RESEARCH

Circular RNA profile in Graves' disease and potential function of hsa_circ_0090364

Zhengrong Jiang, Linghong Huang, Lijun Chen, Jingxiong Zhou, Bo Liang, Xuefeng Bai, Lizhen Wu and **Huibin Huang**[ID]

Department of Endocrinology, The Second affiliated Hospital of Fujian Medical University, Quanzhou, Fujian, China

Correspondence should be addressed to H Huang: huibinhuang@fjmu.edu.cn

Abstract

Background: Graves' disease is a common autoimmune disease. Cytokines and their signalling pathways play a major part in the pathogenesis of Graves' disease; however, the underlying mechanism needs to be clarified.
Aims: The aim of this study was to explore whether circular RNAs participate in the immunological pathology of Graves' disease via cytokine-related signalling pathways.
Methods: Bioinformatics analysis was performed to identify differentially expressed circular RNAs and their targets and associated pathways. A total of three patients with Graves' disease and three sex- and age-matched healthy controls were enrolled for validation with microarray analysis and real-time quantitative PCR (qPCR). An additional 24 patients with Graves' disease and 24 gender- and age-matched controls were included for validation by real-time fluorescent qPCR. Flow cytometry and CCK8 assays were used to detect the apoptotic and proliferative levels of Jurkat cells (T lymphocytes) with the silenced expression of circRNA. ELISA was performed to detect the growth and apoptosis-related proteins. The competition mechanism of endogenous RNA was explored by real-time fluorescence qPCR.
Results: A total of 366 significantly differentially expressed circular RNAs were identified in the Graves' disease group compared to healthy controls. The level of hsa_circ_0090364 was elevated in Graves' disease patients and positively correlated with thyroid-stimulating hormone receptor antibodies. Further analyses suggested that hsa_circ_0090364 may regulate the JAK-STAT pathway via the hsa-miR-378a-3p/IL-6ST/IL21R axis to promote cell growth.
Conclusions: These results provide novel clues into the pathophysiological mechanisms of Graves' disease and potential targets for drug treatment.

Introduction

Graves' disease (GD) is an organ-specific autoimmune disease that commonly manifests as hyperthyroidism and diffuse goitre. The annual incidence of GD is 20–50 cases per 100,000 people worldwide (1, 2). The GD phenotype is becoming milder owing to sensitive diagnostic tests and effective treatments. However, thyroid storms can occur occasionally, and GD treatments such as antithyroid medications, radioiodine therapy, and surgery

(thyroidectomy) may be associated with side effects (2). Thus, further exploration of the molecular mechanisms of GD is essential, which will be beneficial for developing new diagnostic and personalized therapeutic strategies for the disease.

Circular RNAs (circRNAs) are a new type of ncRNA classified into four categories depending on their parental gene, namely exonic RNAs, intronic RNAs, exon-intron

RNAs, and intergenic or fusion RNAs. As more and more circRNAs are reported to be aberrantly expressed in human tissues and associated with disease, the function of these genes has gained increased research attention (3). In particular, the participation of circRNAs in post-transcriptional regulation has become a research hotspot. Among them, the competing endogenous RNA (ceRNA) hypothesis represents a bright spot. As miRNAs bind to their target mRNA transcripts via miRNA recognition elements (MREs), target gene expression is repressed. circRNAs act as molecular sponges by competitively binding to miRNA through MREs and negatively regulating target gene expression. According to the above hypothesis, a new regulatory form is recognized, which is known as the circRNA–miRNA–mRNA or ceRNA network (4, 5). circRNAs are believed to participate in the pathogenesis of several autoimmune diseases through the ceRNA network, such as rheumatoid arthritis, systemic lupus erythematosus, and ulcerative colitis (5, 6, 7, 8). However, there are few studies on the involvement of circRNAs in the mechanisms underlying GD pathogenesis. Sun *et al.* detected a circRNA expression profile in serum exosomes from GD patients, which implicated hsa_circRNA_000102 in the development of GD via a virus infection signalling pathway (9). To date, the functions of other circRNAs in GD remain unclear, and more insight into the relationship between circRNAs and GD is still required.

Cytokines are a group of small molecules secreted by a variety of tissue cells (mainly immune cells), which play a central role in initiating immune cascades through their receptors and multiple signalling pathways (10). In GD patients, pro-inflammatory cytokines are capable of promoting T cell proliferation and differentiation, which trigger the abnormal activation of B cells, resulting in the overproduction of antibodies and ultimately facilitating the occurrence and development of GD (11, 12). Therefore, investigations of the potential molecular mechanisms of cytokine-related signalling pathways in patients with GD are key to study the immunological pathology of the disease.

In this study, we first identified circRNAs expressed in GD patients using a circRNA microarray. Subsequently, enrichment analysis was carried out to detect the potential function of differentially expressed circRNAs (DECs). To gain further understanding of the GD immunological mechanism, we constructed a circRNA–miRNA–mRNA regulation module related to the cytokine pathway. This study enriches our understanding of the immunopathological mechanisms underlying GD and reveals potential targets for both GD prevention and treatment.

Materials and methods

Sample collection

Twenty-seven untreated patients with GD and 27 healthy controls (HCs) were enrolled in this study from January 2022 to June 2022. Among these, three patients with newly diagnosed GD and three sex- and age-matched HCs were recruited for the microarray analysis and real-time quantitative PCR (RT-qPCR) validation, and the remaining 48 participants were included in further RT-qPCR validation only. The research protocols were approved by the Medical Ethics Committee of the Second Affiliated Hospital of Fujian Medical University. All participants were fully informed of the study procedures and signed informed consent forms.

The inclusion criteria for the GD group included the following (13): diffuse goitre, with symptoms of increased sweating, heat intolerance, elevated heart rate, elevated serum thyrotropin receptor antibody (TRAb), and low serum thyroid-stimulating hormone (TSH). The newly diagnosed GD patients had not yet undergone treatment for thyroid disease or ophthalmopathy. The exclusion criteria included hepatic dysfunction, renal dysfunction, infection, other autoimmune disease, tumours, a history of immunosuppressive therapy within the last year, and pregnancy or lactation. The inclusion criteria for the HC group were normal thyroid function and no family history of thyroid diseases. The GD group had elevated FT3, FT4, and TRAb; low TSH; and normal thyroid peroxidase antibody and thyroglobulin antibody levels (P-value < 0.05). The general information of the six participants included in the microarray analysis is listed in Supplementary Table 1 (see section on supplementary materials given at the end of this article), and the basic information of the 48 additional participants included in the validation study is listed in Supplementary Table 2.

Microarray assay

Total RNA was isolated from the plasma of three newly diagnosed GD patients and three HC subjects using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The RNA quality was evaluated using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The circRNA Microarray (Arraystar Inc., Rockville, MD,

USA) was applied to identify the expression profile in GD. First, the RNAs were purified, amplified, and transcribed into complementary RNA (cRNA). Next, the cRNA was combined onto an array slide by hybridization. The array was then scanned, and Agilent Feature Extraction software (version 11.0.1.1; Agilent Technologies) was used to analyse the images from the acquired array. The subsequent data were processed using R software (version 3.22.7; Lucent Technologies, Murray Hill, NJ, USA). DECs in GD were defined as those meeting the following criteria: fold-change > 1.3 and *P*-value <0.05.

