 1                            | Cell death                                   | 1.3     | 1.6      | 1.6     | 1.4      | Bosco et al., 2006          |
| Eff23y | Eurayctotic translation initiation factor 2, subunit 3, Y-linked | Translation                                | -3.5    | 4.1      |         |          |                             |
| Ero1l  | ERO0-like (S. cerevisiae)                     | Protein modification                          | 1.2     | 1.8      | 2.4     | 2.1      | Bosco et al., 2006          |
| F2     | Coagulation factor II (thrombin)              | Cell adhesion, proliferation                 |         |          |         |          |                             |
| Foxo3a | Forkhead box o3a                              | Inhibit migration                             | 0.9     | 1.3      | 1.4     | 1.2      |                             |
| Gadp10 | Ganglioside-induced differentiation-associated-protein 10 | Differentiation                              | 1.4     | 1.1      | 1.7     | 1.2      |                             |
| Glycam | Glycosylation dependent cell adhesion molecule 1 | Cell adhesion                               | 0.8     | 1.7      | 1.6     | 1.2      |                             |
| Gpi1   | Glucose phosphate isomerase 1                 | Glycolysis                                   | 0.7     | 1.2      | 1.8     | 1.1      | Olbryt et al., 2006         |
| Hspa1a | Heat shock protein 1A                         | Protein modification                          | 1.8     | 1.7      | 1.7     | 2.3      | Olbryt et al., 2006         |
| Ier3   | Immediate early response 3                    | Apoptosis                                    | 2.4     | 3        | 1.8      |          |                             |
| Igfbp3 | Insulin-like growth factor binding protein 3  | Apoptosis, growth                            | 1.3     | 3.1      | 1.6     | 2.4      | Tazuke et al., 1998         |
| Kdr    | Kinase insert domain protein receptor          | Vegf signaling                               | 1       | 1        | 1       | 1.1      | Bosco et al., 2006          |
| Ndryg1 | N-myc downstream regulated gene 1             | Differentiation                              | 0.9     | 2.2      | 2.7     | 2.2      | Chen et al., 2006           |
| P4ha1  | Procollagen-proline, 2-oxoglutarate  4-dioxogenase, α 1 polypeptide | Collagen synthesis                          | 0.9     | 1.7      | 1.7     | 1.5      | Bosco et al., 2006          |
| P4ha2  | Procollagen-proline, 2-oxoglutarate  4-dioxogenase, α 1 polypeptide | Collagen synthesis                          | 0.6     | 1.4      | 1.4     | 2        | Bosco et al., 2006          |
| Pbs1f  | Pre-B-cell colony-enhancing factor 1 Phosphorhuctokinase, platelet 4-dioxogenase, α 1 polypeptide | Differentiation | 0.9     | 1.2      | 1.5     | 1.4      | Wang et al., 2005           |
| Pkn2   | Phosphoglucomutase 2                          | Glycolysis                                   | 1.4     | 3.8      | 2.5     | 4.1      | Bosco et al., 2006          |
| Pkp2   | Phakophilin 2                                 | Cell adhesion                                | 0.9     | 1.3      | 1       | 1.2      | Olbryt et al., 2006         |
| Rnf19  | Ring finger protein 19                        | Cytoskeleton biogenesis                      | 0.9     | 1.1      | 1.5     | 1.2      |                             |
| Rora   | RAR-related orphan receptor alpha             | cGMP metabolic process                       | 2.7     | 1.1      | 1.7     |          | Chauvet et al., 2004        |
| Siat4a | ST3 beta-galactoside alpha-2,3-sialytransferase 1 | Protein amino acid glycoylation             | 1       | 1.3      | 1.4     | 1.4      | Koike et al., 2004          |
| Sld2a1 | Solute carrier family 2 (facilitated glucose transporter), member 1 | Glucose transport                          | 0.9     | 1.8      | 1.1     | 1.4      | Bosco et al., 2006          |
| Sld2a3 | Solute carrier family 2 (facilitated glucose transporter), member 3 | Glucose transport                          | 1.4     | 2.1      | 1.7     | 1.9      | Bosco et al., 2006          |
| Ste2   | Stanniocalcin 2                               | Calcium and phosphate transport              | 0.9     | 1        | 1.7     | 1.7      | Ito et al., 2004            |
| Tmem45a| Transmembrane protein 45a                    | Integral to membrane                         | 3.7     | 1.3      | 3.6     |          | Martin-Rendon et al., 2007  |
| Upp1   | Uridine phosphorylase 1                      | Nucleoside metabolic process                | 3.7     | 3.1      | 3.3     | 2.6      | Abramovitch et al., 2004    |
| Vegf   | Vascular endothelial growth factor            | Vegf signaling                              | 1.7     | 2.1      | 2.2     | 2.1      | Bosco et al., 2006          |

Hypoxia-induced genes are indicated in bold with the corresponding references. Folds are shown in log2 ratio.
Data analysis

The GeneChip was scanned and information was extracted using the GeneChip Analysis Suite 5.0 (Affymetrix). The output log2 ratio represents the fold difference between the knockout and control samples. The GeneChip Analysis program gives a present/absent (P/A) call for each spot on the array based on a predetermined signal-to-noise ratio, along with a not changed/increase/marginal increase/decrease/marginal decrease (NC/I/MI/D/MD) call for the comparison. The genes that had an NC/MI/MD call were filtered out. MeV program (MultiExperiment Viewer: http://www.tm4.org/mev.html) was used for clustering and Ingenuity Pathway Analysis (http://www.ingenuity.com) for functional analysis. All relevant microarray data have been deposited in MIAME (Minimum Information About a Microarray Experiment) compliant format in the public repository, ArrayExpress, under accession number E-MTAB-614.

