Transcriptome Analysis of EnJSRV-Env on the Inhibition of Cell Proliferation

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Research

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Abstract

Background

Endogenous retroviruses (ERVs) exert important biological roles, such as mammalian placental development and suppression of the infection of exogenous retrovirus. In addition, ERVs could also inhibit the proliferation of cell. The envelope-protein (Env) of endogenous Jaagsiekte sheep retroviruses (enJSRVs) possesses fusogenic activity, which promoted the formation of nonproliferative multinucleated syncytiotrophoblasts.

Methods

The proliferation of HeLa cells was detected by MTT. Six samples were extracted for RNA-seq transcriptome analysis. Quantitative real-time PCR (qRT-PCR) and western blotting were employed for the validation of interested target.

Results

enJSRV-Env transfection inhibited the proliferation ability of Hela cells and 170 differentially expressed genes (DEGs) were obtained by RNA-seq analysis. Among these, 5 DEGs (BHLHE41, CCN1, DLX2, DUSP6 and SH2D5) were validated by qRT-PCR, which closely related with proliferation. Western blotting analysis showed that the expression of DUSP6 and p-ERK1/2 was decreased, which suggested that ERK1/2 signaling pathway may be involved in enJSRV-Env transfection.

Conclusions

enJSRV-Env transfection inhibited the proliferation of Hela cells, probably via DUSP6 and ERK1/2 signaling pathway.

Background

ERVs are proviruses which present in all vertebrates. They are derived from the ancient infections of the host germ line by exogenous retroviruses. The ovine genome contains approximately 27 copies of endogenous retroviruses that are related to the exogenous Jaagsiekte sheep retroviruses (JSRVs), an oncogenic retrovirus tropic to the lung[1–4]. The enJSRV-Env possesses fusogenic activity, which was thought to induce the fusion of mononuclear trophoblast cells to generate nonproliferative multinucleated syncytiotrophoblasts. Additionally, enJSRV-Env plays a critical role in sheep conceptus development and placental morphogenesis[5, 6].

ERVs in several species has been reported to integrate into the genome of other species, and the Env of ERVs has similar biological functions among species. For example, the Env of bovine endogenous retroviruses (BERV-K1) exhibited fusogenic activity in ovine trophoblast cells[7]. The Env of human endogenous retroviruses (HERV-W) could promote the fusion of quail QT6 cells, hamster BHK21 cells, mouse NIH 3T3 cells, or rat XC cells with HeLa cells [8]. Moreover, the Env of HERV-3 could inhibit the proliferation of BeWo cells[9]. These let us to examine the role of enJSRV-Env in HeLa cells. In this study, the ability of enJSRV-Env on the cell fusion and proliferation will be investigated in Hela cell, and the related mechanisms will be demonstrated by RNA-seq analysis.

Materials And Methods

Cell culture and transfection

Hela cells were purchased from American Tissue Culture Collection (ATCC; Rockville, USA) and cultured in DMEM medium supplemented with 10% FBS in an incubator with 5% CO_2 at 37 °C. The pEGFP-C1/enJSRV-Env vector, used for transient transfections, was previously generated by our laboratory[10], and was transfected by using PEI transfection reagent (Sigma-Aldrich, Saint louis, USA). Cells were harvested 48 h after transfection for the following experiments. The empty plasmid was employed as negative control.

Cell-cell fusion assays

The determination of the fusion activity of the transfected cells was performed 48 h after transfection. Giemsa solutions (Solarbio, Beijing, China) were added to visualize the nuclei of the cells in accordance with the manufacturer's recommendations.

Cell proliferation assay

Cell proliferation was determined by MTT method (Solarbio, Beijing, China). In details, cells were plated in 96-well plates at a density of 5×10^3 per well and incubated at 37 ℃ in the presence of 5% CO_2. After 24, 48 and 72 h incubation, cells were processed by adding 20μL /well (5mg/mL) MTT and incubated for 4 h at 37℃. Then, 150 μL DMSO was added to each well, and the absorbance was examined at a wave length of 490 nm by a microplate reader.

RNA Extraction and cDNA Library Construction

The total RNA was extracted from the cells transfected with pEGFP-C1/enJSRV-Env (Env group) or pEGFP-C1 (NC group) by Trizol, and the mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. First strand cDNA was synthesized using random oligonucleotides and SuperScript II. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA). The sequencing library was then sequenced on a HiSeq platform (Illumina).

Transcriptome Sequencing and Bioinformatics Analysis

Firstly, the HTSeq (0.9.1) was launched to compare the Read Count values on each gene as the original expression of the gene, and then the FPKM was employed to standardize the expression. Then, the DESeq (1.30.0) was used to analyze the DEGs with screened conditions as follows: expression difference multiple |log2FoldChange| > 1, significant P-value < 0.05. Both annotations of GO and KEGG were carried out to identify functional genes.

qRT-PCR analysis

The total RNA from cells was extracted with Trizol reagent (XYGEN, CA, USA) and subsequently reverse transcribed into cDNA by utilizing PrimeScript RT reagent Kit (TaKaRa, Dalian, China). Real-time PCR was carried out with SYBR Premix EX Taq™ II kit (TaKaRa, Dalian, China) on the PCR detection instrument (Opticon CFD-3200). GAPDH were used as reference gene. The quantification of the relative expression of targeted genes was carried out using the $2^{-\Delta\Delta Ct}$ method[11]. The primer sequences are list in table 1.

