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Identification of FHL2-Regulated Genes in Liver by Microarray and Bioinformatics Analysis

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ABSTRACT

FHL2 is a LIM domain protein that is able to form various protein complexes and regulate gene transcription. Recent findings showed that FHL2 is a potential tumor suppressor gene that was down-regulated in hepatocellular carcinoma. In the present study, microarray profiling of gene expression was performed to identify the genes regulated by FHL2 in mouse livers. The differentially expressed genes were further analyzed by bioinformatics tools including DAVID, KEGG, and STRING. Our data illustrate that FHL2 affects genes involved in various functions including signal transduction, responses to external stimulus, cancer-related pathways, cardiovascular function and regulation of actin cytoskeleton. Moreover, a network of differentially expressed genes identified in this study and known FHL2-interacting proteins was constructed. Then, genes identified by bioinformatics tools and most functional relevant to FHL2 were selected for further validation. Finally, the differential expression of *Ar*, *Id3*, *Inhbe*, *Alas1*, *Bcl6*, *Pparδ*, *Angptl4*, and *Erbb4* were confirmed by quantitative real-time PCR. In summary, we have established a database of genes that are potentially regulated by FHL2 and these genes should be future targets for the elucidation of functional roles of FHL2. J. Cell. Biochem. 115: 744–753, 2014.

KEY WORDS: FHL2; KNOCKOUT MICE; GENE REGULATION; MICROARRAY

FHL2 (Four and a Half LIM protein-2) is one of the five members of a small protein family which share a similar protein structure consisting of only cysteine-rich zinc finger LIM domains [Scholl et al., 2000]. FHL proteins are known to function as adaptor proteins that are able to form protein complexes and regulate gene transcription. FHL2, which is the best studied member of the family, has been shown to have diverse biological functions which are related to development, cell differentiation, maintenance of cytoskeleton and cell integrity, regulation of gene expression [Johannessen et al., 2006].

FHL2 is predominantly expressed in the heart tissue. But the expression is also present in a wide range of normal human tissues [Chan et al., 1998; Kong et al., 2001; Muller et al., 2002; Gabriel et al., 2004]. FHL2 has been implicated in many forms of cancers in which its expression was found to be deregulated, either being over-expressed or down-regulated (reviewed in [Kleiber et al., 2007]). The function of FHL2 in cancers is particularly intriguing because FHL2 can function as an oncoprotein or as a tumor suppressor in tissue-specific fashion. Interaction with different protein partners may underlie the functional diversity of FHL2. We have previously reported the interaction of FHL2 with FHL3 [Li et al., 2001], CDC47 [Chan et al., 2000] and NP220 [Ng et al., 2002]. FHL2 can also function as co-activators of several transcription factors including androgen receptor (AR) [Muller et al., 2000], activator protein-1 (AP-1) [Morlon and Sassone-Corsi, 2003], cyclic AMP response element binding protein, cAMP response element modulator [Fimia et al., 2000], breast cancer 1 (BRCA1) [Yan et al., 2003], Wilms′ tumor 1 (WT-1) [Du et al., 2002], nuclear factor-κB (NF-κB) [Stilo et al., 2002; Labalette et al., 2004], and p38 MAPK [Wong et al., 2012]. Moreover, it could function as the co-suppressor for extracellular signal-regulated kinase 2 [Purcell et al., 2004], serum response factor [Philippar et al., 2004], and forkhead box O1 (FOXO1) [Yang et al., 2005]. Previously, it was shown that FHL2 may have a tumor suppressor function in hepatocellular carcinoma (HCC). FHL proteins may suppress HCC cell growth through a TGF-β-like signaling pathway [Ding et al., 2009]. Recently, we suggested the anti-proliferative and anti-apoptotic activities of FHL2 [Ng et al., 2011a] and characterized the FHL2 transcript variants in liver cancer cells [Ng et al., 2011b]. However, the role of FHL2 in cancer development has not been well elucidated.

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Knockout animal model are useful for studying gene functions. FHL2 knockout mice have recently been generated and they appear to be viable and exhibit normal cardiac development and function, but show exaggerated response to cardiac hypertrophic effect following β-adrenergic stimulation in the heart [Kong et al., 2001]. Besides, FHL2 deficiency in mice was found to result in osteopenia due to decreased activity of osteoblasts [Bai et al., 2005] as well as impaired skin wound healing [Wixler et al., 2007]. FHL2 may also play a role in myocardial inflammation and ischemic injury as it is associated with up-regulation of cytokine genes in response to cardiac injury [Wan et al., 2002; Sun et al., 2006]. In this study, we conducted a microarray study to analyze the gene expression in liver of FHL2-deficient mice. A similar microarray study has been reported but in immortalized FHL2$^{-/-}$ mouse embryonic fibroblasts (MEF) [Labalette et al., 2008]. Interestingly, FHL2$^{-/-}$ cells showed a lower rate of proliferation than WT MEFs. FHL2 is shown to play key roles in regulating cell cycle-related proteins and cell proliferation. Since FHL2 interacts with many transcription factors, it may be possible that FHL2 could affect various signaling pathways. The microarray analysis of FHL2 knockout mice could provide information of the genes that are regulated by FHL2 at the whole genome level.

