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Gene Expression Analysis of $G\alpha_{13}^{-/-}$ Knockout Mouse Embryos Reveals Perturbations in $G\alpha_{13}$ Signaling Related to Angiogenesis and Hypoxia

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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 GPCR (G protein coupled receptor) by extracellular stimuli (Oldham and Hamm, 2008). These G proteins are classified into four different families ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ 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 (Riobo and Manning, 2005). Initially, $G\alpha_{12}$ and $G\alpha_{13}$ were shown to induce neoplastic 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 ARHGEF1) 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) (Riobo 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.*,

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2005). Endothelial cells of $G\alpha_{13}$ knockout mice lose the ability to form an organized vascular system, resulting in intrauteric death (Offermanns *et al.*, 1997). Moreover, the head mesenchyme of E8.5-E9.5 (embryo stage 8.5-9.5 day) $G\alpha_{13}$ 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 $G\alpha_{13}$ 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 $G\alpha_{13}$ knockout mouse embryos was carried out in order to identify the role of $G\alpha_{13}$ 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 $G\alpha_{13}$-mediated signaling.

Methods

Generation of $G\alpha_{13}$ knockout mouse embryos

The generation of $G\alpha_{13}$ knockout mouse embryos is described elsewhere (Offermanns *et al.*, 1997). Briefly, the first two exons of the $G\alpha_{13}$ 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 (Ramírez-Solis *et al.*, 1993). Heterozygous mice were interbred to generate homozygous-deficient (knockout) embryos in $G\alpha_{13}$ 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 $G\alpha_{13}$-specific primers. Oligonucleotides used for PCR reaction were 5'-AGC AGC GCA AGT CCA AGG AGA TCG-3' and 5'-AGG AAC ACT CGA GTC TCC 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_PP00000174.pdf?pid=PP00000174). 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 promoter (Genset, USA). An RNA Transcript Labeling Kit (Enzo Diagnostics, USA) was used for the production of hybridizable biotin-labeled RNA targets by *in vitro* 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

Station 400 (Affymetrix, USA). After hybridization, the GeneChip array underwent a series of stringency washes and was stained with streptavidin-conjugated phycoerythrin. Arrays were scanned with a Hewlett-Packard Scanner.

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
Bhlhb2	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 protein 3	Apoptosis	1.5	1.4	1.8	1.8	Bosco *et al.*, 2006
Ccng2	Cyclin G2	Cycle progression	1.3	1.4	2.1	1.6	Bosco *et al.*, 2006
Clcn3	Chloride channel 3	Ion channel	0.6	1.1	1.7	2.3	
Ddit4	DNA-damage-inducible transcript 4	Differentiation	2.9	3.5	3.7	3.3	Schwarzer *et al.*, 2005
Egln1	EGL nine homolog 1	Cell death	1.3	1.6	1.6	1.4	Bosco *et al.*, 2006
Eif2s3y	Eukaryotic 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				4.4	
Foxo3a	Forkhead box o3a	Inhibit migration	0.9	1.3	1.4	1.2	
Gdap10	Ganglioside-induced differentiation-associated-protein 10	Differentiation	1.4	1.1	1.7	1.2	
Glycam1	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	Bosco *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
Ndrg1	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-dioxygenase, α1 polypeptide	Collagen synthesis	0.9	1.7	1.7	1.5	Bosco *et al.*, 2006
P4ha2	Procollagen-proline, 2-oxoglutarate 4-dioxygenase, α1 polypeptide	Collagen synthesis	0.6	1.4	1.4	2	Bosco *et al.*, 2006
Pbef1	Pre-B-cell colony-enhancing factor 1	Differentiation	0.9	1.2	1.5	1.4	Wang *et al.*, 2005
Pfkp	Phosphofructokinase, platelet	Glycolysis	1.4	3.8	2.5	4.1	Bosco *et al.*, 2006
Pgm2	Phosphoglucomutase 2	Glycolysis	0.9	1.9	1.7	1.7	Manjunath *et al.*, 1998
Pkp2	Plakophilin 2	Cell adhesion	0.5	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-sialyltransferase 1	Protein amino acid glycosylation	1	1.3	1.4	1.4	Koike *et al.*, 2004
Slc2a1	Solute carrier family 2 (facilitated glucose transporter), member 1	Glucose transport	0.9	1.8	1.1	1.4	Bosco *et al.*, 2006
Slc2a3	Solute carrier family 2 (facilitated glucose transporter), member 3	Glucose transport	1.4	2.1	1.7	1.9	Bosco *et al.*, 2006
Stc2	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	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 $\log_2$ ratio.