Table 1. The primer sequences for qRT-PCR

Name of primers	Primers sequences (5'–3')	
GAPDH	FORWARD	TGACTTCAACAGCGACACCCA
	REVERSE	CACCCTGTTGCTGTAGCCAAA
BHLHE4	FORWARD	CTCAGCTGAAAGATTTACTGCC
	REVERSE	AGGCGGTTAAAGCTTTTAAGTG
CCN1	FORWARD	CTTGTGAAAGAAACCCGGATTT
	REVERSE	ACTCAAACATCCAGCGTAAGTA
DLX2	FORWARD	TTGAGCCTGAAATTCGGATAGT
	REVERSE	CAGCTGGAAACTGGAGTAGATG
DUSP6	FORWARD	ATGATAGATACGCTCAGACCCG
	REVERSE	GATGTGCGACGACTCGTATAG
SH2D5	FORWARD	AGGAGCTGCCAGAGTCGGAAG
	REVERSE	CGGATCACCTTGCTGCGAATGG
HIST1H4K	FORWARD	GCGGGAAGGGTCTTGGCAAAG
	REVERSE	TCGTAGATGAGGCCGGAGATGC
NKD1	FORWARD	ACCATTGCGTAGATGAGAACAT
	REVERSE	CCAAATTGGGACGTGTAGTTTT

Western blotting

Cells were lysed with RIPA Buffer (Thermo Fisher Scientific, USA), and the protein concentration was determined with the BCA Kit (Beyotime, Beijing, China). The equivalent amounts of proteins were separated on 12% SDS-PAGE and subsequently transferred to PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk dissolved in BSA solution, the membrane was incubated with the primary antibody at 4℃ overnight, followed by incubation with secondary antibody at room temperature for 1 h. Finally, the bands were detected with enhanced chemiluminescence reagent ECL detection kit (Thermo Fisher Scientifc, Inc.) on ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). Antibodies for Erk1/2(cat: 4695, Dilution: 1:1000), Phospho-Erk1/2(cat:4370, Dilution: 1:2000), p38(cat: 9212S, Dilution: 1:1000) and Phospho-p38(cat: 4511P, Dilution: 1:1000) were purchased from Cell Signaling Technology; antibodies for JNK(cat: ab179461, Dilution: 1:1000), Phospho-JNK(cat: ab124956, Dilution: 1:1000), GAPDH(cat: ab8245, Dilution: 1:2000) and DUSP6(cat: ab76310, Dilution: 1:1000) were purchased from Abcam.

Statistical analysis

All data were shown as means ± SD, and at least three independent experiments were performed for each condition. Statistical analysis was conducted by employment of SPSS 21.0 software. Student's t test and one-way ANOVA were applied for comparison of the differences in two groups or more respectively. Differences were defined as statistically significant when $P < 0.05$.

Results

Effect of enJSRV-Env on cell-cell fusion activity and cell viability

Firstly, we evaluated the effect of enJSRV-Env on cell-cell fusion activity. Hela cells transfected with enJSRV-Env did not show the multinucleated phenomenon after 48h (Figure 1A). However, the MTT assay showed that enJSRV-Env can suppress the proliferation of HeLa cells (Figure 1B).

RNA-seq transcriptome analysis for revealing the potential mechanism under enJSRV-Env stimulation

To assess the reliability of the tested samples, the varied degrees among the transfected groups and negative control groups were evaluated by analyzing of the violin plots and principal component (PCA). The expression pattern of all samples was consistent (Figure 2A). Meanwhile, the difference between inter-groups was obvious (Figure 2B). These results suggested that all samples in the present experiment were reliable and suitable for further analysis. Venn diagram showed that 537 specifically expressed genes enriched in Env group, 638 in NC group, and 15070 genes co-expressed in both groups (Figure 2C). DEGs were computed, and 170 genes exhibited a significant difference in their expression levels with the threshold of P-Value≤0.05 FC 1.5 (Figure 2D). Among these DEGs, there are 44 up-regulated and 126 down-regulated genes (Figure 2E), and the DESeq results were shown in supplementary information 1.

Hierarchical clustering and Gene Ontology Analysis of Differentially Expressed Genes

Hierarchical clustering of all DEGs is shown in Figure 3. The up-regulation and down-regulation genes were collected, and the expression patterns of them were different. To characterize the functional consequences of gene expression changes caused by enJSRV-Env, 170 DEGs were selected to perform GO enrichment analysis. Figure 4 illustrated the ranking top 20 GO terms of DEGs, and these genes participated mainly in regulation of multicellular organismal development, cell differentiation and cellular developmental process.

KEGG Pathway Analysis of Differentially Expressed Genes

Through pathway significance enrichment analysis, signal transduction pathways, in which differential genes participated, were determined. Figure 5 showed the top 20 ranked significant pathways in KEGG, and environmental information processing, organismal systems and metabolism were the main functional part. The results showed that the DEGs responding to enJSRV-Env transfection were preferentially in ErbB signaling pathway, MAPK signaling pathway, Wnt signaling pathway and the ABC signaling pathway. These results could provide essential information on the investigation of enJSRV-Env in Hela cells.

Co-expression Network Analysis of Differentially Expressed Genes

In order to systemically analyze the function of DEGs on the Hela cells, we mapped the Co-expression networks of DEGs. As shown in Figure 6, a total of 2401 relationships between 170 genes (nodes) were identified and the node genes with the top 100 ranked degree in Co-expression networks were shown in supplementary information 2. According to the related degree, 7 DEGs (BHLHE41, NKD1, CCN1, HIST1H4K, DLX2, DUSP6 and SH2D5) were exhibited, which closely related with proliferation. These results indicate that enJSRV-Env might affect the proliferation of Hela cells through modulating these target genes.

qRT-PCR Validation of Differentially Expressed Genes

In order to verify the results of transcriptome sequencing, these selected DEGs were validated by qRT-PCR (Figure 7). The expression trends of BHLHE41, CCN1, DLX2, DUSP6 and SH2D5 were found to be in accord with RNA-seq data, suggesting that the RNA-seq data could reliably reflect the gene expression alterations. Combined with the RNA-seq and qRT-PCR results, the expression of BHLHE41, CCN1, DLX2, DUSP6 and SH2D5 was decreased in Hela cells transfected with enJSRV-Env.

Pathway Detection of Differentially Expressed Genes

Based on the analysis of the qRT-PCR results, 5 key genes which high related with proliferation were selected and to reconstruct the co-expression network sub-graph (Figure 8). The MAPK pathway was the main pathway as shown in Figure 8. Strikingly, the DUSP6 was closely related to the MAPK pathway. The DUSP6 protein expression was decreased in enJSRV-Env transfected group. Furthermore, p-ERK1/2 was

lower in the Env group than the NC group. However, the others such as ERK1/2, p-JNK, JNK, p-p38 and p38 were no changes in the two groups(Figure 9). These results suggested that enJSRV-Env could inhibit ERK1/2 pathways. Particularly, DUSP6 might play an important regulatory role in this process, which could be a potential mechanism of suppressing cell proliferation by enJSRV-Env in Hela cells.

