Leucine-rich glioma inactivated 3: Integrative analyses support its role in the cytokine network

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Abstract. Leucine-rich glioma inactivated (LGI)3 is a secreted protein member of LGI family. We previously reported that LGI3 was upregulated in adipose tissues from obese mice and suppressed adipogenesis through its receptor, a disintegrin and metalloproteinase domain-containing protein 23 (ADAM23). We demonstrated that LGI3 regulated tumor necrosis factor-α and adiponectin, and proposed that LGI3 may be a pro-inflammatory adipokine involved in adipose tissue inflammation. In this study, we analyzed adipokine and cytokine profiles in LGI3 knockout mice and demonstrated that multiple factors were increased or decreased in the adipose tissues and plasma of the LGI3 knockout mice. Phosphoprotein array analysis revealed increases in the phosphorylation levels of Akt, AMP-activated protein kinase (AMPK), Bad, extracellular signal-regulated kinase (Erk)1/2, glycogen synthase kinase 3α (GSK3α), phosphatase and tensin homolog (PTEN) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) in the LGI3-treated 3T3-L1 pre-adipocytes. Treatment with LGI3 increased the expression of various inflammatory genes in pre-adipocytes, adipocytes and macrophages. Integrative functional enrichment analysis for all LGI3-regulated gene products suggested their involvement in a number of biological processes, including cancer, inflammatory response, response to wounding, as well as cell proliferation and differentiation. Protein interaction network analysis of LGI3-regulated gene products revealed that 94% of the gene products formed a cluster of interaction networks. Taken together, these results support the critical involvement of LGI3 in the cytokine network by interplaying with multiple adipokines, cytokines and signaling proteins.

Introduction

Leucine-rich glioma inactivated (LGI)3 is a secreted protein of the LGI family that is predominantly expressed in the brain in a developmentally regulated manner (1). LGI3 has been shown to be regulated by neuronal restrictive silencer element and AP-2 at the transcription level (1). We previously reported that LGI3 regulates neuronal exocytosis and differentiation (2,3). Apart from the brain, LGI3 has been shown to be expressed in diverse tissues, including the skin and adipose tissues (4,5). In a previous study, we demonstrated that the ultraviolet B (UVB) irradiation-induced secretion of LGI3 from human keratinocytes promoted cell survival (5). We also demonstrated that LGI3 increased keratinocyte migration and melanocyte pigmentation (6,7).

We have also previously reported that LGI3 is upregulated in the adipose tissues of ob/ob mice and high fat diet-fed mice (4,8). We further demonstrated that LGI3 was downregulated during adipocyte differentiation, and suppressed adipogenesis through its receptor, a disintegrin and metalloproteinase domain-containing protein 23 (ADAM23) (4). LGI3 was shown to downregulate adiponectin, an anti-inflammatory adipokine (8). We also demonstrated that LGI3 increased the levels of pro-inflammatory proteins, including tumor necrosis factor-α (TNF-α) in macrophages (4). LGI3 and TNF-α are upregulated mutually through a key inflammatory transcription factor, nuclear factor-κB (NF-κB) (9). We proposed that LGI3 may be a pro-inflammatory adipokine that functionally interacts with other adipokines and cytokines in metabolic inflammation (4,8,9).

Our previous findings supported the hypothesis that LGI3 may be a multifunctional cytokine and may participate in the cytokine network in metabolic inflammation. LGI3 and its receptor, ADAM23, may transduce intracellular signals through Akt, focal adhesion kinase (FAK), mouse double minute 2 homolog (MDM2), p53, β-catenin and glycogen synthase kinase (GSK)3β (3,5,7).

In order to gain insight into the functional network of LGI3, in this study, we conducted experimental and integrative analyses of the gene products regulated by LGI3 and identified a cluster of protein interaction network that may represent an LGI3-regulated cytokine network.

Materials and methods

Animals and cell culture. All the animal protocols were approved by the Institutional Animal Care and Use Committee. LGI3 knockout mice were generated by Macrogen, Inc. (Seoul, Korea) (8). LGI3 knockout mice and wild-type

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littermates (n=3) were used in this study. White adipose tissues (WATs; epididymal fat) and plasma were obtained from 10-week-old LGI3 knockout mice and wild-type littermates. WAT was isolated after blood collection and after the mice were sacrificed. The culture of 3T3-L1 (ATCC CL-173) and RAW 264.7 (ATCC TIB-71) cells (ATCC, Manassas, VA, USA), and the differentiation of 3T3-L1 cells was carried out as previously described (4). Briefly, 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37°C in 5% CO_2. Two days after reaching confluence, the cells were cultured in DMEM containing 1 μg/ml insulin, 1 μM dexamethasone (Dex), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10% fetal bovine serum (FBS) and antibiotics for 3 days. The cells were then maintained in DMEM containing 1 μg/ml insulin, 10% FBS and antibiotics for 4 days. The medium was changed every 2 days until the cells were harvested. Differentiation was confirmed by Oil Red O staining (data not shown). RAW 264.7 cells were maintained in DMEM supplemented with 10% FBS. The cells were treated with LGI3 (10 ng/ml) or Dulbecco's phosphate-buffered saline (DPBS, control) for 1 or 24 h as indicated in the figure legends.