Data analysis

The GeneChip was scanned and information was extracted using the GeneChip Analysis Suite 5.0 (Affymetrix). The output $\log_2$ 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 $MgCl_2$, 0.5 mM each dNTP, 2.5μM 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 50°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 $G\alpha_{13}^{-/-}$ 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≤

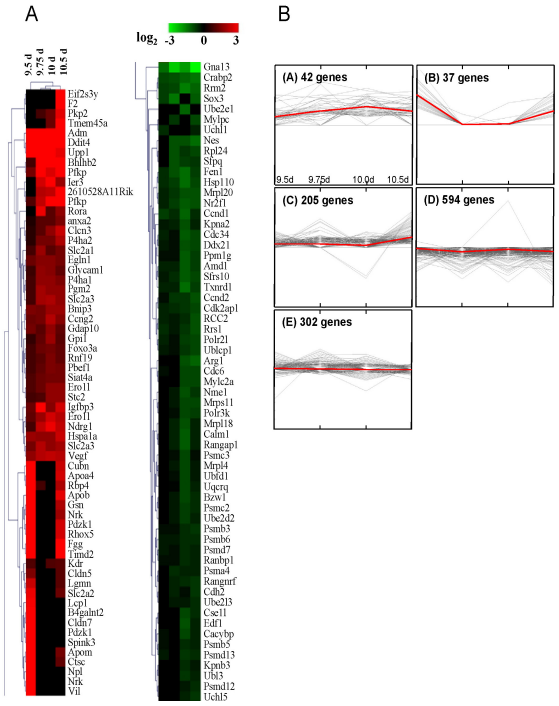

Fig. 1. Clustering analysis. (A) Hierarchical clustering results from MeV. The intensity of color represents the value of upregulation (red) or downregulation (green) in $\log_2$ ratio. (B) K-Means clustering results. Altered genes were classified into 5 clusters by the MeV program. The each line shows changing of each gene over time.

0.01 in either of four time points). The hierarchical clustering result of significantly regulated genes in the $G\alpha_{13}^{-/-}$ mouse embryonic development st ages 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) regulates angiogenesis (Ling *et al*., 2004), and Bnip3 (BCL2/adenovirus E1B interacting protein 3) is related to the reactivity of Vegf (Giatromanolaki *et al*., 2007). Foxo3a (forkhead box O3a) (Potente *et al*., 2005) inhibits tube formation. In addition, other molecules related to 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 between aberrantly expressed molecules as a biological network in $G\alpha_{13}^{-/-}$ embryos. A subset of genes ($\geq$0.5 or $\leq$-0.5 in $\log_2$ 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 $G\alpha_{13}^{-/-}$ 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 Adrm1 (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 (periostin, 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: Col18α1 (collagen, type 18, alpha 1), Col1α1, EfnA1 (ephrin-A1), Meox2 (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, CDKN1C, CLK1, CRABP2, CYBA, Cyclin A, Cyclin E, DCN, DDX17, E2f, FEN1, Hdac, Histone h3, IGF1, ITM2B, MYB, NADPH oxidase, NCOA6, PCSK5, Rar, Rb, REST, RRM2, Scf, SF3B1, SFPQ, SFRS1, SFRS3, SMARCA2, VAMP8, ZNF239
9.75d	44	29	Protein Degradation, Protein Synthesis, Cancer	ADRM1, ANKRD1, CACYBP, CDC34 (includes EG:997), COPS3, DDOST, FBXL3, FBXO8, GPS1 (includes EG:209318), MYBL1, NEDD8, NFkB, PPM1B, Proteasome, Proteasome PA700/20s, PSMB, PSMB3, PSMB5, PSMB6, PSMC2, PSMC3, PSMD, PSMD1, PSMD7, PSMD13, PSMD14, SKP1, SRR, TIMM23, TIMM8A, TXNRD1, UBE2, UBE2D2, UBE2E1, ZNRF1
10d	42	25	Cellular Movement, Skeletal and Muscular System Development and Function, Cancer	ACTL6A, ADM, BHLHB2, BSG, Ck2, COPS2, CTSL2, EIF4B, ERK, ETS2, Fibrin, GATA6, hCG, IGF2R, IGFBP2, IGFBP3, Insulin, Integrin, MAP2K1/2, Mmp, NAMPT, NDRG2, NR2F1, PCK2, PEA15, PEPCK, POSTN, Raf, SAT1, SCARB1, SLC2A3, SPP1, STC2, UPP1, VTN
10.5d	44	29	Cellular Movement, Connective Tissue Disorders, Dermatological Diseases and Conditions	Aldehyde dehydrogenase (NAD), ALDH, ALDH1A2, ALDH3A2, ALDH7A1, CDC34 (includes EG:997), COL18A1, 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