Functional annotation and network analysis

For functional enrichment analysis, we used the Cytoscape plugin, BiNGO version 2.44. We selected GO Biological Process ontology file to annotate the GO term and filtered out only significant GO terms (p-value < 0.0001) according to Hypergeometric test in BiNGO. We generated the hypothetical network using 23 hypoxia-induced genes listed in Table 1. The first interaction neighbors were obtained from human protein-protein interaction database, BioGrid (http://www.thebiogrid.org). To identify the direct regulation of HIF1α (hypoxia-inducible factor 1, alpha subunit) to the hypoxia-induced genes, we performed a literature search which proved the binding of HIF1α to the promoters of the genes. To reconstruct a compact network, first neighbors of hypoxia-induced genes were removed, excluding the neighbors that interacted with two or more hypoxia-induced genes. Numbers of neighbors including removed neighbors were represented by size of the nodes.

Quantitative real-time PCR (QRT-PCR)

QRT-PCR was performed on the GeneAmp 5700 Sequence Detection System (Applied Biosystems, USA) by monitoring of the increase in fluorescence caused by the binding of SYBR Green to double-stranded DNA. Total RNA was prepared from frozen mouse retinas with the use of Trizol reagent (Life Technologies). To prepare cDNA templates, 50-100 ng of total RNA was mixed with 5.5 mM MgCl2, 0.5 mM each dNTP, 2.5 μM random hexamer, 0.4 unit/μl RNase inhibitor, and 1.25 units/μl MultiScribe reverse transcriptase from TaqMan Reverse Transcription Reagents (Applied Biosystems). RT was incubated at 25°C for 10 min and at 48°C for 30 min and inactivated at 95°C for 5 min. The PCR primers were designed with primer express software (Applied Biosystems), and their specificity in gene amplification was confirmed by measurement of the size and purity of the PCR product by 4% NuSieve agarose gel electrophoresis. For a 50-μl PCR, 4 μl cDNA template was mixed with 300 nM each of forward and reverse primers and 2 × SYBR Green PCR Master Mix (Applied Systems). The reaction was first incubated at 95°C for 2 min, then at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each gene-specific PCR was performed in triplicate.

Results

Angiogenesis-related genes were differently regulated in Gα13-/- mouse embryos

Using the Affymetrix GeneChip, gene expressions were measured and significantly regulated genes were selected by statistical filtering (≥1.5 fold and p-value ≤
0.01 in either of four time points). The hierarchical clustering result of significantly regulated genes in the \( G^{\alpha_{13} 13} \) mouse embryonic development stages is shown in Fig. 1A. Using the K-Means method, five expression clusters were obtained (Fig. 1B). Most genes in the cluster A of Fig. 1B were continually and remarkably upregulated from 9.5 day to 10.5 day. Table 1 shows functions of genes in cluster A. Vegf (in our QRT-PCR experiment, Vegf was upregulated by 5.7 fold at 10 day; data not shown) and Kdr (kinase insert domain receptor; Vegf receptor) are key regulators of angiogenesis, Igfbp3 (insulin-like growth factor binding protein 3) (Granata et al., 2007), F2 (coagulation factor 2) (Tsopanoglou and Maragoudakis, 1999) and Adm (adrenomedullin) (Ribatti et al., 2005) induce angiogenesis, Anxa2 (annexin A2) (Potente et al., 2005) inhibits cell differentiation and cell adhesion could get involved in the formation of blood vessels.

### Signal transduction networks

We used Ingenuity Pathways Analysis (IPA) program to show the interactions among aberrantly expressed molecules as a biological network in \( G^{\alpha_{13} 13} \) embryos. A subset of genes (\( \geq 0.5 \) or \( \leq -0.5 \) in log2 ratio) regulated at all time points were analyzed by IPA program and as a result we obtained 4 major networks. The top functions of the molecules in networks and their scores are given in Table 2. The network of 9.5d \( G^{\alpha_{13} 13} \) embryo was related to cell cycle, cancer, and cellular function and maintenance. The names of the molecules in this network which are related to cell cycle include Ccnd (cyclin D1), Cdkn1C (cyclin-dependent kinase inhibitor 1C), Dcn (decorin), Igf1 (insulin-like growth factor 1), Myb (v-myb myeloblastosis viral oncogene homolog) and Smarca2 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2). Subsequently, the network of 9.75d contained molecules involved in protein synthesis, degradation and cancer, such as Adm1 (adhesion regulating molecule 1), Fbxo8 (F-box protein 8), Nedd8 (neural precursor cell expressed developmentally down-regulated 8), Psmb3 (proteasome subunit, beta type 3), Psmb5, Psmc2, Psmd14, Ube2D2 (ubiquitin-conjugating enzyme E2D 2) and Ube2E1. On the other hand, 10d network consisted of Adm, Bsg (basigin), Ctsl2 (cathepsin L2), Ets2 (v-ets erythroblastosis virus E26 oncogene homolog 2), Igfbp2, Postn (peristin, osteoblast specific factor), Spp1 (secreted phosphoprotein 1) and Vtn (vitronectin) marks for cellular movement. In addition, this particular network consisted of the molecules associated with skeletal/muscular system development, connective tissue disorders, and dermatological diseases as well. Top-ranked function of the molecules in 10.5d network is involved in cellular movement: Col18\( \alpha \)1 (collagen, type 18, alpha 1), Col1\( \alpha \)3, Efna1 (ephrin-A1), Moex2 (mesenchyme homeobox 2), Nde1 (nude nuclear distribution gene E homolog 1), Nrp1 (neuropilin 1), Sema3E (sema domain, immunoglobulin domain, short basic domain secreted, 3E), Sparc (secreted protein, acidic, cysteine-rich), TGF-