In the present study, microarray gene expression profiling and bioinformatics analysis was performed to identify the genes regulated by FHL2 in liver. Genes identified by bioinformatics tools and most functional relevant to FHL2 were selected for further validation. Finally, the differential expression of *Ar*, *Id3*, *Inhbe*, *Alas1*, *Bcl6*, *Pparδ*, *Angptl4*, and *Erbb4* were confirmed by quantitative real-time PCR.

MATERIALS AND METHODS

FHL2 KNOCKOUT MICE

FHL2 knockout mice were generated by deletion mutation at the *FHL2* locus as described previously [Chu et al., 2000]. The animals are made on the hybrid Black Swiss-129-SV/J background. Mice were maintained and the heterozygous pairs were bred to generate homozygous knockout (KO) and wild-type (WT) littermates for experiments. The genotypes of mice from all matings were determined by polymerase chain reaction (PCR) on DNA isolated from tail biopsy specimens with the following primers: neo1, 5′-GGATCGGCCATTGAACAAGATG-3′; neo2, 5′-GAGCAAGGT GAGATGACAGG AG-3′; FHL2-P2, 5′-AGCATGACTGAACGCTTTGACT-3′; FHL2-P3, 5′-GGTAACCAG AACAGGGAGAGTG-3′. PCR conditions were as follows: denaturation at 94°C for 4 min, followed by 31 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C. All animals were housed in the Laboratory Animal House of CUHK and maintained under standard conditions.

RNA EXTRACTION

Male WT and KO mice (10–11 weeks old) were sacrificed by cervical dislocation. The whole liver from each of three KO and three WT mice was quickly excised, rinsed in ice-cold PBS, and the different lobes of liver were immediately snap frozen in liquid nitrogen and preserved at −80°C. The left lobe of the liver was used for RNA extraction. Tissues were homogenized using a rotor-stator homogenizer. Total RNA was extracted using Trizol Reagent (Invitrogen) and the RNeasy Mini Kit (Qiagen) according to the manufacturer′s protocol. The concentration and purity of RNA was assessed using Nanodrop ND-1000. The quality and integrity of RNA was checked by agarose gel electrophoresis.

MICROARRAY EXPERIMENT

Aliquots of RNA samples from each of three mice described above were pooled for microarray experiment to minimize individual variability within groups. For gene expression profiling, Affymetrix GeneChip Mouse Gene ST 1.0 Array which contains probe sets for >28,000 mouse genes was used (one microarray for each sample). Briefly, 300 ng of total RNA was reverse transcribed, labeled and hybridized to the arrays according to the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay protocol. Following hybridization, the arrays were washed and stained using the GeneChip fluidics station 450 (Affymetrix) and scanned using GeneArray scanner 3000 (Affymetrix) as described in the protocol. The data was then extracted from the scanned images and checked for quality control with Affymetrix Expression Console software v.1.0. The intensity data between gene chips was normalized using RMA normalization method and expression data analysis was further performed using Partek Genomics Suite v.6.4. The raw and processed data has been deposited in the NCBI Gene Expression Omnibus (GEO) database (Accession Number: GSE26904) in a MIAME-compliant format.

MICROARRAY DATA ANALYSIS

Functional Annotation Clustering. Genes associated with enriched biological pathways were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID). [Huang da et al., 2007, 2009] (http://david.abcc.ncifcrf.gov/). The list of genes with at least 1.5-fold change in gene expression was analyzed using the DAVID Gene Functional Classification Tool to generate a summary of enriched annotation terms ordered by the enrichment *P*-value (EASE score).

KEGG. For pathway analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) module within DAVID was used. Statistical analysis for GO terms and pathways were performed using a modified Fisher Exact Test (EASE score/*P*-value).

Gene Network Analysis. The network analysis of differentially expressed genes were performed using the Search Tool for the Retrieval of Interacting Genes (STRING) v.9.05 (http://string-db.org/) [Franceschini et al., 2013]. STRING is an open-access tool for the analysis of gene-gene or protein–protein interactions by using four different sources to quantitatively integrate interactions. The list of 1.5-fold change genes and 44 known FHL2 interacting proteins was uploaded to STRING. Subsequently, networks with combined score ≥0.8 in STRING were merged and analyzed with Cytoscape v.2.8.3 (http://www.cytoscape.org/), a network visualization platform that enables network analysis and manipulation of a wide variety of plug-ins [Smoot et al., 2011]. To discover more target genes that may be co-regulated with FHL2 interacting partners, a list of 44 well-known FHL2 interacting proteins was retrieved from Biological General Repository for Interaction Datasets (BioGRID) version 3.2.101 (http://thebiogrid.org/) and GeneCards (http://www.genecards.org/; Table I).