βI (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 $G\alpha_{13}^{-/-}$ signaling.

Biological functions enriched with over-expressed genes in $G\alpha_{13}^{-/-}$ mouse embryos

To identify the functional enrichment of upregulated genes in any of the developmental stages of $G\alpha_{13}^{-/-}$ 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 $G\alpha_{13}^{-/-}$ 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 $G\alpha_{13}^{-/-}$ 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.

$G\alpha_{13}$-deletion mimics hypoxia-induced response in $G\alpha_{13}$ 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 $G\alpha_{13}^{-/-}$ 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 $G\alpha_{13}^{-/-}$ mouse was exposed to hypoxic conditions from 9.5-10.5 days. We next performed network analysis to show the interactive relation-

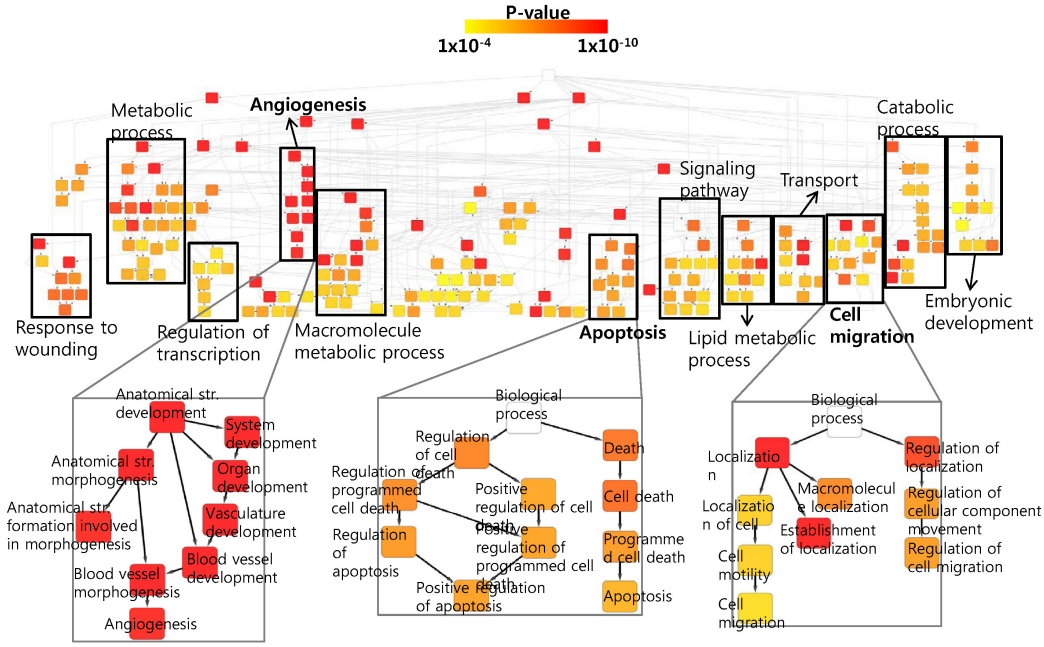

Fig. 2. A GO term tree of over-expressed genes in $G\alpha_{13}^{-/-}$ mouse embryos generated from BiNGO program. Genes with a log2 ratio of 0.5 or greater in any of developmental days were used to perform functional annotation analysis. The brightness of node colors represents the p-value indicating significance of functional enrichment. The cellular processes were categorized into several groups as indicated by black boxes.

