

# Expression Analysis of circRNAs in Human Adipogenesis

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**Purpose:** Adipogenesis is one of the major pathways for generating obesity or overweight that can cause a range of metabolic disorders. Circular RNAs (circRNAs), a specific type of RNAs, have a significant influence on metabolic disorders. This study aims to find differentially expressed circRNAs (DECs) during human subcutaneous adipose tissue (SATs) adipogenesis.

**Patients and Methods:** The human adipose tissue-derived stromal cells (hADSCs) were isolated from human SATs (n = 3), and then induced into adipocytes. Total RNAs were extracted from hADSCs and adipocytes, and the DECs were detected using circRNA microarray. The GO and KEGG pathways of DECs were analyzed by bioinformatic methods, and partial DECs were further validated by quantitative polymerase chain reaction (qPCR).

**Results:** Our study detected a total of 1987 DECs, among which, 1134 were found upregulated and 853 were downregulated. GO analysis showed that the upregulated DECs have catalytic activity in intracellular organelle and cytoplasm, whereas downregulated DECs are enriched in organelle lumen, and are involved in positive regulation of developmental process. In addition, pathway results demonstrated that upregulated DECs are involved in platinum drug resistance and cellular senescence, and downregulated DECs are enriched in proteoglycans in cancer and focal adhesion pathway. Two circRNAs, namely has\_circ\_0001600 and has\_circ\_0001947 were validated to be significantly upregulated in adipocytes compared to hADSCs.

**Conclusion:** Our study explored DECs between hADSCs derived from SATs and adipocytes, and report that two circRNAs named has\_circ\_0001600 and has\_circ\_0001947 might be important factors involved in human adipogenesis, however, the molecular mechanism should be further explored.

**Keywords:** circRNA microarray, subcutaneous adipose tissue, human adipose tissue-derived stromal cells, adipogenesis, SAT

## Introduction

Obesity is a chronic metabolic disease caused by excessive accumulation of adipose tissues and (or) abnormal fat distribution in the body.<sup>1</sup> The human adipogenesis process includes human adipose tissue-derived stromal cells (hADSCs) differentiated to preadipocytes and further differentiated to mature adipocytes.<sup>2, 3</sup> The abnormal hADSCs or preadipocytes proliferation and adipocytes hypertrophy lead to adipogenesis dysfunction are the pathological basis for the occurrence and development of obesity.<sup>4</sup>

Circular RNAs (circRNAs) are a category of endogenous regulatory RNA, of which, the precursor RNA forms a covalent closed-loop structure through the back-splicing site, which makes it difficult to be degraded by RNase R.<sup>5</sup> circRNAs can act as a microRNAs(miRNAs) sponge, which in turn affects the combination with target mRNA and inhibits its activity, thus effectively changing its role in the post-transcriptional regulation of target genes expression.<sup>6, 7</sup> In recent years, circRNAs have been recognized as key molecules in the metabolic diseases due to their regulatory effects on genes including the dynamic balance of sugars and lipids.<sup>8</sup>

There are some studies that explored circRNAs function in adipogenesis.<sup>9</sup> However, only one study used circRNA microarray to analyze circRNAs expression profile from HPA-v cell, isolated from human visceral adipose tissues (VATs), differentiated to adipocytes.<sup>10</sup> In addition, two other studies explored the mouse white and brown adipogenesis, respectively.<sup>11, 12</sup> Furthermore, some studies assessed the differentially expressed circRNAs (DECs) of VATs in human obese compare to lean individuals and VATs compare to subcutaneous adipose tissue (SATs).<sup>8, 13</sup> In other animals, for instance, the circRNAs expression of Yak and Pig of SATs adipogenesis were also evaluated.<sup>14, 15</sup> Up to present, the circRNAs expression studies have been summarized by Zhang et al and Huang et al<sup>16, 17</sup>

Because of the single study information on explored circRNAs expression profile in human VATs adipogenesis,<sup>10</sup> our aim was to clarify the circRNAs expression pattern in human SATs adipogenesis. Firstly, we used circRNA microarray to identify DECs from SATs derived hADSCs differentiated to adipocytes. Secondly, the expression of partially upregulated and downregulated circRNAs were verified by quantitative polymerase chain reaction (qPCR). Lastly, the bioinformatic analysis enabled the prediction of circRNA-miRNA-mRNA networks. Our research findings will offer fresh proof for studying the role of circRNAs in adipogenesis and provide novel targets for the treatment of obesity.