TABLE I. Known FHL2 Interacting Proteins

Gene	Entry	#Exp.	Affinity capture-western	Reconstituted complex	Two-hybrid	FRET	Affinity capture-MS
ADAM17	P78536	2		1	1		
AK1	P00568	1		1			
AR	P10275	3	1	1	1		
ATF3	P18847	2	1		1		
BMPR2	Q13873	2	1		1		
BRCA1	P38398	7	2	2	3		
CARD8	Q9Y2G2	2	1		1		
CKM	P06732	1		1			
CREB1	P16220	2	1		1		
CREBBP	Q92793	2	1		1		
CTNNB1	P35222	3	1	1	1		
EGFR	P00533	2	2				
EIF6	P56537	2	1		1		
EP300	Q09472	1	1				
FHL1	Q13642	1		1			
FHL2	Q14192	3	1		2		
FHL3	Q13643	3			2	1	
FOXO1	Q12778	4	2	1	1		
HAND1	O96004	1	1				
IGFBP5	P24593	3	1	1	1		
ITGA3	P26006	2	1		1		
ITGA7	Q13683	2	1		1		
ITGB1	P05556	2	1		1		
ITGB2	P05107	2	1		1		
ITGB6	P18564	2	1		1		
MAPK1	P28482	3	1	1	1		
PFKM	P08237	1		1			
PSEN2	P49810	2	1		1		
REV1	Q9UBZ9	2	1		1		
RUNX1	Q01196	1	1				
RUNX2	Q13950	2	1	1			
SIRT1	Q96EB6	2	2				
SPHK1	Q9NYA1	2	1		1		
SPHK2	Q9NRA0	1	1				
SRF	P11831	1	1				
STAT3	P40763	2	1		1		
TNFRSF11A	Q9Y6Q6	1	1				
TRAF6	Q9Y4K3	1	1				
TTN	Q8WZ42	2		1	1		
UBC	P0CG48	5					5
WT1	P19544	2	1		1		
ZBTB16	Q05516	3	1	1	1		
ZNF408	Q9H9D4	2	1		1		
ZNF638	Q14966	2			1	1	

Interacting proteins that were only proved by yeast two-hybrid system were not included in the analysis.

VALIDATION OF MICROARRAY DATA BY REAL-TIME PCR

The expression of genes was validated by quantitative real-time PCR (qPCR). The primer sequences used for validation are listed in Table II. Individual RNA samples from each of three KO and three WT mice were analyzed. Briefly, 1 μg of total RNA was converted to cDNA in a 20 μl reaction using QuantiTect Reverse Transcription Kit (Qiagen). Then, 2 μl of 1:10 diluted cDNA was amplified in a volume of 10 μl consisting of 125 nM primer, 1× Power SYBR Green PCR Master Mix (Applied Biosystems) on a ABI 7500 Fast Real-time PCR system (Applied Biosystems). The PCR cycling conditions included an initial denaturation step of 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. Comparative C_T method was used to calculate the relative gene expression normalized to the endogenous control β-actin. No primer dimer was detected in this qPCR after gel electrophoresis examination.

STATISTICAL ANALYSIS

The results shown represent mean ± SD from triplicate samples unless otherwise specified. For comparison between two groups of mice, statistical significance was evaluated by two-tailed Mann–Whitney test. $P < 0.05$ is considered to be statistically significant. Statistical analyses were performed by using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego).

TABLE II. List of Oligonucleotide Primers Used in This Study

Gene name	Forward (F) and reverse (R) primer sequence (5′-3′)	Product size (bp)
Saa2	F-CCCTGCTCCTGGGAGTCTGC	64
	R-GCCCCTTGGAAAGCCTCCCC	
Onecut1	F-CCTGAATGCCCAGGGCCACG	67
	R-GCCGGTCACCGAAGGGTTGG	
Lcn2	F-GCCTTGCCCTGCTTGGGGTC	108
	R-CGCTCCGGAAGTCTGGCTGC	
Meg3	F-TTCCTTCACAGCCCCGGCTTC	127
	R-GGCCTGAGCGAGAGCCGTTC	
Creld2	F-GCAGTTCTCGGTGGGTCGCC	147
	R-GTCCACCAGCGTCCGGCATC	
Ppp1r10	F-CCGCCACCACCTCCATTCCG	110
	R-CGGTGCCCACCACCTCCAAC	
Ccnd1	F-AGAGGGCTGTCGGCGCAGTA	119
	R-GGCTGTGGTCTCGGTTGGGC	
Ar	F-CTGGGAAGGGTCTACCCAC	128
	R-GGTGCTATGTTAGCGGCCTC	
Gja1	F-ACAGCGGTTGAGTCAGCTTG	106
	R-GAGAGATGGGGAAGGACTTGT	
Bcl6	F-GCCAGCCAAGAGCCCCACTG	107
	R-CCTGCTCAGAGCCCTCGGGT	
Inhbe	F-AAAAGCCCAGCTCTGGCTAAT	165
	R-CTGGTTAGGTGCAGTCCCTC	
Id3	F-TCTCCAACATGAAGGCGCTG	125
	R-GCTAAGAGGCTCCTCGGTCG	
Nr4a1	F-CTTCGGCGTCCTTCAAGTTTG	154
	R-GGCTGGAAGTTGGGTGTAGA	
Ppard	F-TCCATCGTCAACAAAGACGGG	109
	R-ACTTGGGCTCAATGATGTCAC	
Alas1	F-TCGCCGATGCCCATTCTTATC	109
	R-GGCCCCAACTTCCATCATCTT	
Erbb4	F-GTGCTATGGACCCTACGTTAGT	102
	R-TCATTGAAGTTCATGCAGGCAA	
Angptl4	F-CATCCTGGGACGAGATGAACT	136
	R-TGACAAGCGTTACCACAGGC	
β-actin	F-AGAGGGAAATCGTGCGTGAC	138
	R-CAATAGTGATGACCTGGCCGT	

RESULTS

MICROARRAY RESULTS AND VALIDATION

The gene expression pattern of FHL2 KO mice and WT mice was compared. There were totally 34 genes which showed twofold up- or down-regulation (Table III). qPCR was used for validating the gene expression data obtained from the microarray experiment. The expression levels of seven representative genes (*Saa2*, *Onecut1*, *Lcn2*, *Meg3*, *Creld2*, *Ppp1r10*, *Ccnd1*) selected by different analysis methods were determined. As shown in Figure 1, the qPCR results were in good concordance with the microarray results.