### Table 2. Scores, top functions and molecules in the networks generated using the IPA program

| Analysis | Score | Focus molecules | Top functions | Molecules in network |
|----------|-------|-----------------|---------------|---------------------|
| 9.5d     | 41    | 25              | Cell Cycle, Cancer, Cellular Function and Maintenance | ANP32A, ASF1B, Caspase, CBX1, CCND1, CDCKN1C, CLK1, CRABP2, CYBA, Cyclin A, Cyclin E, DCN, DDX17, E2f, FEN1, Hdac, Histone h3, IGF1, ITM2B, MYB, NADPH oxidase, NCOA6, PCSK5, Ran, Rb, REST, RRM2, Scr, SRF3B1, SFPQ, SFRS1, SFRS3, SMARCA2, VAMP8, ZNF239 |
| 9.75d    | 44    | 29              | Protein Degradation, Protein Synthesis, Cancer | ADRM1, ANKRD1, CACYBP, CDC34 (includes EG:997), COP3S, DOST, FBXL3, FBXO6, GPS1 (includes EG:209318), MYBL1, NEDD8, NFkB, PPM1A, Proteasome, Proteasome PA700/20s, PSMB5, PSMB6, PSMB7, PSMB8, PSMD1, PSMD2, PSMD3, PSMD4, PSMD6, PSMD7, PSMD13, PSMD14, SKP1, SRR, TIMM3, TIMM3A, TXNRD1, UBE1, UBE2D2, UBE2E1, UBE2F1, ZNF239 |
| 10d      | 42    | 25              | Cellular Movement, Skeletal and Muscular System Development and Function, Cancer | ACTL6A, ADM, BHLHB2, BSG, Ck, COP2S2, CTS1, EIF4B, ERK, ETS2, Fibrin, GATA6, hCG, IGF2R, IGFBP2, IGFBP3, Insulin, Integrin, MAP2K1/2, Mmp, NAMPT, NDRG2, NR2F1, PCK2, PEA15, PECK, POSTN, Raf, SAT1, SCARB1, SLCA2A3, SPP1, STC2, UPP1, VTN |
| 10.5d    | 44    | 29              | Cellular Movement, Connective Tissue Disorders, Dermatological Diseases and Conditions | Aldehyde dehydrogenase (NAD), ALDH, ALDH1A2, ALDH2A2, ALDH7A1, CDC34 (includes EG:997), COL1B3A1, COL1A1, COL1A2, COL3A1, EFNA1, FLI1, MEOX2, NDE1, NFKB, NFYC, NID2, NRP1, P4HA1, P4HA2, Pdgf Ab, PPIF, Proteasome, RNF19A, SEMA3E, SLC2A2, SPARC, TACSTD1, TGFBI, TUBG1, TXNRD1, UBE2, UBE2L3, UBE2S, VEGFC |
β1 (Transforming growth factor, beta-induced, 68 kDa) and VegfC. Thus, our approach on network analyses by IPA revealed potential interactive relationships of aberrantly expressed molecules and defects of Gα13−/− signaling.

**Biological functions enriched with over-expressed genes in Gα13−/− mouse embryos**

To identify the functional enrichment of upregulated genes in any of the developmental stages of Gα13−/− mouse embryos, we generated the GO term tree using BiNGO plugin of Cytoscape program. We arranged the node representing each function to the hierarchical structure. We then categorized the functions into 12 groups such as response to wounding, metabolic process, regulation of transcription, angiogenesis, macromolecule metabolic process, apoptosis, signaling pathway, lipid metabolic process, transport, cell migration, catabolic process and embryonic development (represented as black boxes in Fig. 2). In particular, we observed that the angiogenesis-related genes were highly expressed in Gα13−/− mouse embryos as indicated by strong enrichment of angiogenesis and their upstream GO terms. We also found that biological functions associated with apoptosis, cell migration and transport that are known to be hypoxia-induced responses were also highly enriched. Based on these results, we hypothesized that Gα13−/− mouse embryos suffered hypoxic conditions during developmental stage 9.5-10.5 days, where hypoxia-induced responses such as angiogenesis, apoptosis, transport and cell migration were increased.