Preparation of recombinant LGI3 and protein analysis. The preparation of recombinant LGI3 protein and lysates of cells and tissues were carried out as previously described (3). Briefly, LGI3-His$_6$ protein was expressed in *E. coli* BL21(DE3) using pET28a(+) expression vector (Novagen, Madison, WI, USA) and chaperone system (pGro7) (Takara, Otsu, Japan). The protein was purified by Talon metal affinity resin (Clontech, Mountain View, CA, USA). Protein array analysis was performed using adipokine and cytokine array kits (R&D Systems, Inc., Minneapolis, MN, USA; product nos. ARY013 for 38 adipokines and ARY006 for 40 cytokines) according to the manufacturer's instructions. Briefly, the array membrane was incubated with tissue extracts in RIPA buffer (Sigma, St. Louis, MO, USA) and bound proteins were visualized by detection antibody cocktail and streptavidin-horseradish peroxidase using chemiluminescence detection reagent. Phosphoprotein array analysis was conducted using PathScan array kits (intracellular signaling array and Akt signaling antibody array kits; Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions.

Quantitative PCR. Quantitative PCR was performed as previously described (4). Total RNA was extracted using RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). Thermal cycling using Applied Biosystems StepOne system was, 10 min at 25°C, 120 min at 37°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The assay-on-demand gene expression products (Applied Biosystems, Foster City, CA, USA) were used to measure the mRNA expression levels of the following genes: CD11c, Mm00498698_m1; CD68, Mm03047340_m1; F4/80, Mm00802529_m1; interleukin (IL)-6, Mm00446190_m1; inducible nitric oxide synthase (iNOS, Mm00440502_m1); monocyte chemoattractant protein 1 (MCP-1), Mm00441242_m1); NADPH oxidase (NOX-2, Mm01287743_m1); p22phox, Mm00514478_m1; p47phox, Mm00447921_m1; p67phox, Mm00726636_s1; TNF-α, Mm00443260_g1; and 18S rRNA (endogenous control), Hs99999901_s1.

Functional enrichment and protein-protein interaction network analyses. For functional enrichment of a group of regulated genes, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment analyses were performed using the Database for Annotation, Visualization and Integration Discovery (DAVID) (http://david.abcc.ncifcrf.gov), as previously described (10). The results were ranked by their p-values and 20 entries with the lowest p-values were presented. The protein-protein interaction network was constructed using information provided by the Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org/) (11). Cluster analysis and GO enrichment analysis of subnetwork clusters were performed using ClusterONE application in Cytoscape 3.3.0, as previously described (12).

Statistical analysis. Data are expressed as the means ± standard error of the mean (SEM). Statistical significance between 2 groups was assessed using the Student's t-test. The results were considered statistically significant with a p-value <0.05.

Results

Effect of LGI3 knockout on adipokine and cytokine profiles. Homozygous mice with disruption of the LGI3 gene were viable and fertile, and exhibited no gross abnormalities under normal growth conditions (8). These mice exhibited increased levels of adiponectin in WAT and plasma, as described in our previous study (8). We analyzed cytokine and adipokine profiles using protein arrays in WAT and plasma of the wild-type and LGI3 knockout mice. The results revealed that the levels of multiple cytokines and adipokines were increased or decreased in LGI3 knockout mice (Fig. 1). The factors with increased expression were adiponectin, insulin-like growth factor-binding protein-1 (IGFBP-1), C-reactive protein (CRP), endocan, pre-adipocyte factor-1 (Pref-1), serpin E1, C5/C5a, macrophage colony-stimulating factor (M-CSF) (Fig. 1, a-h). The factors with decreased expression were insulin-like growth factor-1 (IGF-1), insulin-like growth factor binding protein-5 (IGFBP-5), B lymphocyte chemoattractant (BLC), granulocyte colony-stimulating factor (G-CSF), monocyte chemotactic protein 5 (MCP-5), macrophage inflammatory protein-2 (MIP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Fig. 1, i-o). An increase in the levels of adiponectin was observed both in WAT and plasma, as previously observed (8), and alterations in the levels of other factors were observed only in plasma (Fig. 1).

