

# Bioinformatics analysis to screen key genes implicated in the differentiation of induced pluripotent stem cells to hepatocytes

RUI LIN\*, YUFENG WANG\*, KUN JI, ZHONGYAN LIU, SHUAI XIAO,  
DEHUA ZHOU, QUANNING CHEN and BAOMIN SHI

Department of General Surgery, Tongji Hospital, School of Medicine, Tongji  
University Medical School, Shanghai 200065, P.R. China

Received February 1, 2016; Accepted January 27, 2017

DOI: 10.3892/mmr.2018.8385

**Abstract.** Due to the lack of potential organs, hepatocellular transplantation has been considered for treating end-stage liver disease. Induced pluripotent stem cells (iPSCs) are reverted from somatic cells and are able to differentiate into hepatocytes. The present study aimed to investigate the mechanisms underlying iPSC differentiation to hepatocytes. GSE66076 was downloaded from the Gene Expression Omnibus; this database includes data from 3 undifferentiated (T0), 3 definitive endoderm (T5), and 3 early hepatocyte (T24) samples across hepatic-directed differentiation of iPSCs. Differentially expressed genes (DEGs) between T0 and T5 or T24 samples were identified using the linear models for microarray data package in Bioconductor, and enrichment analyses were performed. Using the weighted correlation network analysis package in R, clusters were identified for the merged DEGs. Cytoscape was used to construct protein-protein interaction (PPI) networks for DEGs identified to belong to significant clusters. Using the ReactomeFI plugin in Cytoscape, functional interaction (FI) networks were constructed for the common genes. A total of 433 and 1,342 DEGs were identified in the T5 and T24 samples respectively, compared with the T0 samples. Blue and turquoise clusters were identified as significant gene clusters. In the PPI network for DEGs in the blue cluster, the key node fibroblast growth factor 2 (FGF2) could interact with bone morphogenetic protein 2 (BMP2). Cyclin-dependent kinase 1 (CDK1) was demonstrated to have the highest degree (degree=71) in the PPI

network for DEGs in the turquoise cluster. Enrichment analysis for the common genes, including hepatocyte nuclear factor 4 $\alpha$  (*HNF4A*) and epidermal growth factor (*EGF*), in the FI network indicated that *EGF* and *FGF2* were enriched in the Ras and Rap1 signaling pathways. The present results suggest that *FGF2*, *BMP2*, *CDK1*, *HNF4A* and *EGF* may participate in the differentiation of iPSCs into hepatocytes.

## Introduction

Induced pluripotent stem cells (iPSCs), which are reverted from somatic cells via nuclear transfer and transcription factor-based reprogramming, are pluripotent stem cells that are able to differentiate into all cell types (1). They are successfully derived from somatic cells through viral transduction using the transcription factors sex-determining region Y-box 2, octamer-binding transcription factor 4 (*Oct4*), and either *NANOG* and lineage protein 28 (2) or *c-MYC* and Krüppel-like factor 4 (3,4). The treatment of end-stage liver disease is severely impaired by the shortage of potential organs, therefore, hepatocellular transplantation substituting for whole organ transplant may hold potential as an alternative treatment strategy (5). Similar to embryonic stem cells (ESCs), iPSCs exhibit pluripotent properties and are able to differentiate into all cell lineages *in vitro*, including hepatocytes, suggesting that iPSCs may be a valuable cell source for hepatocellular transplantation (6,7).

Several studies have investigated the mechanisms underlying differentiation of PSCs. The expression of the hepatic marker albumin has been reported to contribute to the efficient differentiation of iPSCs to hepatocyte-like cells (8). Transforming growth factor- $\beta$  has been revealed to correlate with the differentiation of iPSCs into functional endothelial cells, whereas the phosphatase and tensin homolog/Akt pathway targeted by microRNA (miR)-21 can assist the endothelial differentiation of iPSCs (9). E-cadherin and several other crucial cell adhesion molecules, including classic cadherins, heparin sulfate proteoglycans, members of the immunoglobulin (IgG) superfamily and integrins, have been demonstrated to regulate the differentiation and survival of human PSCs, including human ESCs and iPSCs (10,11). Through activating mesenchymal-to-epithelial transition, hepatocyte nuclear factor 4 $\alpha$  (*HNF4A*) may be implicated in the generation of hepatocytes from human ESC-derived hepatoblasts, which may

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*Correspondence to:* Dr Quanning Chen or Dr Baomin Shi, Department of General Surgery, Tongji Hospital, School of Medicine, Tongji University Medical School, 389 Xincun Road, Shanghai 200065, P.R. China  
E-mail: quanning\_chen@sina.com  
E-mail: baomin\_shi@163.com

\*Contributed equally

**Key words:** induced pluripotent stem cells, differentially expressed genes, weighted correlation network analysis, protein-protein interaction network, functional interaction network

represent a favorable pathway for the efficient differentiation of human ESCs and iPSCs into functional hepatocytes (12). Bone morphogenetic protein (*BMP*) is regulated by Brachyury and caudal-related homeobox 2 (*CDX2*), and mainly promotes mouse and human PSC differentiation to mesoderm, not trophoblasts (13). However, the exact mechanisms guiding iPSC differentiation into hepatocytes remain to be elucidated.

