Identification of key genes induced by platelet-rich plasma in human dermal papilla cells using bioinformatics methods

HAIYAN SHEN*, HANXIAO CHENG*, HAIHUA CHEN and JUFANG ZHANG

Department of Plastic Surgery, Hangzhou First People's Hospital, Nanjing Medical University, Hangzhou, Zhejiang 310006, P.R. China

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Abstract. Dermal papilla cells (DPCs) are located at the base of hair follicles, and are known to induce hair follicle regeneration. Platelet-rich plasma (PRP) functions in hair follicle regeneration. To investigate the influence of PRP on DPCs, the present study analyzed RNA-seq data of human hair dermal papilla cells (HHDPCs) that were treated or untreated by PRP. The data included in the RNA-seq were from two normal and two treated HHDPC samples. Following identification by Cuffdiff software, differentially expressed genes (DEGs) underwent enrichment analyses, and protein-protein interaction networks were constructed using Cytoscape software. Additionally, transcription factor (TF)-DEG and TF-long non-coding RNA (lncRNA) regulatory networks were constructed. A total of 178 differentially expressed lncRNA were screened, 365 were upregulated and 142 were downregulated. Notably, upregulated cyclin dependent kinase 1 (*CDK1*) (degree=76), polo-like kinase 1 (*PLK1*) (degree=65), cell division cycle 20 (degree=50), cyclin B1 (degree=49), aurora kinase B (degree=47), cyclin dependent kinase 2 (degree=46) and downregulated v-myc avian myelocytomatosis viral oncogene homolog (*MYC*) (degree=12) had higher degrees in networks. In addition, CCAAT/enhancer binding protein β, E2F transcription factor 1 (*E2F1*), early growth response 1 and *MYC* may be key TFs for their target genes, and were enriched in pathways associated with the cell cycle. They may also be involved in cell proliferation via various interactions with other genes, for example *CDK1-PLK1* and *E2F1→CDK1*. These dysregulated genes induced by PRP may affect proliferation of HHDPCs.

Correspondence to: Dr Jufang Zhang, Department of Plastic Surgery, Hangzhou First People's Hospital, Nanjing Medical University, 261 Huansha Road, Hangzhou, Zhejiang 310006, P.R. China
E-mail: jufangzhangjf@163.com

*Contributed equally

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Introduction

As a type of dermal cell located in the base of the hair follicle, dermal papilla cells (DPCs) have the ability to induce hair follicle regeneration and hair growth (1). Platelet-rich plasma (PRP) is plasma enriched with a higher proportion of platelets, compared with that with that which is usually found in whole blood. α-granules in platelets contain growth factors, indicating that PRP has the ability to promote cell proliferation and differentiation (2). Previous studies have reported that PRP may induce mesenchymal stem cell proliferation and chondrogenic differentiation *in vitro* (3-5). It has been reported that PRP is essential in hair follicle regeneration (6). Thus, it was hypothesized that PRP may contribute to hair follicle regeneration via changes in DPC proliferation levels. Hair follicle regeneration is currently known to have an impact in treating alopecia and dermal wounds (7,8). The initial generation of a hair follicle is intimately linked with signal exchange between mesenchymal and epithelial cells via the formation of hair placodes (9,10). Alopecia is defined as a loss of hair from the body or head, and may be induced by nutritional deficiencies, fungal infection and traumatic damage (11). Increased chronic ulcers, skin disease, trauma caused by burns and accidental skin defects mean the restoration of dermal wounds is becoming an important medical concern (12). Thus, it is important to investigate the influence of PRP on DPC and develop therapeutic strategies for hair follicle regeneration.

Previous studies have investigated mechanisms of hair follicle regeneration. For example, S100 calcium binding protein A4 and S100 calcium binding protein A6 may be important in activating stem cells at the beginning of follicle regeneration (13,14). Wingless-type mouse mammary tumor virus integration site family member 10b promotes hair follicle growth and regeneration via activation of the canonical Wnt signaling pathway and, thus, may be used as therapeutic target in the treatment of hair follicle-associated diseases (15,16). Via the Gpr44 receptor, prostaglandin D2 may function in inhibiting hair follicle regeneration (17). As a crucial ATPase of the BAF chromatin-remodeling complex, brahma-related gene 1 may regulate the processes

of epidermal repair and hair regeneration in bulge stem cells (18).