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 HIF1α directly or indirectly as depicted in Fig. 3 (In our QRT-PCR experiment, *Hif1*α 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), *Igfbp3, 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 ArrrayExpress database: http://www.ebi.ac.uk/microarray-as/aer/result?queryFor=Experiment&eAccession=E-MEXP-392) suggested that apart from hypoxia-related genes such as *Egln1*, 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.

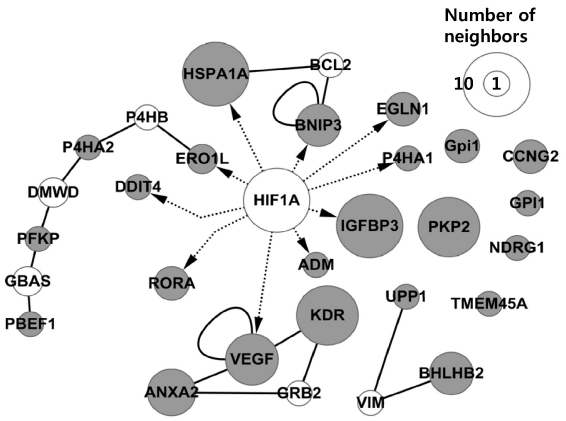

Fig. 3. Graphic view of interactions between hypoxia-induced genes in $G\alpha_{13}^{-/-}$ mouse embryos. Continually increased genes which may be affected by hypoxia were used to construct the hypothetical network. First neighbors were omitted on the basis of interaction with only one hypoxia-induced 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.

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 *Ccnd1, Ccnd2, 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*. Ccnd1 and Ccnd2 modulate the matrix metalloproteinase activity and cell motility. Ccnd1 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). Rangap1 (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 Rangnrf (Ran guanine nucleotide release 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),

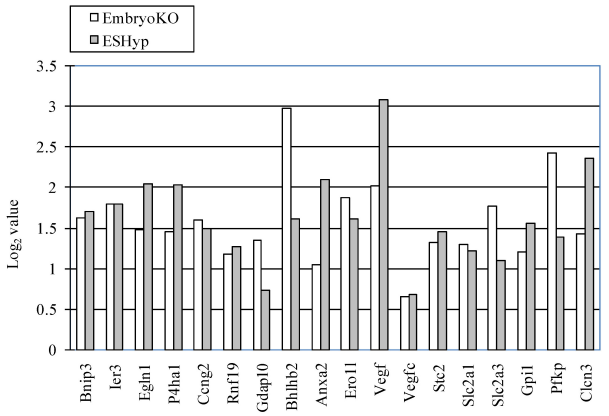

Fig. 4. Comparison of hypoxia or angiogenesis genes upregulated in $G\alpha_{13}^{-/-}$ mouse embryos (EmbryoKO) to those regulated in ES cells under hypoxic conditions (ESHyp).