Enriched functional analysis

Most circRNAs have not yet been functionally annotated, whereas most of the identified mRNAs are well known. Therefore, the ceRNA network was built. Based on this network, we predicted the functions of DECs via enriched functional analysis of their target mRNAs. The specific process was as follows. First, miRNAs matched with DECs (Table 1) and their potential MREs were predicted using Arraystar's homemade miRNA target prediction software based on miRanda and TargetScan. According to the seed matching sequence, the top five MRE and miRNAs were screened. Subsequently, target mRNAs were predicted with TarPmiR (14) and miRDB (15), based on a combination index. The immune-related mRNAs were obtained from the ImmPort database (16). Only target mRNAs recognized by the three databases and reported in the literature as related to autoimmune thyroid diseases were selected as candidate target mRNAs. The ceRNA network was visualized using Cytoscape 3.8.1. Finally, based on the network, Gene Ontology (GO) analysis, including biological process (BP), cell component (CC), and molecular function (MF) annotation, was performed to explore the functions of the DECs. The Kyoto Encyclopedia of Genes and Genomes (KEGG) tool was used to detect the potential pathways of the identified DECs. According to an enrichment score ($-\log_{10}$ (*P*-value)), the top ten GO terms and KEGG pathways were selected and ranked.

Validation with RT-qPCR

RNA from the plasma of the six participants used for microarray sequencing was reverse-transcribed into cDNA by PrimeScript RT Master Mix kit (Takara, Dalian, China). The cDNA samples were amplified with TB Green Premix Ex Taq II kit (Takara). Blood samples were also collected from the remaining 48 participants. Peripheral blood mononuclear cells (PBMCs), rather than plasma, were separated from the blood samples and used to verify the relative expression of the upregulated circRNAs that were validated to be abnormally expressed in the plasma from patients with GD, as functional analysis showed that high expression of DECs was associated with the immune response.

The total RNA extraction from PBMCs and circRNA/mRNA reverse-transcription steps were performed as previously described. Poly (A) Tailing Kit (ShengGong, Shanghai, China) was applied to reverse transcribe the miRNA into cDNA. The expression levels of circRNA, mRNA, and miRNA of PBMC origin were then measured by qPCR. GAPDH was used as an internal reference to normalize the expression levels of circRNAs and mRNAs, whereas the expression levels of miRNAs were normalized to that of U6. All reactions were processed three times for each sample. The primer sequences are listed in Table 2. The generic downstream primers from the Poly (A) Tailing Kit were used as the reverse primers for U6 and miRNA RT-qPCR validation. The relative expression levels of the downstream genes were quantified using the $2^{-\Delta\Delta Ct}$ method. The relationship between the validated circRNAs and miRNAs, TRAb, and downstream mRNAs expression was determined by linear regression analysis.

Cell culture and transfection

The aforementioned bioinformatic analysis and RT-qPCR results suggested that the circRNAs differentially expressed in PBMCs from GD patients might be involved in the JAK-STAT pathway and target downstream mRNAs by a ceRNA regulatory network to play a role in promoting disease

Table 1 Details for the top five upregulated circRNAs.

circRNAs	Gene symbol	Fold change	*P*-value	Regulation
hsa_circ_0090364	JADE3	2.70	0.04	Up
hsa_circ_0001228	MFNG	1.72	0.04	Up
hsa_circ_0091183	HDX	1.79	0.01	Up
hsa_circ_0030131	DGKH	1.75	0.01	Up
hsa_circ_0053141	PREB	1.73	0.04	Up

circRNAs, circular RNAs. Gene symbol, parental gene.

Table 2 Primers for RTqPCR analysis.

Circular RNAs	Primer sequences
hsa_circ_0090364	F:5' ACACCGTTCCACAGCCTTCT3'
	R:5' TGACTTGATCCTATACATTGAGCC3'
hsa_circ _0001228	F:5' AGGAGGACCCCTCCAGTGG 3'
	R:5' AGCCGGATGAGAGCAGATGT3'
hsa-miR-378a-3p	F: 5'ACTGGACTTGGAGTCAGAAGGCAA3'
IL-6ST	F:5' GATTCCTCCTGAAGACACAGCATCC3'
	R: 5' TTCACTCCAGTCACTCCAGTATCCC3'
IL-21R	F: 5' TCCTGTCCTGTGGCTGTGTCTC3'
	R: 5'GAGTGGGTGTCGCTGTTGA3'
JAK1	F: 5' GTCCAGCGACCTTCATTCAGATACC3'
	R: 5' CCTTCAAAGCCACTGCCCAGAC3'
STAT3	F: 5' CACCAAGCGAGGACTGAGCATC3'
	R: 5' AGCCAGACCCAGAAGGAGAAGC3'
GAPDH(HUMAN)	F: 5'GGGAAACTGTGGCGTGAT3'
	R: 5'GAGTGGGTGTCGCTGTTGA3'
U6(HUMAN)	F: 5'AGAGAAGATTAGCATGGCCCCTG3'

F, forward. R, reverse.

development. T-lymphocytes are an important immune cell in PBMCs and their role in autoimmune diseases has been confirmed by several previous studies (17, 18). Therefore, we selected Jurkat cells, which is a T lymphocyte cell line for *in vitro* analysis to further explore the function of the circRNAs. The cells (Jurkat, Clone E6-1, FuHeng, FH0878) were incubated in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). A plasmid expressing an shRNA (Hanbio Biotechnology, Shanghai, China) was used for hsa_circ_0090364 silencing. The shRNA sequence was given as follows: top strand, GATCCGACAGCCTTCTCTCAGGATGCTCCAGTTCAAGAGACTGGAGCATCCTGAGAGAAGGCTGTTTTTTTG; bottom strand, AATTCAAAAAAACAGCCTTCTCTCAGGATGCTCCAGTCTCTTGAACTGGAGCATCCTGAGAGAAGGCTGTCG.

shRNA-negative control (shN) was applied as a negative control. The sequence was top strand, GATCCGTTCTCCGAACGTGTCACGTAATTCAAGAGATTACGTGACACGTTCGGAGAATTTTTTC; bottom strand AATTGAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGAATTACGTGACACGTTCGGAGAACG. Transfection was carried out according to the manufacturer's recommendations.

Proliferation assay

The Cell Counting Kit-8 (CCK-8; Meilun, China) assay was used to measure cell proliferation. The transfected cells were seeded into 96-well plates at a density of 5×10^4 cells/well. At appropriate time points (24, 48, 72, and 96 h), 10 μL of CCK-8 reagent was added to each well. After incubation at 37°C for 2 h, a microplate reader was applied to measure the absorbance at 450 nm.

Flow cytometry

Apoptosis was detected using the AnnexinV FITC ApopDtec kit (BD Pharmaceuticals). All samples were analysed on a BDLSRfortessa™ instrument (BD Biosciences).

Proliferation/apoptosis molecular marker detection by enzyme-linked immunoassay

The concentrations of the proliferation-associated protein proliferating cell nuclear antigen (PCNA) and the apoptosis-associated proteins BCL-XL and BAX were measured using respective ELISA kits (Meimian, Jianshu, China) according to the manufacturer instructions.

Key ceRNA network construction and validation

A ceRNA network was constructed containing the key circRNA (hsa_circ_0090364) and its matched miRNAs and target mRNAs. The data were visualized using Cytoscape 3.8.1.

The expression of hsa_circ_0090364, matched miRNAs, target mRNAs, and key signalling molecules in the JAK-STAT pathway in Jurkat cells transfected with sh-hsa_circ_0090364 or shN was measured by RT-qPCR. The $2^{-\Delta\Delta Ct}$ method was applied for the target gene expression assays in the cell line.

Statistical analyses

SPSS Statistics 23 (IBM) was applied for statistical analysis. The data are expressed as the mean ± S.D. or mean ± S.E.M. Either an unpaired independent sample *t*-test or a non-parametric method such as the Mann–Whitney *U* test was applied for comparisons between groups, as appropriate. A *P*-value < 0.05 was defined as statistically significant.