**Gα13−/−deletion mimics hypoxia-induced response in Gα13 knockout embryos**

A hypoxic condition such as newly formatted tissues or cancer cells needs new blood vessels, a source of energy and oxygen supply. In order to ascertain whether hypoxia-related genes were regulated, we looked for those genes that are known to be induced under hypoxic conditions. The hypoxia-related genes are shown in bold and their references are listed in Table 1. Genes associated with angiogenesis, glucose transporters, and ion regulators were among those that were significantly upregulated. For example, in Gα13−/− mouse embryos at all the time points tested, the expressions of Adm, Bhlhb2 (basic helix-loop-helix domain containing, class B, 2), Bnip3, Ccng2 (cyclin G2), Ddit4 (DNA-damage-inducible transcript 4) and Egln1 (egl nine homolog 1) were higher when compared to normal embryos. Our finding supports that the Gα13−/− mouse was exposed to hypoxic conditions from 9.5-10.5 days. We next performed network analysis to show the interactive relation-

![Image](http://example.com/image.png)
The Knockout of $G_{\alpha 13}$ Perturbs Angiogenesis and Hypoxia

Fig. 3. Graph view of interactions between hypoxia-induced genes in $G_{\alpha 13}^{-/-}$ mouse embryos. Continuously increased genes which may be affected by hypoxia were used to construct the hypothetical network. First neighbors were omitted on the basis of interaction with only one hypoxia-induced gene and number of neighbors was represented by size of nodes. Grey circles are hypoxia-induced genes and white circles represent their neighbors. Solid lines indicate protein-protein interactions and dashed lines indicate transcriptional regulation.

Fig. 4. Comparison of hypoxia or angiogenesis genes upregulated in $G_{\alpha 13}^{-/-}$ mouse embryos (EmbryoKO) to those regulated in ES cells under hypoxic conditions (ESHyp).

A number of transcription and translation factors were downregulated in $G_{\alpha 13}^{-/-}$ knockout embryos

Functions of genes that have more than 50% downregulation in the expression level among filtered genes are summarized in Table 3. The expression of $G_{\alpha 13}$ (also known as $Gna13$) at 9.5d, 9.75d, 10d and 10.5d (log_2 ratio values of -1.3, -1.8, -2.3 and -2.8 respectively) was confirmed. A number of transcription and translation factors were downregulated. Cell cycle regulators including DNA replication effectors were also downregulated. Some of the important down-regulated genes were $Ccdn1$, $Ccdn2$, $Ranbp1$ (RAN binding protein 1), $Ranbp5$ (RAN binding protein 5), $Cdc6$ (cell division cycle 6 homolog), $Cdc34$ (cell division cycle 34 homolog), $Fen1$ (flap structure-specific endonuclease 1), $Sox3$ (SRY-box 3), etc. $Ccdn1$ and $Ccdn2$ modulate the matrix metalloproteinase activity and cell motility. $Ccdn1$ is also involved in the expression of Rac1 (ras-related C3 botulinum toxin substrate 1) that stimulates the dorsal ruffle formation acell motility (Arato-Ohshima and Sawa, 1999; Manes et al., 2003). $Ranap1$ (Ran GTPase activating protein 1) mediated by Ran (member RAS oncogene family) regulates cell division (Arnaoutov and Dasso, 2003). Ran activity-involved proteins such as $Ranbp1$, $Ranbp5$ and $Rangfr$ (Ran guanine nucleotide releasing factor) were marginally downregulated. $Ppm1g$ (protein phosphatase 1G, magnesium-dependent, gamma isoform), $Rcc2$ (regulator of chromosome condensation 2), $Kpna2$ (karyopherin alpha 2), $Cdc6$, $Cdc34$, $Fen1$ and $Rrm2$ (ribonucleotide reductase M2 polypeptide) participate in the processing of cell division. $Cacybp$ (calcyclin binding protein) is involved in the calcium-dependent protein degradation process (Filipek, 2006). $Ubfd1$ (ubiquitin family domain containing 1),

ships of hypoxia-induced genes listed in Table 1. We investigated the interactions between hypoxia-induced genes and their first neighbors, but displayed only the number of interacting neighbors. The network indicates that most of hypoxia-induced genes over-expressed in $G_{\alpha 13}^{-/-}$ embryonic mouse were mainly transcriptionally regulated by HIF1 $\alpha$ directly or indirectly as depicted in Fig. 3. In our QRT-PCR experiment, $Hif1 \alpha$ was down regulated by 3 fold at 10 day; data not shown. We also observed that several genes in network such as $Hspa1A$ (heat shock 70 kDa protein 1A), $lgfbp3$, $Vegf$, $Kdr$ and $Anxa2$ interacted with several neighbor genes, suggesting that these genes play a potent role in $G_{\alpha 13}$ knockout-induced hypoxic responses. Moreover, a comparison of our microarray data with another published data (Accession No. E-MEXP-392 in ArrayExpress database: http://www.ebi.ac.uk/microarray-as/aer/result?queryFor=Experiment&Accession=E-MEXP-392) suggested that apart from hypoxia-related genes such as $Egin1$, genes that were responsible for angiogenesis such as $Vegf$ were upregulated in a similar manner as that of embryonic stem cells under hypoxic conditions (Fig. 4), suggesting that in $G_{\alpha 13}^{-/-}$ embryonic cells, hypoxic condition might exist similarly.
Ubicp1 (ubiquitin-like domain containing CTD phosphatase 1) and other ubiquitin-involved proteins that were marginally downregulated play a role in protein modification. Psmc3 (proteasome 26S subunit ATPase 3) promotes the global cellular ubiquitin-tagged protein degradation process (Demartino and Gillette, 2007). Nes