BIOINFORMATICS ANALYSIS

DAVID. To perform an integrative comparison between gene expression and biological function information, the list of 337 differentially expressed genes with a fold change >1.5 was submitted to DAVID. The genes were classified according to the GO information including biological process, molecular function and cellular component. Results shown in Table IV represent the annotation clusters of GO terms obtained from the Gene Functional Annotation Clustering of DAVID with enrichment score >1.3 (equal to $P<0.05$). Six annotation clusters were significantly enriched in these GO terms: transmembrane receptor activity and signal transduction (cluster 1, 3, and 4), multi-organism responses to external stimulus (cluster 2 and 6) and endoribonuclease activity (cluster 5). These results are consistent with the fact that FHL2 is a co-activator participated in many signaling transduction pathways.

KEGG. To discover more potential genes that may participate in the functional regulation by FHL2, the 337 gene list together with 44 well known FHL2 interacting proteins were submitted for KEGG pathway analysis. Interestingly, when the 337-gene list was compared with the 44 FHL2 interacting proteins, only Ar with a fold change of 1.84 in the 337 gene list was found to be an interacting protein of FHL2 [Muller et al., 2000]. Results shown in Table V represent the significantly

TABLE III. Top 10 Up- and Down-Regulated Genes in FHL2 KO Mice Revealed by the Microarray Experiment

Gene	Gene name	Fold change (KO/WT)
Up-regulated genes		
Xlr4c	X-linked lymphocyte-regulated 4C	4.4591
Xlr4b	X-linked lymphocyte-regulated 4B	4.4163
Mfsd2a	Major facilitator superfamily domain containing 2A	3.5843
Cabyr	Calcium-binding tyrosine-(Y)-phosphorylation regulated (fibrousheathin 2)	3.3398
Lcn2	Lipocalin 2	3.1248
Gm5708	Predicted gene 5708	2.9306
Olfr172	Olfactory receptor 172	2.5791
Alas1	Aminolevulinic acid synthase 1	2.4516
Onecut1	One cut domain, family member 1	2.4069
Zfp42	Zinc finger protein 42	2.3632
Down-regulated genes		
S1pr2	Sphingosine-1-phosphate receptor 2	0.5177
Xlr	X-linked lymphocyte-regulated complex	0.5004
Creld2	Cysteine-rich with EGF-like domains 2	0.4764
Dnahc7b	Dynein, axonemal, heavy chain 7B	0.4681
Hpdl	4-Hydroxyphenylpyruvate dioxygenase-like	0.4677
Chst1	Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	0.4525
Ins1	Insulin I	0.4387
Bglap3	Bone gamma-carboxyglutamate protein 3	0.4014
4921511C20Rik	RIKEN cDNA 4921511C20 gene	0.3978
Bcl6	B cell leukemia/lymphoma 6	0.2539