Results

Significant differences in circRNA expression

Figure 1A shows the volcano plot of the DECs identified between GD patients and HCs. A total of 366 DECs were identified, which included 195 upregulated and 171 downregulated circRNAs (fold-change >1.3, *P*-value <0.05).

The circRNAs were classified into five categories: exonic, sense-overlapping, intronic, antisense, and intergenic. Among the upregulated circRNAs, 173, 10, 9, 2, and 1 were exonic, antisense, intronic, sense overlapping, and intergenic, respectively (Fig. 1B). Among the
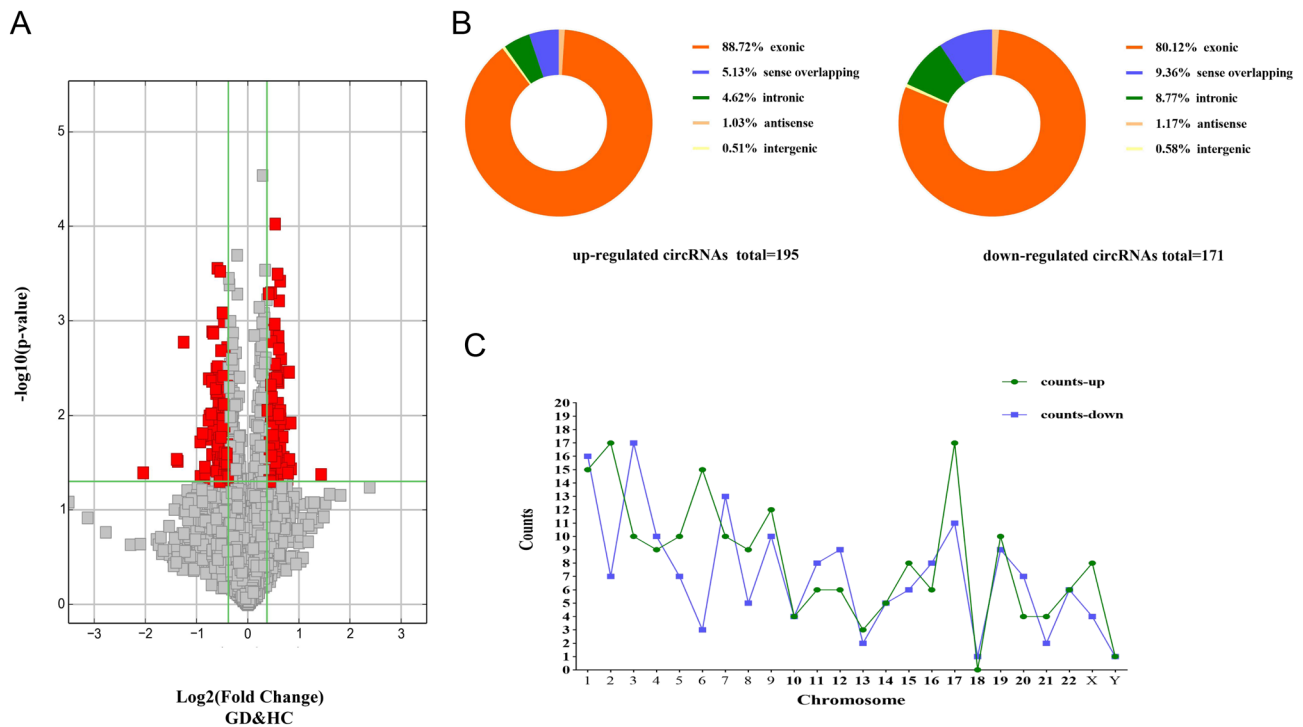
Figure 1
Microarray analysis of the differential expression of circular RNAs between Graves' disease patients and healthy control subjects (*n* = 3:3). (A) Volcano plot showing the differential expression profiles of Graves' disease patients compared to healthy control subjects. The log 2 scale depicted on the x-axis indicates the fold-change and the −log$_{10}$ *P* value is depicted on the y-axis. The red dots in the figure represent the differential expression of circular RNAs. (B) Category characteristics of upregulated differentially expressed circular RNAs. (C) Category characteristics of downregulated differentially expressed circular RNAs. (D) Chromosome distributions of differentially expressed circular RNAs. 'Count' indicates the number of differentially expressed circular RNAs. 'Chromosome' represents different chromosomes. circRNAs, circular RNAs.

downregulated circRNAs, 137, 2, 15, 16, and 1 were exonic, sense-overlapping, intronic, antisense, and intergenic, respectively (Fig. 1C). Among the upregulated circRNAs, 17 were on both chromosome 17 and chromosome 2, whereas 17 of the downregulated circRNAs were located on chromosome 3 (Fig. 1D).

Functional enrichment analysis of upregulated circRNAs

Owing to the large proportion of upregulated DECs in GD patients, we mainly focused on the biological functional annotation of these circRNAs, whereas the downregulated DECs will be used for follow-up research. According to the fold-change and *P*-values, the top five upregulated circRNAs were selected. All of these circRNAs were annotated in CircBase. A ceRNA network was constructed based on these 5 circRNAs, 25 miRNAs, and 135 mRNAs (Fig. 2). The 135 mRNAs in this network were processed for functional enrichment analysis with GO and KEGG tools.

Overall, 192 BPs, 24 CCs, and 59 MFs were enriched (*P*-values <0.05). With respect to BPs, the top five

upregulated circRNAs primarily participated in positive regulation of the ERK1 and ERK2 cascade and regulation of cell migration. The GO analysis of CCs showed that the top five upregulated circRNAs mainly play a role in the plasma membrane, extracellular region, and receptor complex during functional execution. For MFs, these circRNAs were associated with steroid hormone receptor activity, growth factor binding, growth factor activity, cytokine receptor activity, semaphoring receptor binding, and chemorepellent activity. The top 10 BP, CC, and MF terms are listed in Fig. 3A.

