Differential Expression Profiles and Function Prediction of tRNA-Derived Fragments in Fibrous Dysplasia

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Abstract

Background: Fibrous dysplasia (FD) is a benign bone disease which normal bone matrix is replaced by fibrous tissue and immature bone tissue. Transfer RNA (tRNA)-derived RNA fragments (tRFs) and tRNA halves (tiRNAs) are a type of small non-coding RNA in the transcriptome of eukaryotes that produced by specific shearing of mature tRNA. Here, we conducted a comparative analysis of the expression of tRFs/tiRNAs in BMSCs and FD BMSCs using a high-throughput sequencing technique. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to validate the differential expressed tRFs/tiRNAs between two samples.

Results: The results showed that tRF-34-87R8WP9I1EWJIQ [tDR-001276] was significantly upregulated in FD BMSCs, and 3 downregulated tDRs (tRNA-derived small RNAs) (tRF-22-8EKSP1852 [tDR-006826], tRF-18-H9R8B7D2 [tDR-006049] and tRF-33-86V8WPMN1E8Y0E [tDR-001271]) were also detected. Prediction of target genes and gene ontology (GO) and KEGG pathway analysis indicated that the upregulated tRF was mainly involved in regulation of immune response and osteoclast differentiation, which may be the underlying mechanism of FD pathological features. The downregulated tRFs/tiRNAs were related to calcium ion transport (tDR-006826), apoptotic signaling pathway and cell proliferation (tDR-006049) and endocrine system development (tDR-001271). The upregulated tRFs/tiRNAs was related to immune response (tDR-001276). The protein-protein interaction network analysis for predicted target genes established by the STRING database showed that PPP2R5A, ADAMTS1, PPARA, and POLR2C were the most frequently interacted proteins in target genes of tDR-006826, tDR-006049, tDR-001271 and tDR-001276, respectively.

Conclusions: Our findings provided a comprehensive analysis of the expression of tRFs/tiRNAs in FD BMSCs and BMSCs. These differential expression tRFs/tiRNAs may be novel regulatory factors involved in FD BMSCs, and they could serve as potential therapeutic targets.

Background

Fibrous Dysplasia (FD) is a skeletal disorder resulting in local bulging deformities, pain, and pathological fractures, which seriously affect the health and life quality of patients [1, 2]. FD is recognized as a stem cell disease which normal bone tissue is replaced by excessively proliferated fibrous tissue caused by mosaic mutations in GNAS gene [3, 4]. Mutations in the α subunit of activated G protein leading to excessive accumulation of cyclic adenosine monophosphate (cAMP) in FD bone marrow stromal cells (BMSCs), which is the key cause of pathogenesis [5]. The application of osteoclast inhibitory drugs is the main way to relieve symptoms, but there is still a lack of effective and safe treatment methods [6].

Non-coding RNAs (ncRNAs) play significant roles in different disease pathogenesis. Recent advances in high-throughput RNA sequencing technology have revealed a class of ncRNAs which are derived from various RNA species such as ribosomal RNA, messenger RNA (mRNA), and transfer RNA (tRNA) [7-9]. Transfer RNA (tRNA) is an important linker molecule in the process of protein synthesis [10]. Studies have

shown that mature tRNA or precursor tRNA could be specifically cleaved to produce two major categories: tRNA-derived fragments (tRFs) and tRNA halves (tiRNAs) in many species of cells [11-13]. tRFs/tiRNAs are divided because of the difference of restriction sites. tiRNAs are usually 29~50 nt in length and are divided into 5' tiRNA and 3' tiRNA, whereas tRFs are usually shorter than tiRNA, which about 14~30 nt, and are mainly divided into 5' derived tRF (tRF-5), 3' derived tRF (tRF-3), tRF-1, tRF-2, and i-tRF [14, 15].

It has been found that tRFs/tiRNAs are closely related to many human diseases such as tumors, metabolic diseases, neuropsychiatric diseases, and infectious diseases [16]. There have been accumulating studies in tRFs/tiRNAs which are involved in the regulation of biogenesis processes in different types of mammalian stem cells [8]. Recent studies have identified that tRFs play important roles in regulating translation, as well as microRNAs [15, 17, 18]. Moreover, some tRFs are also reported mediating gene silencing by binding to proteins [12, 19]. Study also uncovers a critical function of epigenetic modification of tRFs in directing regulation of translation in stem cells. An additional posttranscriptional layer of regulation has been discovered which may directly affect the spatiotemporal regulation of gene expression during development and diseases [20].

