Characteristics of Circular RNA Expression in Ishikawa Human Endometrial Carcinoma Cells with Progesterone Treatment

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Abstract

Background

Progestins are commonly used as the conservative endocrine treatment of young early endometrial cancer (EC) patients. Circular RNAs (circRNAs) are new group players in multiple cellular functions. This study aims to identify the differentially expressed circular RNAs (DE-circRNAs) in endometrial cancer (EC) cells upon the progesterone treatment, aiding in the understanding of the impact of circRNAs in progestin therapy.

Methods

RNA-seq analysis was used to identify the DE-circRNAs between MPA-treated and -non-treated Ishikawa cell lines. Functional enrichment analysis and circRNA-miRNA interaction network analysis were applied to these DE-circRNAs, validated by qRT-PCR analyses. Moreover, shRNA knockdown was utilized to explore the circRNA function.

Results

A total of 87 circRNAs were differentially expressed in Ishikawa cell lines with MPA treatment compared with that of control cells ($|\text{fold change}| \geq 2.0$, $p < 0.05$). Besides, these circRNAs were mainly enriched in cancer-related pathways, including response to cholesterol, epithelial to mesenchymal transition, and base-excision repair. The competing endogenous RNA (ceRNA) network showed that miR-296-3p, miR-1236-3p, and miR-144-5p were related to cancer. Furthermore, the qPCR results were coincident with the RNA-seq data. Of note, hsa_circ_0001860 was significantly induced upon MPA treatment. Knockdown of hsa_circ_0001860 can partially rescue the MPA-inhibited cell proliferation and invasion and regulate the expression of its interacting miRNAs.

Conclusions

This is the first time to evaluate the circRNA expression profile in progesterone treatment for EC. These circRNAs (*e.g.*, hsa_circ_0001860) may be used as potential diagnostic biomarkers and treatment targets for the evaluation of EC progesterone treatment.

Background

Endometrial cancer (EC) is the most common gynecologic malignancy of the female genital tract (1). Currently, the standard treatment of EC includes total abdominal hysterectomy and bilateral salpingo-oophorectomy (BSO) (2). However, hysterectomy is not an acceptable therapy for young patients without children. With an increasing number of women electing to delay childbearing, there is a critical need for

adequate treatment without hysterectomy for nulliparous women with EC (3). Progestins, are widely used in young early EC patients as conservative endocrine treatment (4). Progesterone, a tumor suppressor in the endometrium, plays a critical role in cell differentiation, cycle, and apoptosis (5). Approximately 90% of young patients can be treated with progestin due to a high expression of progesterone receptor (PR), which often results in a good prognosis (4). A meta-analysis indicates that 72 percent of patients achieved remission by oral progesterone, and the pregnancy outcome is about 35–40% (6, 7). However, a follow-up study shows that 35.4% of patients treated with progesterone have a recurrence after an initial response ($p = 0.03$), and 25.4% of them suffer persistent disease ($p = 0.02$) (8). In addition, according to clinical data, 30 percent of young patients fail to respond to progestin due to the possessed drug resistance during progestin treatment (4), and in turn, receptor-negative patients have been reported to respond to hormone treatment (7, 9). Accordingly, drug resistance is a major challenge during the treatment of endometrial cancer. However, the molecular mechanisms underlying progestin therapy and progestin resistancestill confused.

The recent advances in circular RNA (circRNA), a single-stranded, covalently closed circular molecule acting as microRNA (miRNA) sponges to sequester miRNA of a particular family, opened up a new direction for exploring the pathogenesis of EC. CircRNA is a competitive inhibitor that suppresses one miRNA binding to its mRNA targets and regulates gene expression via functioning as ceRNA in distinct physiological and pathological states (10, 11). CircRNA is more suitable than other RNA types to be a biomarker due to its stable structure, as well as high cell-type-, tissue- and developmental-specific expression (10, 12). In cervical cancer, Wang *et al.* identified 44 novel circRNAs, which may be associated with cancer (11). In EC, Xu *et al.* identified 209 up-regulated circRNAs and 66 down-regulated circRNAs in extracellular vesicles (EVs) of patients with stage III adenocarcinoma endometrial cancer (13). However, no research has investigated the effect of MPA on the expression profiles of circRNAs and the function of MPA-regulated circRNAs.

Herein, this study performed RNA-seq analysis of Ishikawa human endometrial carcinoma cells (ISK) treated with MPA or not. First, 46 up-regulated circRNAs and 41 down-regulated circRNAs were identified in ISK cells treated with MPA compared with the untreated cells. Second, gene functional enrichment analysis was performed, and we identified the top 10 significant GO terms and KEGG pathways. Third, the circRNA-miRNA networks were constructed to investigate the interaction of circRNAs and miRNAs. In addition, we verified the expression levels of selected circRNAs by qRT-PCR and investigated the function of the MPA-induced circRNA.

Methods

Cell culture

ISK are maintained by our laboratory,They were established by Nishida et al (14) from an endometrial adenocarcinoma with characteristics of ER-positive and PR-positive in 1985. ISK cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (HyClone) supplemented with 10% fetal bovine serum

(HyClone) at 37 °C under an atmosphere of 5% CO2. A group of ISK cell lines were treated with MPA (selleck, USA). Untreated ISK cell lines were used as controls. Previous study indicated that there was a positive correlation between the dose-dependent of MPA and reducingthe growth of parental Ishikawa cells (15). Hence, Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, and MPA (selleck, USA) was added 10 μM. The treatment stocks were initially prepared in DMSO (vehicle) with subsequent dilution for experiments of more than 1:1000 (for 10 − 5 M). The presence of a vehicle at such dilutions has previously been demonstrated to have no effect on cell growth.

Rna Extraction And Quality Control

Three samples from each of the ISK/MPA and ISK cell lines were collected, and tRNA isolation Total RNA was isolated by using Trizol reagent (Invitrogen life, USA) following the manufacturer's instructions. The quantity and quality of the RNA samples were determined using the NanoDrop ND-1000 instrument (Thermo Fisher Scientific, Waltham, MA, USA). And then RNA Integrity and gDNA contamination test were conducted by Denaturing Agarose Gel Electrophoresis. Sequencing library was determined by Agilent 2100 Bioanalyzer using the Agilent DNA 1000 chip kit (Agilent, part # 5067 − 1504). The isolated RNA was stored at -80℃ for further experimental verification.

Circrna Rna-seq

CircRNA-Seq high throughput sequencing and subsequent bioinformatics analysis were all performed by Cloud-Seq Biotech (Shanghai, China). The circRNA sequencing library was constructed by the total RNA from each sample. First of all, pretreating 5 μg total RNA by CircRNA Enrichment Kit (Cloud-seq Inc, USA); Secondly, the prepared RNAs was used to construct the RNA libraries; Then, libraries were controlled for quality and quantified using the BioAnalyzer 2100 system. In addition, libraries were denatured as single-stranded DNA molecules, captured on Illumina flow cells, amplified in situ as clusters and finally sequenced for 150 cycles on Illumina HiSeq Sequencer according to the instructions.