Preliminary studies demonstrated that PRP at a concentration of 5% should be used to treat human hair DPCs (HHDPCs) in the present study (data not shown). Using RNA-seq data of HHDPCs from normal samples and samples treated with 5% PRP, the present study aimed to identify differentially expressed genes (DEGs) and predict their possible function using Gene Ontology (GO) and pathway enrichment analyses. The interactions and associations between the DEGs were investigated using protein-protein interaction (PPI) networks. Furthermore, regulatory networks were constructed to screen key genes and transcription factors (TFs).

Materials and methods

PRP preparation. Samples of whole blood (10 ml) were taken from the median cubital vein of each of the 8 male, healthy participants (mean age, 24.9 years) and mixed with 3.2% sodium citrate (vol/vol=10:1). PRP was prepared using a two-step centrifugation method. Firstly, the whole blood was centrifuged at 400 x *g* for 10 min at room temperature, allowing separation of blood into three layers, the topmost platelet-poor plasma layer, an intermediate PRP layer and the bottommost red blood cell layer. Subsequently, the upper two layers were centrifuged again at 3,800 x *g* for 10 min at room temperature. The platelets in PRP were activated by 0.2 ml 10% $CaCl_2$ and 1,000 U bovine thrombin. After a 10 min incubation, PRP was centrifuged at 1,500 x *g* for 5 min at room temperature, and the supernatant was stored at -80˚C. All participants provided informed consent, and the present study was approved by the ethics committee of the Hangzhou First People's Hospital (Hangzhou, China).

HHDPCs cultivation. At 37˚C in a humidified 5% CO_2 incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA), the HHDPCs (Shanghai Hu Zheng Industrial Co., Ltd., Shanghai, China) were cultivated in a medium consisting of 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) and 1% double antibody (Gibco; Thermo Fisher Scientific, Inc.). HHDPCs were passaged at 80-90% confluence and pancreatin was used for digestion (Gibco; Thermo Fisher Scientific, Inc.). Subsequently, the cells were centrifuged at 300 x *g* for 5 min at room temperature and the supernatant was removed. HHDPCs were preserved in a frozen stock solution which consisted of 10% dimethyl sulfoxide, 40% FBS and 50% RPMI-1640.

RNA extraction and RNA-seq library construction. Following cell counting, HHDPCs were spread on 6-well plates (Applied Biosystems; Thermo Fisher Scientific, Inc.; $2x10^5$ cells/well) and starved for 24 h. HHDPCs in the treatment group were treated with 5% PRP whilst the HHDPCs in the control group were treated with RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin solution. Each experiment was repeated twice. Total RNA from HHDPC treatment samples and normal HHDPC samples was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The purity and integrity of total RNA were checked by a spectrophotometer (Merinton Instrument, Ltd., Beijing, China) and 2% agarose gel electrophoresis. Following this, the RNA-seq library was prepared using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Inc. Ipswich, MA, USA) according to the manufacturer's protocols. Briefly, mRNAs were isolated and divided into ~200 nt fragments. Subsequently, double-stranded cDNAs were synthesized and modified, and DNA cluster amplification was performed using a Phusion® Human Specimen Direct Polymerase Chain Reaction kit (Thermo Fisher Scientific, Inc.). The reaction mixture was subjected to the following cycling conditions: An initial denaturation step at 94˚C for 30 sec, followed by 11 cycles of denaturation at 98˚C for 10 sec, annealing at 65˚C for 30 sec and extension at 72˚C for 30 sec, and a final extension step at 72˚C for 5 min. Using Illumina HiSeq 2500 v4 100PE (Illumina, Inc., San Diego, CA, USA), high-throughput sequencing was conducted for the RNA-seq library.