## Materials and Methods

### Human Tissue Specimens

The SATs were collected from three normal weight individuals (BMI:18.5–23.9 kg/m<sup>2</sup>) who underwent abdominal surgery at the Department of Gastrointestinal Surgery, Third Xiangya Hospital, Central South University. The individuals were eliminated from conditions like severe contagious diseases, autoimmune conditions, hematological disorders, malignant tumors, and more. The Ethical Committee of the Third Xiangya Hospital of Central South University in Changsha, China, approved this study, which followed the guidelines stated in the Declaration of Helsinki during its conduction. The consent of every patient was obtained in writing.

### hADSCs Isolation and Differentiation

Approximately 10g of SATs were first washed with phosphate buffer saline (PBS) for three times. Then the sample underwent fragmentation into fragments less than 1 mm<sup>3</sup>, and digested at 37°C with 0.1% (w/v) collagenase I solution (Gibco, Life Technology, China) for 1.5 hours. The digestion was terminated with DMEM/F12 (Gibco, Life Technology, China) and filtered by 100µm cell strainer (NEST Biotechnology Co., Ltd., China), centrifuged at 800 rpm for 5 min, gently poured out the supernatant. Subsequently, 3mL red blood cell lysis buffer (Merck, China) was used to remove red blood cells, and after centrifuged at 800 rpm for 5 min again, the isolated cells were suspended in DMEM/F12 supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technology, Australia), 100U/mL penicillin and 100 µg/mL streptomycin were added to the culture medium. The cells were then incubated at 37°C in an environment containing 5% CO<sub>2</sub>. In order to induce the hADSCs differentiated to adipocytes, when hADSCs grew to almost 100% confluency, the hADSCs were cultured in complete DMEM/F12 medium which contained 1µM dexamethasone, 10µM insulin, 0.5mM isobutylmethylxanthine (IBMX), and 200µM indomethacin (four reagents were purchased from Merck, China); the culture medium was replaced every 48 hours.

### circRNA Microarray Analysis

The circRNA microarray analysis was performed in accordance with our previous studies.<sup>18, 19</sup> Briefly, total RNA was obtained from human hADSCs and adipocytes using the Trizol (Invitrogen, Carlsbad, CA, USA) method, and then, the linear RNA samples were removed by treating with RNase R (Epicentre, Inc., WI, USA), the circRNAs were collected and amplified. The fluorescent complementary RNAs (cRNAs) were transcribed and labeled using a random primer approach (Arraystar, MD, USA). The presence of cRNAs in the Agilent G2565CA Microarray Scanner System (Agilent Technologies, CA, USA) was confirmed. Fold-change filtering identified DECs between two groups. The Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were carried out.

## circRNAs Verification

The 4 most upregulated and 2 most downregulated circRNAs in DEC's data were validated using RT-qPCR. Briefly, RNA was isolated from hADSCs and adipocytes, and then was reverse transcribed into cDNA (Invitrogen, Carlsbad, CA, USA). The relative quantification of circRNAs expression using qPCR was calculated by  $2^{-\Delta\Delta ct}$  method. The primers were designed with circinteractome software (<https://circinteractome.irp.nia.nih.gov/index.html>), and all primers are listed in [Supplementary Table 1](#).

## circRNA-miRNA-mRNA Networks Construction

At first, the circRNAs and miRNAs interaction was analyzed by TargetScan and miRanda. Then, the DIANA tools enabled the prediction of the interaction between miRNAs and mRNA. And finally, the Cytoscape 2.8.2 software was utilized to generate a visual representation of the circRNA-miRNA-mRNA network.

## Statistics Analysis

The data underwent analysis utilizing GraphPad Prism 10 software, after the data was verified to conform to a normal distribution; the data from two groups were compared using a *t*-test, where the significance was defined as  $p < 0.05$ .