Circrna Rna-seq Data Analysis

Paired-end reads were harvested from Illumina HiSeq 4000 sequencer, and quality controlled by Q30. After 3' adaptor-trimming and low quality read removing by cutadapt software (v1.9.3), the reads were aligned to the reference genome/transcriptome by STAR software, and circRNA were detected and annotated by DCC software. CircBase database and circ2Trait disease database were used to annotate the identified circRNA. The junction read counts were normalized, and differentially expressed circRNA were determined using the edgeR package of R software. $P < 0.05$ was set as a threshold. GO, and Pathway enrichment analysis was performed by using the host genes of the differentially expressed circRNA. CircRNA-miRNA interaction analysis was performed, and the network was constructed by Cytoscape software. Cytoscape (https://cytoscape.org/) is an open-source software platform for

visualizing complex networks and integrating these with any attribute data. We generated a Cluster plot to identify the hosting genes of circRNAs by utilizing R3.2.2, which is from the R package – “hclust(method = "single").

Experimental Validation With Quantitative Rt-pcr

We screened 20 circRNAs for experimental validation using quantitative RT-PCR. Total RNA was extracted and reversely transcribed into cDNA using SuperScriptTM III Reverse Transcriptase (Invitrogen). The expression levels of circRNAs was determined by ViiA 7 Real-time PCR System (Applied Biosystems). Triplicates were performed for each sample in three independent experiments. The primers were designed using the “Out-facing” strategy to guarantee the amplifications were from circle template. Relative expression ratio ($\Delta\Delta Ct$) of each circRNA is presented in a log 2 value. miScript PCR Starter Kit (Qigen) was utilized to set-up of miRNA quantification experiment. The sequences of primers for circRNAs and miRNAs was included in Table 3.

Cell Proliferation Assay, Invasion Assay, And Shrna Knockdown Experiment

We utilized CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit (# G3582) from Promega to examine cell proliferation (16). Standard 24-well Boyden control and invasion chambers (BD Biosciences) were used to assess cell migration and invasion following manufacturer suggestions. We cloned shRNAs into pGPU6/GFP/Neo vectors. The sequence of shRNA targeting hsa_circ_0001860-2 is “TTACTGAAGCTTCAAGGTTAC”; and the control shRNA sequence is “TTCTCCGAACGTGTCACGT”.

Data analysis

Statistical analysis Data is presented as the mean standard error (SE) for triplicate measurements. Student’s t test was using to estimate the significance of data (Statistically significant : $P < 0.05$).

Results

Identification of dysregulated circRNAs in ISK cells upon the MPA treatment

The concentration and purity of total RNAs from different samples were determined by NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). All RNA samples showed an OD A260/280 ratio between 1.8 and 2.1. Principal Component Analysis (PCA) of these sequenced samples showed that there was a clear separation relationship between the treated cells and control cells (Supplemental Data 1). RNA-seq analysis showed that a total of 4,814 circRNAs were detected. Among them, 964 circRNAs were novel according to the published studies. We then compared the expression profiles of circRNAs between

MPA-treated-ISK cells and control cells. The results showed 87 differentially expressed circRNAs in MPA-treated-ISK cells compared with control cells (|fold change| ≥2.0, $p < 0.05$), among them 46 up-regulated and 41 down-regulated (Fig. 1). It is reported that circRNA is divided into exotic circular RNA (ecircRNA), circular intronic RNA (ciRNAs), and exonic-intron circular RNA (EiciRNA) (17). In this study, all circRANs are located in exons. Moreover, we conducted a clustering analysis of these circRNAs and their hosting genes (Supplemental Data 2 and 3).

Go And Kegg Pathway Analyses

Gene Ontology (GO) includes biological process classification (BP), cellular component classification (CC), and molecular function classification (MF). Top 10 dysregulated GO analysis of each domain were identified according to enriched up-regulated or down-regulated circRNAs (Figs. 2A-B). GO terms with P-value < 0.05 were selected and ranked by enrichment score (-log10 (P-value)). Among the BP terms, response to cholesterol, epithelial to mesenchymal transition, and base-excision repair have been reported to be associated with tumor (6, 18–21). In MF terms, WNT-activated receptor activity and WNT-protein binding might play a role in tumors.

KEGG analysis showed that the down-regulated circRNAs were mainly enriched in renal cell carcinoma, thyroid hormone signaling pathway, and pathways in cancer (Fig. 2C). While the up-regulated circRNAs were mainly enriched in protein processing in the endoparasitic reticulum, chronic myeloid leukemia, and RNA degradation (Fig. 2D).

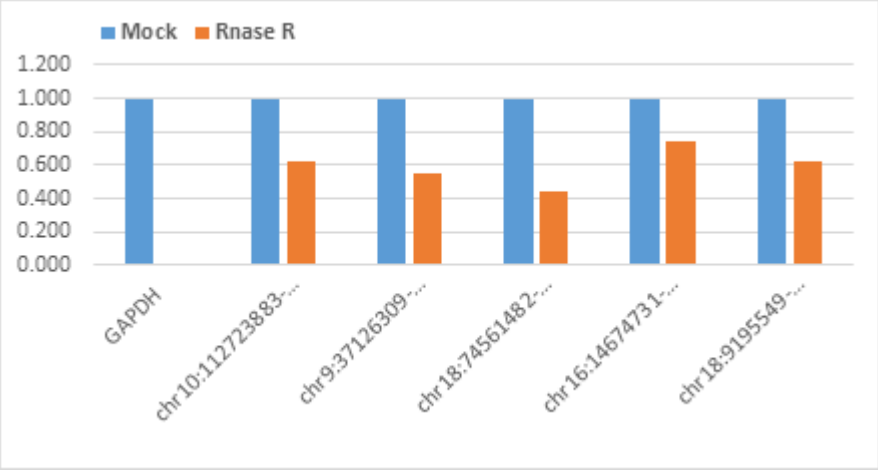
Competing Endogenous Rna (cerna) Network Analysis