DEGs screening. RNA-seq data were preprocessed by the Next Generation Sequencing Quality Control Toolkit (19). Any sequences containing >20% bases with a quality value <20 were filtered out. Using TopHat2 software (ccb.jhu.edu/software/tophat/index.shtml) (20), RNA-seq data was aligned to human genome hg19, which was downloaded from the University of California, Santa Cruz website (genome.ucsc.edu) (21). The parameter was set as no-mixed and other parameters used default settings. Cuffdiff software (cufflinks.cbcb.umd.edu/) (22) was used to identify the DEGs between RNA-seq data of normal HHDPCs and treated HHDPCs. The adjusted $P<0.05$ and $|\log_2 \text{fold change (FC)}|>1$ served as the cut-off criteria.

Functional and pathway enrichment analysis. GO analysis aims to describe subcellular location, molecular function and the biological processes of gene products (23). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database introduces functions of molecules or genes (24). Using the GOFunction (25) of bioconductor (www.bioconductor.org/), as well as annotation files GO.db (26) and org.Hs.eg.db (27), GO and KEGG pathway enrichment analyses were performed for the DEGs. $P<0.05$ and ≥2 genes enriched in one pathway served as the cut-off criteria.

PPI network construction. The STRING online software (28) was applied to screen interactions of proteins encoded by DEGs, and the Cytoscape software (www.cytoscape.org/) (29) was used to visualize the PPI network. A combined score >0.7 was used as the cut-off criterion.

Regulatory network construction. Using information provided by the ENCODE website (genome.ucsc.edu/ENCODE/encode.hg17.html) regarding TF binding sites, (30), in addition to genetic and long non-coding RNAs (lncRNAs) location information on the genome, differentially expressed TF-DEG pairs and differentially expressed TF-differentially expressed lncRNA pairs were screened. A certain amount of overlapping occurred between TF binding sites and the cut-off criterion was defined as 1,000 bp upstream to 500 bp downstream of the transcription start site.

Table I. Pathways enriched for upregulated genes.

Category	Term	Description	Gene number	Gene symbol	P-value
KEGG	4110	Cell cycle	25	*BUB1, BUB1B*	<0.0001
KEGG	4914	Progesterone-mediated oocyte maturation	12	*CCNA2, CCNB1*	$8.13x10^{-8}$
KEGG	4114	Oocyte meiosis	13	*AURKA, BUB1*	$2.12x10^{-7}$
KEGG	5130	Pathogenic *Escherichia coli* infection	8	*ACTB, ACTG1*	$1.10x10^{-5}$
KEGG	4115	p53 signaling pathway	8	*CCNB1, CCNB2*	$4.72x10^{-5}$
KEGG	4145	Phagosome	11	*ACTB, ACTG1*	0.000182
KEGG	4540	Gap junction	8	*CDK1, PDGFRB*	0.000346
KEGG	3030	DNA replication	5	*FEN1, MCM3*	0.000614
KEGG	4974	Protein digestion and absorption	7	*COL18A1, COL2A1*	0.000971
KEGG	4510	Focal adhesion	11	*ACTB, ACTG1*	0.001748

KEGG, Kyoto Encyclopedia of Genes and Genomes; *BUB1*, BUB1 mitotic checkpoint serine/threonine kinase; *BUB1B*, BUB1 mitotic checkpoint serine/threonine kinase B; *CCNA2*, cyclin A2; *CCNB1*, cyclin B1; *AURKA*, aurora kinase A; *ACTB*, β-actin; *ACTG1*, γ-actin 1; *CCNB2*, cyclin B2; *CDK1*, cyclin dependent kinase 1; *PDGFRB*, platelet derived growth factor receptor β; *FEN1*, flap structure-specific endonuclease 1; *MCM3*, mini chromosome maintenance complex component 3; *COL18A1*, collagen type XVIII α1 chain; *COL2A1*, collagen type II α 1 chain.

Table II. Pathways enriched for downregulated genes.