Discussions

ERVs are the remnants of past infections of the germ line by ancestral retroviruses[12–15]. Indeed, sequences of ERV make up 8% of the human genome[15], 10% of the mouse genome[16], and 18% of the bovine genome[17]. ERVs may be species-specific genes. However, ERV originated from a species could integrated in the genomes of other species. When a bovine embryo was transplanted in a sheep uterus, the enJSRV could incorporate in bovine DNA[18]. Additionally, porcine endogenous retroviruses (PERVs) can be transmitted from pig to human cells and also transmitted among human cells in vitro[19–21]. The enJSRV-Env has been reported to involve in the formation of trophectoderm cells in sheep placenta, which plays a major role in sheep reproduction[6]. Therefore, we assumed to investigate the effect of enJSRV-Env on the fusion process of Hela cells. However, we did not observe the fusion phenomenon by enJSRV-Env transfection, indicating that enJSRV-Env could not promote the fusion of other types of cells like the Env of HERV-W. The explanation could be that Hela cells lack of the specific enJSRV-Env targeting cell surface receptor, since the fusogenicity of retroviral envelopes must be activated upon their interaction with specific cell surface receptors [22].

Interestingly, the proliferation of Hela cells was inhibited by enJSRV-Env. To further explore the mechanism of enJSRV-Env on inhibiting the proliferation of HeLa cells, we analyzed the gene expression and cell signal pathway. The proliferation related genes, BHLHE41, CCN1, DLX2, DUSP6 and SH2D5 were collected, and the MAPK pathway was indicated as the main signaling pathway that related with the DEGs.

The ERK1/2 signaling pathway regulates many cellular functions, including proliferation, differentiation, and transformation[23]. DUSP6, a member of the MAPK phosphatase family, interacts with and specifically targeted to ERK 1/2 via a negative feedback regulation [24]. However, the ERK1/2-DUSP6 interplay is complex, as active ERK1/2 upregulating DUSP6 mRNA, but by favoring protein degradation, thereby downregulating DUSP6 protein level[25]. Here, our results showed a decreased protein expression of DUSP6 and p-ERK1/2 in Env group, giving that enJSRV-Env may inhibit Hela cell proliferation via MAPK-ERK1/2 signaling pathway. This is consistent with the study in surgical samples of undifferentiated anaplastic thyroid carcinoma [26].

Although the role of DUSP6 in neoplastic transformation was poorly defined, either up- or down-regulation of this phosphatase has been reported in different tumors[26]. To date, the tumor-suppressive role of DUSP6 has been explored in esophageal squamous cell, nasopharyngeal carcinoma, ovarian cancer, non-small cell lung cancer, and pancreatic cancer. Moreover, the pro-oncogenic role of DUSP6 has been observed in human glioblastoma, breast cancer, thyroid carcinoma, and acute myeloid carcinoma. Both

of these roles have also been documented in malignant melanoma depending on the histological subtype of the cancer[27]. Interestingly, it was noted that DUSP6 had pro-survival functions in HeLa cell via inhibiting apoptosis [28]. Upon this, we inferred that DUSP6 has a positive regulatory effect on the proliferation of Hela cells.

Conclusions

In summary, the proliferation of Hela cells was inhibited by enJSRV-Env transfection, and probably via DUSP6 and ERK1/2 signaling pathway. In future, the investigation of the functions of enJSRV in human cancers would also be desirable.

Abbreviations

ERVs: Endogenous retroviruses

Env: Envelope-protein

enJSRVs : Jaagsiekte sheep retrovirus

qRT-PCR: Quantitative real-time PCR

DEGs: Differentially expressed genes

JSRVs: Exogenous Jaagsiekte sheep retroviruses

BERVs: Bovine endogenous retrovirus

HERVs: Human endogenous retrovirus

PERVs: Porcine endogenous retrovirus

Declarations

Ethics approval and consent to participate

This study does not involve any human or animal samples. All manipulations conformed to the experimental practices and standards approved by the animal welfare and research ethics committee of Inner Mongolia Agricultural University (Approval ID:2020007).

Consent for publication

All authors have reviewed the final version of the manuscript and approve it for publication.

Availability of data and materials

All data generated or analysed during this study are included in this published article (and its supplementary information files).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Xiaojuan Wang performed the experiments, analyzed the data and wrote the manuscript. Liang Zhang conceived the experiments and wrote the manuscript. Shuying Liu conceived the experiments. All authors critically reviewed the content and approved the final version of the manuscript.

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Not applicable.

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Figures

Figure 1

Effect of enJSRV-Env on the HeLa cells. (A) Determination of the fusion activity of Hela cells after transfection with pEGFP-C1/enJSRV-Env (Env group) and pEGFP-C1 (NC group). (B) MTT analysis of the proliferation of Env group and NC group. Five replicates were carried out in proliferation assays (n = 5).