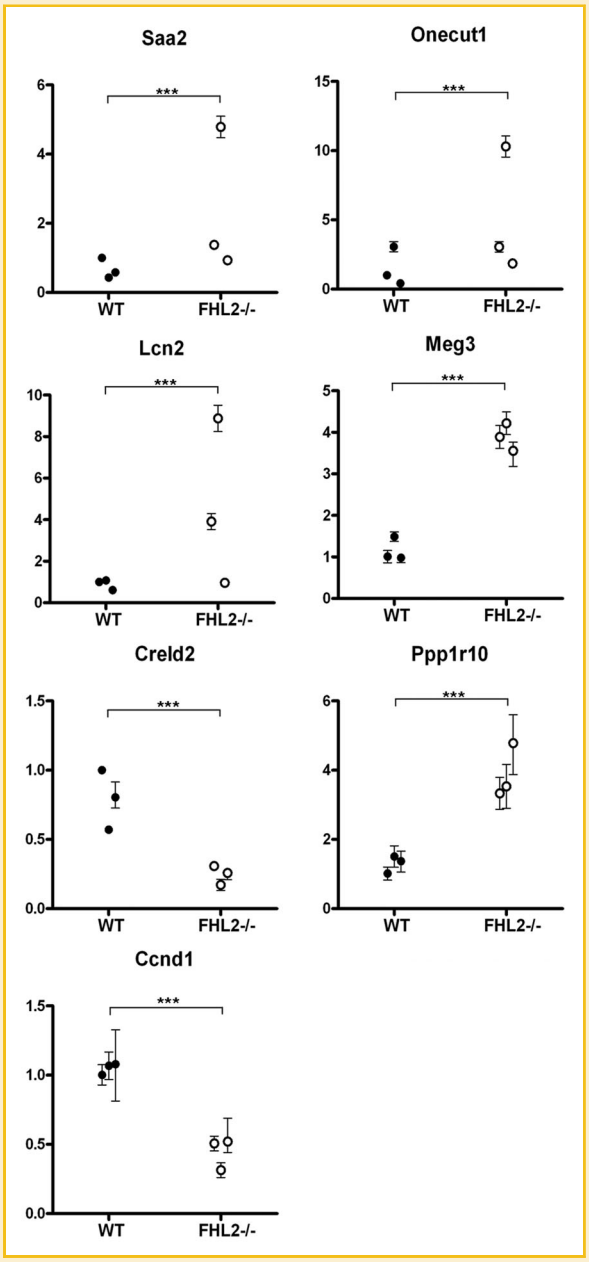

Fig. 1. Real-time PCR analysis for comparing gene expression between WT and KO mice. The transcript levels for the indicated genes of individual RNA samples from both WT and KO mice were determined. Each point represents the mean value of individual mouse ± SD of two independent experiments performed in triplicate wells. Triple asterisks (***) indicates $P < 0.001$ versus control group by two-tailed Mann–Whitney test.

enriched pathways and they are ordered by the enrichment *P*-value. All nine pathways showed the involvement of genes from the microarray gene list as well as the genes coding for the FHL2 interacting proteins. Besides *Ar*, several new target genes that may be regulated by FHL2 were found in the previously reported FHL2-related pathways, for example, pathways related to cancer—*Ins1* and *Pparδ*; cardiovascular function—*Gja1* and *Ighg*; regulation of actin cytoskeleton—*Abi2*, *Bdkrb1* and *Ins1*; acute myeloid leukemia—*Pparδ*; and TGF-β signaling pathway—*Inhbe* and *Id3*.

STRING. To explore the possible protein–protein interaction within these genes, the 337-gene list and 44 FHL2 interacting proteins were submitted to STRING. To better visualize the network and separate genes from different sources, the list of individual interactions with score was generated from STRING and subsequently analyzed by Cytoscape. The gene network shown in Figure 2 contains all the interactions with STRING combined score ≥0.80. Yellow and green colors denote the FHL2 interacting partners and genes from microarray result, respectively. As shown in the figure, many targets from the microarray gene list showed their interaction with known FHL2 interacting proteins.

Experimental Validation of FHL2-Regulated Genes by RT-PCR. To select genes for experimental validation, we overlapped genes in this network with those that could be found in KEGG pathway analysis. Besides *Ar*, four of them were identified in both STRING and KEGG analysis—*Ins1* (prostate cancer and regulation of actin cytoskeleton), *Pparδ* (pathways in cancer and acute myeloid leukemia), *Gja1* (arrhythmogenic right ventricular cardiomyopathy) and *Bdkrb1* (regulation of actin cytoskeleton). Then, genes in the top 10 up- and down-regulated gene list were overlapped with genes in the protein-protein interaction network. *Bcl6*, *Ins1*, and *S1pr2* in the list of top 10 down-regulated genes and *Alas1* in list of top 10 up-regulated genes were found in the protein-protein interaction network. Finally, genes linked to more than one node were also chosen because of their pivotal roles in the network. Five genes were selected by these criteria: *Nr4a1*, *Hey1*, *Angptl4*, *Erbb4*, and *Lif*. qPCR was used for validating the gene expression data obtained from the microarray experiment. The expression levels of 10 representative genes (*Id3*, *Inhbe*, *Alas1*, *Ar*, *Bcl6*, *Pparδ*, *Gja1*, *Angptl4*, *Erbb4*, and *Nr4a1*) selected by different analysis methods were determined. As shown in Figure 3A, the qPCR results were in good concordance with the microarray results. The qPCR results for each gene between the WT and FHL2 KO mice were shown in Figure 3B. Eight out of ten genes (*Ar*, *Id3*, *Inhbe*, *Alas1*, *Bcl6*, *Pparδ*, *Angptl4*, and *Erbb4*) showed remarkable changes in expression levels in FHL2 KO mice when compared with the WT mice, implicating the regulation of these eight genes by FHL2.

DISCUSSION

From the functional annotation analysis, we found that FHL2 affects various genes involved in (i) signal transduction (three clusters); (ii) multi-organism response to external stimulus (two clusters); and (iii) endoribonuclease activity (one cluster). It has been previously reported that FHL2 participates in diverse biological processes including signal transduction [Johannessen et al., 2006]. Moreover, deficiency in FHL2 can impair skin wound healing and increase virus replication, demonstrating a weakened ability to cope with external stimulus [Nordhoff et al., 2012; Wixler et al., 2007]. New functions of FHL2 in biological processes have been implicated but these functions need to be confirmed by further investigation. In addition, four KEGG pathways were enriched but only one pathway (Biosynthesis of unsaturated fatty acids) was found to be significant with $P < 0.05$.