Wilson *et al.* (14) investigated the differentially expressed genes (DEGs) in iPSCs derived from patients with liver disease and healthy subjects upon *in vitro* differentiation to hepatocytes, and identified 419 DEGs at false discovery rate (FDR) <0.25 and 85 DEGs at FDR<0.1. In the present study, using the more restrictive thresholds of adjusted P-value, i.e. FDR<0.01 and  $\log_2$ fold change (FC) $\geq 2$ , the DEGs between undifferentiated samples and definitive endoderm or early hepatocyte samples were identified, and their potential functions were predicted using enrichment analyses. Subsequently, the DEGs between the two groups were merged, and weighted correlation network analysis (WGCNA) was performed to identify gene clusters for the merged DEGs. Furthermore, the protein-protein interaction (PPI) networks for the DEGs belonging to the significant gene clusters were constructed, the common genes between the two comparison groups were identified, and their functional interaction (FI) network was analyzed.

## Materials and methods

**Microarray data.** The GSE66076 expression profile ([http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE\\_66076](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE_66076)) deposited by Wilson *et al.* (14), was downloaded from the National Center for Biotechnology Information Gene Expression Omnibus database, which was based on the GPL6244 [HuGene-1\_0-st] Affymetrix Human Gene 1.0 ST Array [transcript (gene) version] platform. To study the differentiation mechanisms of iPSCs to hepatocytes, iPSCs across three stages of hepatic-directed differentiation were selected from GSE66076, including 3 undifferentiated (T0), 3 definitive endoderm (T5), and 3 early hepatocyte (T24) samples.

**Data preprocessing and DEG screening.** Following the download of GSE66076, raw data was preprocessed with background correction, normalization and expression calculation by Oligo package (15) in Bioconductor. The org.Hs.eg.db (16) and hugene10sttranscriptcluster.db (17) annotation packages were used to transform probe identifications (IDs) into gene symbols. For one gene symbol corresponding to several probe IDs, the mean value of probes was used as the final gene expression value.

The linear models for microarray data (limma) package (18) in Bioconductor was applied to identify the DEGs between T0 and T5 or T24 samples. The P-values for the DEGs were calculated using the t-test method in the limma package and were then adjusted using the method described by Benjamini and Hochberg (19). An FDR<0.01 and  $\log_2$ FC $\geq 2$  were considered as the thresholds for significance.

**Functional and pathway enrichment analysis.** The ToppGene database (<https://toppgene.cchmc.org/>) (20) integrates pathway information in BioSystems [including BioCyc, Kyoto Encyclopedia of Genes and Genomes (KEGG), REACTOME, WikiPathways], GenMAPP, MSigDB C2 (including BioCarta,

SigmaAldrich and Signaling Gateway), PantherDB, Pathway Ontology and Small Molecule Pathway Database databases, and can be used for functional and pathway enrichment analyses. Gene Ontology (GO, <http://www.geneontology.org/>) describes functions of genes and their products in molecular function (MF), biological process (BP) and cellular component (CC) aspects (21). The KEGG (<http://www.genome.jp/kegg/>) database integrates chemical, genomic and systemic functional information of biological systems (22). Combined with the ToppGene database, GO functional and KEGG pathway enrichment analyses were carried out for the DEGs between T0 and T5 samples, as well as those between T0 and T24 samples. An FDR $\leq 0.05$  and the involvement of at least 2 genes were used as the cut-off criteria.

**WGCNA analysis.** WGCNA is usually applied for identifying highly correlated gene clusters, for summarizing the clusters using the intramodular hub gene or module eigengene, for linking modules to other modules and to external sample characteristics, and for calculating module membership measures (23). The DEGs in the T0 vs. T5 and the T0 vs. T24 comparison groups were merged. Subsequently, the WGCNA package (23) in R was used to identify gene clusters for the merged DEGs. The clusters with  $|Correlation\ coefficient| > 0.8$  and  $P < 0.05$  were identified as significant gene clusters.

**PPI network construction.** The Search Tool for the Retrieval of Interacting Genes (STRING) database contains easily accessed and uniquely comprehensive experimental and predicted interaction information (24). The STRING database (<http://string-db.org/>) (24) was used to identify PPI relationships among the significant gene clusters, and a required confidence (combined score) $> 0.7$  was set as the cut-off criterion. Subsequently, the PPI network was visualized using the Cytoscape software (<http://www.cytoscape.org/>) (25). The proteins in the network were represented as nodes, whereas their degrees corresponded to the number of edges associated with that node.

**Common gene analysis.** The Venny 2.0 online tool (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>) was used to identify the common genes between the two comparison groups. The gene FI network was constructed by merging interactions predicted using a machine learning approach with interactions extracted from human curated pathways (24). ReactomeFI can be used for network-based data analysis through the highly reliable Reactome FI network (26). According to the expression profiles data, the ReactomeFI plugin (26) in Cytoscape was used to analyze the FI network for the common genes.

## Results

**DEG analysis.** Using a threshold of FDR<0.01 and  $\log_2$ FC $\geq 2$ , the DEGs between T0 and T5 or T24 samples were investigated. Compared with T0 samples, 433 (including 268 upregulated and 165 downregulated genes) and 1,342 (including 729 upregulated and 613 downregulated genes) DEGs were identified in the T5 and T24 samples, respectively.

**Functional and pathway enrichment analysis.** The upregulated genes in T5 samples were significantly enriched in

Table I. Top 3 functions and pathways enriched for differentially expressed genes in T5 samples.