Category	Term	Description	Gene number	Gene symbol	P-value
KEGG	4115	p53 signaling pathway	6	*BBC3, CCND2*	<0.0001
KEGG	250	Alanine, aspartate and glutamate metabolism	3	*ASNS, GLS2, GOT1*	0.002425
KEGG	5219	Bladder cancer	3	*CDKN1A, MYC, VEGFA*	0.005288
KEGG	4010	MAPK signaling pathway	7	*DDIT3, DUSP2*	0.00709
KEGG	330	Arginine and proline metabolism	3	*GLS2, GOT1, SAT1*	0.010639
KEGG	910	Nitrogen metabolism	2	*ASNS, GLS2*	0.016056
KEGG	4964	Proximal tubule bicarbonate reclamation	2	*GLS2, PCK2*	0.016056
KEGG	4110	Cell cycle	4	*CCND2, CDKN1A*	0.020687
KEGG	4612	Antigen processing and presentation	3	*CIITA, HLA-DQA1, HLA-DQA2*	0.026471
KEGG	5310	Asthma	2	*HLA-DQA1, HLA-DQA2*	0.026583

KEGG, Kyoto Encyclopedia of Genes and Genomes; *BBC3*, BCL2 binding component 3; *CCND2*, cyclin D2; *ASNS*, asparagine synthetase (glutamine-hydrolyzing); *GLS2*, glutaminase 2; *GOT1*, glutamic-oxaloacetic transaminase 1; *CDKN1A*, cyclin dependent kinase inhibitor 1A; *MYC*, v-myc avian myelocytomatosis viral oncogene homolog; *VEGFA*, vascular endothelial growth factor A; *DDIT3*, DNA damage inducible transcript 3; *DUSP2*, dual specificity phosphatase 2; *SAT1*, spermidine/spermine N1-acetyltransferase 1; *PCK2*, phosphoenolpyruvate carboxykinase, mitochondrial; *CIITA*, class II major histocompatibility complex transactivator; *HLA-DQA1*, major histocompatibility complex, class II, DQ α 1; *HLA-DQA2*, major histocompatibility complex, class II, DQ α 2; MAPK, mitogen-activated protein kinase.

Results

DEGs analysis. Compared with normal HHDPC samples, there were 365 upregulated and 142 downregulated genes screened in the treated HHDPC samples. Furthermore, 178 differentially expressed (including 131 upregulated and 47 downregulated) lncRNAs were identified in the treated HHDPC samples.

Functional and pathway enrichment analysis. The enriched KEGG pathways for upregulated genes were listed in Table I, including cell cycle (P<0.0001), progesterone-mediated oocyte maturation (P<0.0001) and DNA replication (P=0.000614). The enriched KEGG pathways for downregulated genes included p53 signaling pathway (P<0.0001), mitogen-activated protein kinase signaling pathway (P=0.00709) and cell cycle (P=0.020687; Table II). In addition, no GO functions were enriched for the DEGs.

PPI network analysis. The PPI network for upregulated genes had 192 nodes and 1,017 interactions (Fig. 1). In this network,