## Results

### The DEC's Analysis of hADSCs and Adipocytes

The DEC's profiles between hADSCs and adipocytes were investigated by human circRNA microarray. The Box Plot result revealed that the log2 ratio distribution was similar in 6 samples ([Figure 1A](#)). There were 1987 DEC's in two group, among them, 1134 DEC's were upregulated and 853 DEC's were downregulated in adipocytes compare to hADSCs. The most significantly upregulated and downregulated 10 circRNAs are showed in [Table 1](#) and [Table 2](#), respectively. The Hierarchical Clustering showed partially significant difference in two groups ([Figure 1B](#)). In addition, the partial DEC's were filtered according to the fold change  $\geq 0.5$  and *p* values  $< 0.05$ . The results are showed in Scatter Plot and Volcano Plot ([Figure 1C](#) and [D](#)). Furthermore, the chromosome distribution of DEC's is displayed in [Figure 1E](#).

### GO and KEGG Pathway Analysis of the DEC's

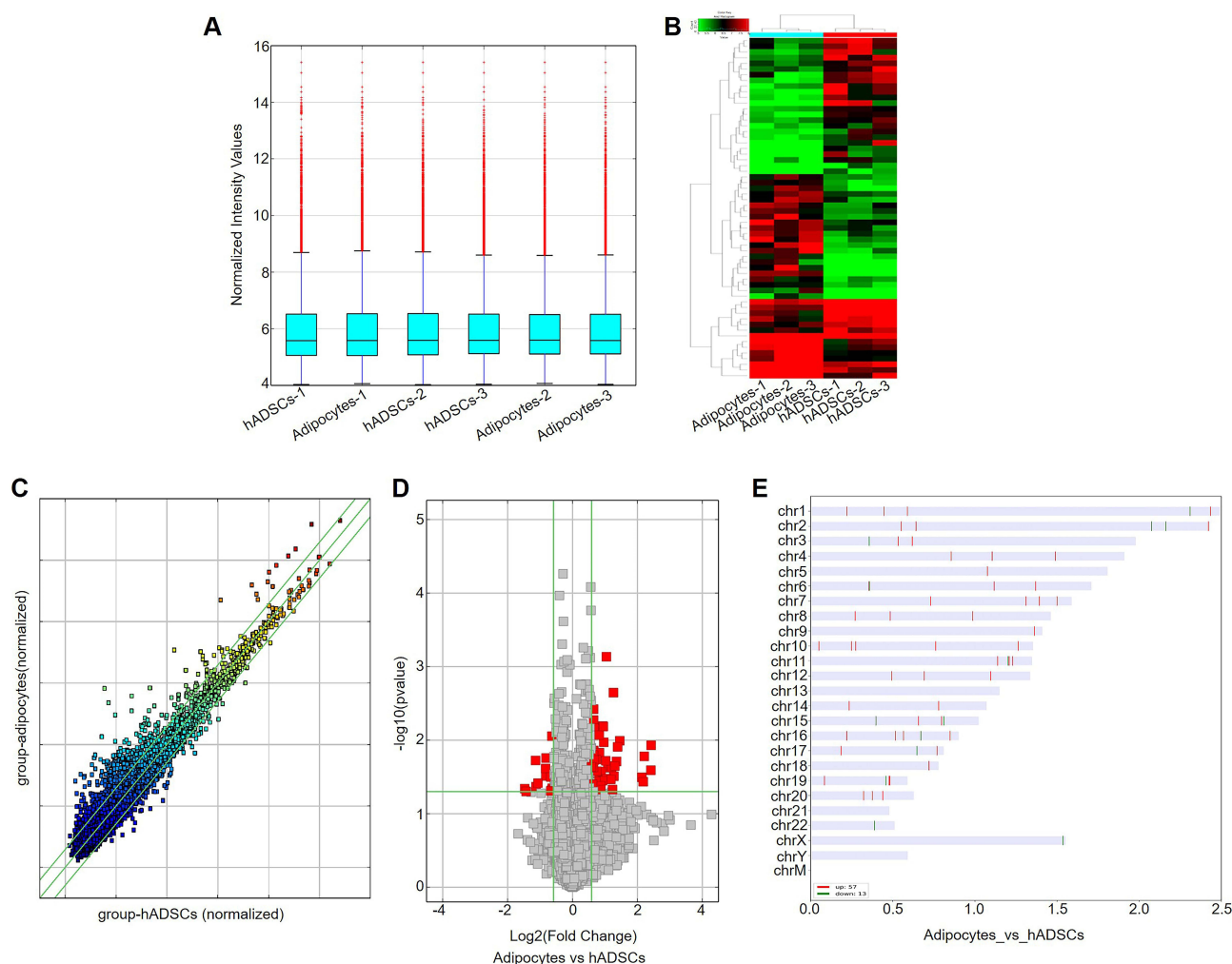
The GO and KEGG pathway were analyzed according to the host genes of circRNAs. The GO results of upregulation of DEC's demonstrated that intracellular organelle, cytoplasm and catalytic activity were significantly enriched. In addition, in Biological Process (BP) analysis, some host genes of circRNAs were found related to steroid metabolic process, regulation of steroid biosynthetic process, cellular response to fatty acid, etc. ([Figure 2A](#)). In downregulation of DEC's, organelle lumen, positive regulation of developmental process and membrane-enclosed lumen, etc. were enriched ([Figure 2B](#)). The pathway analysis of upregulated circRNAs were enriched in platinum drug resistance, cellular senescence and bladder cancer ([Figure 2C](#)), however, in downregulated circRNAs, proteoglycans in cancer, focal adhesion and PI3K-Akt signaling pathway were significantly enriched ([Figure 2D](#)).