The functions of messenger RNAs in FD have been well studied, however, there is no research on the role of tRFs in FD. In the present study, small RNA (miRNA) sequencing analysis was performed to detect tRFs/tiRNAs in BMSCs and FD BMSCs, and the differential expression tRFs/tiRNAs were further validated by real-time quantitative polymerase chain reaction (qRT-PCR). Our findings indicate that tDR-006826, tDR-006049 and tDR-001271 are downregulated in FD BMSCs, whereas tDR-001276 is upregulated. Moreover, the biological functions of four tiRNAs were evaluated to reveal potential pathological mechanisms of FD. Taken together, our findings might provide a theoretical basis for exploring novel therapeutic targets of FD.

Results

Expression profiles of tRFs/tiRNAs in FD BMSCs and normal BMSCs

We used a high-throughput sequencing technique to detect expression profiles of tRFs/tiRNAs in FD BMSCs and normal BMSCs. For the six libraries, an average of 10 million raw reads per library was obtained, whereas adapter-trimmed reads (length >= 16nt) were about 8.8 million (88.79%) (Additional file 1). Among the mapped reads, miRNAs were the most common with an average of 3154113 reads (to adapter-trimmed reads, 35.54%), and tRFs had an average of 1117973 reads (to adapter-trimmed reads, 12.60%) (Additional file 1). The copy number and sequence were recorded for each unique read and showed in the sequence read length distribution (Fig. 1a, b). tRNAs with different sequences may have the same anticodon and transfer the same amino acid. Stacked plot showed the number of tRFs/tiRNAs derived from the same anticodon tRNA in FD BMSCs and normal BMSCs (Fig. 1c, d).

Differential expression of tRFs/tiRNAs in FD BMSCs and BMSCs

The differential expression tRFs/tiRNAs analysis was performed between FD BMSCs and BMSCs, and the results were shown in the heat map (Fig. 2a). The principal component analysis (PCA), which showed distinguishable tRFs/tiRNAs expression profiles among 6 samples, was used to reduce the dimensionality of the data sets (Fig. 2b). The scatter plot assessed tRFs/tiRNAs expression variation between FD BMSCs and BMSCs (Fig. 2c), and the upregulated and downregulated tRFs/tiRNAs are showed in Fig. 2d. The Venn diagram exhibited that there were 797 types of tRFs/tiRNAs specifically in BMSCs, and 17 types of tRFs/tiRNAs specifically in FD BMSCs (Fig. 2d). We analyzed the percentage of each subtype of the tRFs/tiRNAs expressed in FD BMSCs and BMSCs, and the pie chart revealed that FD BMSCs mainly increased the expression of tiRNA-5, whereas decreased the expression of tRF-1 and tRF-3 (Fig. 2e).

Validation of differential expression tRFs/tiRNAs in BMSCs and FD BMSCs

In order to validate the differential expression, 3 significantly downregulated tRFs (tRF-22-8EKSP1852 [tDR-006826], tRF-18-H9R8B7D2 [tDR-006049] and tRF-33-86V8WPMN1E8Y0E [tDR-001271]) and 1 upregulated tRF (tRF-34-87R8WP9I1EWJIQ [tDR-001276]) in BMSCs and FD BMSCs were measured by qRT-PCR (Fig. 3a). The four tRFs positions on the cloverleaf secondary structure of derived tRNA were shown in Fig. 3b.

Prediction of target genes with bioinformatics tool

To further investigate the function of the differential expression tRFs/tiRNAs in FD BMSCs and BMSCs, we first performed target gene prediction of these tRFs (tDR-006826, tDR-006049, tDR-001271, and tDR-001276) via TargetScan and miRanda databases and showed the target components in Fig. 4a, b, c, d. tDR-006826, tDR-006049, tDR-001271 and tDR-001276 were predicted to 107, 78,148, and 275 target transcripts, respectively (Fig. 4 a, b, c, d).