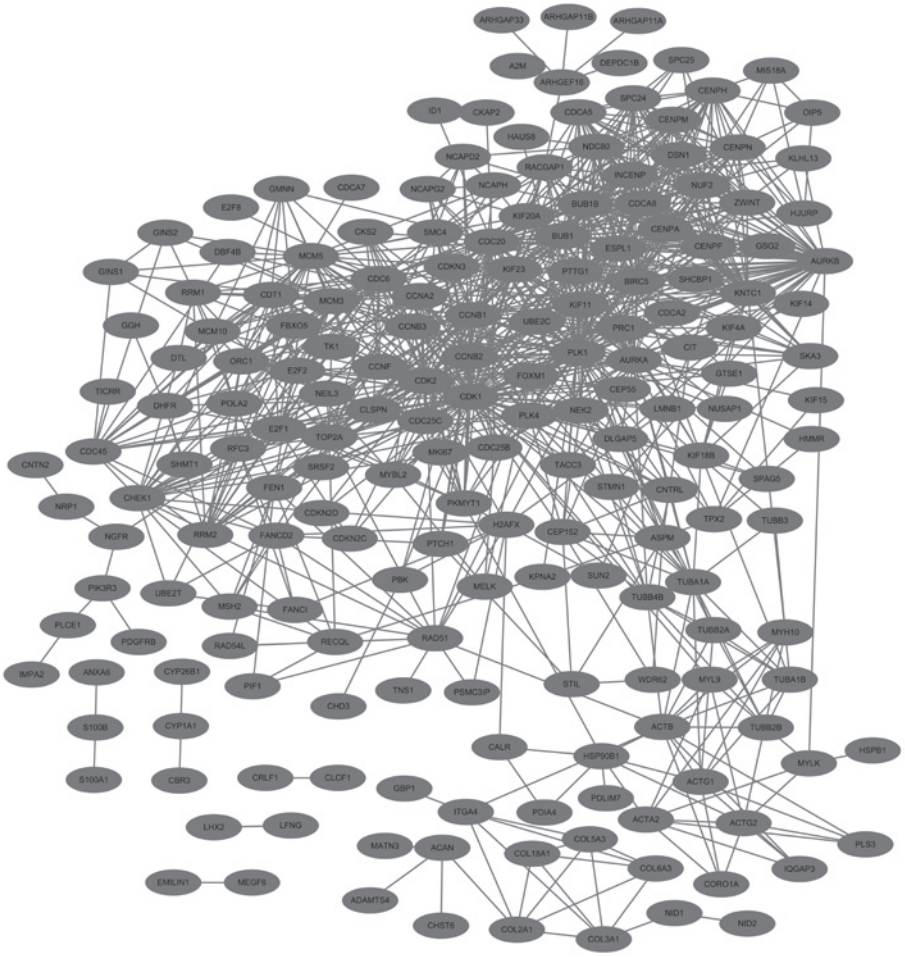

Figure 1. Protein-protein interaction network for upregulated genes. The gray nodes represent the upregulated genes.

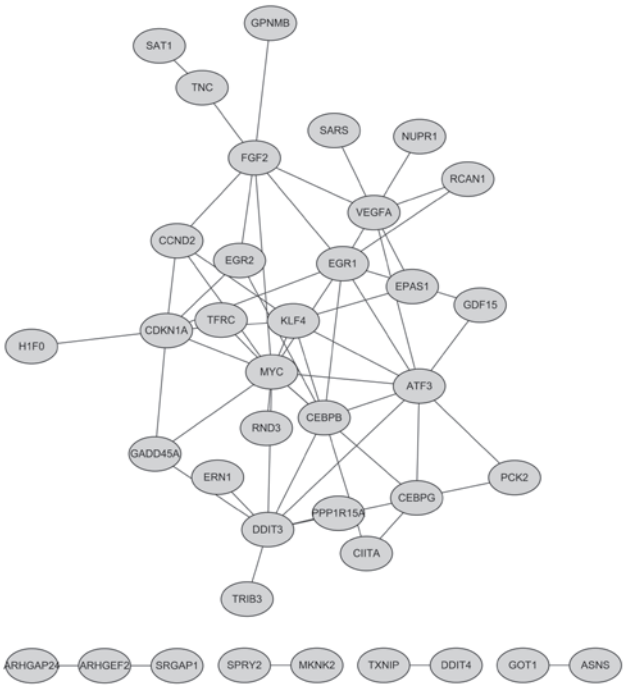

Figure 2. Protein-protein interaction network for downregulated genes. The gray nodes indicate the downregulated genes.

cyclin-dependent kinase 1 (CDK1, degree=76), polo-like kinase 1 (PLK1, degree=65), cell division cycle 20 (CDC20, degree=50), cyclin B1 (CCNB1, degree=49), aurora kinase B (AURKB, degree=47) and cyclin-dependent kinase 2 (CDK2, degree=46) demonstrated higher degrees. In addition, these genes interacted with each other for example, *CDK1-PLK1*, *CDC20-CCNB1*, *AURKB-CDK2* and *CDK1-AURKB* in the PPI network.

The PPI network for downregulated genes had 38 nodes and 58 interactions (Fig. 2). In this network, c-myc (*MYC*, degree=12), activating transcription factor 3 (degree=9), DNA-damage-inducible transcript 3 (degree=8) and early growth response 1 (*EGR1*, degree=8) indicated higher degrees.

Regulatory network analysis. A total of 453 TF-DEG pairs and 530 TF-lncRNA pairs were identified. The TF-DEG and TF-lncRNA regulatory networks are presented in Figs 3 and 4. Notably, the target genes of TFs CCAAT/enhancer binding protein-β (*C/EBP*-β), E2F transcription factor (*E2F*) *1*, *EGR1* and *MYC* were enriched in pathways associated with the cell cycle. In the TF-DEG regulatory network, these TFs targeted *CDK1*, *PLK1*, *CCNB1* and *AURKB* (for example, *MYC→PLK1*, *MYC→CCNB1*, *MYC→AURKB*, *CEBPB→AURKB*, *E2F1→CDK1* and *E2F1→CCNB1*).