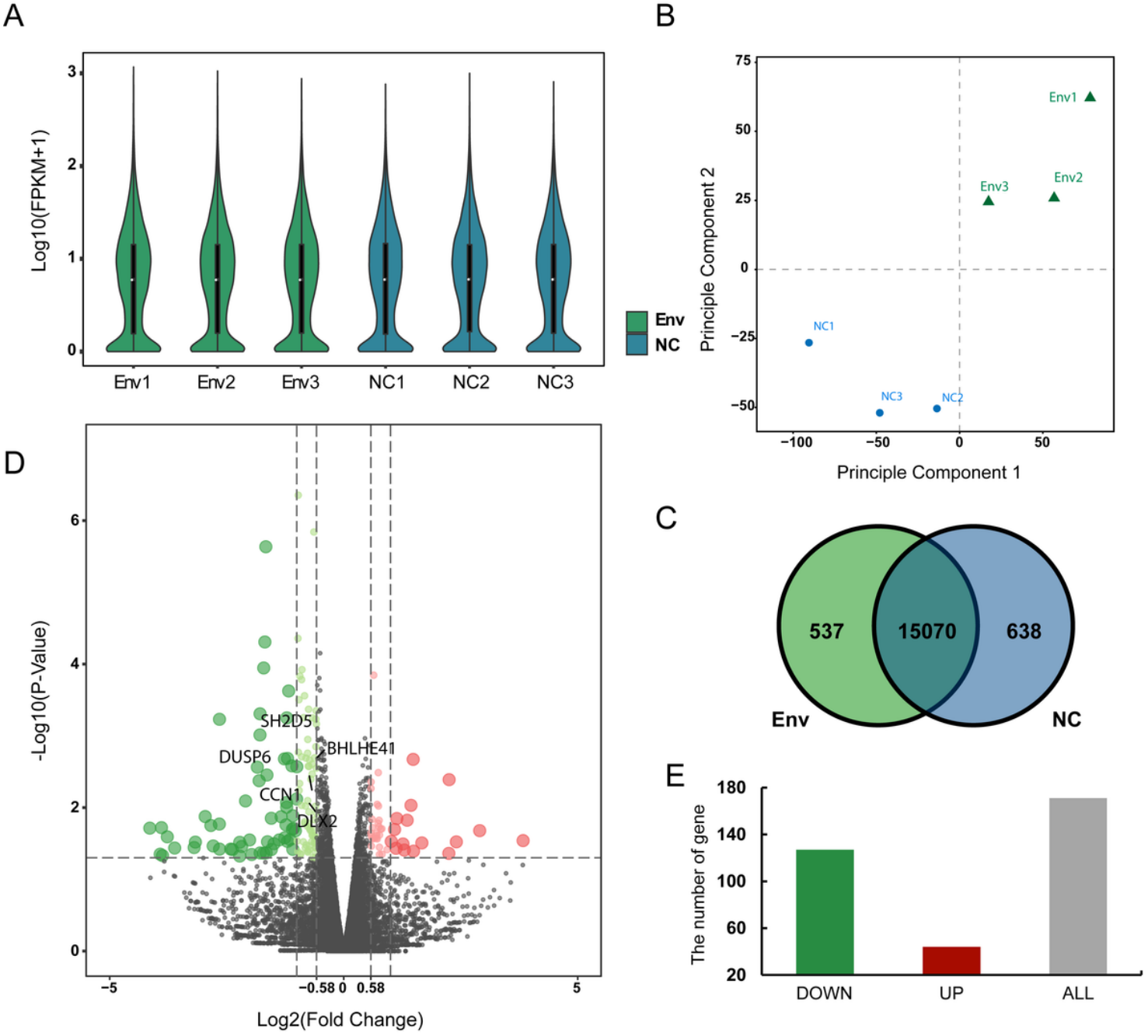

Figure 2

Overview of DEGs between Env group and NC group. A: Violin plots of NC group and Env group. The NC group was blue and the Env group was green. B: Principal component analysis was performed using read counts from Env group and NC group. C: Venn diagram showing the specifically expressed genes between NC group and Env group. D: Volcano plots depict DEGs for each group. Grey dots represent

genes with no significant discrepancy, red dots and green dots represent genes significantly up-regulated and down-regulated respectively. E: The number of DEGs.

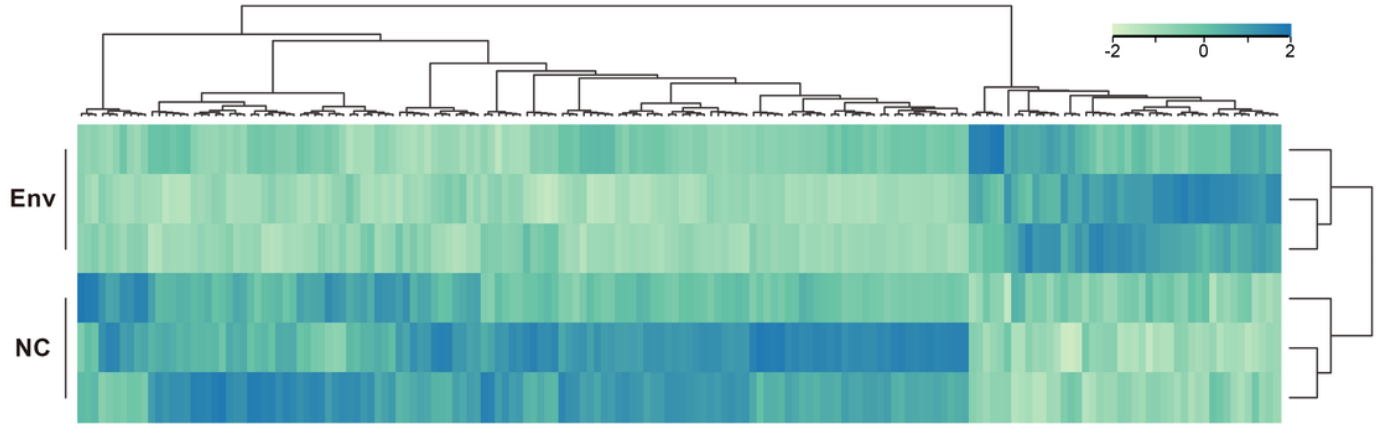

Figure 3

Hierarchical clustering of DEGs. Hierarchical clustering based on normalized gene counts of DEGs in samples derived from NC group and Env group. The up-regulated and down-regulated genes were indicated by blue and green respectively.