### Partial DEC's Validation by qPCR

To confirm our circRNA microarray results, the 4 most upregulated and 2 most downregulated circRNAs were chosen for qPCR validation. hsa\_circ\_0001600 and hsa\_circ\_0001974 were significantly upregulated in adipocytes compare to hADSCs ([Figure 3A](#) and [B](#)), however, there was no notable difference observed for hsa\_circ\_0076155 and hsa\_circ\_0054947 ([Figure 3C](#) and [D](#)). Among downregulated circRNAs, hsa\_circ\_0004585 and hsa\_circ\_0034435 also showed no significant difference ([Figure 3E](#) and [F](#)). The back-splice junction sequence of hsa\_circ\_0001600 and hsa\_circ\_0001974 were further verified using Sanger sequencing of PCR product ([Figure 3G](#) and [H](#)).

### Prediction of circRNA-miRNA-mRNA Interaction Networks

In order to gain a deeper comprehension of the potential possible molecular mechanism of hsa\_circ\_0001600 and hsa\_circ\_0001974 in adipogenesis, a construction of circRNA-miRNA-mRNA interaction networks was carried out



**Figure 1** DECs in hADSCs and adipocytes. **(A)** The box plot depicted the distribution of normalized intensity for the entire data set. **(B)** Hierarchical clustering analysis was performed to generate maps of DECs in hADSCs and adipocytes. Red indicates a relatively elevated level of expression. Green indicates a relatively low level of expression. **(C)** Scatter plot was utilized to evaluate alterations in the expression of circRNAs between hADSCs and adipocytes. The presence of circRNAs above the upper green threshold and below the lower green threshold signifies a quantity greater than 1.5-fold DECs between the two samples being compared. **(D)** The volcano plot revealed a substantial fold change indicating statistically significant differential expression comparisons between hADSCs and adipocytes (fold change >0.5,  $P < 0.05$ ). **(E)** Characterization of the classification and distribution of DEGs across human chromosomes.

using TargetScan, miRanda, and DIANA TOOLS software. The results indicated that certain miRNAs (hsa-miR-619-5p, hsa-miR-20b-3p, hsa-miR-367-3p, hsa-miR-153-5p, and hsa-miR-665) potentially target has\_circ\_0001600. Moreover, hsa-miR-668-3p, hsa-miR-194-3p, hsa-miR-182-5p, hsa-miR-588 and hsa-miR-494-5p were predicted as target miRNAs of has\_circ\_0001947 (Figure 4).