Effect of LGI3 treatment on the phosphorylation of signaling proteins. As shown in our previous study, LGI3 and its receptor ADAM23 was predominantly expressed at the pre-adipocyte stage in 3T3-L1 cells (4). Thus, LGI3 may transduce intracellular signals primarily in pre-adipocytes. To explore proteins that convey LGI3 signaling, we conducted phosphoprotein array analysis using the extracts from 3T3-L1 pre-adipocytes treated with LGI3 protein. The results revealed increases in the phosphorylation levels of extracellular signal-regulated kinase (Erk)1/2 (Thr202/Tyr204), Akt (Ser473), AMP-activated protein kinase (AMPK; Thr172), GSK3α (Ser21), Bad (Ser112), phosphatase and tensin homolog (PTEN) (Ser380) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Thr37/46) in the LGI3-treated cells (Fig. 2, a-g).
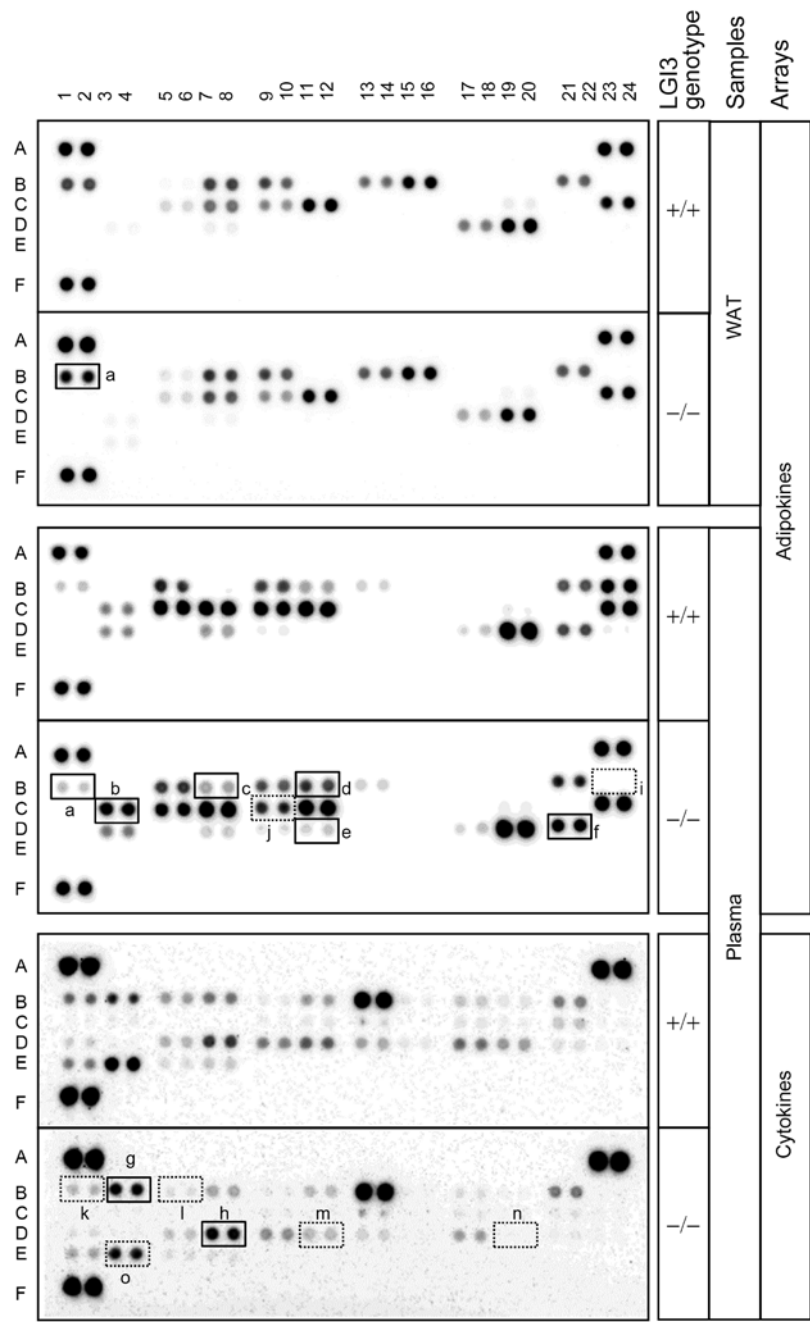
Figure 1. Effect of leucine-rich glioma inactivated 3 (LGI3) knockout on adipokine and cytokine profiles. White adipose tissue (WAT) and plasma from the wild-type (+/+) and homozygous LGI3 knockout (-/-) mice were analyzed by adipokine and cytokine arrays. Solid line box, increased proteins in knockout mice; dotted line box, decreased protein in knockout mice. a, adiponectin; b, IGFBP-1; c, C-reactive protein; d, endocan; e, pref-1; f, serpin E1; g, C5/C5a; h, M-CSF; i, IGF-1; j, IGFBP-5; k, BLC; l, G-CSF; m, MCP-5; n, MIP-2; o, TIMP-1. Numbers and capital letters indicate array coordinates.
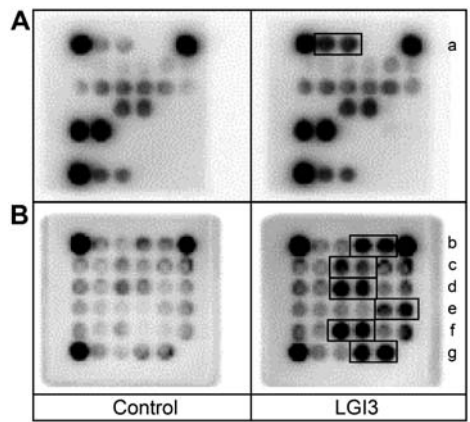