Category	ID	Description	FDR	Gene no.	Gene symbol	
Upregulated	GO_BP	GO:0009888	tissue development	2.70E-13	72	<i>HHEX, ARHGAP24, FOXQ1.....</i>
		GO:0072359	circulatory system development	2.88E-11	46	<i>EPHB3, HHEX, ARHGAP24.....</i>
		GO:0072358	cardiovascular system development	2.88E-11	46	<i>ADAM19, GATA4, GATA6.....</i>
	GO_CC	GO:0005615	extracellular space	2.85E-07	47	<i>PRSS2, RELN, ABCA1.....</i>
		GO:0002116	semaphorin receptor complex	2.12E-04	4	<i>NRP2, NRP1, PLXNA2, PLXNA4</i>
		GO:0009897	external side of plasma membrane	5.50E-04	15	<i>ABCA1, DLK1, ITGA5.....</i>
	GO_MF	GO:0060089	molecular transducer activity	2.50E-04	50	<i>EPHB3, ABCA1, WLS.....</i>
		GO:0004871	signal transducer activity	2.50E-04	50	<i>HNF4A, IL18R1, RXRG.....</i>
		GO:0004872	receptor activity	7.51E-04	46	<i>SORCS1, FZD4, FZD8.....</i>
	KEGG pathway	119526	other semaphorin interactions	5.42E-03	5	<i>SEMA6D, PLXNA2, SEMA5A.....</i>
576262		extracellular matrix organization	1.10E-02	15	<i>PRSS2, MATN3, ITGA5.....</i>	
198832		adipogenesis	5.42E-03	11	<i>SPOCK1, CYP26A1, GATA4.....</i>	
Downregulated	GO_BP	GO:0007267	cell-cell signaling	1.20E-02	28	<i>SOX2, LPAR3, SFRP2.....</i>
		GO:0007268	synaptic transmission	2.03E-02	20	<i>LPAR3, CHRNA9, RASGRF2.....</i>
		GO:0045766	positive regulation of angiogenesis	4.82E-02	7	<i>FLT1, SFRP2, VASH2.....</i>
	GO_CC	GO:0045202	synapse	1.42E-03	18	<i>NMNAT2, CHRNA9, GAP43.....</i>
		GO:0097060	synaptic membrane	1.42E-03	11	<i>CHRNA9, GABRQ, CNKSR2.....</i>
		GO:0045211	postsynaptic membrane	1.42E-03	10	<i>LRRTM3, VRL3, MET.....</i>

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function; FDR, false discovery rate.

567 GO\_BP terms, 12 GO\_CC terms, 29 GO\_MF terms and 7 KEGG pathways. The top 3 functions and pathways are presented in Table I, including tissue development (GO\_BP, FDR=2.70E-13), extracellular space (GO\_CC, FDR=2.85E-07), molecular transducer activity (GO\_MF, FDR=2.50E-04; which involved *HNF4A*) and extracellular matrix organization (pathway, FDR=1.10E-02). Meanwhile, the downregulated genes in T5 samples were significantly enriched in 3 GO\_BP terms and 15 GO\_CC terms, including cell-cell signaling (GO\_BP, FDR=1.20E-02) and synapse (GO\_CC, FDR=1.42E-03).

Upregulated genes in T24 samples were significantly enriched in 1,145 GO\_BP terms, 88 GO\_CC terms, 146 GO\_MF terms and 142 KEGG pathways. The top 3 functions and pathways are presented in Table II, including extracellular matrix organization (GO\_BP, FDR=1.39E-21), extracellular space (GO\_CC, FDR=8.05E-44), receptor binding (GO\_MF, FDR=1.16E-11; which involved *HNF4A*) and complement and coagulation cascades (pathway, FDR=1.27E-16). Meanwhile, downregulated genes in T24 samples were significantly enriched in 317 GO\_BP terms, 70 GO\_CC terms, 41 GO\_MF terms and 152 KEGG pathways. The top 3 functions and

pathways are presented in Table II, including cell cycle (GO\_BP, FDR=1.55E-51), chromosome (GO\_CC, FDR=1.18E-44), ribonucleotide binding (GO\_MF, FDR=1.40E-07) and cell cycle (pathway, FDR=1.67E-49).

*WGCNA analysis.* The DEGs in the T0 vs. T5 and T0 vs. T24 comparison groups were merged and 1,569 DEGs were obtained. Based on WGCNA, 3 gene clusters were identified, including blue (correlation coefficient, -0.98; P=3.07E-06), green (correlation coefficient, 0.25; P=5.16E-01), and turquoise (correlation coefficient, 0.89; P=1.14E-03) clusters (Fig. 1). Blue and turquoise gene clusters were significant.

The 504 DEGs in the blue cluster were significantly enriched in 274 GO\_BP terms, 36 GO\_CC terms and 33 KEGG pathways. The top 5 functions and pathways are presented in Table III, including regulation of multicellular organismal development (GO\_BP; FDR=5.97E-06; which involved *BMP2*), chromosome (GO\_CC, FDR=3.79E-03) and systemic lupus erythematosus (pathway, FDR=1.00E-03). Meanwhile, the 833 DEGs in the turquoise cluster were significantly enriched in 802 GO\_BP terms, 87 GO\_CC terms, 78 GO\_MF terms and 166 KEGG pathways. The

Table II. Top 3 functions and pathways enriched for differentially expressed genes in T24 samples.