It was reported that mRNAs and circRNAs act as ceRNAs, or miRNA sponges to communicate with each other by competing for miRNA-binding through common miRNA response elements (22). In addition, circRNAs have been reported to sequester relevant miRNAs via miRNA response elements to regulate gene expression post-transcriptionally (22). To investigate circRNA regulation in MPA-treated EC cells, we investigated miRNA-circRNA interactions. We identified miRNAs that paired with the differentially expressed circRNAs. And in total, we obtained 25 miRNA-circRNA interaction pairs. And then, we constructed the circRNA-miRNA network (Fig. 3). The parameter of the network was listed in Supplemental Data 4. The top 5 differentially expressed circRNAs that interacts with multiple miRNA partners were hsa_circ_0020028, hsa_circ_0001860, hsa_circ_0001993, hsa_circ_0105045, and hsa_circ_0046843. The five circRNAs were treated with RNase R, and qPCR analyses were used to compare the gene expression level changes pre- and post-treatment. In contrast to the > 10000-fold change of control GAPDH, all the five circRNAs had less than a 3-fold change, proving that they are circle RNA (Table 1). In addition, Sanger sequencing was performed to determine the cyclization site of those circRNAs (Supplemental Data 5). The network analysis showed that different circRNA bound to the same miRNAs, suggesting that regulation of miRNAs by circRNA was complicated. These five circRNAs were all

paired with hsa-miR-4753-3p (Fig. 3). Some of the miRNAs have been reported to be associated with cancer treatment or progression, including miR-296-3p (23), miR-1236-3p (24), and miR-144-5p (25).

Table 1
qPCR analysis of gene expression level changes before and after treatment.

					Relative Ratio	
Gene	Rnase R	Mock	Ct(RnaseR)-Ct(Mock)	Fold Enrichment	Mock	Rnase R
GAPDH	30.777	17.474	13.303	10107.434	1.000	0.000
chr10:112723883–112745523+ (hsa_circ_0020028)	24.332	23.637	0.695	1.619	1.000	0.618
chr9:37126309–37126939+ (hsa_circ_0001860)	24.553	23.682	0.871	1.829	1.000	0.547
chr18:74561482–74583781+ (hsa_circ_0001993)	22.440	21.255	1.185	2.274	1.000	0.440
chr16:14674731-14721193- (hsa_circ_0105045)	28.187	27.753	0.434	1.351	1.000	0.740
chr18:9195549–9221997+ (hsa_circ_0046843)	20.533	19.859	0.674	1.596	1.000	0.627



Validation Of The Expression Levels Of Circrnas Using Qrt-pcr

In order to confirm the RNA-seq data, we screened 20 differentially expressed circRNAs to validate their expression levels by qRT-PCR, including ten up-regulated circRNAs and ten down-regulated circRNAs (Table 2). In addition, the expression levels of the five circRNAs in the network were also detected. The qRT-PCR analysis revealed that the expression of these circRNAs showed either the same up-regulation pattern or the same down-regulation pattern as the RNA-seq data. Particularly, hsa_circ_0020028 (circRNA-1) and hsa_circ_0001860 (circRNA-7) were up-regulated in ISK cell line treated with progesterone compared with untreated cells, while hsa_circ_0001993 (circRNA-8), hsa_circ_0105045 (circRNA-12) and hsa_circ_0046843 (circRNA-13) were down-regulated, which is consistent with our RNA-seq results (Fig. 4). These findings indicated that the results of qRT-PCR were well consistent with RNA-seq results, suggesting the high reliability of the RNA-seq expression results.

Table 2
Twenty differentially expressed circRNAs were validated by qRT-PCR

number	CircRNA-ID	logFC	PValue	circBase-ID	GeneName
circRNA-1	**chr10:112723883-112745523+**	**2.871**	**0.029**	**hsa_circ_0020028**	**SHOC2**
circRNA-2	chr14:62187100-62188541+	-3.883	0.035	hsa_circ_0006393	HIF1A
circRNA-3	chr13:20534098-20568059+	3.920	0.040	/	ZMYM2
circRNA-4	chr20:39721112-39729993+	-3.038	0.028	hsa_circ_0115215	TOP1
circRNA-5	chr10:70196768-70229920-	-4.157	0.020	hsa_circ_0018524	DNA2
circRNA-6	chr3:56626998-56628056+	3.957	0.031	hsa_circ_0001313	CCDC66
circRNA-7	**chr9:37126309-37126939+**	**2.767**	**0.044**	**hsa_circ_0001860**	**ZCCHC7**
circRNA-8	**chr18:74561482-74583781+**	**-3.260**	**0.008**	**hsa_circ_0001993**	**ZNF236**
circRNA-9	chr2:74834182-74867454-	-3.803	0.046	/	M1AP
circRNA-10	chr7:157009561-157024021+	4.104	0.030	hsa_circ_0133805	UBE3C
circRNA-11	chr4:88104355-88116842-	-4.279	0.015	hsa_circ_0006866	KLHL8
circRNA-12	**chr16:14674731-14721193-**	**-3.851**	**0.038**	**hsa_circ_0105045**	**PARN**
circRNA-13	**chr18:9195549-9221997+**	**-3.917**	**0.034**	**hsa_circ_0046843**	**ANKRD12**
circRNA-14	chr2:239090706-239093928-	3.071	0.017	hsa_circ_0001116	ILKAP
circRNA-15	chr10:126097111-126100769-	-3.955	0.033	hsa_circ_0008898	OAT
circRNA-16	chr6:108225833-108246136-	4.390	0.012	hsa_circ_0009144	SEC63
circRNA-17	chr4:178274462-178274882+	3.170	0.010	hsa_circ_0001459	NEIL3
circRNA-18	chr15:55835782-55837423-	3.824	0.049	/	PYGO1
circRNA-19	chr4:151719233-151738409-	4.472	0.009	hsa_circ_0008618	LRBA
circRNA-20	chr18:45391430-45423180-	-2.020	0.025	hsa_circ_0000847	SMAD2
Note: / means a novel circRNA which has not been in circBase					

Investigation Of The Representative Mpa-regulated Circrna (hsa_circ_0001860)

To examine the role of these identified MPA-regulated circRNAs, we have hsa_circ_0001860 as a representative one for the functional analysis. First, we validated the induction of hsa_circ_0001860 by the MPA treatment of ISK cells (Fig. 5A). As expected, MPA treatment of ISK cells inhibits the ISK cell

proliferation, and this inhibition can be attenuated by the shRNA knockdown of hsa_circ_0001860 (Fig. 5B). It suggested that MPA inhibited cell proliferation partially through hsa_circ_0001860. Similar effects were observed in the cell invasion experiments upon the MPA treatment and the hsa_circ_0001860 knockdown (Fig. 5C). Moreover, the knockdown of hsa_circ_0001860 can regulate its interacting miRNAs, which were shown in the network analysis (Fig. 5D). Taken together, hsa_circ_0001860, which can be induced by MPA, inhibits cell proliferation and invasion, probably by regulating its interacting miRNAs.