Figure 2. Effect of leucine-rich glioma inactivated 3 (LGI3) treatment on the phosphorylation of various signaling proteins. 3T3-L1 pre-adipocytes were treated with DPBS (control) or 10 ng/ml LGI3 for 1 h and the cell extracts were analyzed by PathScan phosphoprotein array kits [(A) intracellular signaling array; (B) Akt signaling antibody array]. Box, proteins with increased phosphorylation. a, Erk1/2 (Thr202/Tyr204); b, Akt (Ser473); c, AMPK (Thr172); d, GSK3α (Ser21); e, Bad (Ser112); f, PTEN (Ser380); g, 4E-BP1 (Thr37/46).
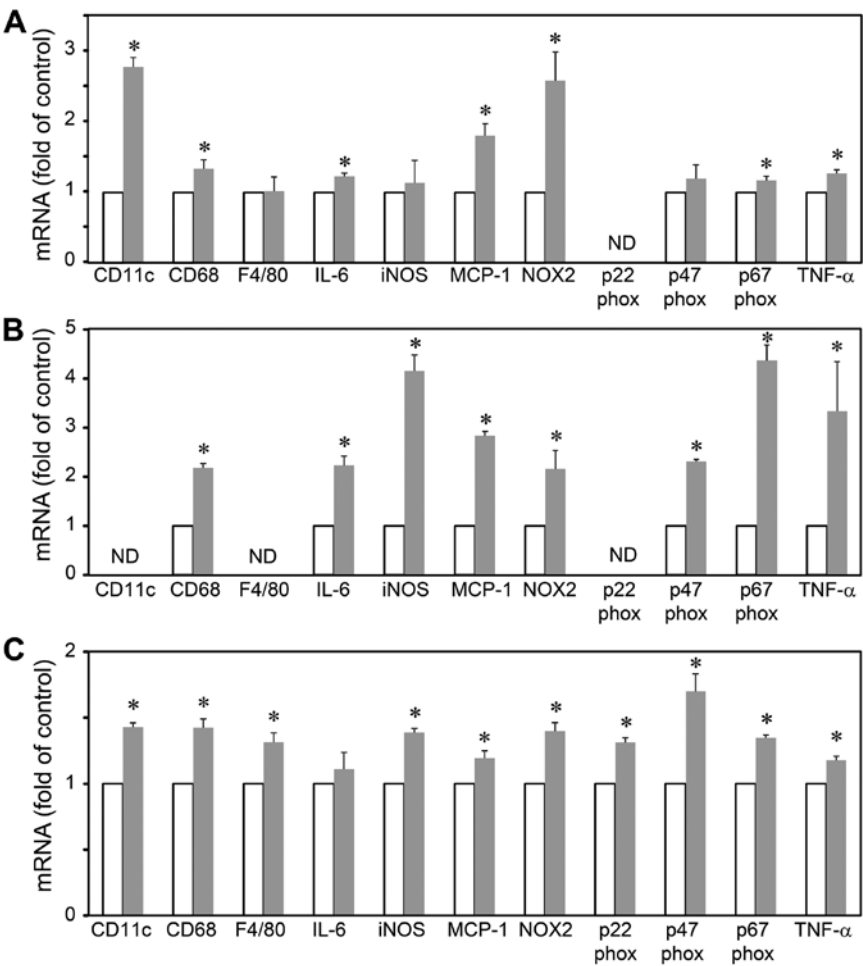
Figure 3. Effect of leucine-rich glioma inactivated 3 (LGI3) treatment on the expression of various inflammatory genes. (A) 3T3-L1 pre-adipocytes, (B) 3T3-L1 adipocytes and (C) RAW 264.7 cells were treated with DPBS (open bar, control) or 10 ng/ml LGI3 for 24 h (closed bar) and the cell extracts were analyzed by quantitative PCR. ND, not detected. $^*p<0.05$ vs. control.
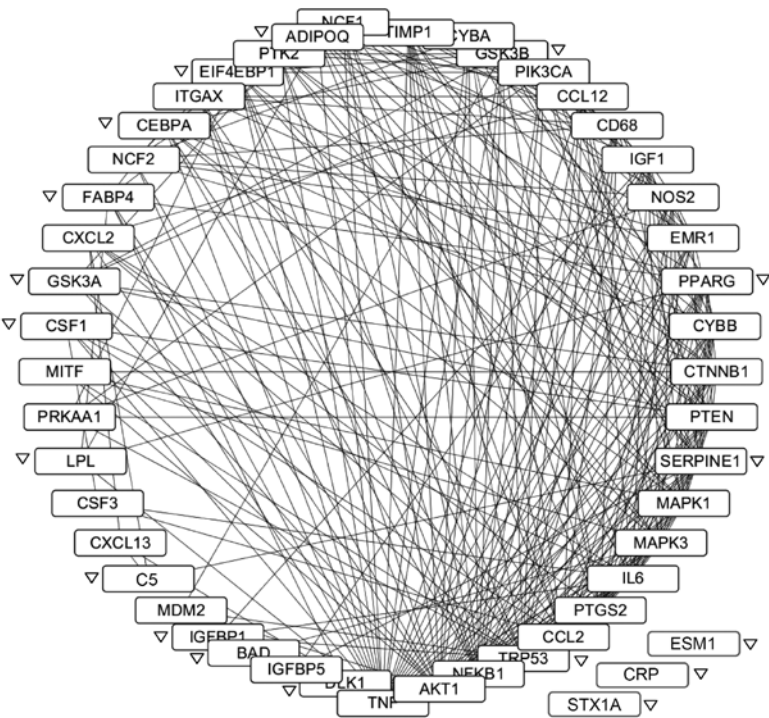