Category	ID	Description	FDR	Gene no.	Gene symbol	
Upregulated	GO_BP	GO:0030198	extracellular matrix organization	1.39E-21	66	<i>TTR, FAP, MF12.....</i>
		GO:0043062	extracellular structure organization	1.39E-21	66	<i>FBNI, EFEMP1, HPN.....</i>
	GO_CC	GO:0009611	response to wounding	6.97E-19	122	<i>CFH, EPHX2, SERPINA3.....</i>
		GO:0005615	extracellular space	8.05E-44	165	<i>ABCA1, IL32, FSTL3.....</i>
		GO:0031012	extracellular matrix	1.34E-18	63	<i>SERPINF1, CHI3L1, F2.....</i>
	GO_MF	GO:0005578	proteinaceous extracellular matrix	2.12E-17	55	<i>SERPINA1, FBNI, EFEMP1.....</i>
		GO:0005102	receptor binding	1.16E-11	113	<i>EPHX2, ABCA1, IL32.....</i>
		GO:1901681	sulfur compound binding	6.03E-09	34	<i>CFH, ACADL, HNF4A.....</i>
	KEGG pathway	GO:0050839	cell adhesion molecule binding	7.59E-09	29	<i>NDRG1, FGA, FGB.....</i>
		83073	complement and coagulation cascades	1.27E-16	28	<i>CFH, F2, F3.....</i>
198880		complement and coagulation cascades	7.64E-15	23	<i>SERPINA1, FGB, PLG.....</i>	
	M4470	extrinsic prothrombin activation pathway	7.08E-13	12	<i>FGB, FGG, SERPINC1.....</i>	
Downregulated	GO_BP	GO:0000278	mitotic cell cycle	8.81E-55	141	<i>NUSAP1, CDKN3, KIF18A.....</i>
		GO:0007049	cell cycle	1.55E-51	180	<i>BRIP1, MIS18BP1, CENPW.....</i>
		GO:0022402	cell cycle process	1.06E-48	151	<i>CENPW, CENPE, CENPF.....</i>
	GO_CC	GO:0005694	chromosome	1.18E-44	116	<i>NUSAP1, CHAF1B, MIS18BP1.....</i>
		GO:0044427	chromosomal part	1.44E-36	97	<i>CHAF1B, MIS18BP1, CENPW.....</i>
		GO:0032993	protein-DNA complex	6.76E-32	62	<i>MIS18BP1, CENPW, CENPE.....</i>
	GO_MF	GO:0032559	adenyl ribonucleotide binding	1.40E-07	94	<i>KIF18A, BRIP1, CENPE.....</i>
		GO:0005524	ATP binding	1.40E-07	92	<i>ATAD5, MARK1, MCM2.....</i>
		GO:0030554	adenyl nucleotide binding	1.52E-07	94	<i>MCM4, PFAS, MCM5.....</i>
	KEGG pathway	530733	cell cycle	1.67E-49	105	<i>KIF18A, PTTG1, MIS18BP1.....</i>
		105765	cell cycle, mitotic	1.57E-35	81	<i>KIF18A, PTTG1, CENPE.....</i>
		105750	G2/M checkpoints	7.54E-21	24	<i>MCM2, MCM3, MCM4.....</i>

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function; FDR, false discovery rate.

top 5 functions and pathways are presented in Table IV, including mitotic cell cycle [GO\_BP; FDR=3.93E-28; which involved cyclin-dependent kinase 1 (*CDK1*)], extracellular space (GO\_CC, FDR=3.10E-23), receptor binding (GO\_MF, FDR=2.47E-04) and cell cycle (pathway, FDR=4.76E-16; which involved *CDK1*).

**PPI network analysis for genes in the blue and turquoise clusters.** The PPI network for DEGs in the blue cluster demonstrated 218 nodes and 388 interactions (Fig. 2). In the PPI network, fibroblast growth factor 2 (*FGF2*, degree=14) and *BMP2* (degree=12) were the nodes with the higher degrees, and *FGF2* had interactions with *BMP2* in the PPI network. Furthermore, the PPI network for DEGs in the turquoise cluster demonstrated 488 nodes and 1,803 interactions (Fig. 3).

Notably, *CDK1* (degree=71) was the node with the highest degree in the PPI network.

**Common gene analysis.** A total of 202 common genes, including *HNF4A*, epidermal growth factor (*EGF*) and *FGF2* were identified between the two comparison groups, of which 100 were upregulated and 102 were downregulated (Fig. 4). According to the expression profile data of the common genes, a gene FI network was constructed (Fig. 5A). The top 11 most significant pathways enriched for the genes in the FI network are presented in Fig. 5B, and include the Ras signaling pathway (K), the Rap1 signaling pathway (K) and actions of nitric oxide in the heart (B). Notably, *EGF* and *FGF2* were enriched in the Ras (K) and Rap1 signaling pathways (K).