## Discussion

Increased adipose tissues are strongly associated with some metabolic diseases, for instance, hypertension, diabetes, hyperlipidemia, etc.<sup>20</sup> In a previous study, Sun et al have detected circRNAs expression during the human preadipocytes derived from VATs differentiated to adipocytes,<sup>10</sup> and in this study, we explored human SATs adipogenesis according to hADSCs differentiated to adipocytes.

Our findings indicate significant difference of circRNAs expression between SATs and VATs; the data differences might be due to different race, gender, age, or limited sample size. In our study, 1987 DECs between hADSCs and adipocytes were identified, and among them, 1134 DECs were found upregulated and 853 DECs were found down-regulated. In GO analysis, intracellular organelle, cytoplasm and catalytic activity were found significantly enriched,

**Table 1** Upregulated of circRNAs in Human Adipocytes Vs hADSCs

circRNA	P-value	FDR	FC	Best Transcript	Gene Symbol
hsa_circ_0001600	0.025662501	0.876236021	5.3354614	NM_001145776	FKBP5
hsa_circ_0076155	0.016607831	0.876236021	4.6263981	NM_001145777	FKBP5
hsa_circ_0054947	0.032060295	0.876236021	4.3993268	NM_006759	UGP2
hsa_circ_0001974	0.010186452	0.876236021	2.7463416	NM_001135055	TKT
hsa_circ_0008758	0.019498067	0.876236021	2.56242	NM_001144932	PSMB5
hsa_circ_0070284	0.023463071	0.876236021	2.3048021	NM_001263	CDS1
hsa_circ_0058992	0.022518763	0.876236021	2.1382801	NM_002712	PPP1R7
hsa_circ_0027491	0.023740104	0.876236021	2.1235198	NM_002392	MDM2
hsa_circ_0083756	0.006439968	0.876236021	1.9016188	NM_171982	TRIM35
hsa_circ_0007184	0.026785899	0.876236021	1.8429111	NM_006323	SEC24B

**Abbreviations:** FDR, false discovery rate; FC, fold change.

**Table 2** Downregulated of circRNAs in Human Adipocytes Vs hADSCs

circRNA	P-value	FDR	FC	Best Transcript	Gene Symbol
hsa_circ_0004585	0.045691205	0.876236021	2.7784119	NM_018689	KIAA1199
hsa_circ_0034435	0.049816225	0.876236021	2.6928044	NM_003246	THBS1
hsa_circ_0001281	0.018912045	0.876236021	2.1986082	NM_016300	ARPP21
hsa_circ_0076177	0.042975956	0.876236021	2.1799198	NR_034069	SRPK1
hsa_circ_0016873	0.038453215	0.876236021	2.0918932	NM_001136494	Clorf198
hsa_circ_0008615	0.024364647	0.876236021	1.776247	NM_001142502	PPP1R13L
hsa_circ_0007580	0.030770798	0.876236021	1.7669337	NM_002737	PRKCA
hsa_circ_0091840	0.017506796	0.876236021	1.7590539	NM_001110556	FLNA
hsa_circ_0024604	0.049131184	0.876236021	1.593505	NM_015313	ARHGEF12
hsa_circ_0004795	0.031767785	0.876236021	1.5914283	NM_007068	DMC1