Figure 4. Protein-protein interaction network of leucine-rich glioma inactivated 3 (LGI3)-regulated gene products. The network was depicted by nodes (gene products) and lines (pairwise protein interactions) sorted by interaction degree. Downregulated gene products are indicated by downward arrowheads.

Table I. Summary of LGI3-regulated gene products from the present study and our previous studies.

Upregulated genes	Downregulated genes
Adipokine/cytokine arrays[a]	Adipokine/cytokine arrays[a]
BLC (CXCL13)	Adiponectin (ADIPOQ) (8)
G-CSF (CSF3)	C5/C5a (C5, HC)
IGF-1 (IGF1)	C-reactive protein (CRP)
IGFBP-5 (IGFBP5)	Endocan (ESM1)
MCP-5 (CCL12)	IGFBP-1 (IGFBP1)
MIP-2 (CXCL2)	M-CSF (CSF1)
TIMP-1 (TIMP1)	Pref-1 (DLK1)
	Serpin E1 (SERPINE1)
PathScan	PathScan[b]
Akt (AKT1) (3)	4E-BP1 (EIF4EBP1)
AMPKα (PRKAA1)	Bad (BAD)
Erk1/2 (MAPK3/1)	GSK3α (GSK3A)
PTEN (PTEN)	
Quantitative PCR	Previous studies
CD11c (ITGAX)	C/EBPα (CEBPA) (4)
CD68 (CD68)	FABP4 (FABP4) (4)
F4/80 (EMR1)	GSK3β (GSK3B) (7)
IL-6 (IL6)	LPL (LPL) (4)
iNOS (NOS2) (4)	p53 (TP53) (5)
MCP-1 (CCL2) (9)	PPARγ (PPARG) (4)
NOX-2 (CYBB)	Syntaxin 1 (STX1A)[c] (2)
p22phox (CYBA)	
p47phox (NCF1)	
p67phox (NCF2)	
TNF-α (TNF) (4,9)	
Previous studies	
β-catenin (CTNNB1) (7)	
Cox-2 (PTGS2) (4)	
FAK (PTK2) (3)	
MDM2 (MDM2) (5)	
MITF (MIFT) (6)	
NF-κB (NFKB1) (9)	
PI3K (PIK3CA) (3)	

[a]Upregulated proteins in LGI3 knockout mice were presumed to be downregulated proteins by LGI3 and vice versa for downregulated proteins in LGI3 knockout mice; [b]proteins that are downregulated by phosphorylation; [c]syntaxin 1 was presumed to be functionally down-regulated by LGI3, as in our previous study (2). Names in parenthesis are official gene symbols. Numbers in parenthesis correspond to reference numbers.

Effect of LGI3 treatment on the expression of inflammatory genes. As shown in our previous study, treatment with LGI3 protein increased the level of TNF-α in 3T3-L1 pre-adipocytes, and those of iNOS, cyclooxygenase (Cox)-2 and TNF-α in RAW 264.7 macrophages (4,9). We thus hypothesized that LGI3 may upregulate inflammatory genes in adipose tissues from obese mice (4). We thus examined the effects of LGI3

treatment on expression of various inflammatory genes in pre-adipocytes, adipocytes and macrophages (Fig. 3). The results revealed that LGI3 increased the levels of CD11c, CD68, IL-6, MCP-1, NOX-2, p67phox and TNF-α in the 3T3-L1 pre-adipocytes (Fig. 3A). In the 3T3-L1 adipocytes, LGI3 increased the expression of CD68, IL-6, iNOS, MCP-1, NOX-2, p47phox, p67phox and TNF-α (Fig. 3B). The LGI3-treated RAW 264.7 cells exhibited an increased expression of CD11c, CD68, F4/80, iNOS, MCP-1, NOX-2, p22phox, p47phox, p67phox and TNF-α (Fig. 3C).