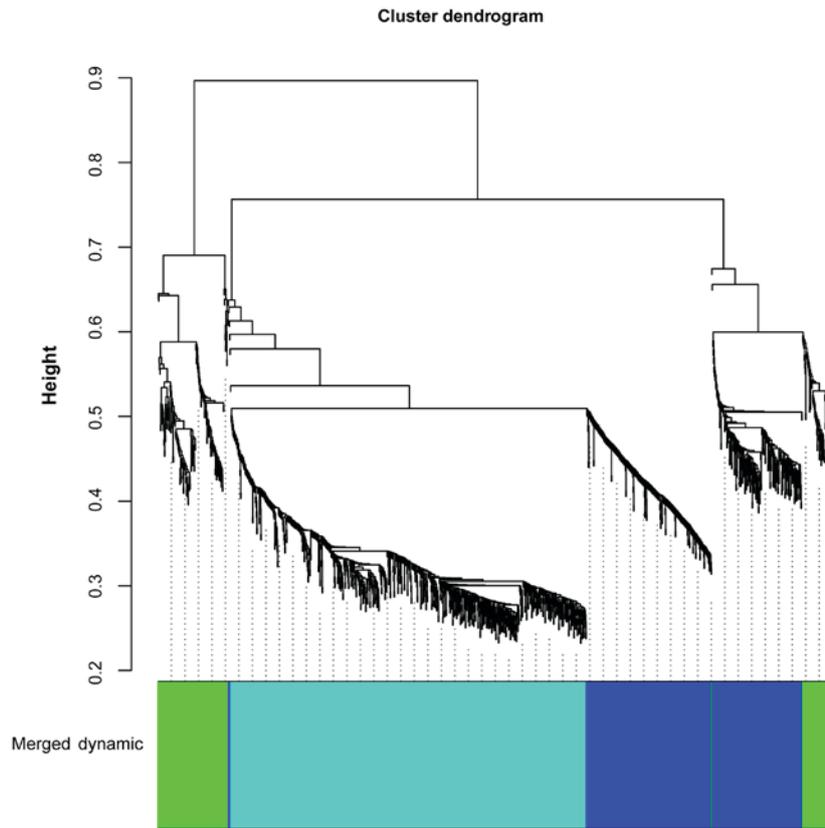


Figure 1. Dendrogram for identifying gene clusters for the merged differentially expressed genes using weighted correlation network analysis.

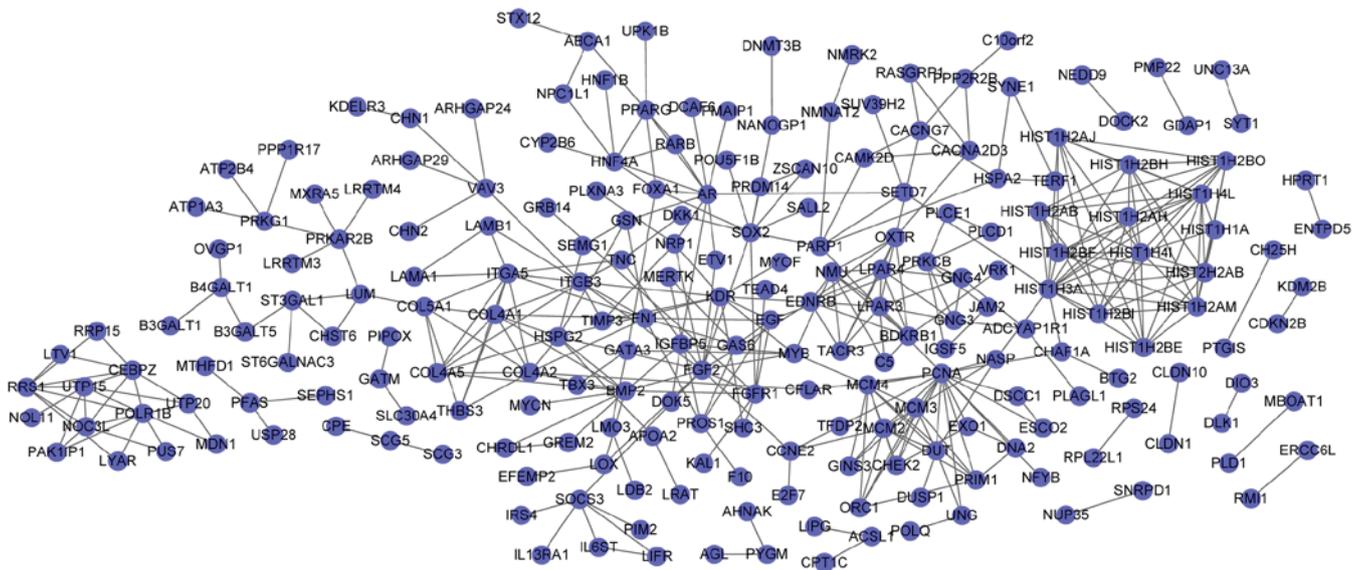


Figure 2. Protein-protein interaction network for differentially expressed genes in the blue cluster.

**Discussion**

In the present study, 433 DEGs were identified between the T5 and T0 samples, including 268 up- and 165 downregulated genes, whereas 1,342 DEGs were identified between the T24 and T0 samples, including 729 up- and 613 downregulated genes. Based on WGCNA, blue and turquoise clusters were identified as significant gene clusters. A total of 202 common

genes, including 100 up- and 102 downregulated genes, were identified between the two comparison groups, and a gene FI network was constructed.

In the PPI network for DEGs in the blue cluster, upregulated FGF2 (degree=14) and downregulated BMP2 (degree=12) were the nodes with the higher degrees. Exogenous FGF2 has been reported to enhance the role of intracrine FGF2 signaling in the maintenance of pluripotency; conversely, a downregulation

Table III. Top 5 functions and pathways enriched for differentially expressed genes in the blue cluster.