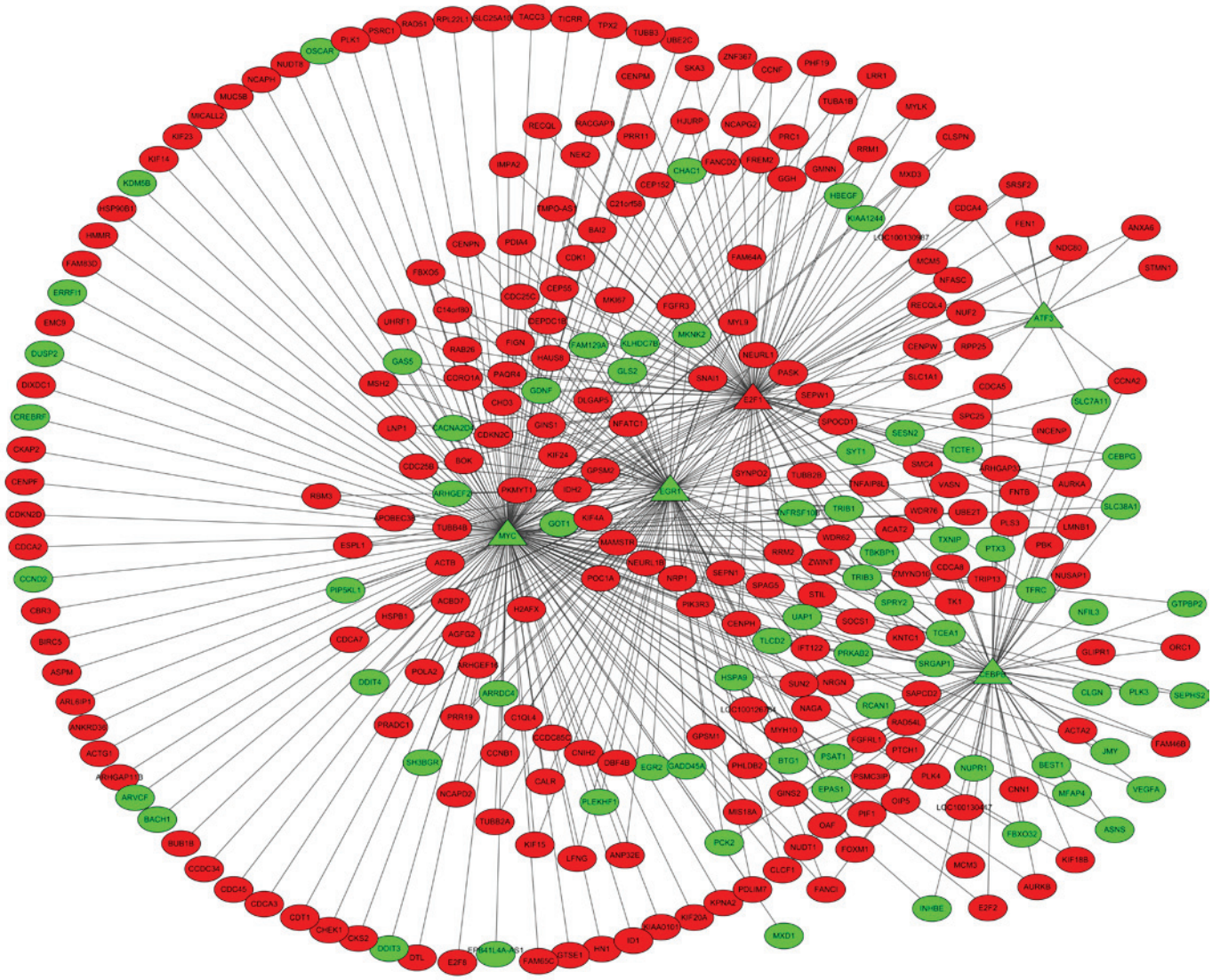

Figure 3. TF-DEG regulatory network. The red and green nodes represent upregulation and downregulation, respectively. The circles and triangles indicate for DEGs and TFs, respectively. TF, transcription factor; DEG, differentially expressed gene.