The KEGG pathway analysis showed that the top five upregulated circRNAs principally participated in the JAK-STAT, PI3K-AKT, T cell receptor, Rap1, Ras, and natural killer cell-mediated cytotoxicity signalling pathways. The top ten KEGG pathways are listed in Fig. 3B. The JAK-STAT signalling pathway is a crucial cytokine-related pathway and was the most meaningful enriched pathway. Therefore, we defined it as a candidate pathway for follow-up research. According to the results of functional enrichment analysis, we selected two upregulated circRNAs (hsa_circ_0090364 and hsa_circ_0001228)
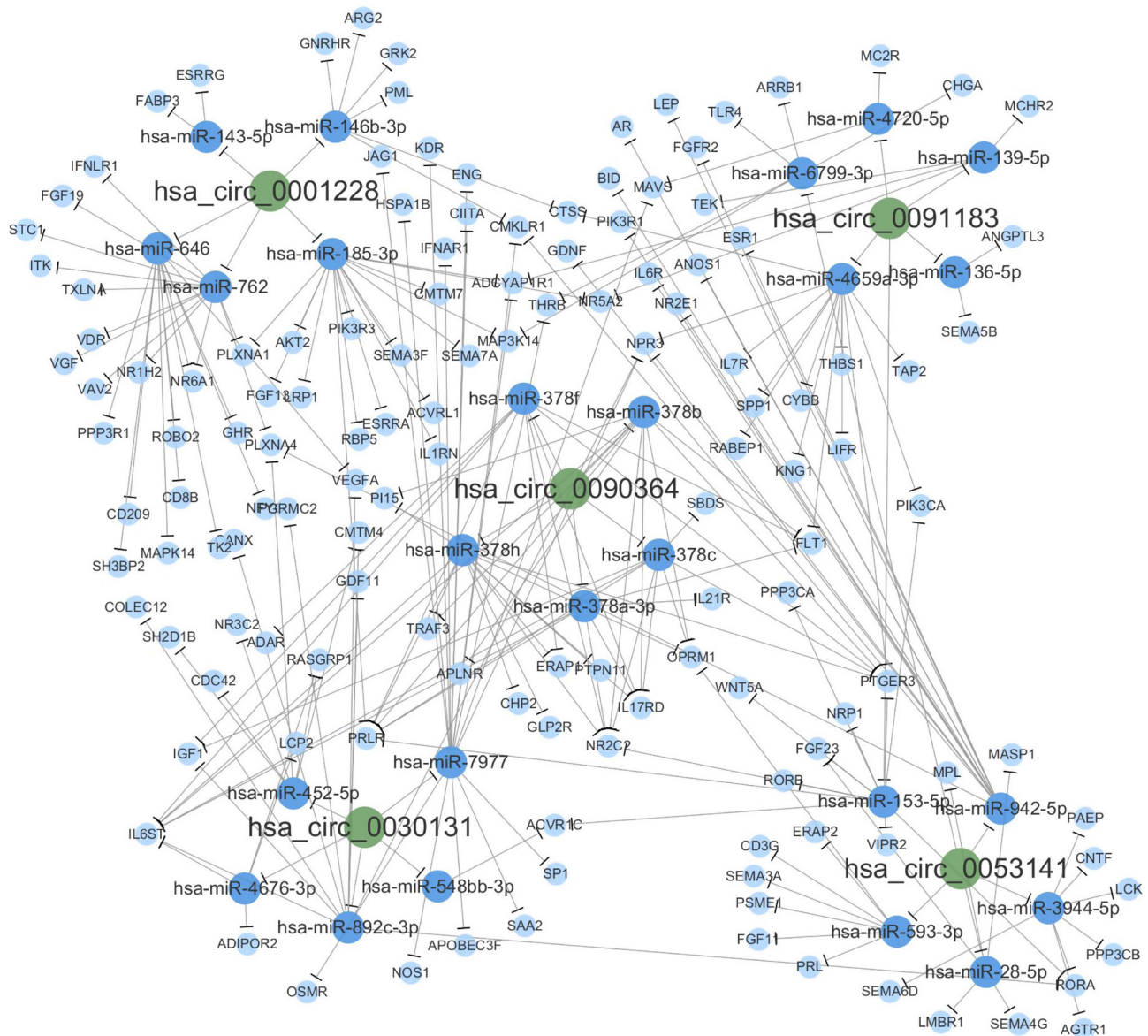
Figure 2
The ceRNA network of the top five upregulated circular RNAs in Graves' disease patients. The circular RNA that was validated (green) is displayed in a network diagram. The top five miRNA regulated by the top five upregulated circular RNAs are shown in blue. mRNAs are indicated in light blue. circRNA, circular RNA; miRNA, microRNA.

involved in JAK-STAT signalling for circRNA expression validation (Table 1).

Validation of circRNA expression

Only the transcript expression of hsa_circ_0090364 was verified to be highly expressed in the plasma of GD patients (Supplementary Fig. 1), and hsa_circ_0090364 was also differentially highly expressed ($P < 0.05$) in PBMCs from GD patients in the expanded validation sample (Fig. 4A). Furthermore, the expression level of hsa_circ_0090364 was

positively correlated with the serum TRAb concentration ($R^2 = 0.554$, $P < 0.01$; Fig. 4B). These results revealed that hsa_circ_0090364 might be related to the immune mechanism of GD.

Knockdown of hsa_circ_0090364 inhibited cell proliferation

To further probe the immunological function of hsa_circ_0090364, an shRNA-hsa_circ_0090364 plasmid was transfected into Jurkat cells. The knockdown efficiency
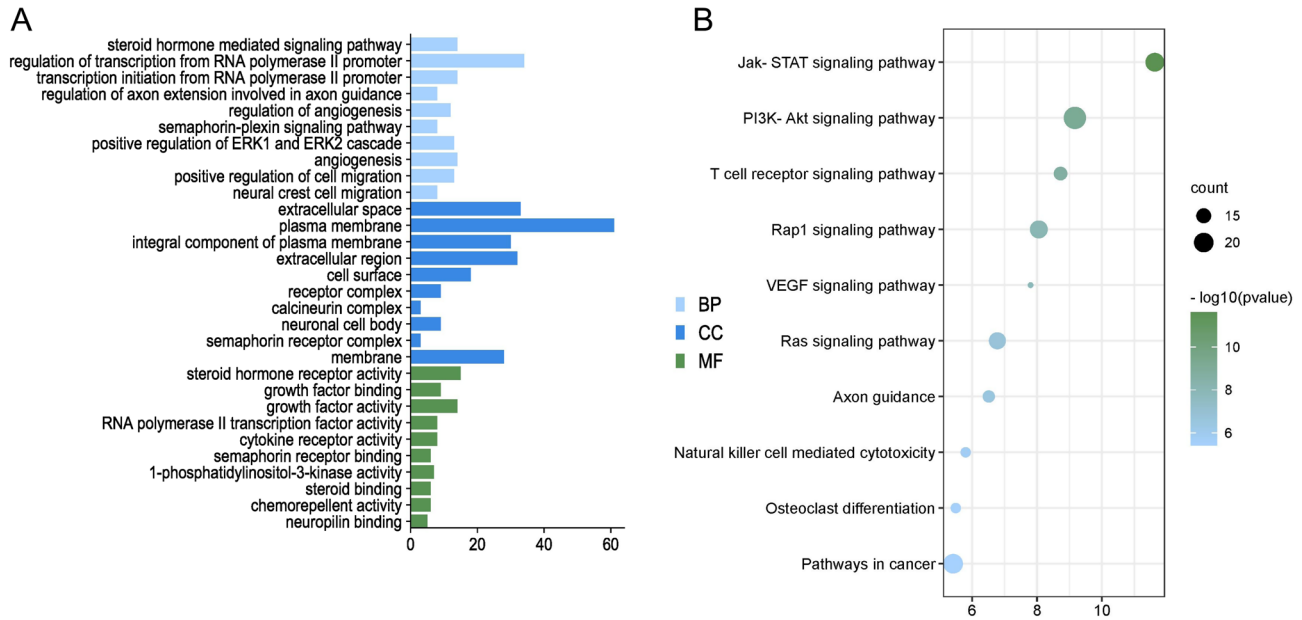
Figure 3

Functional analyses of the top five upregulated circular RNAs in Graves' disease patients. (A) The significantly enriched terms by Gene Ontology (GO) analysis of the top five upregulated circular RNAs in Graves' disease patients. GO analysis is divided into three categories: biological processes, cellular components, and molecular functions. (B) The top ten significantly enriched pathways by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

of shRNA-hsa_circ_0090364 was confirmed, as shown in Fig. 5A ($P = 0.002$). The CCK-8 assay further showed that knockdown of hsa_circ_0090364 resulted in the inhibition of Jurkat cell proliferation (Fig. 5B, $P < 0.05$). The flow cytometry assay revealed no difference in cell apoptosis in the shRNA-hsa_circ_0090364 group and shN group. In addition, the expression of the proliferation-associated

protein PCNA was downregulated in hsa_circ_0090364 knockdown cells (Fig. 5C), whereas the expression levels of the apoptosis-associated proteins BCL-XL and BAX are not different between the shRNA-hsa_circ_0090364 and shN groups (Fig. 5D, E and F). Collectively, these results demonstrated that hsa_circ_0090364 knockdown inhibited the proliferation of Jurkat cells but did not affect apoptosis in the cells.
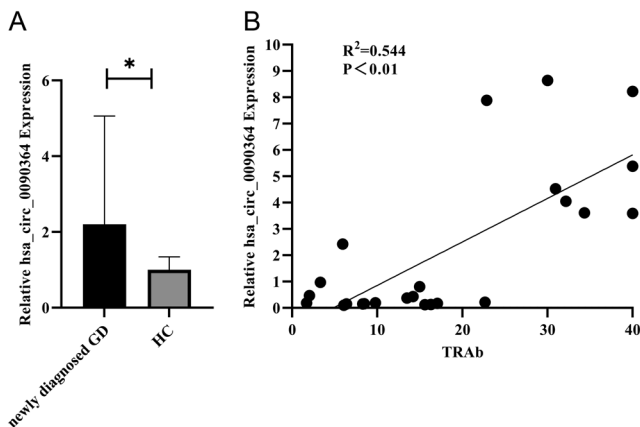
The ceRNA network of hsa_circ_0090364 related to JAK-STAT pathway