Table 3. Functions of downregulated genes

| Symbol | Gene name | Function | 9.5 day | 9.75 day | 10 day | 10.5 day |
|--------|-----------|----------|---------|----------|--------|----------|
| Amd1   | S-adenosylmethionine decarboxylase 1 | Morphology, metabolism | -0.5 | -1.1 | -0.5 | -0.8 |
| Anp32  | Acidic nuclear phosphoprotein 32 | | -0.9 | -0.6 | -0.6 | -0.6 |
| Arg1   | Arginase 1, liver | Arginine metabolism, nervous system development | -0.9 | -1 | -1.1 | -1.2 |
| Bzr1   | Basic leucine zipper and W2 domains 1 | Transcription | -0.9 | -0.4 | -0.6 |
| Cacybp | Calcytin binding protein | | -0.3 | -0.9 | -0.7 | -0.5 |
| Calm1  | Calmodulin 1 | Calcium signaling, migration | -1.1 | -0.5 | -0.5 |
| Ccd1   | Cyclin D1 | Division, migration | -0.8 | -1.2 | -0.6 | -0.6 |
| Ccd2   | Cyclin D2 | Division, migration | -0.3 | -0.7 | -0.7 | -1 |
| Cdc34  | Cell division cycle 34 homolog (S. cerevisiae) | Ubiquitin, DNA replication | -0.5 | -1.1 | -0.3 | -0.9 |
| Cdc6   | Cell division cycle 6 homolog (S. cerevisiae) | DNA replication | -0.3 | -0.9 | -0.9 | -0.9 |
| Cad2   | Cadherin 2 (N-cadherin) | Adhesion, vascular morphogenesis | -0.2 | -0.3 | -0.5 | -0.7 |
| Cdk2ap1| CDK2 (cyclin-dependent kinase 2)-associated protein 1 | Growth suppress | -0.5 | -0.7 | -0.6 | -0.9 |
| Crabp2 | Cellular retinoic acid binding protein II | Transport, differentiation | -0.9 | -1.2 | -1 | -1.4 |
| Cse1l  | Chromosome segregation 1-like (S. cerevisiae) | Transport, proliferation | -0.9 | -0.8 | -0.6 |
| Ddx21  | DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 21 (RNA helicase I/Gu) | Translation | -0.4 | -1 | -0.3 | -0.9 |
| Edf1   | Endothelial differentiation-related factor 1 | Differentiation | -1 | -0.8 | -0.5 |
| Elavl1 | ELAV (embryonic lethal, abnormal vision, (Hu)) | Destabilizes mRNAs | -0.4 | -0.7 | -0.4 | -0.6 |
| Fen1   | Flap structure specific endonuclease 1 | DNA replication | -0.6 | -1.3 | -1 | -0.8 |
| Gna13  | Guanine nucleotide binding protein, alpha 13 | G protein signaling | -1.3 | -1.8 | -2.3 | -2.8 |
| Hsp110 | Heat shock protein 110 | Protein modification | -0.6 | -1.3 | -0.8 | -0.9 |
| Kpn2a  | Karyopherin (importin) alpha 2 | DNA recombination, transport | -0.4 | -1 | -0.5 | -0.5 |
| Mpr18  | Mitochondrial ribosomal protein 118 | Translation | -1 | -0.5 | -0.5 |
| Mpr20  | Mitochondrial ribosomal protein 120 | Translation | -0.5 | -1 | -0.7 | -1 |
| Mpr4   | Mitochondrial ribosomal protein 14 | Translation | -0.9 | -0.4 | -0.4 |
| Mps11  | Mitochondrial ribosomal protein s11 | Translation | -0.9 | -0.4 | -0.9 |
| Mylpc  | Myosin light chain, phosphorylatable, Cardiac ventricles | Adhesion, heart development | -0.7 | -0.9 |
| Nes    | Nestin | Intermediate filament protein, central nervous system development | -1 | -1 | -1 | -1.3 |
| Nme1   | Expressed in non-metastatic cells 1, protein (NM23A) (nucleoside diphosphate kinase) | Nucleotide metabolic | -1 | -0.4 | -0.7 |
| Nrr2f1 | Nuclear receptor subfamily 2, group F, member 1 | Neuron migration, forebrain development | -0.5 | -1.1 | -0.8 | -1 |
| Poph2l | Polymerase (RNA II) (DNA directed) polypeptide L | Transcription | -0.5 | -0.8 | -0.3 | -0.9 |
| Poph3k | Polymerase (RNA III) (DNA directed) polypeptide K | Transcription | -0.9 | -0.3 | -0.8 |
| Ppm1g  | Protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform | Cell cycle | -0.3 | -1.1 | -0.3 | -0.8 |
| Psmc3  | Proteasome (prosome, macropain) 26S subunit, ATPase 3 | Protein catabolic | -0.9 | -0.3 | -0.3 |
| Rangap1| RAN GTPase activating protein 1 | Ran GTPase, division | -1.1 | -0.5 | -0.5 |
| RCC2   | Regulator of chromosome condensation 2 | Division | -0.5 | -0.8 | -0.4 | -1 |
| Rpl24  | Ribosomal protein L24 | Translation | -0.7 | -1 | -0.9 |
| Rnm2   | Ribonucleotide reductase M2 | DNA replication | -0.8 | -1.5 | -0.9 | -1.2 |
| Rrs1   | RRS1 ribosome biogenesis regulator homolog (S. cerevisiae) | Transcription | -0.6 | -0.7 | -0.4 | -1 |
| Stfplq | Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated) | Translation | -0.8 | -1 | -0.7 |
Table 3, Continued

| Symbol | Gene name | Function | 9.5 day | 9.75 day | 10 day | 10.5 day |
|--------|-----------|----------|---------|----------|--------|---------|
| Sfrs10 | Splicing factor, arginine/serine-rich 10 | Translation | -0.4 | -1.1 | -0.6 | -0.9 |
| Slmo2  | Slowmo homolog 2 (Drosophila) | Unknown | -0.5 | -0.7 | -0.5 | -0.9 |
| Sox3   | SRY-box containing gene 3 | Transcription, central nervous system development | -0.8 | -1.1 | -1.4 | -1.1 |
| Txnrd1 | Thioredoxin reductase 1 | Electron transport, proliferation | -0.5 | -1.3 | -0.5 | -0.8 |
| Ublcp1 | Ubiquitin-like domain containing CTD phosphatase 1 | Protein modification | -0.4 | -0.7 | -0.3 | -0.9 |
| Uqcrq  | Ubiquinol-cytochrome c reductase, complex III subunit VII | Electron transport | -0.9 | -0.2 | -0.5 |

Folds are shown in log2 ratio.