Discussion

The present study screened 178 differentially expressed lncRNA, 365 upregulated genes and 142 downregulated genes in treated HHDPC samples compared with normal HHDPC samples. Only selected DEGs and lncRNAs have been presented in the present study. DEGs enriched in pathways have been listed in Tables I and II. Pathway enrichment indicated that these upregulated and downregulated genes were enriched in proliferation-associated pathways, including those involved in the cell cycle and DNA replication, indicating that PRP may affect cell proliferation of HHDPCs. The DEGs and lncRNAs that were key nodes in the networks have been mentioned. In the PPI networks, upregulated *CDK1* (degree=76), *PLK1* (degree=65), *CDC20* (degree=50), *CCNB1* (degree=49), *AURKB* (degree= 47), *CDK2* (degree=46) and downregulated *MYC* (degree=12) had higher degrees. Furthermore, *CEBPB*, *E2F1*, *EGR1* and *MYC*, which may be key TFs for their target genes, were enriched in pathways associated with the cell cycle.

CDK1 activity is continuously required to ensure continuing end resection and to maintain a double-strand break-induced checkpoint. Furthermore, *CDK1* is involved in later stages of homologous recombination (31). It has been demonstrated that *CDK1* is the only crucial cell cycle CDK and is able to drive cell division alone (32). As an important moderator of cell division (33), *PLK1* is essential for mitotic entry at the appropriate time (34). *PLK1* appears to be required for centrosome-mediated microtubule activities and spindle assembly. In addition, siRNAs targeted against *PLK1* may be potential anti-proliferative agents that inhibit neoplastic cells (35,36). This suggests that *CDK1* and *PLK1* may be associated with cell proliferation.

CDC20 can activate anaphase-promoting complex (APC/C) in metaphase of the cell cycle, and its C terminus contains a WD40 repeat domain that regulates protein-protein interactions (37,38). Suppression of *CCNB1*, which is a cyclin controlling mitotic entry, induces a marked arrest of G_2/M phase in HCT116 and SW480 cells, preventing the expression