Integrative functional enrichment analysis of LGI3-regulated genes. The results of adipokine and cytokine arrays, phospho-protein arrays and quantitative PCR revealed 23 gene products upregulated and 11 gene products that were downregulated by LGI3 in this study (Table I and Figs. 1-3). The proteins that were increased or decreased in LGI3 knockout mice were presumed to be the proteins downregulated or upregulated by LGI3, respectively. The findings of our previous studies indicated that 14 genes were regulated positively or negatively by LGI3 (Table I) (2-7). We conducted functional enrichment analysis for LGI3-regulated gene products using integrative database of functional annotations (Table II). GO terms of upregulated genes with the highest significance were associated with various types of cancer, inflammatory responses, apoptosis and response to wounding (Table IIA). Downregulated genes were categorized with the highest significance into the terms of cell differentiation, growth and homeostasis (Table IIB). Functional annotation clustering analysis of all LGI3-regulated genes revealed that the 5 functional clusters with the highest scores, included response to endogenous and hormone stimuli, regulation of cell proliferation, response to wounding, inflammatory response and regulation of apoptosis (Table III).

Protein-protein interaction network of LGI3-regulated gene products. The protein-protein interaction network of LGI3-regulated genes was constructed (Fig. 4). All gene products except for endocan, CRP and syntaxin 1A formed an interaction cluster. A total of 270 interactions were observed involving 45 gene products. TNF-α, Akt, NF-κB, p53 and CCL2 (MCP-1) were the gene products with the highest degree of interactions. All the genes upregulated by LGI3 and 83% (15/18) of the downregulated genes participated in the network. Four subnetworks were identified by ClusterONE (p<0.01, data not shown). Functional enrichment analysis indicated that the subnetworks were associated with developmental process, the regulation of apoptosis, response to hormone stimuli and response to stress (data not shown).

Discussion

LGI3 has been postulated to participate in the adipokine network through the regulation of adiponectin and TNF-α in obesity-associated metabolic inflammation (8,9). Among the multiple molecular mass forms of LGI3 (75-, 60- and 35-kDa), 60-kDa LGI3 has been shown to be selectively increased in obese adipose tissues and has been suggested to be a major adipokine form (4,8). LGI3 knockout mice exhibit a selective ablation of 60-kDa LGI3 (8). The alteration of various

Table II. Functional enrichment analysis of the gene products regulated by LGI3.

A, Gene products regulated positively by LGI3

Category	Term	P-value
KEGG_PATHWAY	mmu05200:Pathways in cancer	1.24E-12
KEGG_PATHWAY	mmu05215:Prostate cancer	3.67E-12
KEGG_PATHWAY	mmu04062:Chemokine signaling pathway	2.03E-10
KEGG_PATHWAY	mmu05218:Melanoma	6.76E-10
KEGG_PATHWAY	mmu05213:Endometrial cancer	2.56E-09
KEGG_PATHWAY	mmu04621:NOD-like receptor signaling pathway	9.11E-09
KEGG_PATHWAY	mmu04510:Focal adhesion	1.58E-07
GOTERM_BP_FAT	GO:0006954-inflammatory response	2.74E-07
KEGG_PATHWAY	mmu05214:Glioma	3.74E-07
GOTERM_CC_FAT	GO:0005829-cytosol	6.18E-07
GOTERM_BP_FAT	GO:0042981-regulation of apoptosis	9.91E-07
KEGG_PATHWAY	mmu04370:VEGF signaling pathway	1.05E-06
KEGG_PATHWAY	mmu05220:Chronic myeloid leukemia	1.05E-06
GOTERM_BP_FAT	GO:0043067-regulation of programmed cell death	1.10E-06
GOTERM_BP_FAT	GO:0010941-regulation of cell death	1.15E-06
KEGG_PATHWAY	mmu05222:Small cell lung cancer	2.04E-06
KEGG_PATHWAY	mmu05210:Colorectal cancer	2.19E-06
KEGG_PATHWAY	mmu04012:ErbB signaling pathway	2.34E-06
KEGG_PATHWAY	mmu04150:mTOR signaling pathway	4.22E-06
GOTERM_BP_FAT	GO:0009611-response to wounding	4.97E-06