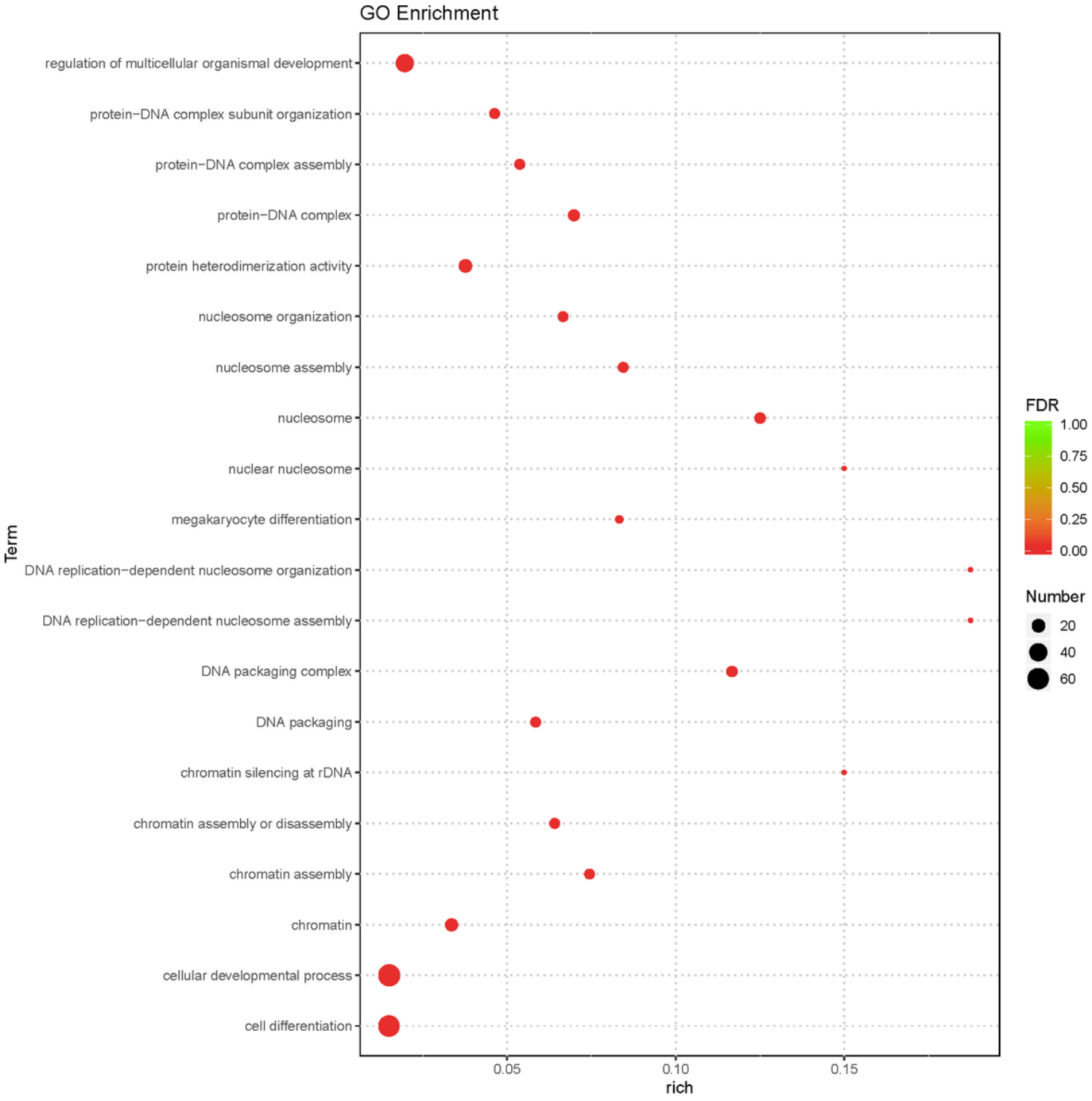

Figure 4

Bubble diagram of top 20 ranked GO terms of DEGs. The vertical axis indicates GO terms and the horizontal axis represents the Rich factor. The enrichment degree was stronger with a bigger Rich factor. The size of dots indicates the number of genes in the GO term.