**Discussion**

The G protein $G_{a13}$ plays a key role in the mouse embryonic development. Several lines of evidence indicate that $G_{a13}$ knockout mice die due to the abnormality of angiogenesis, but the molecular mechanism has not yet been completely elucidated (Offermanns et al., 1997). In order to determine the outcome of $G_{a13}$ deletion in mouse embryos, whole gene expression was analyzed using the DNA microarray method. As shown in Fig. 1A and Table 1, the genes that participated in the processes of cell differentiation, cell-cell adhesion and angiogenesis, were all continually overexpressed from 9.5d to 10.5d. As shown in Table 3, downregulated genes were related to the cell cycle, DNA replication, protein modification and cell-cell dissociation. This gene level analysis supported the morphological observation of blood vessel sprouting and branching defects due to $G_{a13}$ deficiency. The lack of oxygen as well as the energy and nutrient depletion due to vascular system defects caused the abnormality of organ development in developing mouse embryos. Blood vessels were formed from progenitor cells as $G_{a13}$ embryos grew, but division, movement and migration of endothelial cells of blood vessels that are dependent on the $G_{a13}$ signaling pathway did not occur, thereby inhibiting angiogenesis. Based on our data, a primitive model was constructed as shown in Fig. 5.

The analysis of the whole genome expression data using Ingenuity Pathway Analysis showed statistically significant gene alterations, pertaining to different functional categories and occurring at various time points measured in the knockout mouse embryos (Fig. 6). As shown, the deletion of $G_{a13}$ resulted in not only abnormalities of cardiovascular system but also severe impediments in various other categories including metabolic pathway, movement, cancer, proliferation, cell cycle, etc. The regulation of cancer-related genes is not surprising when we consider the importance of the $G_{a13}$/$G_{a12}$ family members in cancer biology (Spiegelberg and Hamm, 2007).

Taken together, we present the results that were obtained from the whole genome gene expression of $G_{a13}$ mouse embryos compared to wild type littermate embryos, in an attempt to ascertain the roles of $G_{a13}$ during embryonic development. The results presented here...
throw light on the possible mechanisms of $G_{\alpha 1}$ that are involved in the regulation of angiogenesis and cytoskeletal rearrangements during embryonic development. Moreover, the potential characteristics of $G_{\alpha 12}$ and $G_{\alpha 13}$ in causing neoplasia (Voyno-Yasenetskaya et al., 1994) as well as their roles in metastasis (Spiegelberg and Hamm, 2007) cannot be ignored, and our study further provides clues to understanding the roles of these enigmatic proteins in cancer angiogenesis.

Acknowledgements
This work was supported by the Basic Science Research Program through the NRF of Korea funded by the MEST (2010-0015356). This work was also partly supported by the grant from Korea Food & Drug Administration (10182KFDA992) and the Priority Research Centers Program (NRF 2011-0022978).

References
Abramovitch, R., Tavor, E., Jacob-Hirsch, J., Zeira, E., Amariglio, N., Pappo, O., Rechav, G., Galun, E., and Honigman, A. (2004). A pivotal role of cyclic AMP-responsive element binding protein in tumor progression, Cancer Res, 64, 1338-1346.

Arano-Ohshima, T. and Sawa, H. (1999). Over-expression of cyclin D1 induces glioma invasion by increasing matrix metalloproteinase activity and cell motility, Int. J. Cancer, 38, 373-382.

Armentano, M., Filosa, A., Andolfi, G., and Studer, M. (2006). COUP-TFI is required for the formation of commissural projections in the forebrain by regulating axonal growth, Development, 133, 4151-4162.

Aramaoutov, A. and Dasso, M. (2003). The Ran GTAPase regulates kinetochore function, Dev. Cell, 5, 99-111.

Berestetskaya, Y.V., Faure, M.P., Ichijo, H., and Voyno-Yasenetskaya, T.A. (1998). Regulation of apoptosis by alpha-subunits of G12 and G13 proteins via apoptosis signal-regulating kinase-1, J. Biol. Chem, 273, 27816-27823.

Bosco, M.C., Puppo, M., Santangelo, C., Anfosso, L., Pfeffer, U., Fardin, P., Battaglia, F., and Varesio, L. (2006). Hypoxia modifies the transcriptome of primary ovarian cancer cells and induces hypoxia-inducible factor 1a pathway and prognosis, J. Clin. Pathol, 61, 213-220.

Buhl, A.M., Johnson, N.L., Dhanasekaran, N., and Johnson, G.L. (1995). G alpha 12 and G alpha 13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly, J. Biol. Chem, 270, 24631-24634.

