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

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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, including hepatocyte nuclear factor 4α (HNF4A) and epidermal growth factor (EGF), in the FI network. Enrichment analysis for the common genes, including hepatocyte nuclear factor 4α (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-β 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 4α (HNF4A) may be implicated in the generation of hepatocytes from human ESC-derived hepatoblasts, which may

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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 log₂fold change (FC)≥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) 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 log₂FC ≥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≤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 log₂FC ≥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
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), and molecular transducer activity (GO_MF, FDR=2.50E-04; which involved HNF4A). 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), and receptor binding (GO_MF, FDR=1.16E-11; which involved HNF4A) and complement and coagulation cascades (pathway, FDR=1.27E-49). 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), and ribonucleotide binding (GO_MF, FDR=1.40E-07).

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.

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 cell cycle (GO_BP, FDR=1.55E-51), chromosome (GO_CC, FDR=1.18E-44), and ribonucleotide binding (GO_MF, FDR=1.40E-07) and cell cycle (pathway, FDR=1.67E-49).

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), and ribonucleotide binding (GO_MF, FDR=1.40E-07) and cell cycle (pathway, FDR=1.67E-49).

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  | tissue development                   | 2.70E-13 | 72       | HHEX, ARHGAP24, FOXQ1,… |
|          | GO_BP  | circulatory system development       | 2.88E-11 | 46       | EPHB3, HHEX, ARHGAP24,… |
|          | GO_BP  | cardiovascular system development    | 2.88E-11 | 46       | ADAM19, GATA4, GATA6,… |
|          | GO_CC  | extracellular space                  | 2.85E-07 | 47       | PRSS2, RELN, ABCA1,… |
|          | GO_CC  | semaphorin receptor complex          | 2.12E-04 | 4        | NRP2, NRP1, PLXNA2, PLXNA4 |
|          | GO_CC  | external side of plasma membrane     | 5.50E-04 | 15       | ABCA1, DLK1, ITGA5,… |
|          | GO_MF  | molecular transducer activity        | 2.50E-04 | 50       | EPHB3, ABCA1, WLS,… |
|          | GO_MF  | signal transducer activity           | 2.50E-04 | 50       | HNF4A, IL18R1, RXRG,… |
|          | GO_MF  | receptor activity                    | 7.51E-04 | 46       | SORC1S, FZD4, FZD8,… |
| KEGG pathway |       | other semaphorin interactions        | 5.42E-03 | 5        | SEMA6D, PLXNA2, SEMA5A,… |
|          |       | extracellular matrix organization    | 1.10E-02 | 15       | PRSS2, MATN3, ITGA5,… |
|          |       | adipogenesis                         | 5.42E-03 | 11       | SPOCK1, CYP26A1, GATA4,… |
| Downregulated | GO_BP  | cell-cell signaling                  | 1.20E-02 | 28       | SOX2, LPAR3, SFRP2,… |
|          | GO_BP  | synaptic transmission                | 2.03E-02 | 20       | LPAR3, CHRNA9, RASGRF2,… |
|          | GO_BP  | positive regulation of angiogenesis  | 4.82E-02 | 7        | FLT1, SFRP2, VASH2,… |
|          | GO_CC  | synapse                              | 1.42E-03 | 18       | NMNAT2, CHRNA9, GAP43,… |
|          | GO_CC  | synaptic membrane                    | 1.42E-03 | 11       | CHRNA9, GABRQ, CNKS5R2,… |
|          | GO_CC  | postsynaptic membrane                | 1.42E-03 | 10       | LRRTM3, VRL3, MET,… |