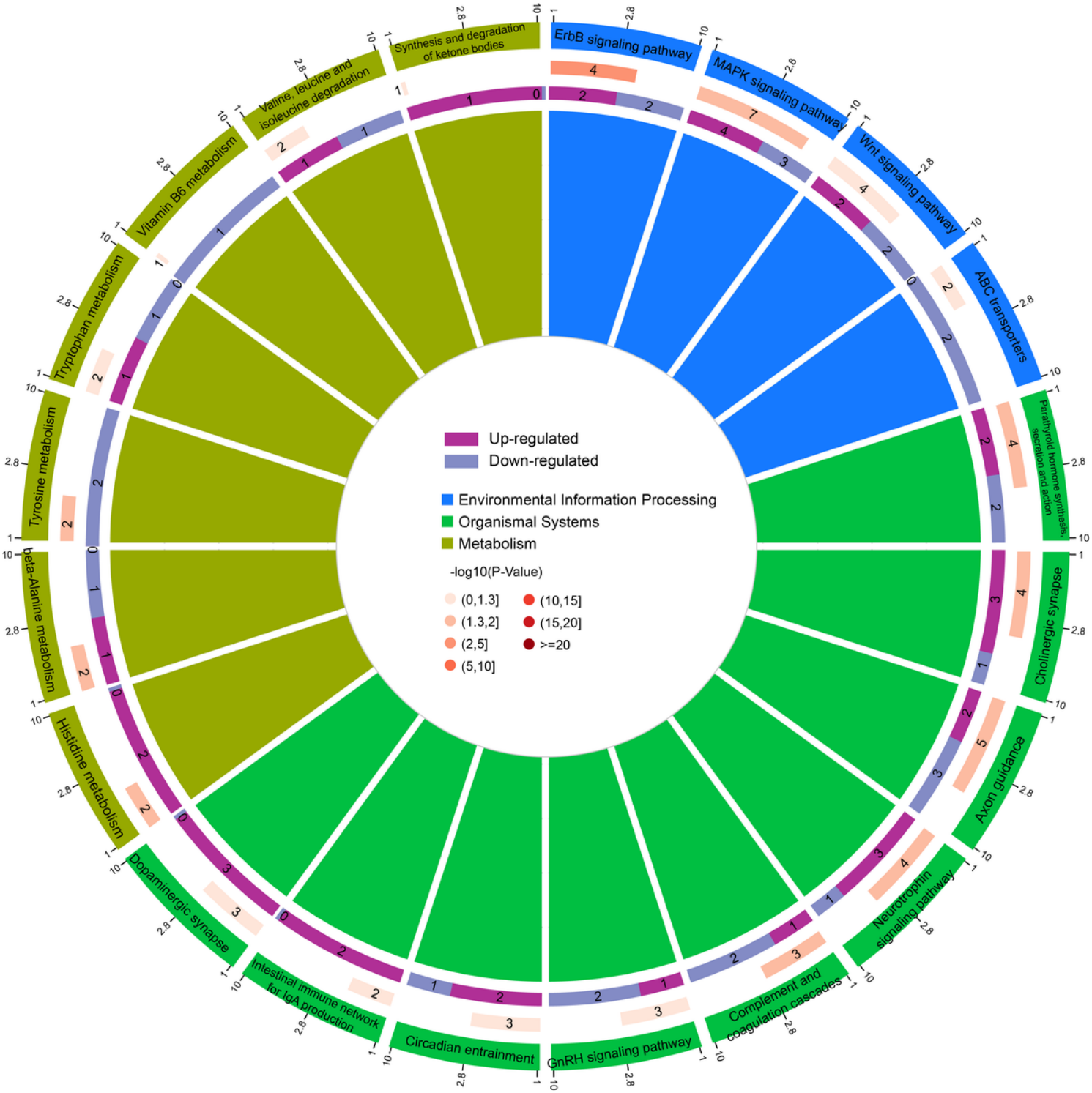

Figure 5

KEGG pathway analysis of DEGs. Three main level were shown: Environmental Information Processing (blue), Metabolism (Yellow green), Organismal Systems (green). The up-regulated genes with purple and down-regulated genes with light blue were shown, and the P-value was increasing with the red gradually get dark.

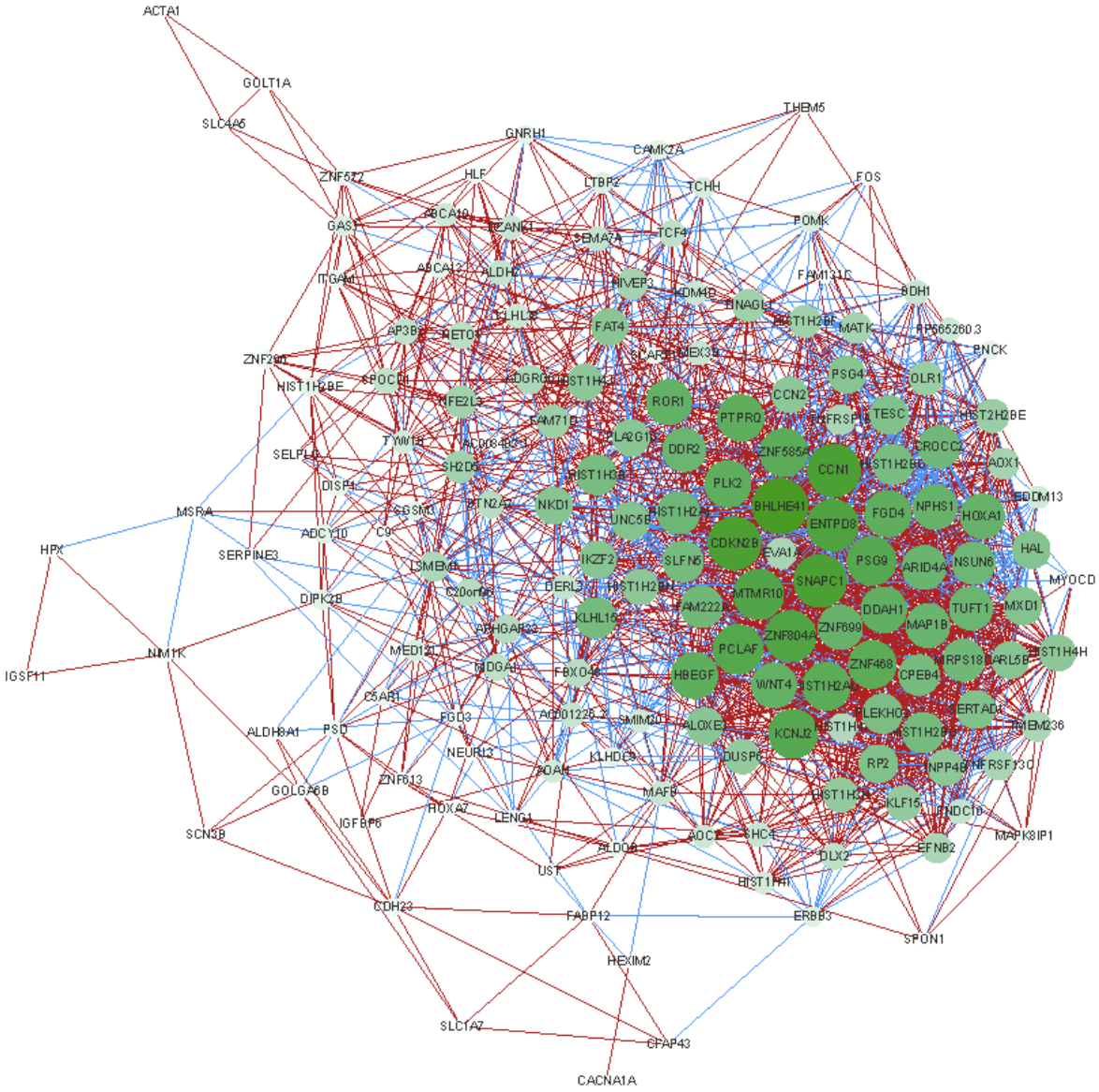

Figure 6

Co-expression network analysis of DEGs.