Category	ID	Description	FDR	Gene no.	Gene symbol
GO_BP	GO:2000026	regulation of multicellular organismal development	5.97E-06	78	<i>CDKN2B, LPAR3, RAMP2</i> .....
	GO:0001763	morphogenesis of a branching structure	1.17E-05	24	<i>FOXA1, FGF2, FGFR1</i> .....
	GO:0048589	developmental growth	1.17E-05	30	<i>LPAR3, BCL11A, DRAXIN</i> .....
	GO:0061138	morphogenesis of a branching epithelium	1.17E-05	23	<i>FOXA1, FGF2, FGFR1</i> .....
	GO:0048754	branching morphogenesis of an epithelial tube	1.17E-05	21	<i>FOXA1, FGF2, COL4A1</i> .....
GO_CC	GO:0044420	extracellular matrix component	1.46E-03	15	<i>COL4A1, COL4A2, COL4A5</i> .....
	GO:0000785	chromatin	2.27E-03	26	<i>MCM2, HIST1H2AJ, HIST1H2AB</i> .....
	GO:0005604	basement membrane	2.36E-03	12	<i>COL4A1, COL4A2, COL4A5</i> .....
	GO:0005694	chromosome	3.79E-03	40	<i>MCM2, MCM3, HIST1H4I</i> .....
	GO:0044427	chromosomal part	4.60E-03	35	<i>MCM2, MCM3, HIST1H2AJ</i> .....
KEGG pathway	106540	telomere maintenance	7.80E-04	13	<i>HIST1H4I, HIST1H2AJ, HIST1H2AB</i> .....
	366238	amyloids	9.81E-04	13	<i>HIST1H4I, HIST1H2AJ, HIST1H2AB</i> .....
	106548	packaging of telomere ends	9.81E-04	10	<i>HIST1H4I, HIST1H2AJ, HIST1H2AB</i> .....
	477134	meiotic synapsis	9.81E-04	12	<i>HIST1H4I, HIST1H2AJ, HIST1H2AB</i> .....
	83122	systemic lupus erythematosus	1.00E-03	16	<i>HLA-DOA, HIST1H4I, HIST1H2AJ</i> .....

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; FDR, false discovery rate.

of endogenous *FGF2* has been demonstrated during the differentiation of human ESCs, whereas its knockdown has been revealed to contribute to hESC differentiation (27,28). It has previously been reported that FGF2 signaling controls *BMP4*-mediated hESCs differentiation by maintaining levels of *NANOG* via the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway (29). In the present study, functional enrichment of the DEGs in the blue cluster demonstrated that *BMP2* was enriched in the regulation of multicellular organismal development. Previous studies have reported that *BMP2* may participate in hESC differentiation through the control of an important early commitment step, which may provide the route for differentiation of pluripotent cells into neural precursors (30). It has been revealed that *BMP-2/6* was more successful in inducing hESCs differentiation than *BMP-2* or *BMP-6*, and it was able to substitute these BMPs during *in vitro* differentiation guidance (31). In addition, the FGF pathway serves an important role in directing the *BMP4*-induced generation of syncytiotrophoblasts from hESCs (32). These findings suggested that *FGF2* and *BMP2* may serve key roles in the differentiation of iPSCs. In the PPI network for DEGs in the blue cluster, FGF2 could interact with *BMP2*, suggesting that *FGF2* may participate in iPSC differentiation through interacting with *BMP2*.

Upregulated *CDK1* (degree=71) was the node with the highest degree in the PPI network for DEGs in the turquoise

cluster. In human mesenchymal stem cells (MSCs), *CDK1* activation has been reported to facilitate the differentiation of MSCs into osteoblasts by phosphorylating the enhancer of zeste homologue 2 at Thr 487 (33). Through promoting the binding between *Oct4* and the trophectoderm marker *CDX2*, *CDK1* has been demonstrated to prevent the generation of trophectoderm from ESCs and accordingly maintain stemness (34). *CDK1* suppression conferred by p57, as well as the inhibition of the DNA damage response caused by p21, can trigger the differentiation of trophoblast stem cells into giant cells (35). *CDK1/2* have been considered critical for the regulation of self-renewal and lineage specification of hESCs (36). *CDK1* expression has been reported to markedly decrease during ESC differentiation, whereas its knockdown reduced the colony formation potential and proliferation of ESCs, suggesting that *CDK1* may contribute to maintaining the self-renewing and unique undifferentiated state of mouse ESCs (37). In the present study, enrichment analysis for DEGs in the turquoise cluster revealed that *CDK1* was enriched in mitosis and cell cycle pathways. Therefore, it may be hypothesized that *CDK1* is involved in iPSC differentiation.