B, Gene products regulated negatively by LGI3

Category	Term	P-value
GOTERM_BP_FAT	GO:0010743-regulation of foam cell differentiation	1.63E-07
GOTERM_BP_FAT	GO:0040008-regulation of growth	2.40E-05
GOTERM_BP_FAT	GO:0001558-regulation of cell growth	4.64E-05
GOTERM_BP_FAT	GO:0010558-negative regulation of macromolecule biosynthetic process	8.43E-05
GOTERM_BP_FAT	GO:0031327-negative regulation of cellular biosynthetic process	8.97E-05
GOTERM_BP_FAT	GO:0009890-negative regulation of biosynthetic process	1.01E-04
INTERPRO	IPR000867:Insulin-like growth factor-binding protein, IGFBP	1.43E-04
KEGG_PATHWAY	bta03320:PPAR signaling pathway	1.89E-04
GOTERM_BP_FAT	GO:0010605-negative regulation of macromolecule metabolic process	1.95E-04
GOTERM_BP_FAT	GO:0050873-brown fat cell differentiation	2.59E-04
GOTERM_MF_FAT	GO:0005520-insulin-like growth factor binding	2.89E-04
SMART	SM00121:IB -insulin growth factor-binding protein homologues	3.64E-04
GOTERM_BP_FAT	GO:0042127-regulation of cell proliferation	3.81E-04
GOTERM_CC_FAT	GO:0005576-extracellular region	5.76E-04
GOTERM_BP_FAT	GO:0045444-fat cell differentiation	6.34E-04
GOTERM_BP_FAT	GO:0042592-homeostatic process	9.81E-04
GOTERM_MF_FAT	GO:0019838-growth factor binding	1.90E-03
GOTERM_MF_FAT	GO:0046983-protein dimerization activity	2.46E-03
GOTERM_BP_FAT	GO:0048878-chemical homeostasis	2.81E-03
GOTERM_BP_FAT	GO:0045596-negative regulation of cell differentiation	4.73E-03

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adipokines and cytokines in LGI3 knockout mice supports the widespread functional interaction of adipokines and cytokines with LGI3 (Fig. 1). The increased levels of adipokines and cytokines in LGI3 knockout mice may represent compensatory

Table III. Functional annotation clustering analysis of LGI3-regulated gene products.

Category	Term	Count	P-value
Annotation cluster 1	Enrichment score: 10.13		
GOTERM_BP_FAT	GO:0009719-response to endogenous stimulus	19	3.63E-13
GOTERM_BP_FAT	GO:0009725-response to hormone stimulus	18	7.76E-13
GOTERM_BP_FAT	GO:0048545-response to steroid hormone stimulus	14	1.85E-11
GOTERM_BP_FAT	GO:0010033-response to organic substance	20	1.22E-10
GOTERM_BP_FAT	GO:0051384-response to glucocorticoid stimulus	9	1.27E-08
GOTERM_BP_FAT	GO:0031960-response to corticosteroid stimulus	9	1.95E-08
Annotation cluster 2	Enrichment score: 9.36		
GOTERM_BP_FAT	GO:0042127-regulation of cell proliferation	19	4.52E-12
GOTERM_BP_FAT	GO:0048545-response to steroid hormone stimulus	14	1.85E-11
GOTERM_BP_FAT	GO:0008284-positive regulation of cell proliferation	11	9.64E-07
Annotation cluster 3	Enrichment score: 8.51		
GOTERM_BP_FAT	GO:0009611-response to wounding	16	1.06E-11
GOTERM_BP_FAT	GO:0006954-inflammatory response	11	4.10E-09
GOTERM_BP_FAT	GO:0006952-defense response	13	1.15E-08
GOTERM_BP_FAT	GO:0006955-immune response	12	1.84E-07
Annotation cluster 4	Enrichment score: 7.49		
GOTERM_BP_FAT	GO:0042981-regulation of apoptosis	17	7.48E-10
GOTERM_BP_FAT	GO:0043067-regulation of programmed cell death	17	9.07E-10
GOTERM_BP_FAT	GO:0010941-regulation of cell death	17	9.67E-10
GOTERM_BP_FAT	GO:0043066-negative regulation of apoptosis	13	1.49E-09
GOTERM_BP_FAT	GO:0043069-negative regulation of programmed cell death	13	1.77E-09
GOTERM_BP_FAT	GO:0060548-negative regulation of cell death	13	1.83E-09
GOTERM_BP_FAT	GO:0043065-positive regulation of apoptosis	9	2.13E-05
GOTERM_BP_FAT	GO:0043068-positive regulation of programmed cell death	9	2.22E-05
GOTERM_BP_FAT	GO:0010942-positive regulation of cell death	9	2.37E-05
Annotation cluster 5	Enrichment score: 4.81		
GOTERM_BP_FAT	GO:0040007-growth	10	6.21E-08
GOTERM_BP_FAT	GO:0031099-regeneration	7	3.77E-06
GOTERM_BP_FAT	GO:0042060-wound healing	6	4.02E-04
GOTERM_BP_FAT	GO:0042246-tissue regeneration	4	5.73E-04

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upregulation under LGI3 deficiency or may be the factors that are negatively regulated by LGI3 in wild-type mice (Fig. 1, a-h). Downregulated factors in LGI3 knockout mice suggested that LGI3 may cooperate with these factors by positive regulation. These results revealed the adipokine and cytokine network regulated by LGI3 that may play a role in fine-tuning responses to metabolic perturbation and other stimuli.