To further investigate the molecular mechanism underlying the effects of hsa_circ_0090364 on the JAK-STAT pathway, a ceRNA network was established based on the aforementioned network. A ceRNA network is a many-to-many regulatory network, where a gene is considered to be a key gene when it is able to regulate more downstream genes than its counterpart. Therefore, based on the above concept, we further refined the regulatory relationships shown in Table 3 to obtain a hub ceRNA regulatory network. In brief, hsa-miR-378a-3p and two target mRNAs (IL-6ST and IL21R) were integrated into this ceRNA network. The network showed that hsa_circ_0090364 could alter the expression of its target genes by acting as a sponge for hsa-miR-378a-3p via their binding sites.
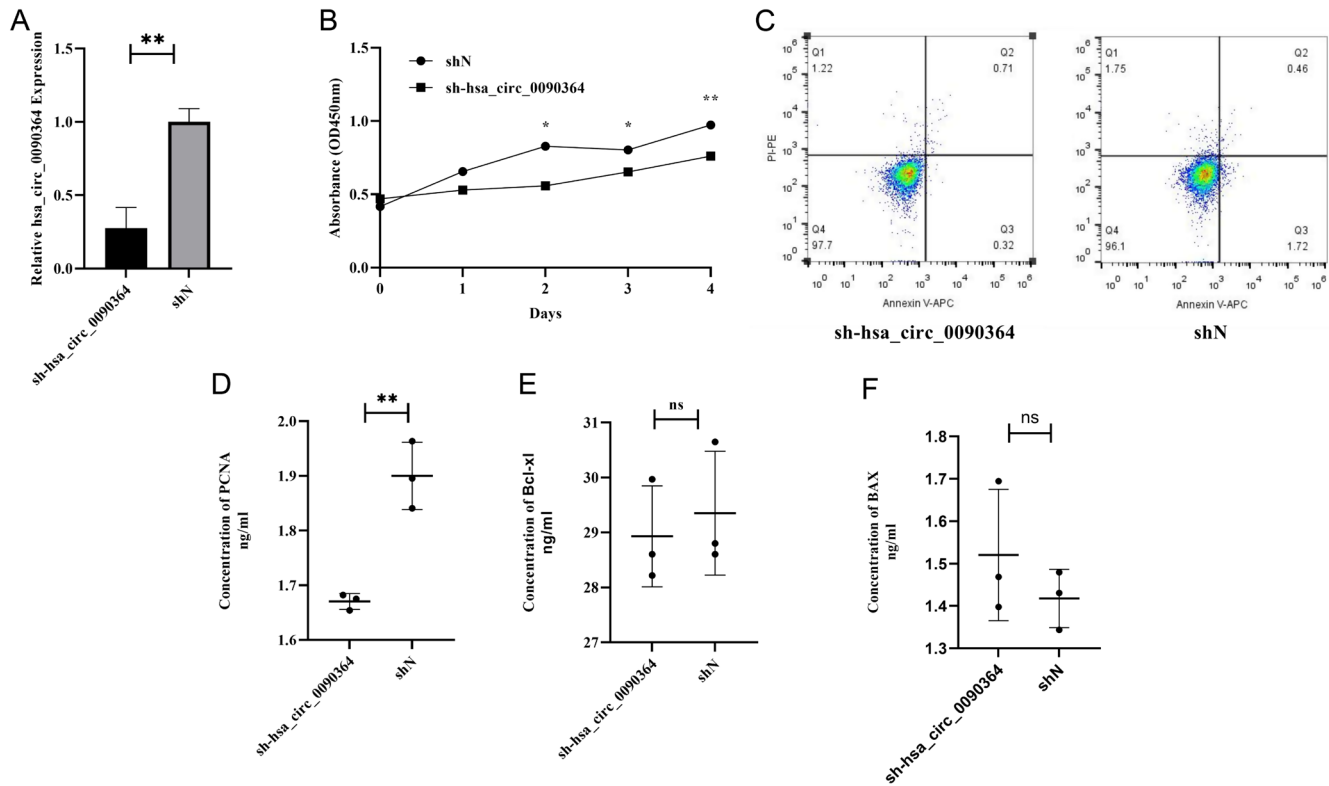

Figure 4

The validation of hsa_circ_0090364. (A) Relative expression of hsa_circ_0090364 in PBMCs ($n = 24:24$) by real-time qPCR. *GAPDH* was used as an internal reference gene. (B) The transcript expression of hsa_circ_0090364 was significantly correlated with TRAb levels in Graves' disease patients' PBMCs.

Figure 5

hsa_circ_0090364 promotes Jurkat cell proliferation but does not affect apoptosis. (A) hsa_circ_0090364 knockdown efficiency after shRNA-hsa_circ_0090364 or shRNA negative control (shN) transfection in Jurkat cells. (B) Seventy-two hours after transfection, the CCK8 assay was applied to detect the function of hsa_circ_0090364 on cell proliferation. (C) Seventy-two hours after transfection, flow cytometry was used to assess the effect of hsa_circ_0090364 on cell apoptosis. (D) Seventy-two hours after transfection, ELISA was used to evaluate the levels of proliferation-associated and apoptosis-associated proteins.

As shown in Fig. 6A and B, the hsa-miR-378a-3p expression level was lower in the GD group ($P=0.006$) and was negatively correlated with the hsa_circ_0090364 expression level ($P=0.023$). Knockdown of hsa_circ_009036 resulted in elevated miRNA expression (Fig. 6C, $P=0.002$). IL-6ST and IL21R are downstream mRNAs of the hsa_circ_0090364-hsa-miR-378a-3p axis and are also involved in the JAK-STAT pathway. Our study revealed that the transcript levels of IL-6ST and IL21R were higher in the GD group (Fig. 6D and G). Meanwhile, the expression of hsa_circ_0090364 was positively correlated with the expression of IL-6ST and IL21R transcripts (Fig. 6E and H).

Knockdown of hsa_circ_009036 resulted in the decreased expression of IL-6ST and IL21R (Fig. 6F and I).

JAK1 and STAT3 were the key molecules of the JAK-STAT pathway, as shown in Fig. 7, and the expression of JAK1 mRNA and STAT3 mRNA was higher in GD group. The mRNA levels of these genes were positively correlated with the expression of hsa_circ_0090364. To determine whether hsa_circ_0090364 affects the expression of JAK1 and STAT3 mRNA, Jurkat cells were transfected with shRNA-hsa_circ_009036. Decreased expression of JAK1 and STAT3 mRNA was detected in transfected Jurkat cells.

Table 3 A key ceRNA network related to JAK-STAT pathway.

circRNAs	miRNA	mRNA
hsa_circ_0090364	hsa-miR-378a-3p	IL-6ST IL-21R

circRNAs, circular RNAs.