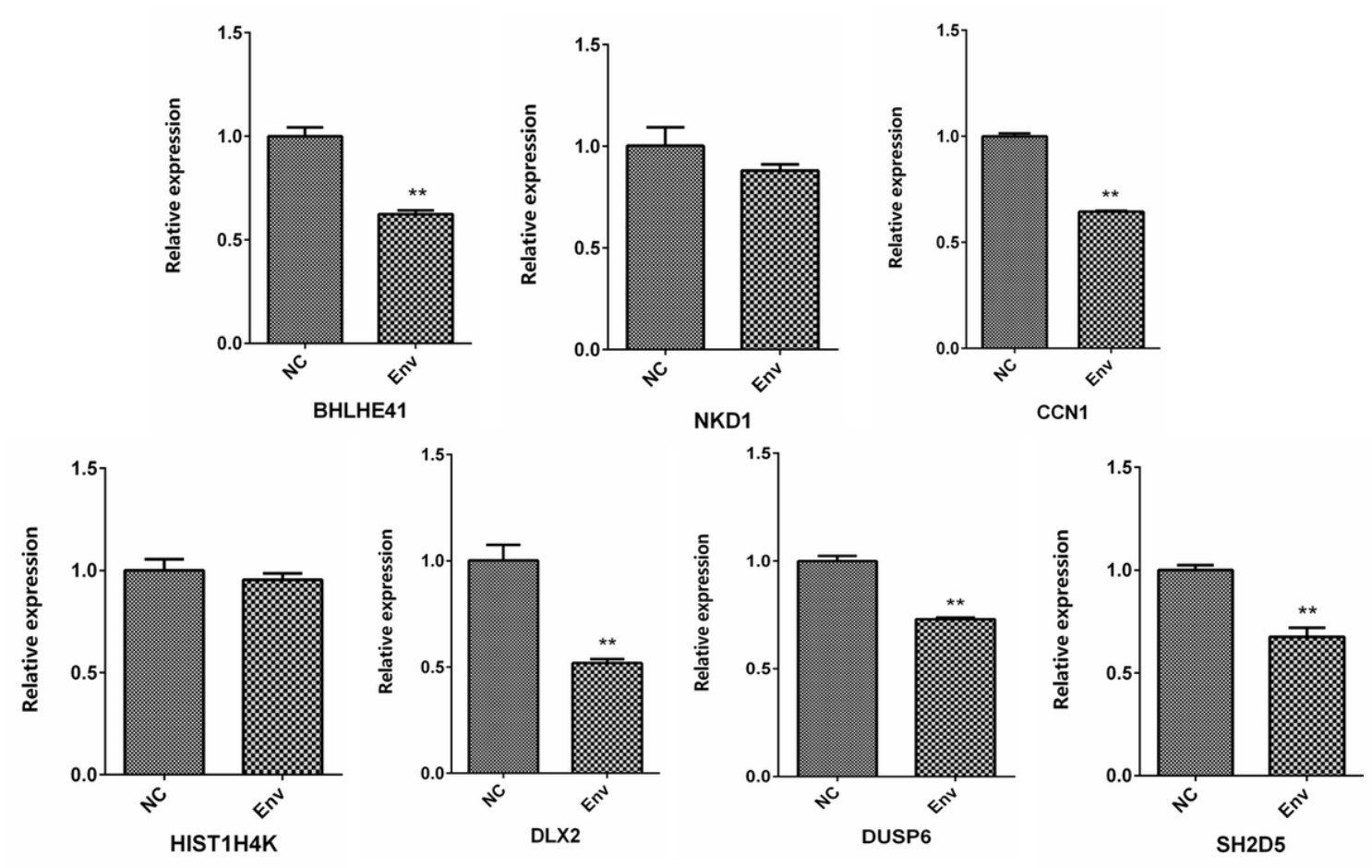

Figure 7

qRT-PCR analysis of the expression of 7 DEGs. Three replicates were carried out in the qRT-PCR analysis (n = 3). Bars represent means ± SD, *, $p < 0.05$; **, $p < 0.01$.