TABLE IV. DAVID GO Term Enrichment Analysis of Genes With A Minimal Fold Change of 1.5 as Revealed by the Microarray Experiment (Enrichment Score >1.3)

Cluster	Enrichment score	ID	Category	Count	*P*-value
1	2.18	GO:0004888	Transmembrane receptor activity	54	3.06E−05
		GO:0004930	G-protein coupled receptor activity	48	3.66E−05
		GO:0007186	G-protein coupled receptor protein signaling pathway	47	6.10E−04
		GO:0004872	Receptor activity	59	1.05E−03
		GO:0007165	Signal transduction	60	2.06E−03
		GO:0060089	Molecular transducer activity	61	3.27E−03
		GO:0004871	Signal transducer activity	61	3.27E−03
		GO:0007606	Sensory perception of chemical stimulus	31	4.17E−03
		GO:0050890	Cognition	36	5.34E−03
		GO:0007600	Sensory perception	34	7.33E−03
		GO:0007166	Cell surface receptor linked signal transduction	53	8.87E−03
		GO:0007608	Sensory perception of smell	28	1.09E−02
		GO:0004984	Olfactory receptor activity	28	1.22E−02
		GO:0050877	Neurological system process	37	2.07E−02
		GO:0003008	Ssystem process	37	5.58E−02
		GO:0016021	Integral to membrane	95	8.57E−02
		GO:0031224	Intrinsic to membrane	98	8.72E−02
		GO:0044425	Membrane part	107	3.16E−01
		GO:0016020	Membrane	114	3.84E−01
2	1.72	GO:0051704	Multi-organism process	14	3.30E−03
		GO:0009615	Response to virus	5	2.84E−02
		GO:0051707	Response to other organism	9	2.85E−02
		GO:0009607	Response to biotic stimulus	10	5.01E−02
3	1.44	GO:0007165	Signal transduction	60	2.06E−03
		GO:0050794	Regulation of cellular process	107	6.54E−02
		GO:0050789	Regulation of biological process	110	1.05E−01
		GO:0065007	Biological regulation	115	1.18E−01
4	1.39	GO:0044421	Extracellular region part	19	3.56E−02
		GO:0005615	Extracellular space	14	3.80E−02
		GO:0005576	Extracellular region	34	4.87E−02
5	1.31	GO:0004521	Endoribonuclease activity	5	7.71E−03
		GO:0004540	Ribonuclease activity	5	1.93E−02
		GO:0016891	Endoribonuclease activity, producing 5′-phosphomonoesters	3	6.61E−02
		GO:0004519	Endonuclease activity	5	7.01E−02
		GO:0016893	Endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5′-phosphomonoesters	3	9.26E−02
		GO:0004518	Nuclease activity	5	2.03E−01
6	1.31	GO:0006953	Acute-phase response	4	1.04E−02
		GO:0009611	Response to wounding	12	1.72E−02
		GO:0009605	Response to external stimulus	18	1.81E−02
		GO:0006954	Inflammatory response	9	2.17E−02
		GO:0002526	Acute inflammatory response	5	3.48E−02
		GO:0006952	Defense response	11	1.45E−01
		GO:0050896	Response to stimulus	44	1.59E−01
		GO:0006950	Response to stress	18	6.33E−01

The insensitivity of pathway detection may probably due to the background noises in the gene list, which contains large proportion of genes not participating in any well-known pathways, or the fact that FHL2 regulated genes are scattered in different pathways so that functional enrichment cannot be detected.

To further identify FHL2-related genes in the 337-gene list, we tried to include known FHL2 interacting proteins in the analysis. The sensitivity of pathway analysis was greatly improved and nine pathways were detected and showed the participation of genes from the 337-gene list. Among these pathways, most of the pathways/functions were previously shown to be related to FHL2, such as prostate cancer [Kahl et al., 2006b], TGF-β signaling pathway [Xia et al., 2013], and dilated cardiomyopathy [Arimura et al., 2007]. The new genes found in the analysis should help broaden our understanding on the roles of FHL2 in these pathways and facilitate future research on FHL2.

The same combined gene list was then used for protein–protein interacting network analysis. Ar served as a positive control because it is a well-known interacting partner of FHL2 and was found during the pathway and network analysis. Some overlapping genes were also found in this pattern. Finally, 10 candidate genes potentially regulated by FHL2 were chosen for qPCR validation and eight of them showed the significant differential expressions in FHL2 KO mice.

TABLE V. KEGG Pathway Analysis of Genes With Minimal Fold Change of 1.5 and FHL2 Interacting Proteins ($P < 0.05$)

KEGG Pathway Term	Count	*P*-value	Genes[a]
Prostate cancer	9	5.99E−04	EGFR, MAPK1, AR, EP300, CREB1, CREBBP, FOXO1, INS1, CTNNB1
Biosynthesis of unsaturated fatty acids	4	1.89E−02	ACOT2, ELOVL6, ACOT4, ACOT3
Pathways in cancer	14	1.91E−02	EGFR, PPARD, AR, CREBBP, FOXO1, ITGA3, ZBTB16, ITGB1, STAT3, CTNNB1, MAPK1, EP300, RUNX1, TRAF6
Arrhythmogenic right ventricular cardiomyopathy	6	2.12E−02	ITGB6, ITGA7, GJA1, ITGA3, ITGB1, CTNNB1
Acute myeloid leukemia	5	3.23E−02	MAPK1, PPARD, ZBTB16, RUNX1, STAT3
TGF-β signaling pathway	6	3.71E−02	MAPK1, EP300, INHBE, CREBBP, BMPR2, ID3
Ribosome	6	4.03E−02	GM5516, GM5502, UBC, GM7059, GM4974, RPL12
Regulation of actin cytoskeleton	10	4.07E−02	EGFR, MAPK1, ITGB6, ITGA7, ABI2, BDKRB1, ITGA3, ITGB2, INS1, ITGB1
Dilated cardiomyopathy	6	4.55E−02	IGHG, ITGB6, ITGA7, ITGA3, TTN, ITGB1

[a]Black and red color denotes the FHL2 interacting proteins and genes from microarray result, respectively.