Discussion

Our microarray analysis indicated a differential expression profile in the plasma of GD patients containing five of the most significantly upregulated circRNAs. Bioinformatics analysis showed that the five circRNAs were mainly related
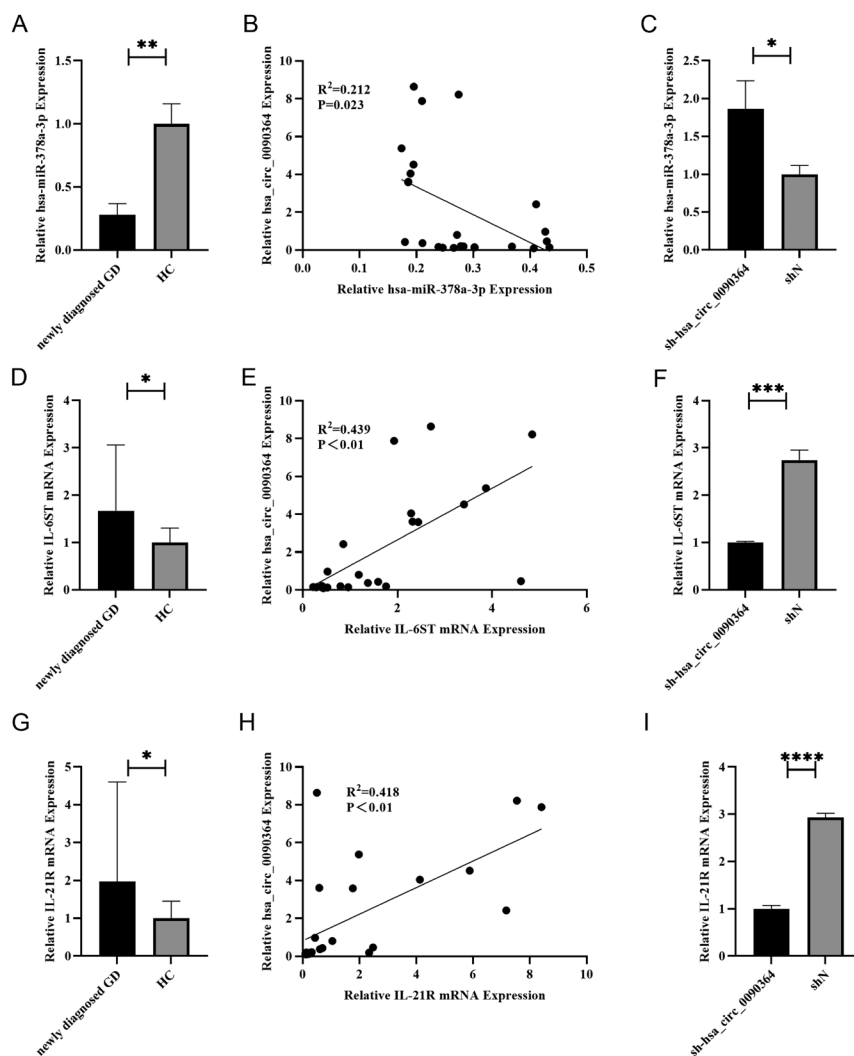
Figure 6

The ceRNA network of hsa_circ_0090364 (A and B) hsa-miR-378a-3p expression is lower in the GD group and is negatively correlated with hsa_circ_0090364 expression in Graves' disease patients' PBMCs. (C) The relative expression levels of hsa-miR-378a-3p after shRNA-hsa_circ_0090364 or shRNA negative control transfection in Jurkat cells. (D, E and F) IL-6ST mRNA expression levels are higher in the Graves' disease patients' PBMCs and are positively correlated with hsa_circ_0090364 expression in Graves' disease patients' PBMCs. After shRNA-hsa_circ_0090364 or shRNA negative control transfection, the mRNA expression of IL-6ST *is decreased.* (G, H and I) IL-21R mRNA expression levels are higher in the Graves' disease patients' PBMCs and are positively correlated with hsa_circ_0090364 expression in Graves' disease patients' PBMCs. After shRNA-hsa_circ_0090364 or shRNA negative control (shN) transfection, the mRNA expression of IL-21R is decreased. The results are indicated as the mean ± s.d. of three independent experiments; horizontal lines show the mean (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

to immune and inflammatory responses. By RT-qPCR, hsa_circ_0090364 was verified as the key upregulated molecule in GD patients' plasma and PBMCs. Further, functional studies confirmed that hsa_circ_0090364 promotes the proliferation of Jurkat cells. Finally, ceRNA network analysis and validation revealed that hsa_circ_0090364 might absorb hsa-miR-378a-3p and regulate downstream IL-6ST and IL-21R mRNAs to participate in the molecular mechanisms of GD through the JAK-STAT signalling pathway.

Excess production of thyroid hormones is induced by the thyroid-stimulating antibody mediated by the TSH receptor located in the thyroid gland. The production of pathogenic auto-antibodies by B lymphocytes depends on the abnormal proliferation and differentiation of CD4^{+} T cells (19). Some cytokines such as IFN-γ, IL-4, and IL6 lead to the activation, proliferation, and differentiation of CD4^{+} T cells. The dysfunction of CD4^{+} T cells in turn leads to

inflammatory cytokine maintenance, which enhances the autoimmune response (20). In patients with GD, cytokines act as messengers to transfer information from one cell to another, promoting transformation between T cell subsets and initiating a cascade of inflammatory responses via pro-inflammatory cytokine signalling pathways. However, many aspects of this process still need to be further clarified.

Benefiting from the development of high-throughput genomic technology, the differential expression profile analysis of circRNAs has been used to study the molecular mechanisms of a variety of autoimmune diseases. Sun *et al.* identified a 15-circRNA differential expression profile from the serum exocrine response (9). Our microarray analysis indicated 195 upregulated and 171 downregulated plasma circRNAs in the GD patients. Although these DECs are derived from different tissues, this finding confirms a crucial role of circRNAs as key molecules in the pathogenesis of GD.
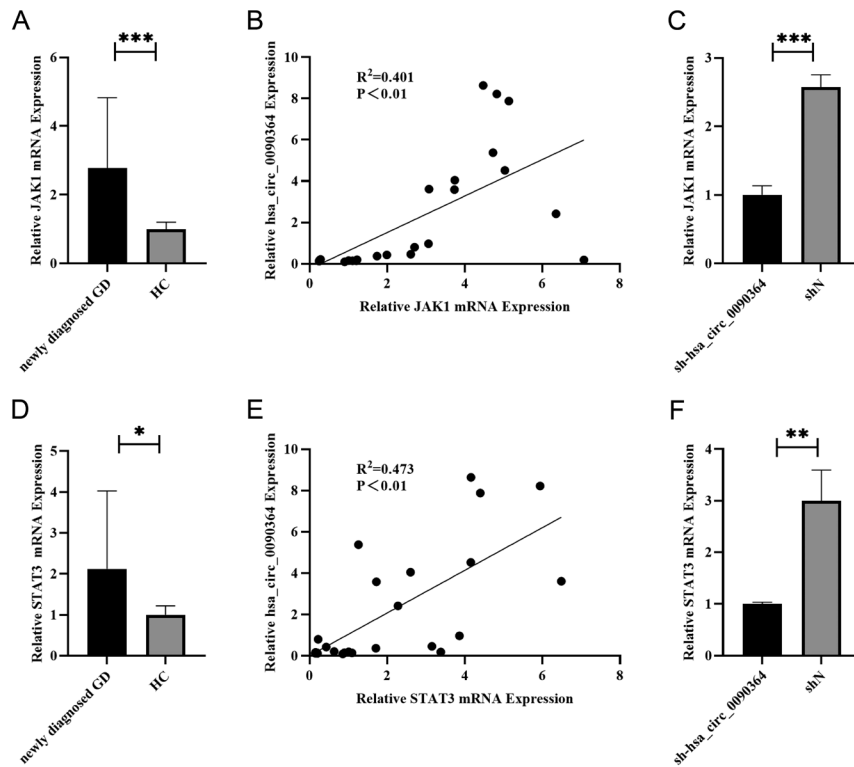
Figure 7