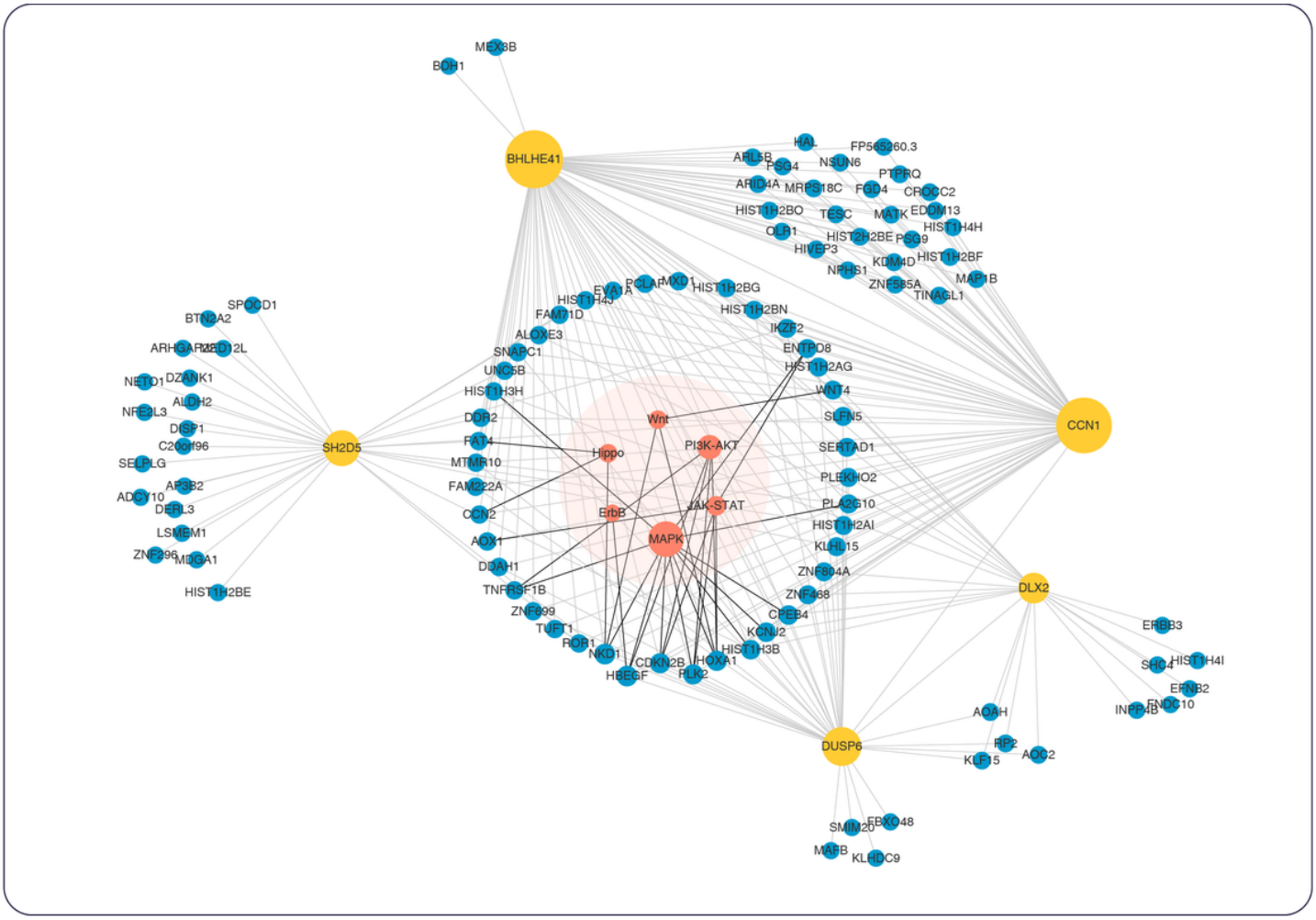

Figure 8

Co-expression network sub-graph of 5 DEGs. The yellow circles represent the 5 selected DEGs, the blue circles are the associated genes with the 5 DEGs, and the red circles show the signaling pathway enriched by the associated genes. The larger the dot, the higher the node degree.

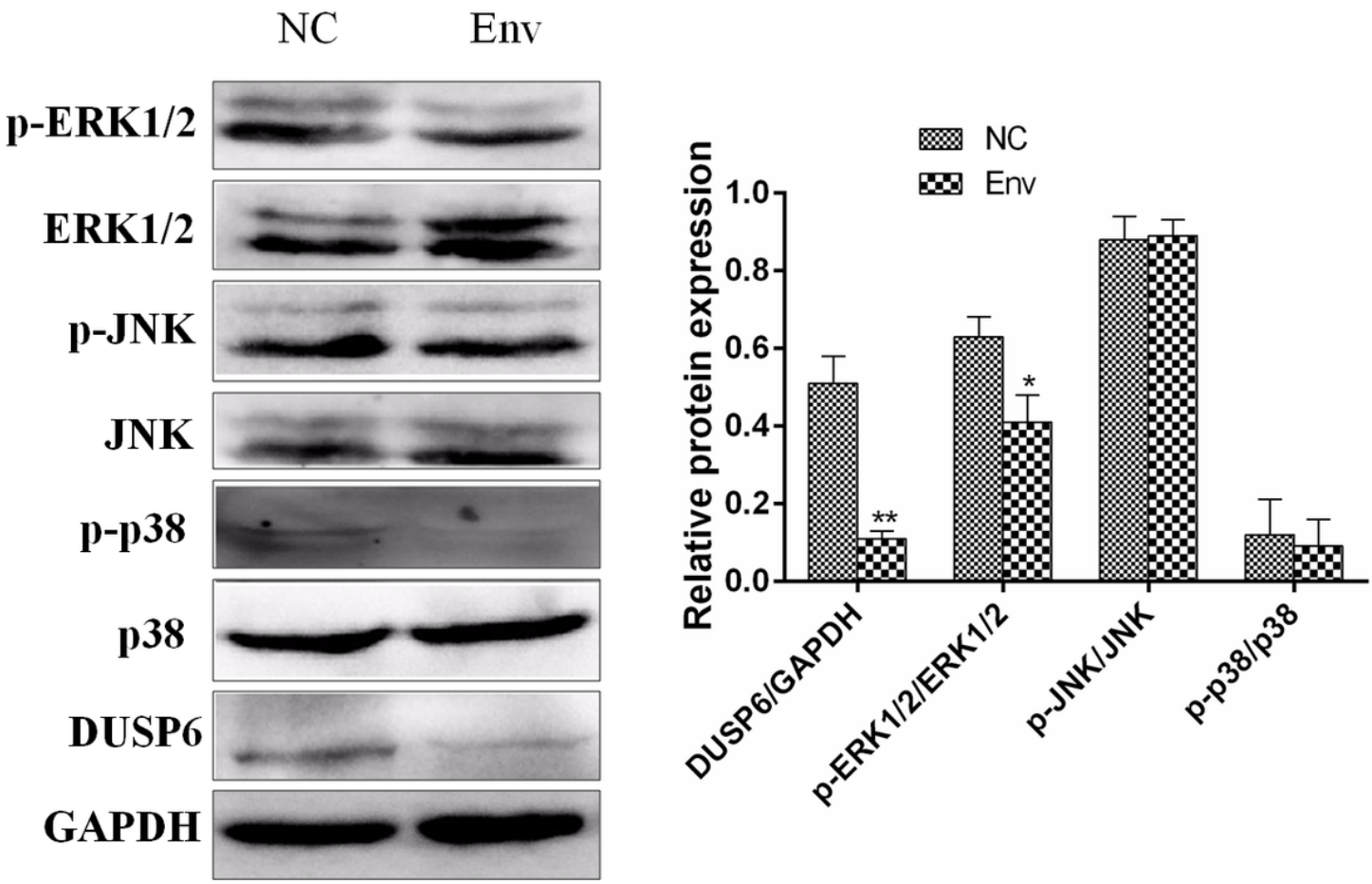

Figure 9

Western blotting analysis of DUSP6 and key proteins involved in MAPK pathways in Hela cells after transfected for 48 h. The quantification analysis of protein expression is shown in the histogram. Three replicates were carried out in each condition (n = 3). Bars represent means ± SD. *, $p < 0.05$; **, $p < 0.01$.

Supplementary Files

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- supplementaryinformation2.xlsx