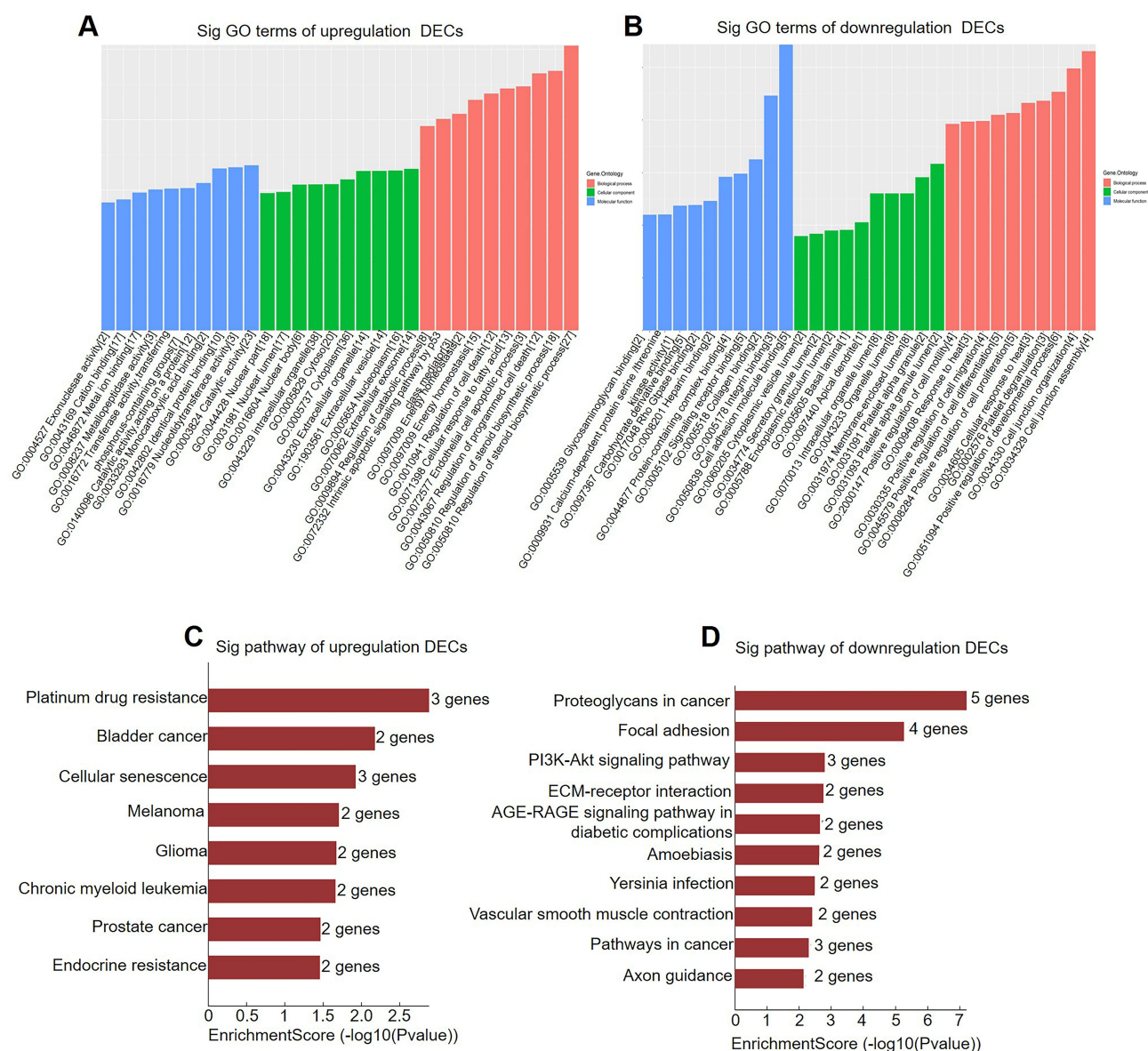
**Abbreviations:** FDR, false discovery rate; FC, fold change.

especially in BP, some lipid metabolic related processes including steroid metabolic process, regulation of steroid biosynthetic process, cellular response to fatty acid, etc. also were enriched. Thus, our results demonstrated that some circRNAs might play important role in adipogenesis or lipogenesis.

A series of studies have confirmed the molecular mechanisms of some circRNAs in adipogenesis, for instances, hsa\_circH19 is highly expressed in blood of metabolic syndrome patients, and silencing of hsa\_circH19 promoted hADSCs adipogenesis via regulation of PTBP1.<sup>21</sup> Moreover, circSAMD4A showed positive correlation and upregulated in VATs of obese patients, and knockdown of circSAMD4A blocked human preadipocytes adipogenesis through sponging with miR-138-5p and further repressing EZH2.<sup>13</sup> Besides, decrease of circRNA CDR1as attenuate human bone marrow mesenchymal stem cells (BMSCs) adipogenesis but enhance osteogenic differentiation through regulation of miR-7-5p/WNT5B signaling.<sup>22</sup> In addition, in bovine model, circFUT10 prevented adipogenesis and circFLT1 accelerated adipogenesis via regulation of let-7c/let-e and miR-93, respectively.<sup>23, 24</sup> Overall, the recent studies have been summarized by Zhang et al and Huang et al<sup>16, 17</sup>