The ceRNA network of hsa_circ_0090364 related to JAK-STAT pathway. (A, B, D and E) JAK1 mRNA and STAT3 mRNA expression are high in the GD group and are positively correlated with hsa_circ_0090364 expression in Graves' disease patients' PBMCs. (C and F) The relative expression levels of JAK1 mRNA and STAT3 mRNA after shRNA-hsa_circ_0090364 or shRNA negative control (shN) transfection in Jurkat cells. The results are indicated as the mean ± s.d. of three independent experiments; horizontal lines show the mean ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

To demonstrate the possible role of circRNAs in GD, we analysed the classification and chromosome distribution of DECs in patients with GD. Exonic circRNAs accounted for the majority of the DECs (84.27%), which might be associated with their function. The exonic circRNA is a covalently closed continuous loop generated from exons via back-splicing, utilizing the 3′ and 5′ sites of the same or upstream exon. This structure makes an exonic circRNA more inclined to regulate its linear counterpart. The DECs were predominantly localized to chromosomes 17, 2, and 3. A previous study showed that a chromosome 17 disorder might induce cell proliferation and differentiation (21). We speculate that DECs in GD may be involved in the cell processes of proliferation.

In this study, upregulated DECs accounted for a larger proportion than downregulated DECs. To explore the potential function of misregulated circRNAs intuitively, we screened the top five upregulated circRNAs, and GO and KEGG analyses were used to detect the potential mechanisms of these circRNAs' target genes. The GO function of these circRNAs was mainly related to the regulation of cell proliferation and migration, and these transcripts mainly function on the cell membrane. At the same time, to determine meaningful pathways, the KEGG tool was used in pathway analysis, and the results showed

that these abnormal transcripts are mainly involved in the JAK-STAT, PI3K-AKT, T cell receptor, and Rap1 signalling pathways. Song *et al.* reported that a specifically expressed protein of thyroid-associated ophthalmopathy patients was enriched in the JAK-STAT signalling pathway (22). Egwuagu *et al.* pointed out that the JAK-STAT signalling pathway plays a role in Th17 differentiation from naive T cells (23). The results of these enrichment analyses reveal that the top five upregulated circRNAs might promote the pathogenesis of GD through a pro-inflammatory pathway; however, this hypothesis needs to be confirmed in further research. Most cytokines accomplish their function through the JAK-STAT signalling pathway, which was proven to be the most meaningful pathway in this study. Therefore, we further explored the circRNAs that might play a role in the pathogenesis of GD through the JAK-STAT signalling pathway.

Microarray analysis showed that hsa_circ_0090364 and hsa_circ_0001228 were upregulated in patients with GD. Pathway analysis revealed that these two circRNAs might participate in the JAK-STAT signalling pathway. Hsa_circ_0090364 was verified to be upregulated in plasma and PBMCs from patients with GD by RT-qPCR. Further, Pearson correlation analysis suggested that the expression of hsa_circ_0090364 transcripts in PBMCs was

positively correlated with that of TRAb. All of these results indicated that hsa_circ_0090364 may play a role in the immunological pathogenesis of GD.

To investigate the function of hsa_circ_0090364, loss-of-function assays were performed in the Jurkat cell line. The results demonstrated that knockdown of hsa_circ_0090364 inhibited cell proliferation. Further studies showed that apoptosis was not a factor in promoting cell growth.

CircRNAs act as 'sponges', targeting miRNAs to regulate certain mRNAs, which are related to the mechanism of some diseases. To further explore the potential molecular regulatory function of hsa_circ_0090364, a ceRNA network was established. Intriguingly, we found that hsa_circ_0090364 might bind closely to members of the hsa-miR-378 family. Dubois-Camacho *et al.* found that decreased expression levels of the hsa-miR-378 family might enhance the expression of cytokines such as IL-33 and could play a pro-inflammatory role in the autoimmune disease ulcerative colitis (24). GD is an immune-mediated disease; thus, we speculated that the hsa-miR-378 family is related to the occurrence and development of GD. In this study, the expression level of mature hsa-miR-378a-3p from this miRNA family was validated to be lower in GD patients and negatively correlated with hsa_circ_0090364 expression. Further experiments confirmed that hsa-miR-378a-3p expression was upregulated in hsa_circ_0090364-knockdown Jurkat cells. Together, this evidence suggests that hsa_circ_0090364 negatively regulates hsa-miR-378a-3p and that the hsa_circ_0090364–hsa-miR-378a-3p axis might play a regulatory role in the pathogenesis of GD. Two mRNAs (IL6ST and IL21R) are downstream genes of the above circRNA-miRNA axis. All of these mRNAs were related to the JAK-STAT signalling pathway. IL6ST is a member of the interleukin-6 cytokine family, which is capable of forming a complex with IL6 receptor (IL6R) and assists in IL6R activation, thereby initiating signal transduction cascades in multiple autoimmune diseases (25, 26, 27). It has been reported that IL21 binds to IL21R, affecting the immune system via the JAK-STAT pathway, which plays an important role in both innate and acquired immunity (28, 29). Our study showed that IL6ST and IL21R mRNA expression levels were higher in GD patients and positively correlated with hsa_circ_0090364 expression. In Jurkat cells with hsa_circ_0090364 knockdown, the mRNA expression levels of IL6ST and IL21R were reduced compared with those of controls. These results suggest that hsa_circ_0090364 may act as a competitive sponge of the hsa-miR-378 family and then affect the expression of associated mRNAs. The JAK-STAT pathway was the key pathway identified in this study, and we further found that the expression levels of JAK1

and STAT3, key molecules of this pathway, were elevated in GD and positively correlated with hsa_circ_0090364 expression. Further, the positive correlation between hsa_circ_0090364 and the JAK-STAT pathway was verified in cells with hsa_circ_0090364 knockdown.

Our study had some limitations. The number of samples used could have been higher and the range of DECs in GD patients could have been further narrowed. The analyses in this study were confined to the differential expression profile of circRNAs and the detection of their ability to act as miRNA sponges; however, the specific mechanism is still unclear. Future studies should aim for more functional validation.

Conclusions

Our results suggest that hsa_circ_0090364 may promote cell proliferation through sponge adsorption and act as a ceRNA to regulate the JAK-STAT signalling pathway in GD patients. These findings provide insights into uncovering the molecular mechanism of GD. In the future, we will focus on investigating the potential function of dysregulated circRNAs in GD patients.