Table 3

Sequence of qPCR primers for the detection of circRNAs and miRNAs

number	CircRNA-ID	circBase-ID	Primers (5’-3’)
circRNA-1	chr10:112723883-112745523+	hsa_circ_0020028	Forward: CCAACCTTGACTTGCAGC Reverse: TGCCCATTTCTGATCATTCA
circRNA-2	chr14:62187100-62188541+	hsa_circ_0006393	Forward: TCCATGTGACCATGAGGAAA Reverse: GAGATCTGGCTGCATCTCG
circRNA-3	chr13:20534098-20568059+	/	Forward: TGAATGTGGCAGGAGACG Reverse: AGGGCTGAAGGCGATTCT
circRNA-4	chr20:39721112-39729993+	hsa_circ_0115215	Forward: GTGGAAAGAAGTCCGGCA Reverse: TGGTGGGGCAAATACTGG
circRNA-5	chr10:70196768-70229920-	hsa_circ_0018524	Forward: CATGGTGCCATACCTGTCA Reverse: CCAGGCGCTTTTCACAGT
circRNA-6	chr3:56626998-56628056+	hsa_circ_0001313	Forward: TGCTCTCTTGGACCCAGC Reverse: GAAAGGGTGCTCCAGCAGT
circRNA-7	chr9:37126309-37126939+	hsa_circ_0001860	Forward: GCTGGATGCTACTGGGATG Reverse: TGGGCATAATGAATTTGGCT
circRNA-8	chr18:74561482-74583781+	hsa_circ_0001993	Forward: CACGTATTCGTGTCCGCA Reverse: TGGCGTTGAAACTGGGAT
circRNA-9	chr2:74834182-74867454-	/	Forward: CGTGGACTCAGCGTCTCC Reverse: CCAGTAGTTCGCCCAGGA
circRNA-10	chr7:157009561-157024021+	hsa_circ_0133805	Forward: CCCAACCAGGGGTTCTTT Reverse: CAGCCAGTGGTTTGGAGG
circRNA-11	chr4:88104355-88116842-	hsa_circ_0006866	Forward: TCCATTCGGACTACCCCA

			Reverse: CCCACTCAACTGCCATTGT
circRNA-12	chr16:14674731-14721193-	hsa_circ_0105045	Forward: ACGAGGCAGGCTACGATG Reverse: TGCAAAGGCCAAACTGAA
circRNA-13	chr18:9195549-9221997+	hsa_circ_0046843	Forward: GGAGCGTCCAGTGGATGTA Reverse: TCCTTGCTCACATCACTTCG
circRNA-14	chr2:239090706-239093928-	hsa_circ_0001116	Forward: CTCGGAGATAGTCGGGCA Reverse: TCGAATTCCTCCATGTCCA
circRNA-15	chr10:126097111-126100769-	hsa_circ_0008898	Forward: GGCTGGAGAGACTGCCTG Reverse: GGAGGGCCTTGGACTGTT
circRNA-16	chr6:108225833-108246136-	hsa_circ_0009144	Forward: AATAGTAATGGCCCGGAACC Reverse: GAACAATGCCCATCCTGC
circRNA-17	chr4:178274462-178274882+	hsa_circ_0001459	Forward: AAAAGCTGCAACCCTGGA Reverse: CCAAATGCAGTTTTTCTGTTG
circRNA-18	chr15:55835782-55837423-	/	Forward: TTAAGTTTCAGTAGTTAGCTGT Reverse: TTCCTCATCTGCAATATGTCC
circRNA-19	chr4:151719233-151738409-	hsa_circ_0008618	Forward: AGCCATGGACAAGAACTGCT Reverse: AAGGTGGAAGGTGAGACTGC
circRNA-20	chr18:45391430-45423180-	hsa_circ_0000847	Forward: TATTCCAGAAACGCCACCTC Reverse: TTCCATCCCAGCAGTCTCTT
Note: / means a novel circRNA which has not been in circBase. Actin is the internal control. The forward primer of actin is (5’-3’)GTGGCCGAGGACTTTGATTG. The reverse primer of actin is (5’-3’) CCTGTAACAACGCATCTCATATT			

U6 (5’-3’: GCTTCGGCAGCACATATACTAAAAT) is the internal control of qPCR analysis of miRNAs.

miRNAs	Forward primers (5’-3’)
hsa-miR-6821-3p	AACAAGTGACCTCTCCGCTCC
hsa-miR-29a-5p	AACACGCACTGATTTCTTTTGGTG
hsa-miR-1236-3p	AACAAGCCTCTTCCCCTTGTCT
hsa-miR-144-5p	ACGCCGGGATATCATCATATACTGT
hsa-miR-4753-3p	AACACGCTTCTCTTTCTTTAGCCTT

Discussion

CircRNAs are initially reported by Hsu and Coca-Prados (26). Nevertheless, these CircRNAs were thought to be transcription errors. Now, researchers have discovered thousands of circRNAs which may serve as diagnostic or predictive biomarkers, such as hsa_circ_0005075 in hepatocellular carcinoma (27), as well as hsa_circ_002059, hsa_circ_0000190, and circPVT1 in gastric carcinoma (28–31), and all of this were benefited from bioinformatics analysis.

CircRNAs can regulate many cellular processes, including sponging miRNAs (32, 33), the assembly and transport of cellular proteins (34), alternative splicing and gene expression (35), possessing protein-coding activity, modulating generation of rRNAs/tRNAs (35), and antiviral immunity (36). Specifically, ciRS-7 is the first identified CircRNA sponging miR-7 with direct binding (32, 33). Foxo3 circular RNA (circ-Foxo3) binds to ID-1 (inhibitor of differentiation-1), the transcription factor E2F1, HIF-1α (hypoxia-inducible factor-1α) and FAK (focal adhesion kinase), and inhibits their translocation (34). The knockdown of circRNA EIF3J or circRNA PAIP2 significantly reduces the expression level of their parental genes (37). circRasGEF1B, which is conserved between human and mouse circRNA, positively regulates lipopolysaccharide (LPS) response (36).

However, no study has focused on circRNA regulation in EC with progesterone treatment. Therefore, in this study, we applied RNA-seq to investigate the differentially expressed circRNA in EC with progesterone treatment, and the results revealed that a total of 87 circRNAs were differentially expressed in MPA-treated-ISK cells compared control cells ($|\text{fold change}| \geq 2.0$, $p < 0.05$). Furthermore, the expression levels of the top 20 significant expressed circRNAs were validated by qRT-PCR. Particularly, hsa_circ_0046843 was significantly down-regulated in the ISK cell line treated with progesterone, while hsa_circ_0001860 was significantly up-regulated. In addition, we performed GO and KEGG pathways analysis. Most of the BP terms in our study have been reported to be closely associated with cancer, including response to cholesterol, epithelial to mesenchymal transition, and base-excision repair. Firstly, cholesterol has been reported to play a role in the synthesis of estrogen and progesterone. 3β-Hydroxysteroid-Δ24 reductase (DHCR24), the final enzyme in the cholesterol biosynthetic pathway, has been demonstrated to aggravate cancer invasion and progesterone resistance in EC (18). Additionally, dietary cholesterol consumption, including saturated fatty acid, unsaturated fatty acid, and cholesterol intake, influences EC risk by regulating the production, metabolism, and excretion of endogenous hormones (6). Secondly, Epithelial-

to-Mesenchymal Transition (EMT) is an important step towards the invasion and metastasis of cancer (19). Hsu et al. demonstrated that epithelial cell adhesion molecule (EpCAM)-regulated transcription exerted influences on nanomechanical properties of EC cells, which promotes EMT (20). Last, a hospital-based case-control study examined the association between various polymorphisms in base excision repair (BER) DNA pathway genes (OGG1, MUTYH, XRCC1, APEX1, and PARP1) among Japanese postmenopausal women with and without endometrial cancer and found some worthful interaction (21). In MF terms, WNT-activated receptor activity and WNT-protein binding were significantly enriched.