In our study, hsa\_circ\_0001600 (circBank ID: hsa\_circFKBP5\_002) and hsa\_circ\_0001974 (circBank ID: hsa\_circTKT\_008) were validated and found to be dramatically upregulated in adipocytes compared to hADSCs. In mouse model, both of circ\_FKBP5 and its host gene, FKBP prolyl isomerase 5 (FKBP5), were validated to be upregulated during adipogenesis,<sup>11</sup> although it was not clear whether hsa\_circ\_0001600 participate in adipogenesis, but FKBP5 was an important factor to regulate lipolysis, lipogenesis and adipogenesis. FKBP5 was found upregulated in SATs of Type 2 diabetes compared to non-diabetic individuals and positive correlation with blood sugar level.<sup>24</sup> FKBP5 of SATs has significant positive relation with adipocytes size and HOMA-IR and blood insulin level.<sup>25</sup> Knockout of FKBP5 inhibited mice adipogenesis and fat-diet induced obesity in normoxia and hypoxic stress state.<sup>26</sup>

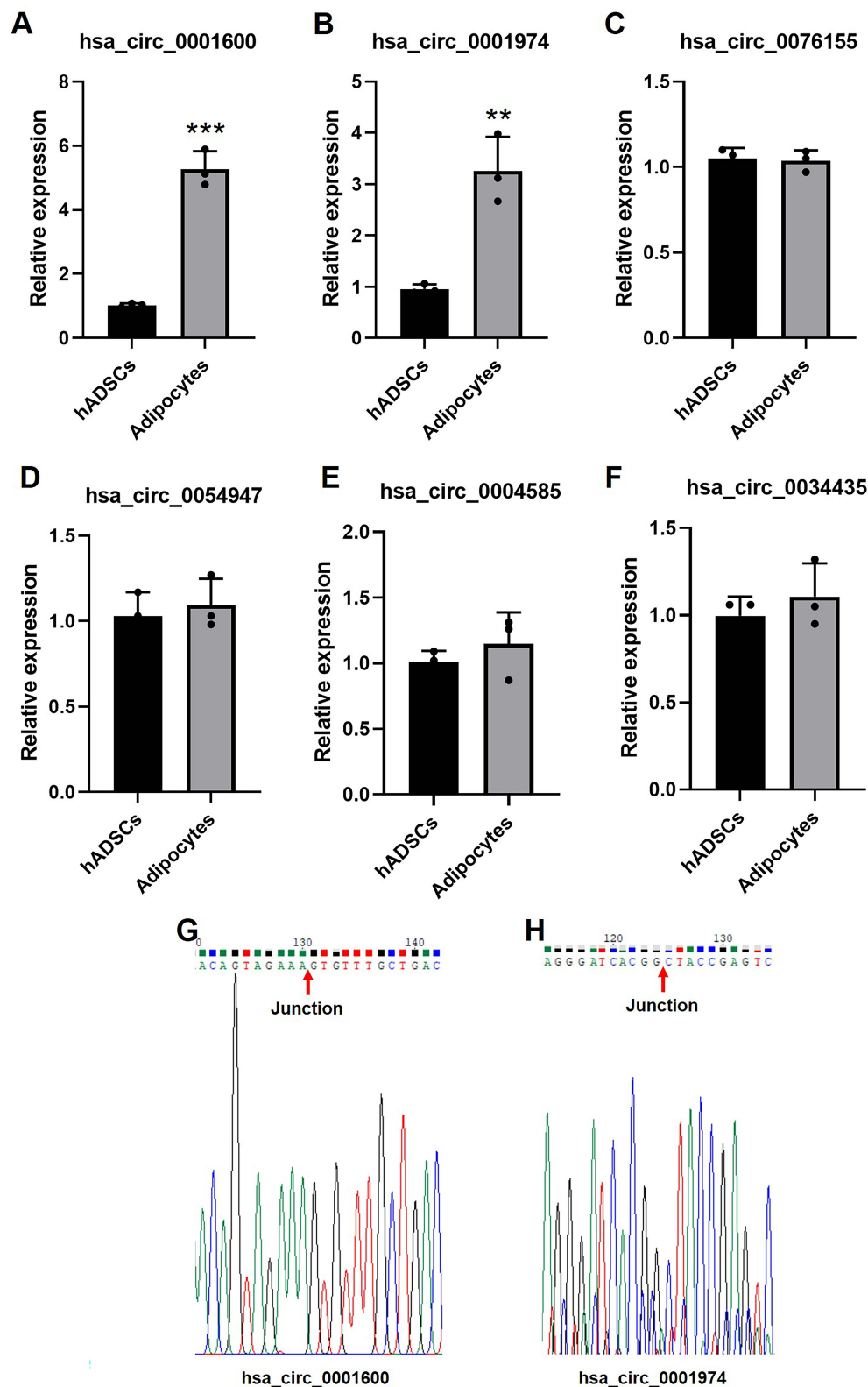




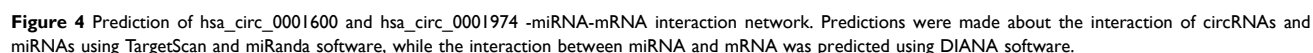
**Figure 2** GO and KEGG pathway analysis of DECs according to host genes in hADSCs and adipocytes. GO analysis was used to compare the expression of upregulated (**A**) and downregulated (**B**) DECs in adipocytes versus hADSCs. KEGG pathway analysis of upregulated (**C**) and downregulated (**D**) DECs in adipocytes versus hADSCs.

In miRNA prediction target of hsa\_circ\_0001600, miR-20b was found increased in obesity with normal blood sugar compared to obesity with type 2 diabetes in VATs.<sup>27</sup> miR-20b-5p derived from serum exosomal repress 3T3-L1 adipogenesis,<sup>28</sup> however, the expression of hsa-miR-665 was found decreased during adipogenesis.<sup>29</sup> So, we speculate that hsa\_circ\_0001600 might regulate adipogenesis via miR-20b-5p or hsa-miR-665.