References

1 Smith TJ & Hegedüs L. Graves' disease. *New England Journal of Medicine* 2016 **375** 1552–1565. (https://doi.org/10.1056/NEJMra1510030)

2 Bartalena L, Masiello E, Magri F, Veronesi G, Bianconi E, Zerbini F, Gaiti M, Spreafico E, Gallo D, Premoli P, *et al.* The phenotype of newly diagnosed Graves' disease in Italy in recent years is milder than in the past: results of a large observational longitudinal study. *Journal of Endocrinological Investigation* 2016 **39** 1445–1451. (https://doi.org/10.1007/s40618-016-0516-7)

3 Patop IL, Wüst S & Kadener S. Past, present, and future of circRNAs. *EMBO Journal* 2019 **38** e100836. (https://doi.org/10.15252/embj.2018100836)

4 Le TD, Zhang J, Liu L & Li J. Computational methods for identifying miRNA sponge interactions. *Briefings in Bioinformatics* 2017 **18** 577–590. (https://doi.org/10.1093/bib/bbw042)

5 Yang Y, Liu M, Yang F, Wang X, Bai X, Mu S, Liu Y & Hu D. Circular RNA expression profiles following negative pressure wound therapy in burn wounds with experimental Pseudomonas aeruginosa infection. *Bioengineered* 2022 **13** 4122–4136. (https://doi.org/10.1080/21655979.2021.2006965)

6 Zhou Z, Sun B, Huang S & Zhao L. Roles of circular RNAs in immune regulation and autoimmune diseases. *Cell Death and Disease* 2019 **10** 503. (https://doi.org/10.1038/s41419-019-1744-5)

7 Taheri M, Eghtedarian R, Dinger ME & Ghafouri-Fard S. Dysregulation of non-coding RNAs in rheumatoid arthritis. *Biomedicine and Pharmacotherapy* 2020 **130** 110617. (https://doi.org/10.1016/j.biopha.2020.110617)

8 Xue G, Hua L, Zhou N & Li J. Characteristics of immune cell infiltration and associated diagnostic biomarkers in ulcerative colitis: results from bioinformatics analysis. *Bioengineered* 2021 **12** 252–265. (https://doi.org/10.1080/21655979.2020.1863016)

9 Sun Y, Wang W, Tang Y, Wang D, Li L, Na M, Jiang G, Li Q, Chen S & Zhou J. Microarray profiling and functional analysis of differentially expressed plasma exosomal circular RNAs in Graves' disease. *Biological Research* 2020 **53** 32. (https://doi.org/10.1186/s40659-020-00299-y)

10 Li Q, Wang B, Mu K & Zhang JA. The pathogenesis of thyroid autoimmune diseases: new T lymphocytes – cytokines circuits beyond the Th1-Th2 paradigm. *Journal of Cellular Physiology* 2019 **234** 2204–2216. (https://doi.org/10.1002/jcp.27180)

11 Shao S, Yu X & Shen L. Autoimmune thyroid diseases and Th17/Treg lymphocytes. *Life Sciences* 2018 **192** 160–165. (https://doi.org/10.1016/j.lfs.2017.11.026)

12 Yin Q, Jin Z, Zhou Y, Song D, Fu C, Huang F & Wang S. lncRNA:mRNA expression profile in CD4+ T cells from patients with Graves' disease. *Endocrine Connections* 2020 **9** 1202–1211. (https://doi.org/10.1530/EC-20-0373)

13 Ross DS, Burch HB, Cooper DS, Greenlee MC, Laurberg P, Maia AL, Rivkees SA, Samuels M, Sosa JA, Stan MN, *et al.* 2016 American Thyroid Association guidelines for diagnosis and management of hyperthyroidism and other causes of thyrotoxicosis. *Thyroid* 2016 **26** 1343–1421. (https://doi.org/10.1089/thy.2016.0229)

14 Ding J, Li X & Hu H. TarPmiR: a new approach for microRNA target site prediction. *Bioinformatics* 2016 **32** 2768–2775. (https://doi.org/10.1093/bioinformatics/btw318)

15 Chen Y & Wang X. miRDB: an online database for prediction of functional microRNA targets. *Nucleic Acids Research* 2020 **48** D127–D131. (https://doi.org/10.1093/nar/gkz757)

16 Huang R, Mao M, Lu Y, Yu Q & Liao L. A novel immune-related genes prognosis biomarker for melanoma: associated with tumor microenvironment. *Aging* 2020 **12** 6966–6980. (https://doi.org/10.18632/aging.103054)

17 Jeurink PV, Vissers YM, Rappard B & Savelkoul HF. T cell responses in fresh and cryopreserved peripheral blood mononuclear cells: kinetics of cell viability, cellular subsets, proliferation, and cytokine production. *Cryobiology* 2008 **57** 91–103. (https://doi.org/10.1016/j.cryobiol.2008.06.002)

18 Sakaguchi S, Mikami N, Wing JB, Tanaka A, Ichiyama K & Ohkura N. Regulatory T cells and human disease. *Annual Review of Immunology* 2020 **38** 541–566. (https://doi.org/10.1146/annurev-immunol-042718-041717)

19 Fallahi P, Ferrari SM, Ragusa F, Ruffilli I, Elia G, Paparo SR & Antonelli A. Th1 chemokines in autoimmune endocrine disorders. *Journal of Clinical Endocrinology and Metabolism* 2020 **105**. (https://doi.org/10.1210/clinem/dgz289)

20 Zhu C, Ma J, Liu Y, Tong J, Tian J, Chen J, Tang X, Xu H, Lu L & Wang S. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *Journal of Clinical Endocrinology and Metabolism* 2012 **97** 943–950. (https://doi.org/10.1210/jc.2011-2003)

21 Ecsedi S, Rákosy Z, Vízkeleti L, Juhász A, Sziklai I, Adány R & Balázs M. Chromosomal imbalances are associated with increased proliferation and might contribute to bone destruction in cholesteatoma. *Otolaryngology: Head and Neck Surgery* 2008 **139** 635–640. (https://doi.org/10.1016/j.otohns.2008.07.019)

22 Song RH, Wang B, Yao QM, Li Q, Jia X & Zhang JA. Proteomics screening of differentially expressed cytokines in tears of patients with Graves' ophthalmopathy. *Endocrine, Metabolic and Immune Disorders Drug Targets* 2020 **20** 87–95. (https://doi.org/10.2174/1871530319666190618142215)

23 Egwuagu CE. STAT3 in CD4+ T helper cell differentiation and inflammatory diseases. *Cytokine* 2009 **47** 149–156. (https://doi.org/10.1016/j.cyto.2009.07.003)

24 Dubois-Camacho K, Diaz-Jimenez D, De la Fuente M, Quera R, Simian D, Martínez M, Landskron G, Olivares-Morales M, Cidlowski JA, Xu X, *et al.* Inhibition of miR-378a-3p by inflammation enhances IL-33 levels: A novel mechanism of alarmin modulation in ulcerative colitis. *Frontiers in Immunology* 2019 **10** 2449. (https://doi.org/10.3389/fimmu.2019.02449)

25 Wang RX, Yu CR, Mahdi RM & Egwuagu CE. Novel IL27p28/IL12p40 cytokine suppressed experimental autoimmune uveitis by inhibiting autoreactive Th1/Th17 cells and promoting expansion of regulatory T cells. *Journal of Biological Chemistry* 2012 **287** 36012–36021. (https://doi.org/10.1074/jbc.M112.390625)

26 Jones SA & Jenkins BJ. Recent insights into targeting the IL-6 cytokine family in inflammatory diseases and cancer. *Nature Reviews: Immunology* 2018 **18** 773–789. (https://doi.org/10.1038/s41577-018-0066-7)

27 Čokić VP, Mitrović-Ajtić O, Beleslin-Čokić BB, Marković D, Buač M, Diklić M, Kragujac-Kurtović N, Damjanović S, Milenković P, Gotić M, *et al.* Proinflammatory cytokine IL-6 and JAK-STAT signaling pathway in myeloproliferative neoplasms. *Mediators of Inflammation* 2015 **2015** 453020. (https://doi.org/10.1155/2015/453020)

Z Jiang *et al.*

28 Zhang H, Watanabe R, Berry GJ, Tian L, Goronzy JJ & Weyand CM. Inhibition of JAK-STAT signaling suppresses pathogenic immune responses in medium and large vessel vasculitis. *Circulation* 2018 **137** 1934–1948. (https://doi.org/10.1161/CIRCULATIONAHA.117.030423)

29 Davis ID, Skak K, Smyth MJ, Kristjansen PE, Miller DM & Sivakumar PV. Interleukin-21 signaling: functions in cancer and autoimmunity. *Clinical Cancer Research* 2007 **13** 6926–6932. (https://doi.org/10.1158/1078-0432.CCR-07-1238)