Circular RNAs act as ceRNAs to regulate the expression of their targeted miRNAs. Additionally, miRNAs promote the degradation of target messenger RNAs (mRNAs) or inhibit their translations by recognizing specific binding sites on the 3'-untranslated region (UTR) of mRNAs in either a completely or partially complementary fashion (38). Therefore, these three elements constitute an associated pathway to modulate physiological function. The previous study has reported many miRNAs dysregulated in EC, which may be used as prognostic markers, treatment assessment markers, or treatment targets (39). miRNAs play essential roles in the ontogenetic processes of EC, including cell proliferation, migration, and metastasis (40). For example, mir-505 suppresses EC cell proliferation, invasion, and metastasis by targeting TGFA (41). miR-30c enhances the proliferation of EC cells, and the low expression level of miR-194 contributes to poor prognosis. The expression levels of miR-200c and miR-205 are significantly increased in EC compared with normal tissue (42–44). The silence of miR-124, a novel tumor suppressor miRNA, reverses EMT, and the invasive properties, by attenuating the expression of IQGAP1 (IQ Motif Containing GTPase Activating Protein 1) oncogene (45). Thus, to further understand the impact of the circRNA-related ceRNA crosstalk on MPA-treated EC cells, we used miRNA-circRNA interaction data to construct a circRNA-miRNA network. Hsa_circ_0046843, hsa_circ_0001860, hsa_circ_0020028, hsa_circ_0105045 and hsa_circ_0001993 were the top 5 significant circRNAs in EC cells treated with MPA. Each circRNA paired with five miRNAs, and all these circRNAs could regulate the expression of hsa-miR-4753-3p. Among the miRNAs, miR-296-3p, miR-144-5p, and miR-1236-3p have been reported to play a role in tumor progression. miR-1236-3p is associated with ovarian cancer metastasis (24). miR-296-3p plays a critical role in cell growth and multi-drug resistance in glioblastoma by targeting ether-à-go-go (EAG1) (23). As is known to all, drug resistance and recurrence are the limitations of the clinical application of progesterone treatment. The objective response rate of patients with advanced or recurrent EC is approximately 15 to 20% (46). Thus, miR-296-3p can be further used for progesterone-induced drug resistance. miR-144-5p acts as a tumor suppressor in bladder cancer cells by regulating CCNE1 and CCNE2 (25). miR-296-3p pairs with hsa_circ_0105045, while miR-144-5p and miR-1236-3p pair with hsa_circ_0001860, suggesting the two circRNAs may be associated with cancer progression or drug resistance.

Understanding the mechanism of circRNA-miRNA may provide critical insights into potential strategies for overcoming the shortcomings of progesterone treatment in EC. However, this study also has some limitations. First, this study examined the circRNAs in small EC cell lines treated with progesterone. In order to increase the accuracy of the results, tissues and plasma levels should also be studied respectively in EC patients treated with progesterone. Second, the mechanisms of circRNA-miRNA

interaction are intricate. However, we only constructed a circRNA-miRNA network using five circRNAs. Further study should be concentrated on this interaction. If breakthrough this bottleneck, we can get great achievements in the progesterone treatment of EC.

Conclusion

All in all, we acquired 87 differentially expressed circRNAs in EC cell -lines treated with MPA, including 46 up-regulated circRNAs and 41 down-regulated circRNAs. These differentially expressed circRNAs were mainly enriched in many pathways related to cancer, such as response to cholesterol, epithelial to mesenchymal transition, and base-excision repair. Hsa_circ_0046843, hsa_circ_0001860, hsa_circ_0020028, hsa_circ_0105045, and hsa_circ_0001993 were associated with progesterone treated EC, and they can be used as novel biomarkers for EC progression, treatment, or drug resistance. This study improves our knowledge of the molecular mechanisms of progesterone applied in EC, which benefit to further study the roles of circRNAs in EC prognosis, diagnosis, and progesterone therapy.

Abbreviations

circRNA
circular RNA
EC
endometrial cancer
MPA
medroxyprogesterone acetate
RNA-seq
high-throughput RNA sequencing
qRT-PCR
quantitative real-time reverse transcription
EMT
Epithelial-to-Mesenchymal Transition
ceRNA
competing endogenous RNA
ciRNAs
circular intronic RNA
ecircRNA
exotic circular RNA
EiciRNA
exonnic-intron circular RNA
GO
Gene Ontology
BP
biological process

CC
cellular component
MF
molecular function
HIF1α
hypoxia-inducible factor

Declarations

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Availability of data and material:

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Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

PZ contributed to design and experimental data collection; WC contributed to data collection; XS and WG contributed to statistical analysis; YW and LW contributed to data analysis and interpretation, and manuscript writing. All authors finally approve the manuscript.