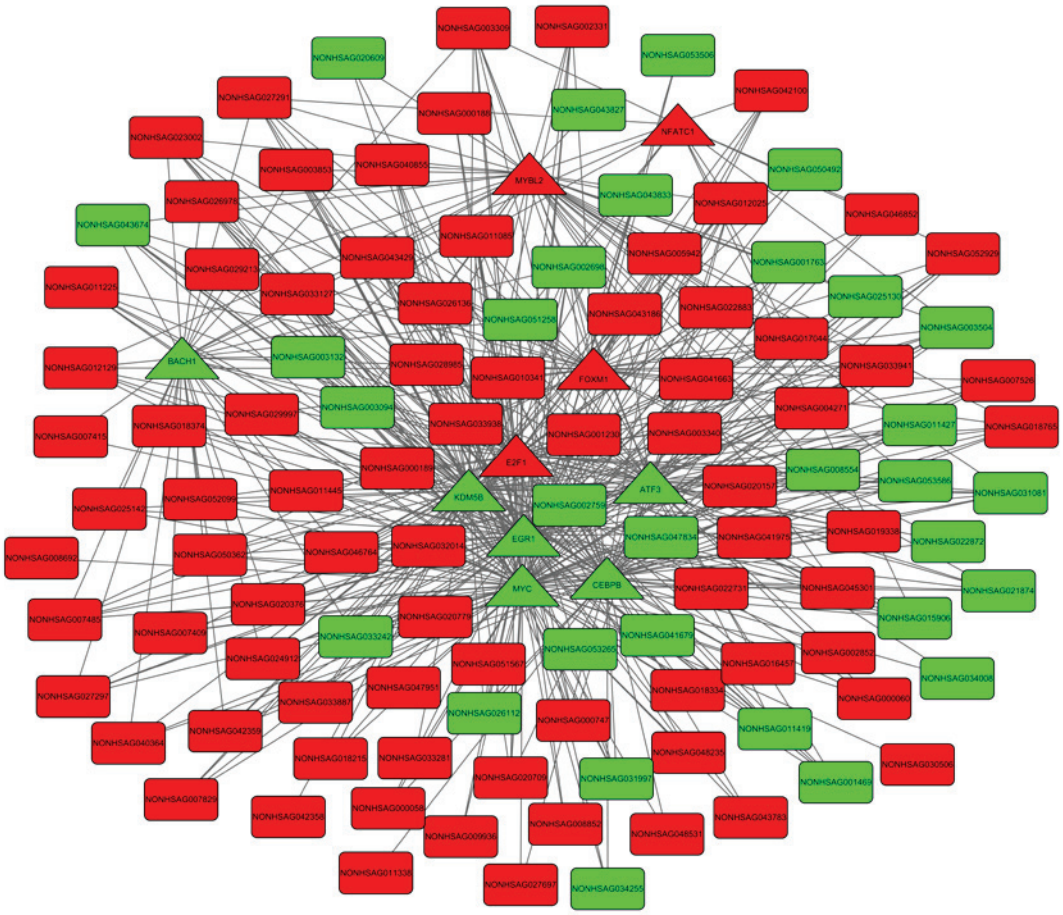

Figure 4. TF-lncRNA regulatory network. The red and green nodes represent upregulation and downregulation, respectively. The circles and rectangles indicate TFs and lncRNAs, respectively. TF, transcription factor; lncRNA, long non-coding RNA.

of *CDK1* and cell division cycle 25C (39,40). AURKB functions in chromosome bi-orientation, spindle assembly and cytokinesis, and may be degraded by APC/C bound to cadherin 1 in G_1 and in late mitosis (41). In animal cells, CDK2/cyclin E can trigger initiation of centrosome duplication by targeting substrate nucleophosmin/B23 (42-44). Thus, the expression levels of *CDC20*, *CCNB1*, *AURKB* and *CDK2* may be associated with cell proliferation. In the PPI network for upregulated DEGs, it was observed that numerous DEGs demonstrated a certain level of interaction with each other, for example, *CDK1-PLK1*, *CDC20-CCNB1*, *AURKB-CDK2* and *CDK1-AURKB*. Therefore, it may be other genes, including *PLK1* and *CCNB*, which had interaction with *CDC20*, *CCNB1*, *AURKB* and *CDK2*, may also function in cell proliferation.

The proto-oncogene *MYC* encodes transcription factor c-myc which is associated with regulation of cell proliferation and differentiation (45). Conditional activation or constitutive expression of wild-type *CEBPB* may promote differentiation and suppress proliferation of 32D-BCR/ABL cells, which is contrary to a DNA binding-deficient *CEBPB* mutant (46). It is reported that *E2F1*, *E2F2* and *E2F3* function as transcriptional activators in the process of the G_1/S transition (47). As a member of the Egr transcription factor family, *EGR1* is involved in cell growth, proliferation and stress responses in various types of tissues (48). This may suggest the expression levels of *CEBPB*, *E2F1*, *EGR1* and *MYC* were associated with cell proliferation. In the TF-DEG regulatory network, it was observed that TFs could target *CDK1*, *PLK1*, *CCNB1* and *AURKB*, for example *MYC→PLK1*, *MYC→CCNB1*, *MYC→AURKB*, *CEBPB→AURKB*, *E2F1→CDK1* and *E2F1→CCNB1*, suggesting that TFs may be involved in cell proliferation via regulation of *CDK1*, *PLK1*, *CCNB1* and *AURKB*.

In conclusion, the present study conducted a comprehensive bioinformatics analysis of genes that may be associated with cell proliferation. A total of 178 differentially expressed lncRNA were identified, and additionally, 365 upregulated and 142 downregulated genes were screened. It was indicated that *CDK1*, *PLK1*, *CDC20*, *CCNB1*, *AURKB*, *CDK2*, *CEBPB*, *E2F1*, *EGR1* and *MYC* may be associated with cell proliferation of HHDPCs. Thus, PRP may be important in HHDPCs. Further investigation is required in order to elucidate the functional mechanisms of these genes in HHDPCs. In addition, the proliferative ability of HHDPCs treated with PRP will be investigated in future work.

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