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function; FDR, false discovery rate.
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       | TTR, FAP, MF12...... |
|          | GO:0043062   | extracellular structure organization       | 1.39E-21| 66       | FBN1, EFEMP1, HPN... |
|          | GO:0009611   | response to wounding                       | 6.97E-19| 122      | CFH, EPHX2, SERPINA3 |
|          | GO:0005615   | extracellular space                        | 8.05E-44| 165      | ABCA1, IL32, FSTL3... |
|          | GO:0031012   | extracellular matrix                      | 1.34E-18| 63       | SERPIN1F1, CHI3L1, F2... |
|          | GO:0005578   | proteinaceous extracellular matrix         | 2.12E-17| 55       | SERPIN1A, FBN1, EFEMP1... |
| GO_CC    | GO:0005102   | receptor binding                           | 1.16E-11| 113      | EPHX2, ABCA1, IL32... |
|          | GO:1901681   | sulfur compound binding                    | 6.03E-09| 34       | CFH, ACADL, HNF4A... |
|          | GO:0050839   | cell adhesion molecule binding             | 7.59E-09| 29       | NDRG1, FGA, FGB...   |
| KEGG     | 83073        | complement and coagulation cascades        | 1.27E-16| 28       | CFH, F2, F3...      |
|          | 198880       | complement and coagulation cascades        | 7.64E-15| 23       | SERPIN1A, FGB, PLG... |
|          | M4470        | extrinsic prothrombin activation pathway   | 7.08E-13| 12       | FGB, FGG, SERPINC1... |
| Downregulated GO_BP | GO:000278   | mitotic cell cycle                         | 8.81E-55| 141      | NUSAP1, CDK3, KIF18A... |
|          | GO:0007049   | cell cycle                                | 1.55E-51| 180      | BRIP1, MIS18BP, CENPW... |
|          | GO:0022402   | cell cycle process                        | 1.06E-48| 151      | CENPW, CENPE, CENPF... |
|          | GO:0005694   | chromosome                                | 1.18E-44| 116      | NUSAP1, CHAF1B, MIS18BP... |
|          | GO:0044427   | chromosomal part                          | 1.44E-36| 97       | CHAF1B, MIS18BP, CENPW... |
|          | GO:0032993   | protein-DNA complex                        | 6.76E-32| 62       | MIS18BP, CENPW, CENPE... |
|          | GO:0032559   | adenyl ribonucleotide binding              | 1.40E-07| 94       | KIF18A, BRIP1, CENPE... |
|          | GO:0005524   | ATP binding                               | 1.40E-07| 92       | ATAD5, MARK1, MCM2... |
|          | GO:0030554   | adenyl nucleotide binding                  | 1.52E-07| 94       | MCM4, FFAS, MCM5...   |
| KEGG     | 53073        | cell cycle                                | 1.67E-49| 105      | KIF18A, PTTG1, MIS18BP... |
|          | 105765       | cell cycle, mitotic                        | 1.57E-35| 81       | KIF18A, PTTG1, CENPE... |
|          | 105750       | G2/M checkpoints                          | 7.54E-21| 24       | MCM2, MCM3, MCM4...   |

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

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).
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
has been demonstrated during the 

were common genes between the 

was 

and the trophectoderm marker 

mediated hESCs differentiation by maintaining 

or 

interacting with 

that 

the blue cluster, FGF2 could interact with BMP2, suggesting 

in the differentiation of iPSCs. In the PPI network for DEGs in 

findings suggested that 

generation of syncytiotrophoblasts from hESCs (32). These 

serves an important role in directing the 

differentiation guidance (31). In addition, the FGF pathway 

and it was able to substitute these BMPs during 

inducing hESCs differentiation than 

It has been revealed that 

differentiation of human ESCs, whereas its knockdown has 

been revealed to contribute to hESC differentiation (27,28). 