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Figures

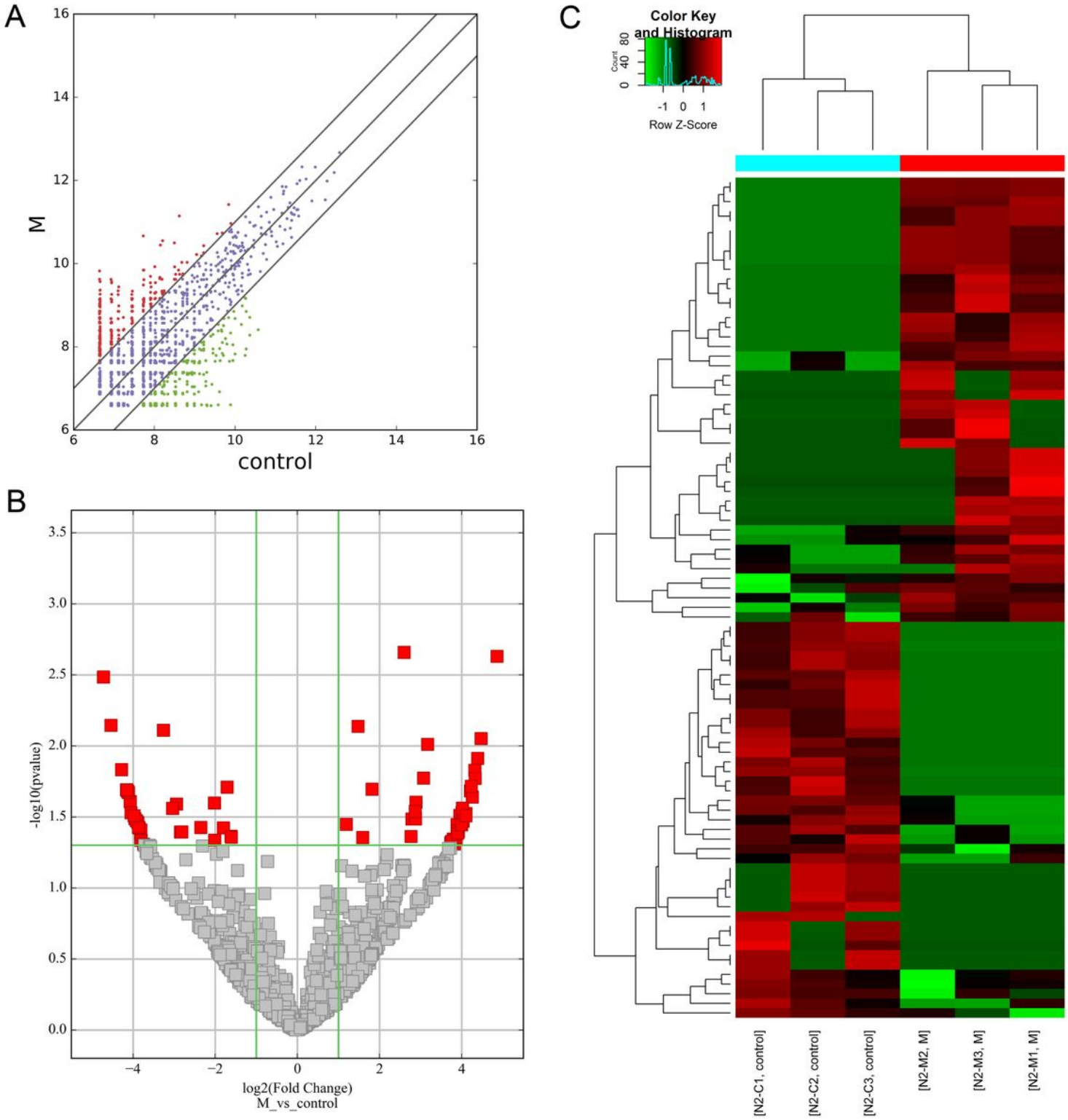

Figure 1

Bioinformatic analysis of circRNA expression pattern in ISK cell lines treated with MPA compared with untreated. A: Scatter plot was conducted to exhibit the circRNA expression distribution. The scatter plot is a visualization method used for assessing the variation in circRNA expression between ISK cell lines treated with MPA and untreated cells. The values corresponding to the X- and Y-axes in the scatter plot are the normalized signal values of the samples (log2 scaled). The lines represent the default significant

fold change (2.0). B: Volcano plot was created to show significantly dysregulated circRNAs. The vertical green lines corresponded to two-fold up- and down-regulation (log2 scaled), and the horizontal green line represented a p value of 0.05. The red points represented significantly dysregulated circRNAs (fold change ≥2.0, p < 0.05). C: Heat map evaluation of the circRNA expression patterns among cells treated with MPA and untreated cells. Each column represents the expression profile of a cell line sample, and each row corresponds to a circRNA. “Red” indicates higher expression level, and “green” indicates lower expression level