Intracellular signaling pathways of LGI3 were explored in our previous studies. In neuronal cells, LGI3-promoted neurite outgrowth was mediated by Akt and FAK (3). MDM2 and p53 were shown to be involved in LGI3-promoted survival under UVB irradiation in keratinocytes (5). Keratinocyte migration induced by LGI3 was shown to employ β-catenin and GSK3β (7). The present study on pre-adipocytes demonstrated that LGI3 treatment regulated a multitude of signaling proteins (Erk1/2, AMPK, GSK3α, Bad, PTEN and 4E-BP1, as well as previously observed Akt) (Fig. 2, a-g) (3,7). These results suggested that the intracellular signaling of LGI3 may consist of common

and unique signaling components in different target cell types. The suppressive effect of LGI3 on adipogenesis was shown to be mediated by its receptor, ADAM23 (4). ADAM22, other putative LGI3 receptor abundantly expressed in brain, was not expressed in adipose tissues and 3T3-L1 cells (4). Thus, LGI3 and ADAM23 may transduce intracellular signaling through these proteins (Fig. 2, a-g) in pre-adipocytes in adipose tissues.

We previously proposed that LGI3 may be a pro-inflammatory adipokine based on its upregulating effect on COX-2, iNOS, MCP-1, TNF-α and NF-κB (4,9). In this study, LGI3 increased the expression of multiple inflammatory genes differentially in pre-adipocytes, adipocytes and macrophages that are the predominant cell types in adipose tissues (Fig. 3). All of the LGI3-regulated inflammatory gene products have been previously reported to be associated positively with obesity-associated metabolic disorders (13-23). In this study, following treatment with LGI3, the significant upregulation of CD68, MCP-1, NOX-2, p67phox and TNF-α was observed in 3 cell types. F4/80 and p22phox

were only upregulated in RAW 264.7 macrophages (Fig. 3C). As LGI3 was previously shown to be secreted predominantly by pre-adipocytes and macrophages (4), these results suggested that LGI3 may act as an autocrine and paracrine adipokine that conveys pro-inflammatory stimuli on target cells.

The findings of the present study and those of previous studies demonstrated that an array of proteins were regulated by LGI3 in different cell types (Table I). As expected, functional enrichment analysis revealed that LGI3-regulated gene products were associated with inflammatory responses and related pathways, such as chemokines, apoptosis, focal adhesion (Table IIA) and with cell growth and differentiation (Table IIB). It is noted that GO terms of the genes upregulated by LGI3 were associated with various cancers including prostate, melanoma, endometrial and glioma cancers (Table IIA). A previous study demonstrated that LGI3 was expressed at high levels in glioma, neuroblastoma, melanoma, colon and breast cancer cells (24). Among four LGI family members, LGI3 was the only member expressed at very high levels in gliomas, melanomas and neuroblastoma cells (24). Cytokine networks play regulatory roles in cancer through inflammatory immune responses to tumors (25,26) or chronic and carcinogenic inflammation (25). TNF-α and adiponectin have been described to be risk factors and potential prognostic biomarkers in various types of cancer (26,27). Since LGI3 has been shown to regulate adiponectin and TNF-α (8,9), LGI3 may also be associated with cytokine networks in cancer. Furthermore, functional annotation clustering analysis supported the notion that LGI3 may act as a pleiotropic cytokine in various biological processes such as hormonal stimuli, proliferation, wounding and inflammatory responses and apoptosis (Table III).

It is remarkable that a majority of LGI3-regulated gene products formed a cluster of protein-protein interaction network (Fig. 4). The upregulation of LGI3 in obese adipose tissues has been proposed to contribute to perturbation of cytokine network in metabolic inflammation (4,8). The LGI3-regulated protein interaction network may account for the complex mechanisms in dysregulation of adipose tissue homeostasis in obesity. This notion was supported by the proteins with the highest degree of interactions in the network (TNF-α, Akt, NF-κB, p53, CCL2, IL-6, Erk2, serpin E1, PTEN, PPARγ and IGF-1) that were previously reported to be involved in obesity-associated metabolic disorders (15,16,28-34). Since NF-κB, a crucial inflammatory transcription factor, was shown to mediate mutual upregulation of LGI3 and TNF-α in adipose tissues (9), TNF-α and NF-κB may be the major players that mediate the effect of LGI3 on this network.

In conclusion, the present study presented an integrative insight into LGI3-regulated gene products identified in this study and our previous studies. High degree interactions within the network of LGI3-regulated gene products suggested that LGI3 may be profoundly involved in a wide variety of biological processes regulated by cytokines. These findings supported our hypothesis that LGI3 is an adipokine that participates in cytokine networks involved in metabolic inflammation and related disorders.

Acknowledgements

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