Although hsa\_circ\_0001974 is not being reported to participate in adipogenesis, its host gene, transketolase (TKT), is a critical molecule to regulate lipolysis, and knockout of TKT in mice adipose tissue decreased fat-diet induced obesity through increase lipolysis.<sup>30</sup> In addition, TKT was found downregulated in omental tissues of obese vs non-obese and upregulated during 3T3-L1 adipogenesis.<sup>31, 32</sup> In miRNA target prediction of hsa\_circ\_0001974, hsa-miR-194 impedes adipogenesis through COUP-TFII axis in murine C3H10T1/2 mesenchymal cells and primary bone marrow stromal cells.<sup>33</sup> Moreover, miR-182 acts as negative regulatory factor that prevented 3T3-L1 and human preadipocytes adipogenesis through combining with CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ).<sup>34</sup> In another study, miR-182 impaired adipogenesis in Graves' orbitopathy through regulation of thyrotropin receptor.<sup>35</sup> miR-182 also could increase beige



**Figure 3** Validate of partial DECs by qPCR. The expression of has\_circ\_0001600 (**A**), has\_circ\_0001974 (**B**), has\_circ\_0076155 (**C**), has\_circ\_0054947 (**D**), has\_circ\_0004585 (**E**) and has\_circ\_0034435 (**F**) in hADSCs and adipocytes,  $n = 3$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Sanger sequencing map of hsa\_circ\_0001600 (**G**) and hsa\_circ\_0001974 (**H**). The red arrow represents back-splice junction sequence of circRNAs.



## Conclusion

## Abbreviations

circRNAs, circular RNAs; DEC, differentially expressed circRNAs; SATs, subcutaneous adipose tissue; hADSCs, human adipose tissue-derived stromal cells; qPCR, quantitative polymerase chain reaction; miRNAs, microRNAs;



cRNAs, fluorescent complementary RNAs; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; BMSCs, human bone marrow mesenchymal stem cells; C/EBP $\alpha$ , CCAAT/enhancer-binding protein  $\alpha$ .

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## Disclosure

The authors affirm that there are no competing interests relating to this study.

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