Chauvet, C., Bois-Joyeux, B., Berra, E., Pouyssegur, J., and Danan, J.L. (2004). The gene encoding human retinoic acid-receptor-related orphan receptor alpha is a target for hypoxia-inducible factor 1, Biochem, J, 384, 79-85.

Chen, B., Nelson, D.M., and Sadovsky, Y. (2006). N-myc down-regulated gene 1 modulates the response of term human trophoblasts to hypoxic injury, J. Biol, Chem, 281, 2764-2772.

Collignon, J., Sockanathan, S., Hacker, A., Cohen-Tannoudji, M., Norris, D., Rastan, S., Stevanovic, M., Goodfellow, P.N., and Lovell-Badge, R. (1996). A comparison of the properties of Sox-3 with Sry and two related genes, Sox-1 and Sox-2, Development, 122, 509-520.

Cormier-Regard, S., Nguyen, S.V., and Claycomb, W.C. (1998). Adrenomedullin gene expression is developmentally regulated and induced by hypoxia in rat ventricular cardiac myocytes, J. Biol, Chem, 273, 17787-17792.

Demartino, G.N. and Gillette, T.G. (2007). Proteasomes: machines for all reasons, Cell, 129, 659-662.

Dhanasekaran, D.N. (2006). Transducing the signals: a G protein takes a new identity, Sci. STKE, 2006, pe31.

Dhanasekaran, N., Prasad, M.V., Wadsworth, S.J., Dermott, J.M., and van Rossum, G. (1994). Protein kinase C-dependent and -independent activation of Na+/H+ exchanger by G alpha class of G proteins, J. Biol, Chem, 269, 11802-11806.

Dutt, P., Nguyen, N., and Toksoz, D. (2004). Role of Lbc RhoGEF in Galphalpha12/13-induced signals to Rho GTAPase, Cell, Signal, 16, 201-209.

Filipek, A. (2006). S100A6 and CacyBP/SIP - two proteins discovered in ehrlich ascites tumor cells that are potentially involved in the degradation of beta-catenin, Chemotherapy, 52, 32-34.

Fukuhara, S., Murga, C., Zohar, M., Igishi, T., and Gutkind, J.S. (1999). A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho, J. Biol, Chem, 274, 5868-5879.

Girotomalolaki, A., Koulourakis, M.I., Gatter, K.C., Harris, A.L., and Sviridis, E. (2007). BNIP3 expression in endometrial cancer relates to active hypoxia inducible factor 1a pathway and prognosis, J. Clin, Pathol, 61, 217-220.

Gohla, A., Harhammer, R., and Schultz, G. (1998). The G-protein G13 but not G12 mediates signaling from lysophosphatidic acid receptor via epidermal growth factor receptor to Rho, J. Biol, Chem, 273, 4653-4659.

Gohla, A., Offermanns, S., Wilkie, T.M., and Schultz, G. (1999). Differential involvement of Galphalpha12 and Galphalpha13 in receptor-mediated stress fiber formation, J. Biol, Chem, 274, 17901-17907.

Granata, R., Trovato, L., Lupia, E., Sala, G., Settanni, F., Camussi, G., Ghidoni, R., and Ghigo, E. (2007). Insulin-like growth factor binding protein-3 induces angiogenesis through IGF-I- and SphK1-dependent mechanisms, J. Thromb, Haemost, 5, 835-845.

Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W.D., Gilman, A.G., Sternweis, P.C., and Bollag, G. (1998). Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galphalpha13, Science, 280, 2112-2114.

Hoang, V.M., Foulk, R., Clauser, K., Burlingame, A., Gibson, B.W., and Fisher, S.J. (2001). Functional proteomics: examining the effects of hypoxia on the cytotrophoblast protein repertoire, Biochemistry, 40, 4077-4086.

Huang, J.S., Dong, L., Kozasa, T., and Le Breton, G.C.
(2007). Signaling through G(alpha)13 switch region I is essential for protease-activated receptor 1-mediated human platelet shape change, aggregation, and secretion, J. Biol. Chem., 282, 10210-10222.

Ito, D., Walker, J.R., Thompson, C.S., Moroz, I., Lin, W., Veselits, M.L., Hakim, A.M., Fienberg, A.A., and Thinakaran, G. (2004). Characterization of stanniocalcin 2, a novel target of the mammalian unfolded protein response with cytoprotective properties, Mol. Cell. Biol., 24, 9456-9469.

Jho, E.H. and Malbon, C.C. (1997), Galpha12 and Galpha13 mediate differentiation of P19 mouse embryonal carcinoma cells in response to retinoic acid, J. Biol. Chem., 272, 24461-24467.

Kim, M.S., Lee, S.M., Kim, W.D., Ki, S.H., Moon, A., Lee, C.H., and Kim, S.G. (2007). G alpha 12/13 basally regulates p53 through Mdm4 expression, Mol. Cancer Res, 5, 473-484.

Kitamura, K., Singer, W.D., Star, R.A., Muallem, S., and Galpha12 and Galpha13. Science 280, 2109-2111.

(1998). p115 RhoGEF, a GTPase activating protein for response with cytoprotective properties. Mol. Cell. Biol. 24, 1997. Vascular system defects and impaired cell chemokinesis as a result of Galpha13 deficiency, Science 275, 533-536.

Oldham, W.M. and Hamm, H.E. (2004). Heterotrigenic G protein activation by G-protein-coupled receptors, Nat, Rev, Mol Cell Biol, 9, 60-71.