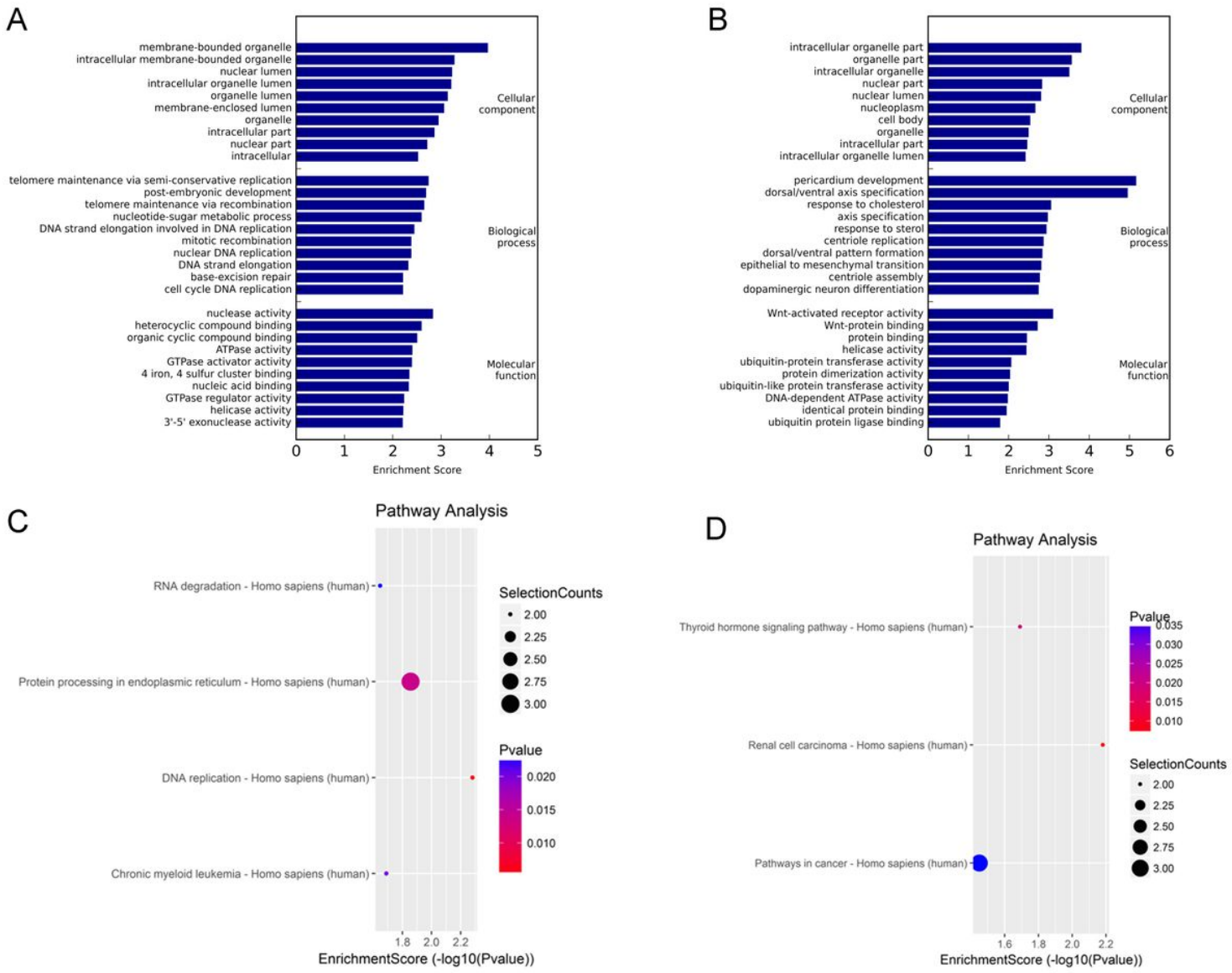

Figure 2

CircRNAs functional enrichment analysis. A: Gene ontology (GO) terms for the parental genes of upregulated circRNAs. B: Gene ontology (GO) terms for the parental genes of down-regulated circRNAs. C: KEGG pathways for the parental genes of up-regulated circRNAs. D: KEGG pathways for the parental genes of down-regulated circRNAs