Interestingly, S1pr2, Cxcr1, P2ry14, Bdkrb1, S1pr4, Ccr9 interact with each other and form a hexagonal network. To check their interrelationship, genes of these six candidates along with Ltb4r1 and Sphk1, which also link together, were submitted to DAVID. Annotation result shown that these eight genes enriched G-protein coupled receptor protein signaling pathway with $P < 0.001$. Five of them (*S1pr2*, *Ltb4r1*, *P2ry14*, *S1pr4*, *Bdkrb1*) significantly enriched the KEGG pathway in neuroactive ligand–receptor interaction. This might be the supporting evidence showing the importance of FHL2 in signal transduction.

Among genes predicted by KEGG pathway, *Id3* and *Inhbe* were found to be enriched in the TGF-β signaling pathway and both of them were significantly up-regulated in FHL2 KO mice. It has been showed that FHL2 could suppress HCC cell growth through a TGF-β-like signaling pathway and physically interact with Smad2, Smad3, and Smad4 in this pathway [Ding et al., 2009]. Furthermore, FHL2 could activate this pathway by regulating ubiquitination of the E3 ligase Arkadia [Xia et al., 2013]. Based on the pathway analysis as well as the significantly increased expression in FHL2 KO mice, *Id3* and *Inhbe* are likely novel downstream genes of FHL2. INHBE, which plays a role in pancreatic exocrine cell growth and proliferation [Hashimoto et al., 2006], has not been reported to relate to FHL2. The human protein of the other candidate, ID3, which participates in the progression of prostate and breast cancer, was shown to be interacted

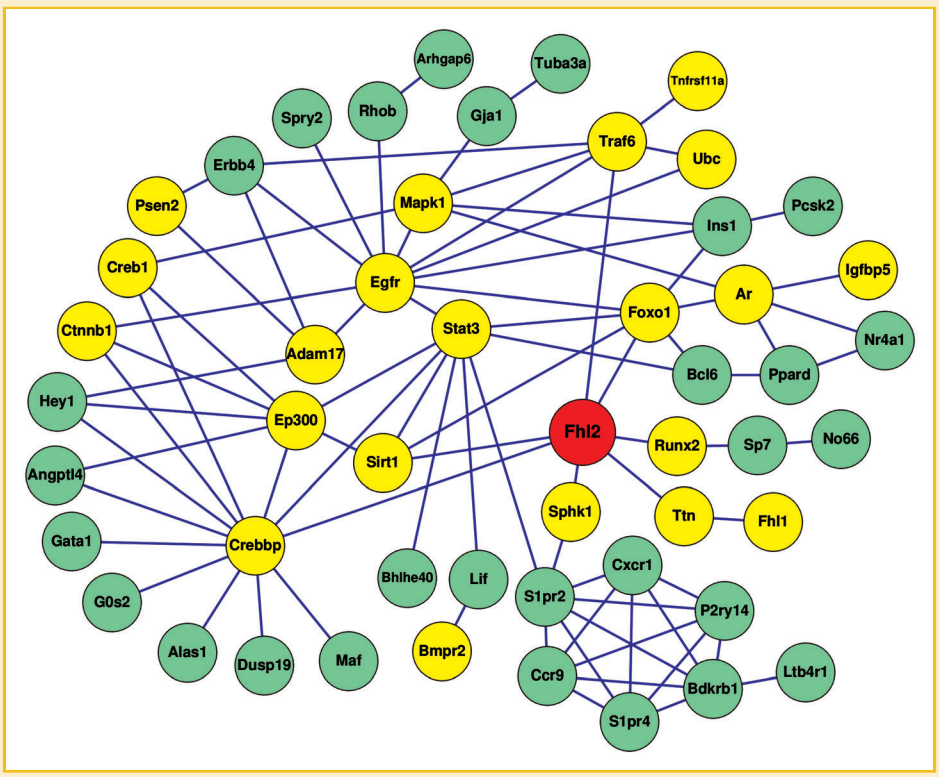

Fig. 2. Gene network obtained from STRING with individual interaction combined score ≥0.80. Yellow and green colors denote the FHL2 interacting proteins and genes from microarray result, respectively.