*HNF4A* and *EGF* were common genes between the two comparison groups, as they were revealed to be consistently downregulated in T5 and T24 samples. *HNF4A* serves an important role in specifying hepatic progenitor cells from hPSCs, via establishing the expression of the transcription factor network regulating the initiation of

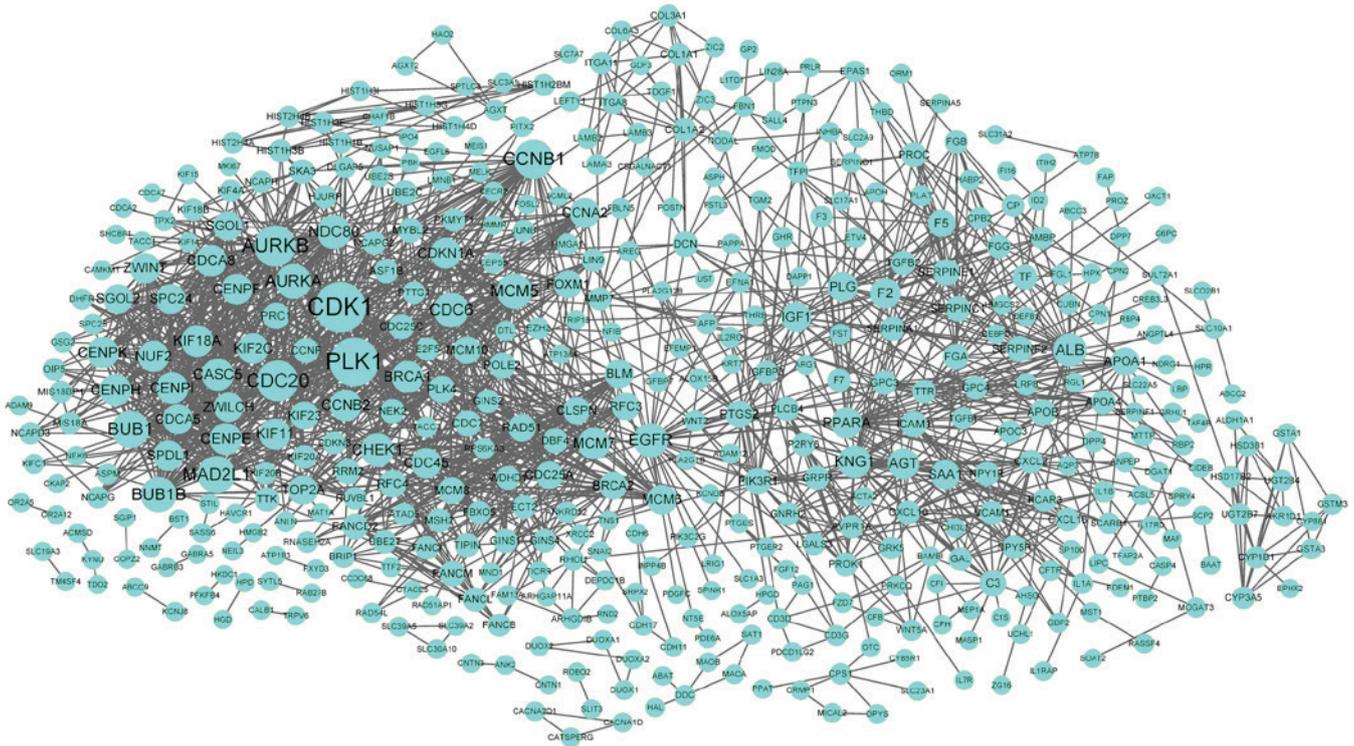


Figure 3. Protein-protein interaction network for differentially expressed genes in the turquoise cluster.

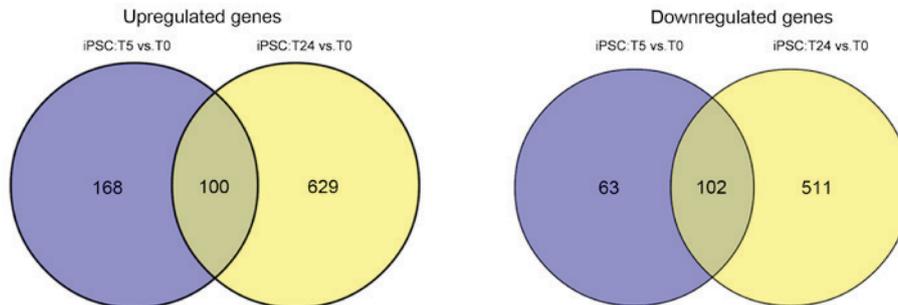


Figure 4. Venn diagram for identifying the common genes between the two comparison groups: T5 vs. T0 and T24 vs. T0.