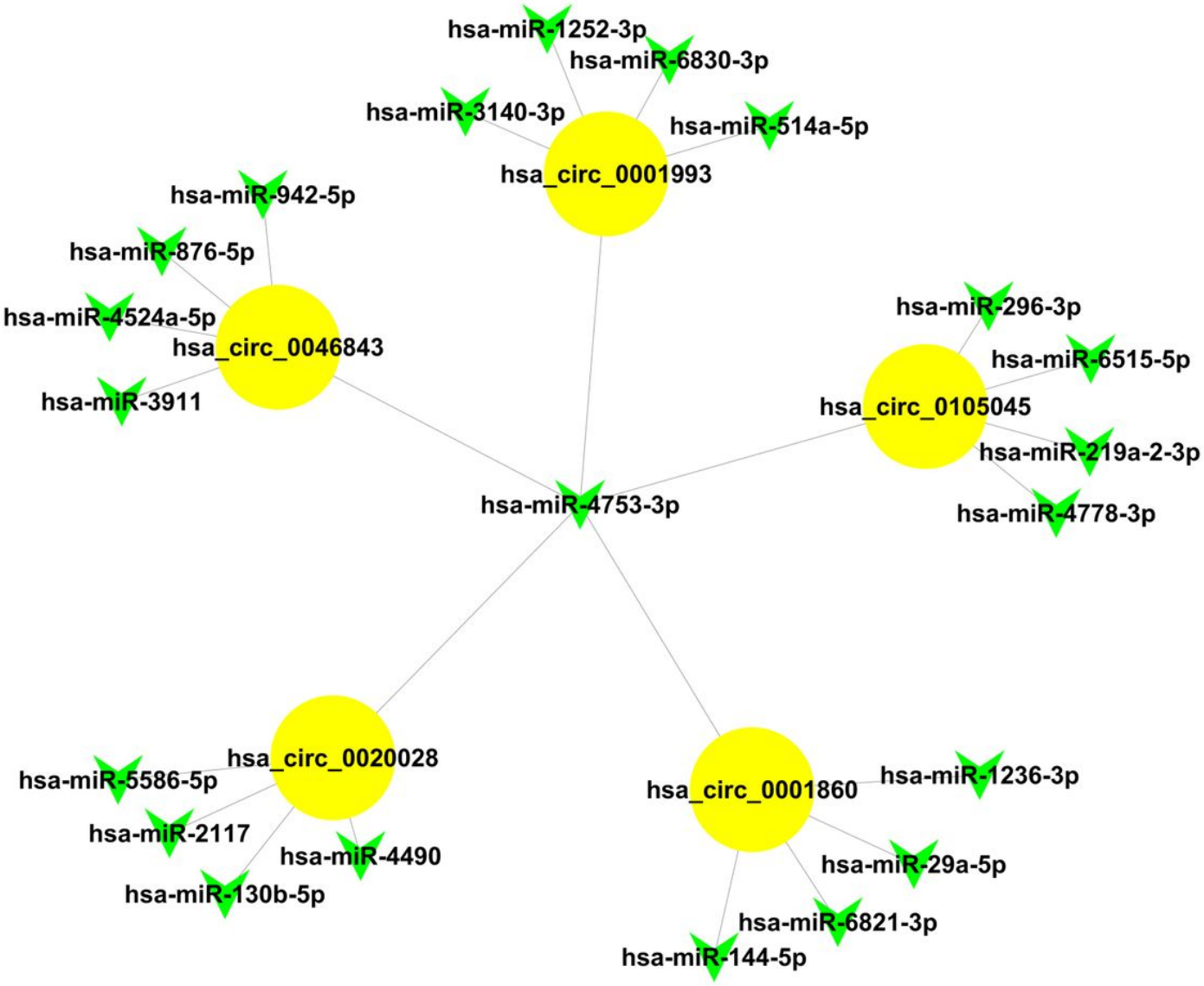

Figure 3

CircRNA-miRNA interaction network

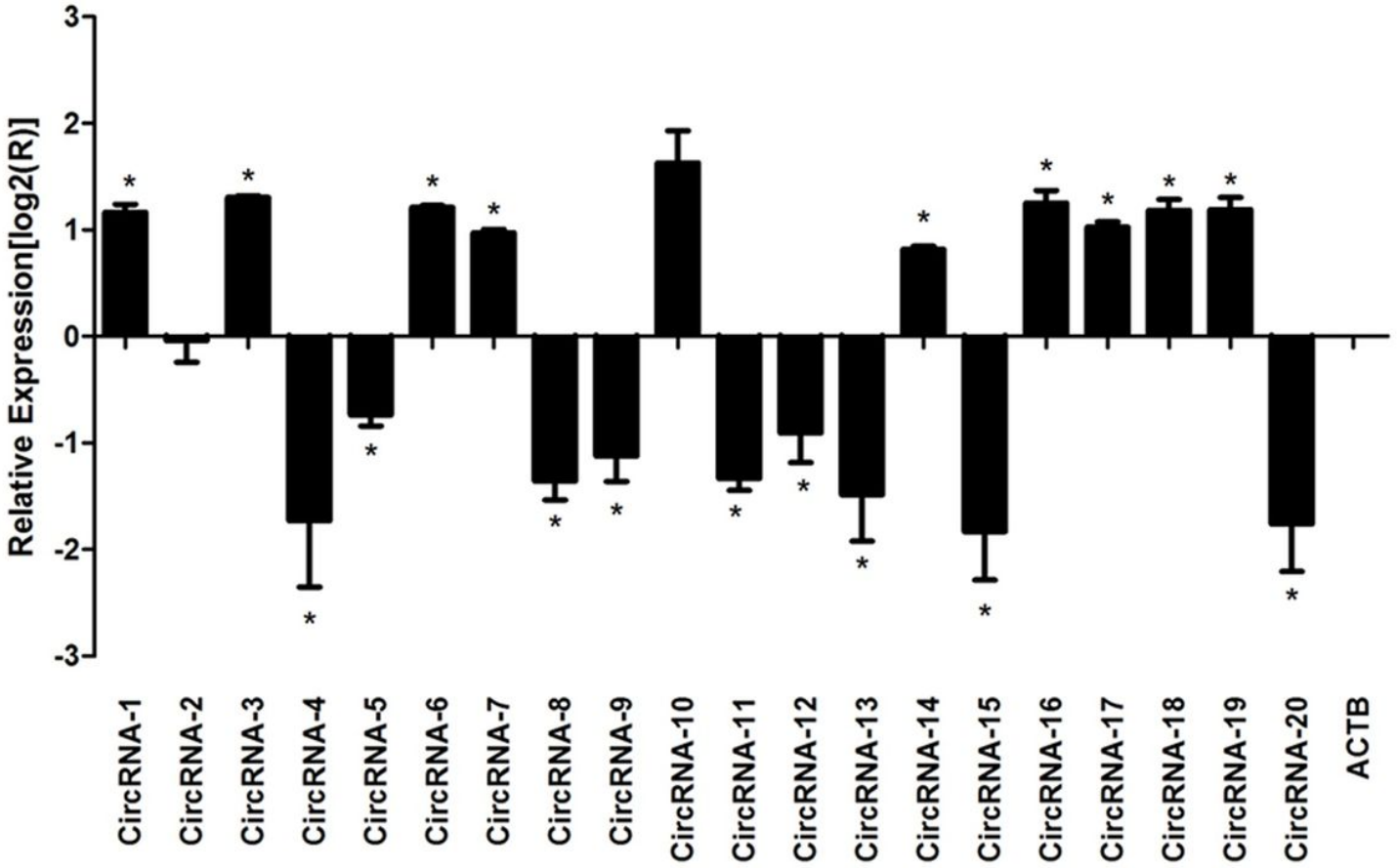

Figure 4

Validation of the differentially expressed circRNAs by real-time PCR assay. The values are mean ± SE (n = 3). *: P < 0.05

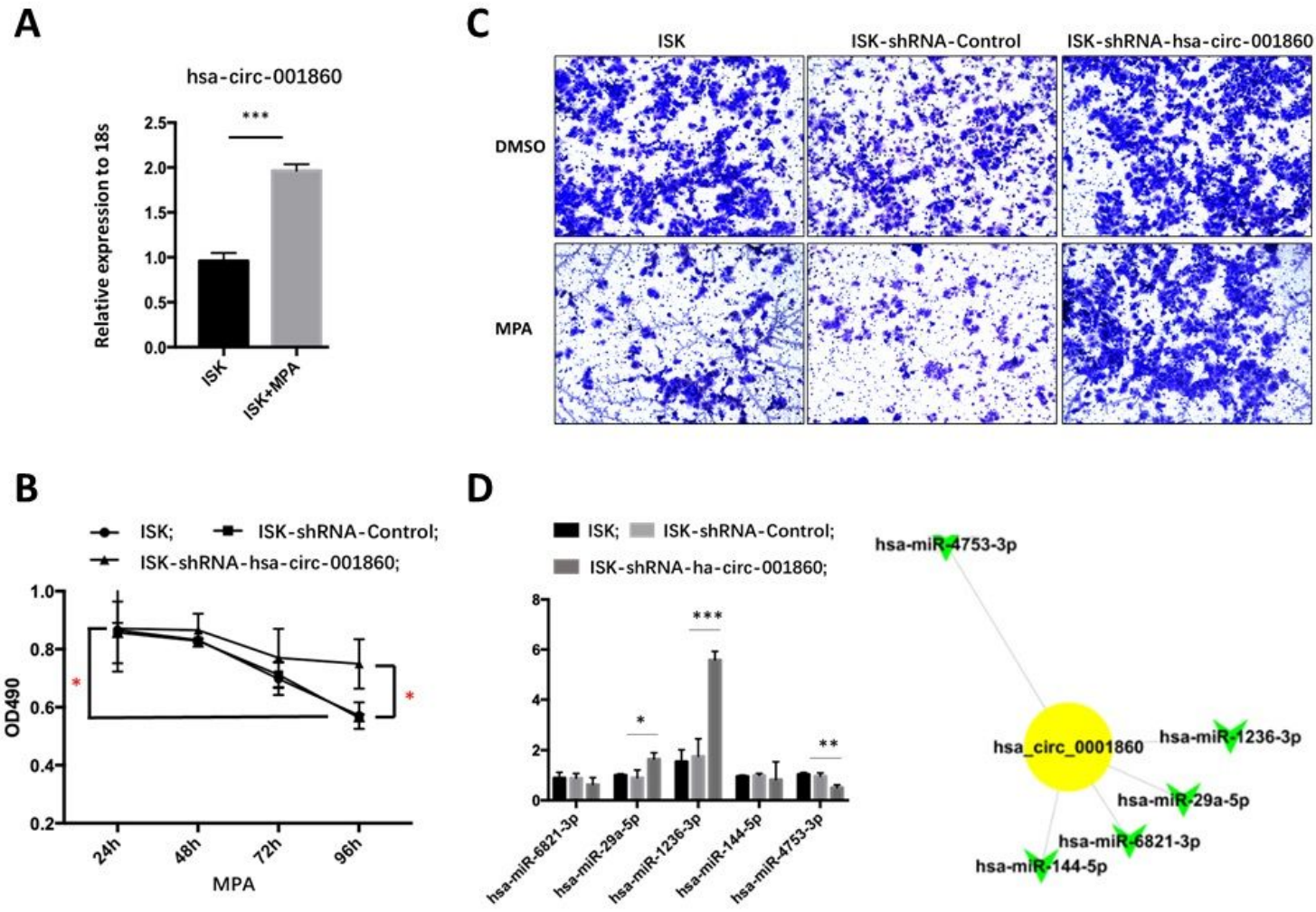

Figure 5

5A. The analysis of effects of MPA treatment and of knockdown of circ-001806 in ISK cells. A. qRT-PCR analysis of relative circRNA expression. B. Cell proliferation analysis using MTS assay. *, P < 0.05. C. Invasion assay using invasion chamber and 4% PFA fixed cells were labelled using 0.1% Crystal violet dye (Blue color). D. qRT-PCR analysis of miRNA expression in ISK cells.

Supplementary Files

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- SupplementalData4networkparameter.xlsx
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