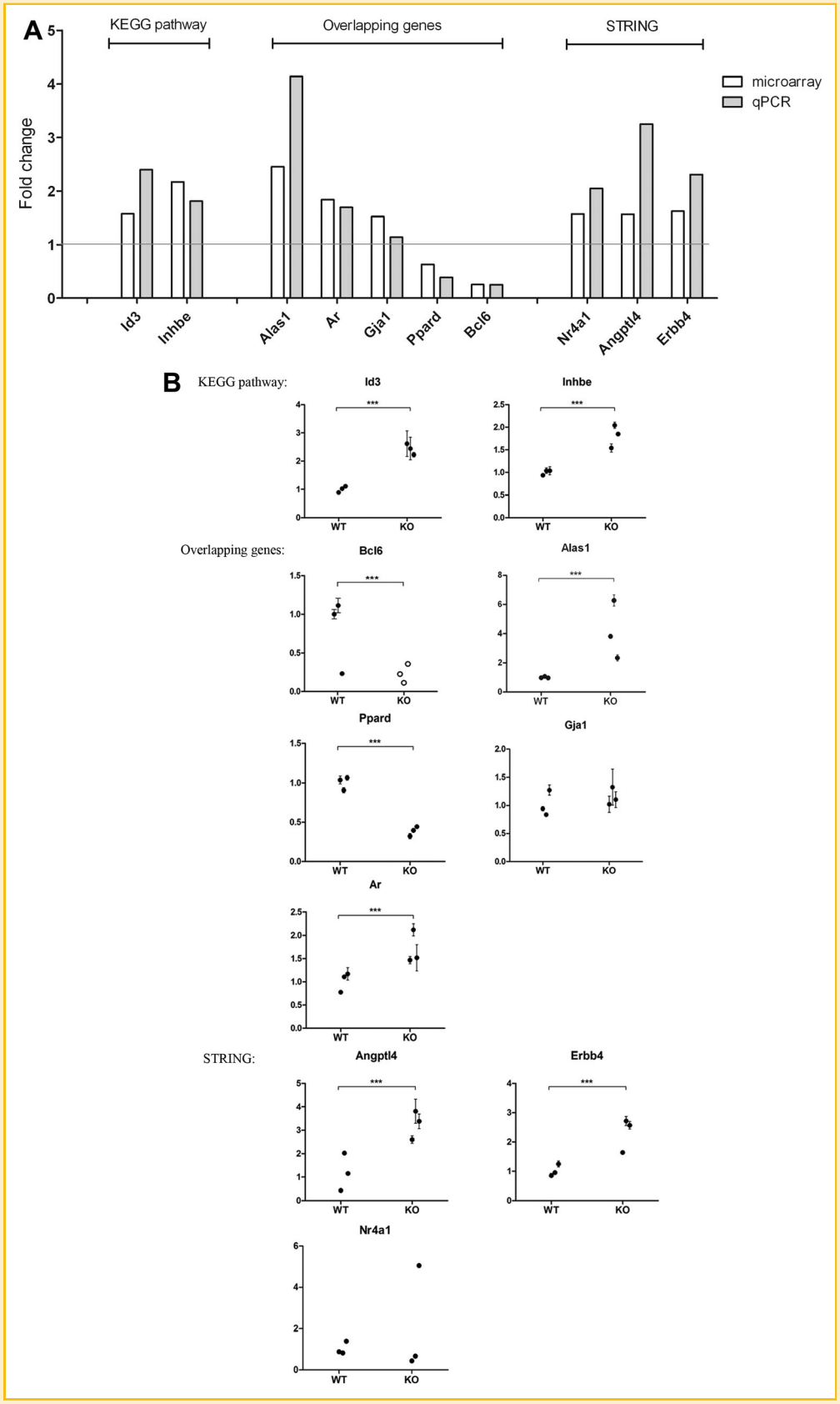

Fig. 3. qPCR validation of genes from microarray results and bioinformatics analysis. The qPCR experiments were performed in three individual mice. Panel A: This figure summarizes the microarray data and the mean values of qPCR results of each gene (KO/WT). There genes are divided into three groups: (i) genes only found in KEGG pathway analysis (KEGG pathway), (ii) genes found in both pathway analysis or top-10 gene list and network analysis (overlapping genes), (iii) genes only found in STRING network analysis (STRING). Panel B: The transcription levels of each indicated genes were determined in three individual mice. Each point represents the mean ± SD obtained from experiment in triplicate. Triple asterisks (***) indicates $P < 0.001$ versus WT group by two-tailed Mann–Whitney test.

with and inhibited by FHL2 in MCF-7 breast cancer cells [Chen et al., 2012]. The previous finding for the relationship between FHL2 and ID3 demonstrated the efficacy of our analysis method in excavating target genes of FHL2.

PPARδ was found to participate in cancer-related pathways, forming a sub-cluster with AR, FOXO1, STAT3, and BCL6 in the network while remarkably down-regulated in FHL2 KO mice. The PPAR families, which are the nuclear hormone receptors that bind peroxisome proliferators, mediate a variety of biological processes including diabetes, obesity, atherosclerosis, and cancer. The expression of PPARδ was previously found to be elevated in colorectal cancer cells [Yang et al., 2006]. In addition, previous findings indicated that agonist-activated PPARδ interfered with interleukin-6-induced acute phase reaction in the liver by inhibiting the transcriptional activity of STAT3 (signal transducer and activator of transcription 3) [Kino et al., 2007]. Interestingly, FHL2 also regulates interleukin-6 expression and interacts with AR, FOXO1 and STAT3 which were included in the sub-cluster with PPARδ [Wong et al., 2012]. Taken together, *PPAR*δ is very likely a gene regulated by FHL2. However, the involvement of this gene in HCC development requires further explorations.

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