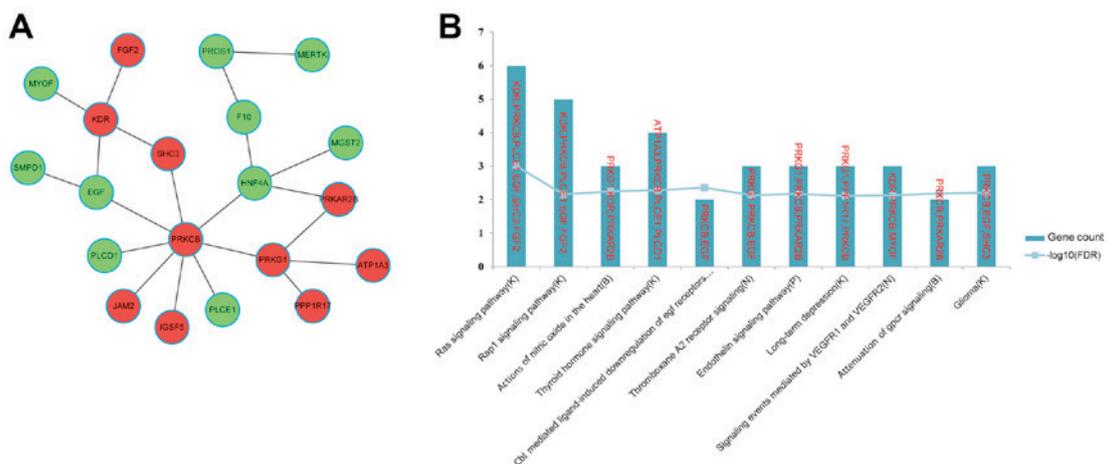


Figure 5. FI network for the common genes and the top 11 most significant pathways enriched for genes in the network. (A) FI network for the common genes. The red and green nodes represent up- and downregulated genes, respectively. (B) Top 11 most significant pathways enriched for the genes in the FI network. The y-axis represents the number of genes enriched in each pathway. The x-axis represents the various pathways. Common symbols for the genes involved in each pathway are shown in red. The database sources of the various pathways are included in brackets. FI, functional interaction; C, CellMap; R, Reactome; K, Kyoto Encyclopedia of Genes and Genomes; N, National Cancer Institute Pathway Interaction Database; P, Panther; and B, BioCarta.

Table IV. Top 5 functions and pathways enriched for differentially expressed genes in the turquoise cluster.

Category	ID	Description	FDR	Gene no.	Gene symbol
GO_BP	GO:0000278	mitotic cell cycle	3.93E-28	127	<i>NUSAP1, KIF18A, MIS18BP1</i> .....
	GO:0007067	mitotic nuclear division	1.58E-26	77	<i>CENPW, SPC25, SGOL1</i> .....
	GO:0000280	nuclear division	1.61E-25	90	<i>FANCD2, NDC80, MKI67</i> .....
	GO:1903047	mitotic cell cycle process	8.19E-25	111	<i>MCM10, RRM2, EZH2</i> .....
	GO:0048285	organelle fission	7.14E-24	90	<i>SPC24, RUVBL1, TPX2</i> .....
GO_CC	GO:0005615	extracellular space	3.10E-23	141	<i>SERPINA3, FSTL3, ACTA2</i> .....
	GO:0000775	chromosome, centromeric region	1.99E-16	39	<i>MIS18BP1, CENPW, HJURP</i> .....
	GO:0005694	chromosome	9.93E-16	90	<i>CENPW, HJURP, SPC25</i> .....
	GO:0000793	condensed chromosome	3.71E-15	40	<i>SPC24, CDCA5, CENPK</i> .....
	GO:0000779	condensed chromosome, centromeric region	7.92E-15	28	<i>BUB1, BUB1B, KIF2C</i> .....
GO_MF	GO:0005102	receptor binding	2.47E-04	100	<i>EPHX2, S100A14, F2</i> .....
	GO:0030414	peptidase inhibitor activity	2.47E-04	24	<i>SERPINA3, RPS6KA3, CD109</i> .....
	GO:0004867	serine-type endopeptidase inhibitor activity	2.47E-04	17	<i>SERPINA3, CD109, AGT</i> .....
	GO:0004866	endopeptidase inhibitor activity	2.47E-04	23	<i>SERPINA3, RPS6KA3, CD109</i> .....
	GO:0061135	endopeptidase regulator activity	3.74E-04	23	<i>AGT, AHSG, AMBP, SERPINA11</i> .....
KEGG pathway	530733	cell cycle	4.76E-16	80	<i>KIF18A, MIS18BP1, HJURP</i> .....
	105765	cell cycle, mitotic	1.16E-14	68	<i>KIF18A, SPC25, MCM5</i> .....
	83073	complement and coagulation cascades	1.44E-13	26	<i>FGB, FGG, SERPINC1</i> .....
	105815	mitotic prometaphase	2.98E-12	31	<i>KIF18A, SPC25, SGOL1</i> .....
	198880	complement and coagulation cascades	8.46E-12	21	<i>FGB, SERPINC1, CIS</i> .....

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function; FDR, false discovery rate.

hepatocyte differentiation (38). The miR-122/forkhead box A1/*HNF4A*-positive feedback loop has been reported to promote maturation and differentiation of mouse ESCs into hepatocytes, via controlling the balance between epithelial-to-mesenchymal and mesenchymal-to-epithelial transition, as well as the balance between differentiation and proliferation (39,40). Previous studies demonstrated that *EGF* promoted proliferation of mouse ESCs through  $Ca^{2+}$  influx, phospholipase C-protein kinase C, and p44/42 mitogen-activated protein kinases signaling pathways, via the phosphorylation of the EGF receptor (41,42). Heparin-binding epidermal growth factor-like growth factor can induce proliferation, as well as inhibit the adipogenic, chondrogenic and osteogenic differentiation of ESCs (43). In the present study, enrichment analysis for genes in the FI network revealed that *EGF* was enriched in the Ras (K) and Rap1 signaling pathways (K). These results suggested that *HNF4A* and *EGF* may also be implicated in the differentiation of iPSCs into hepatocytes.

In conclusion, in the present study, a comprehensive bioinformatics analysis was performed to investigate the

mechanisms involved in the differentiation of iPSCs to hepatocytes. A total of 433 and 1,342 DEGs were identified in T5 and T24 samples respectively, compared with T0 samples. The results indicated that *FGF2*, *BMP2*, *CDK1*, *HNF4A* and *EGF* may participate in the differentiation of iPSCs into hepatocytes. However, further experiments are required to elucidate their exact roles in the generation of hepatocytes from iPSCs.

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