Ublcp1 (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
Bzw1	Basic leucine zipper and W2 domains 1	Transcription		-0.9	-0.4	-0.6
Cacybp	Calcyclin binding protein	Ubiquitin	-0.3	-0.9	-0.7	-0.5
Calm1	Calmodulin 1	Calcium signaling, migration		-1.1	-0.5	-0.5
Ccnd1	Cyclin D1	Division, migration	-0.8	-1.2	-0.6	-0.6
Ccnd2	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
Cdh2	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 II/Gu)	Translation	-0.4	-1	-0.3	-0.9
Edf1	Endothelial differentiation-related factor 1	Differentiation		-1	-0.8	-0.5
Elavl1 (Hur)	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	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
Kpna2	Karyopherin (importin) alpha 2	DNA recombination, transport	-0.4	-1	-0.5	-0.5
Mrpl18	Mitochondrial ribosomal protein l18	Translation		-1	-0.5	-0.5
Mrpl20	Mitochondrial ribosomal protein l20	Translation	-0.5	-1	-0.7	-1
Mrpl4	Mitochondrial ribosomal protein l4	Translation		-0.9	-0.4	-0.4
Mrps11	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
Nr2f1	Nuclear receptor subfamily 2, group F, member 1	Neuron migration, forebrain development	-0.5	-1.1	-0.8	-1
Polr2l	Polymerase (RNA) II (DNA directed) polypeptide L	Transcription	-0.5	-0.8	-0.3	-0.9
Polr3k	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	-0.6	-0.4	-1
Rpl24	Ribosomal protein L24	Translation		-0.7	-1	-0.9
Rrm2	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
Sfpq	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
Ubfd1	Ubiquitin family domain containing 1	Protein modification		-0.9	-0.3	-0.4
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 $\log_2$ ratio.

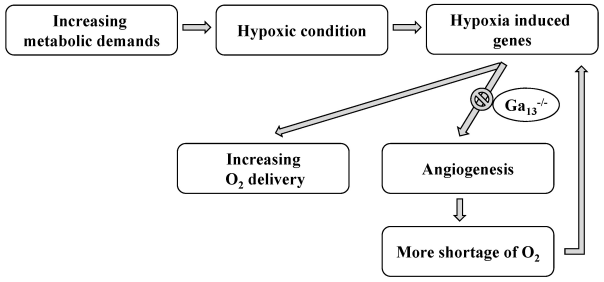

Fig. 5. Mechanisms for the role of $G\alpha_{13}$ in the angiogenesis and the induction of hypoxic conditions.

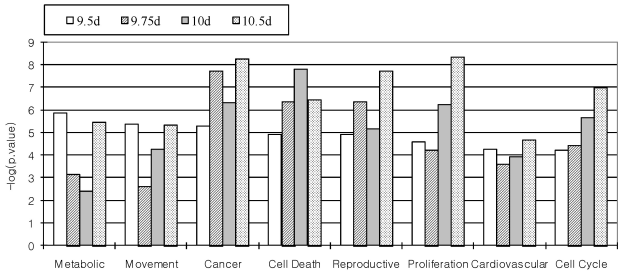

Fig. 6. Significantly affected physiological pathways in $G\alpha_{13}^{-/-}$ embryos revealed by Ingenuity Pathway Analysis.

(nestin) (Yang *et al*., 1997), Nr2f1 (nuclear receptor subfamily 2, group F, member 1) (Armentano *et al*., 2006), Sox3 (Collignon *et al*., 1996) and Arg1 (arginase, liver) (Yu *et al*., 2002) are required for the development of mouse central nervous system.

Discussion

The G protein $G\alpha_{13}$ plays a key role in the mouse embryonic development. Several lines of evidence indicate that $G\alpha_{13}$ 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 $G\alpha_{13}^{-/-}$ 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 $G\alpha_{13}$ 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 $G\alpha_{13}^{-/-}$ embryos grew, but division, movement and migration of endothelial cells of blood vessels that are dependent on the $G\alpha_{13}$ 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 $G\alpha_{13}$ 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 $G\alpha_{12}$/$G\alpha_{13}$ 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 $G\alpha_{13}^{-/-}$ mouse embryos compared to wild type littermate embryos, in an attempt to ascertain the roles of $G\alpha_{13}$ during embryonic development. The results presented here

throw light on the possible mechanisms of $G\alpha_{13}$ that are involved in the regulation of angiogenesis and cytoskeletal rearrangements during embryonic development. Moreover, the potential characteristics of $G\alpha_{13}$ and $G\alpha_{12}$ 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.

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