have 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

| Category | ID          | Description                                           | FDR    | Gene no. | Gene symbol                      |
|----------|-------------|-------------------------------------------------------|--------|----------|----------------------------------|
| GO_BP    | GO:2000026  | regulation of multicellular organismal development    | 5.97E-06 | 78       | CDKN2B, LPAR3, RAMP2……          |
|          | GO:0001763  | morphogenesis of a branching structure                 | 1.17E-05 | 24       | FOXA1, FGF2, FGFR1……            |
|          | GO:0048589  | morphogenesis of a branching epithelium development    | 1.17E-05 | 30       | LPAR3, BCL11A, DRAxin……         |
|          | GO:0061138  | morphogenesis of an epithelial tube                    | 1.17E-05 | 23       | FOXA1, FGF2, FGFR1……            |
|          | GO:0048754  | branching morphogenesis of an epithelial tube          | 1.17E-05 | 21       | FOXA1, FGF2, COL4A1……           |
| GO_CC    | GO:0044420  | extracellular matrix component                         | 1.46E-03 | 15       | COL4A1, COL4A2, COL4A5……        |
|          | GO:0000785  | chromatin                                             | 2.27E-03 | 26       | MCM2, HIST1H2A1, HIST1H2AB……    |
|          | GO:0005604  | basement membrane                                     | 2.36E-03 | 12       | COL4A1, COL4A2, COL4A5……        |
|          | GO:0005694  | chromosome                                            | 3.79E-03 | 40       | MCM2, MCM3, HIST1H4I……          |
|          | GO:0044427  | chromosomal part                                      | 4.60E-03 | 35       | MCM2, MCM3, HIST1H2A1……         |
| KEGG     | 106540      | telomere maintenance                                   | 7.80E-04 | 13       | HIST1H4I, HIST1H2AJ, HIST1H2AB……|
| pathway  | 366238      | amyloids                                              | 9.81E-04 | 13       | HIST1H4I, HIST1H2AJ, HIST1H2AB……|
|          | 106548      | packaging of telomere ends                            | 9.81E-04 | 10       | HIST1H4I, HIST1H2AJ, HIST1H2AB……|
|          | 477134      | meiotic synapsis                                      | 9.81E-04 | 12       | HIST1H4I, HIST1H2AJ, HIST1H2AB……|
|          | 83122       | systemic lupus erythematosus                          | 1.00E-03 | 16       | HLA-DOA, HIST1H4I, HIST1H2AJ……  |

GO: Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; FDR, false discovery rate.
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.
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 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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**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      | NUSAP1, KIF18A, MIS18BP1… |
|          | GO:0007067 | mitotic nuclear division            | 1.58E-26 | 77       | CENPW, SPC25, SGOL1…  |
|          | GO:000280  | nuclear division                    | 1.61E-25 | 90       | FANCD2, NDC80, MKI67… |
|          | GO:1903047 | mitotic cell cycle process          | 8.19E-25 | 111      | MCM10, RRM2, E2F2…  |
|          | GO:0048285 | organelle fission                   | 7.14E-24 | 90       | SPC24, RUVBL1, TPX2… |
| GO_CC    | GO:0005615 | extracellular space                 | 3.10E-23 | 141      | SERPINA3, FSTL3, ACTA2… |
|          | GO:0000775 | chromosome, centromeric region      | 1.99E-16 | 39       | MIS18BP1, CENPW, HJURP… |
|          | GO:0005694 | chromosome                          | 9.93E-16 | 90       | CENPW, HJURP, SPC25… |
|          | GO:0000793 | condensed chromosome                | 3.71E-15 | 40       | SPC24, CDCA5, CENPK… |
|          | GO:0000779 | condensed chromosome                | 7.92E-15 | 28       | BUB1, BUB1B, KIF2C…  |
| GO_MF    | GO:0005102 | receptor binding                    | 2.47E-04 | 100      | EPHX2, S100A14, F2…  |
|          | GO:0030414 | peptidase inhibitor activity        | 2.47E-04 | 24       | SERPINA3, RP56K3A, CD109… |
|          | GO:0004867 | serine-type endopeptidase inhibitor activity | 2.47E-04 | 17 | SERPINA3, CD109, AGT… |
|          | GO:0004866 | endopeptidase inhibitor activity    | 2.47E-04 | 23       | SERPINA3, RP56K3A, CD109… |
|          | GO:0061135 | endopeptidase regulator activity    | 3.74E-04 | 23       | AGT, AHSG, AMBP, SERPINA11… |
| KEGG     | 53073   | cell cycle                          | 4.76E-16 | 80       | KIF18A, MIS18BP1, HJURP… |
| pathway  | 105765  | cell cycle, mitotic                 | 1.16E-14 | 68       | KIF18A, SPC25, MCM5… |
|          | 83073   | complement and coagulation cascades | 1.44E-13 | 26       | FGB, FGG, SERPINC1… |
|          | 105815  | mitotic prometaphase                | 2.98E-12 | 31       | KIF18A, SPC25, SGOL1… |
|          | 198880  | complement and coagulation cascades | 8.46E-12 | 21       | FGB, SERPINC1, C1S… |

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. 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. 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. Heparin-binding epidermal growth factor-like growth factor can induce proliferation, as well as inhibit the adipogenic, chondrogenic and osteogenic differentiation of ESCs.
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