Pandya, N.M., Dhalla, N.S. and Santani, D.D. (2006). Angiogenesis—a new target for future therapy, Vascul. Pharmacol., 44, 265-274.

Potente, M., Urbich, C., Sasaki, K., Hofmann, W.K., Heeschen, C., Aicher, A., Kollipara, R., DePinho, R.A., Zeiher, A.M., and Dimmeler, S. (2005). Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization, J. Clin, Invest, 115, 2382-2392.

Radhika, V., Onesime, D., Ha, J.H., and Dhanasekaran, N. (2004). Galpha13 stimulates cell migration through contact-inhibiting protein Hax-1, J. Biol. Chem, 279, 49406-49413.

Ramirez-Solis, R., Davis, A.C., and Bradley, A. (1993). Gene targeting in embryonic stem cells, Meth. Enzymol., 225, 855-878.

Ribatti, D., Nico, B., Spinazzi, R., Vacca, A., and Vassudorfer, G.G. (2005). The role of adrenomedullin in angiogenesis, Peptides 26, 1670-1675.

Riobo, N.A. and Manning, D.R. (2005). Receptors coupled to heterotrigenic G proteins of the G12 family, Trends Pharmacol. Sci. 26, 146-154.

Ruppel, K.M., Willison, D., Kataoka, H., Wang, A., Zheng, Y.W., Cornelissen, I., Yin, L., Xu, S.M., and Coughlin, S.R. (2005). Essential role for Galpha13 in endothelial cells during embryonic development, Proc, Natl, Acad Sci, U.S.A, 102, 8281-8286.

Schwarz, R., Tondera, D., Arnold, W., Giese, K., Klippel, A., and Kaufmann, J. (2005). REDD1 integrates hypoxia-mediated survival signaling downstream of phosphatidylinositol 3-kinase, Oncogene 24, 1138-1149.

Seghezzi, G., Patel, S., Ren, C.V., Gualandris, A., Pintucci, S., Robbins, E.S., Shapiro, R.L., Galloway, A.C., Rifkin, D.B. and Mignatti, P. (1998). Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis, J. Cell Biol, 141, 1659-1673.
Shan, D., Chen, L., Wang, D., Tan, Y.C., Gu, J.L., and Huang, X.Y. (2006). The G protein G alpha(13) is required for growth factor-induced cell migration, *Dev. Cell* 10, 707-718.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis, *Nature* 359, 843-845.

Simon, M.I., Strathmann, M.P., and Gautam, N. (1991). Diversity of G proteins in signal transduction, *Science* 252, 802-808.

Spiegelberg, B.D. and Hamm, H.E. (2007). Roles of G-protein-coupled receptor signaling in cancer biology and gene transcription, *Curr. Opin. Genet. Dev.* 17, 40-44.

Suzuki, N., Nakamura, S., Mano, H., and Kozasa, T. (2003). Galpha 12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. *Proc. Natl. Acad. Sci. U.S.A.* 100, 733-738.

Tai, Y.T., Podar, K., Gupta, D., Lin, B., Young, G., Akiyama, M., and Anderson, K.C. (2002). CD40 activation induces p53-dependent vascular endothelial growth factor secretion in human multiple myeloma cells, *Blood* 99, 1419-1427.

Tazuke, S.I., Mazure, N.M., Sugawara, J., Carland, G., Faessen, G.H., Suen, L.F., Irwin, J.C., Powell, D.R., Giaccia, A.J., and Giudice, L.C. (1998). Hypoxia stimulates insulin-like growth factor binding protein 1 (IGFBP-1) gene expression in HepG2 cells: a possible model for IGFBP-1 expression in fetal hypoxia, *Proc. Natl. Acad. Sci. U.S.A.* 95, 10188-10193.

Tsopanoglou, N.E., and Maragoudakis, M.E. (1999). On the mechanism of thrombin-induced angiogenesis, Potentiation of vascular endothelial growth factor activity on endothelial cells by up-regulation of its receptors, *J. Biol. Chem.* 274, 23969-23976.

Voyno-Yasenetskaya, T., Conklin, B.R., Gilbert, R.L., Hooley, R., Bourne, H.R., and Barber, D.L. (1994). G alpha 13 stimulates Na-H exchange, *J. Biol. Chem.* 269, 4721-4724.

Voyno-Yasenetskaya, T.A., Pace, A.M., and Bourne, H.R. (1994). Mutant alpha subunits of G12 and G13 proteins induce neoplastic transformation of Rat-1 fibroblasts, *Oncogene* 9, 2559-2565.

Wang, L., Kwak, J.H., Kim, S.I., He, Y., and Choi, M.E. (2004). Transforming growth factor-beta1 stimulates vascular endothelial growth factor 164 via mitogen-activated protein kinase kinase 3-p38alpha and p38delta mitogen-activated protein kinase-dependent pathway in murine mesangial cells, *J. Biol. Chem.* 279, 33213-33219.

Wang, V., Davis, D.A., Haque, M., Huang, L.E., and Yarchoan, R. (2005). Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells, *Cancer Res.* 65, 3299-3306.

Yang, J., Bian, W., and Jing, N.H. (1997). Nestin mRNA expression during the development of mouse central nervous system, *Sheng Li Xue Bao* 49, 657-665.

Yu, H., Iyer, R.K., Yoo, P.K., Kern, R.M., Grody, W.W., and Cederbaum, S.D. (2002). Arginase expression in mouse embryonic development, *Mech. Dev.* 115, 151-155.