GO enrichment and KEGG pathway analysis

The Gene Ontology (GO) analysis, which including biological process (BP), cellular components (CC), and molecular function (MF), was performed for the functional analysis for target genes. The target genes were most involved in phosphate ion binding (GO: 0042301, tDR-006826) (Fig. 5a), transcription corepressor activity (GO: 0003714, tDR-006049) (Fig. 5c), Transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding (GO: 0001077, tDR-001271) (Fig. 5e), and four-way junction DNA binding (GO: 0000400, tDR-001276) (Fig. 5g). By BP assay, it is found that tDR-006826 was involved in Chromosome, centromeric region (GO: 0000775) (Fig. 5a), tDR-006049 in intrinsic component of plasma membrane (GO: 0031226) (Fig. 5c), tDR-001271 in intracellular membrane-bounded organelle (GO: 0043231) and tDR-001276 in nucleoplasm part (GO: 0044451) (Fig. 5e, g), and their molecular functions included calcium ion transmembrane import into cytosol (GO: 0097553), regulation of plasma membrane organization (GO: 1903729), endocrine system development (GO: 0035270) and isoprenoid biosynthetic process (GO: 0008299), respectively (Fig. 5a, c, e, g). After mapping the targeted genes to terms in the Kyoto Encyclopedia of Genes and Genomes (KEGG)

database, we found that the target genes participated in Th17 cell differentiation (tDR-006826), FoxO signaling pathway (tDR-006049), cellular senescence (tDR-001271), and osteoclast differentiation (tDR-001276) (Fig. 5b, d, f, h).

PPI network analysis

Protein-protein interaction (PPI) network for the target genes was established using the STRING database (https://www.string-db.org) (Fig. 6). We analyzed the PPI results and found that PPP2R5A, ADAMTS1, PPARA and POLR2C were the most frequently interacted proteins in target genes of tDR-006826, tDR-006049, tDR-001271 and tDR-001276, respectively.

Discussion

FD is a slowly progressing bone tumor-like lesion which main pathological manifestation is that normal bone tissue is replaced by hyperproliferative fibrous tissue and dysplastic immature trabecular bone [21]. Existing studies suggest that FD is a stem cell disease [22]. Mutation of activated guanine nucleotide binding protein G protein α subunit (Gsα) in local FD leads to over-accumulation of the second messenger cAMP and activates downstream signaling pathways, which further causes the formation of amounts of fibrous tissue and immature bone tissue [23]. The clinical symptoms of FD which occur in cranio-maxillofacial bones often show local bulging deformities, occlusal disorders, and pathological fractures [24]. Although studies are gradually deepening, there is still a lack of effective and safe treatment methods for FD.

tRNA is considered to be an adaptor molecule that helps ribosomes to decode mRNA and synthesize protein [25]. Recent studies have revealed that tRNAs are a major source of small non-coding RNAs that possess varied functions [26]. Studies have shown that in many species of cells, mature tRNA or precursor tRNA is specifically sheared to produce tRNA-derived fragments (tRFs) or tRNA half-molecules (tiRNAs) [27, 28]. tRFs/tiRNAs are widely present in tissue cells of various organisms and have tissue specificity and disease relevance [29]. Although the specific biological functions of tRFs/tiRNAs have not been fully elucidated, more and more evidence suggests that they have a variety of biological functions, such as binding protein to affect the stability of mRNA, interaction with cytochrome C regulating apoptosis, changing the gene transcription cascade process of offspring, exerting negative gene regulation in the form of miRNA [13]. It has been found that tRFs/tiRNAs are closely related to many human diseases [30]. In our present study, high-throughput RNA sequencing detected four differential expression tRFs/tiRNAs in FD BMSCs and BMSCs. Meanwhile, one high-expression tiRNAs and three low-expression tRFs/tiRNAs were verified via qRT-PCR, which were consistent with the high-throughput RNA sequencing data. Furthermore, bioinformatics analysis was performed to demonstrate the biological functions of the differential expression tRFs/tiRNAs.

In order to investigate the functions of tRFs/tiRNAs targets, we employed GO and KEGG analysis. Most of the results were enriched in processes related to calcium ion transport (tDR-006826), apoptotic signaling pathway and cell proliferation (tDR-006049), endocrine system development (tDR-001271), and immune

response (tDR-001276). In our previous study, we have found that FD is relating to the proliferation of BMSCs which contributes to abnormal fibrous tissue proliferation [31]. Meanwhile, FD BMSCs was also exhibited weak apoptotic and strong proliferation ability [31]. When FD presents with skin pigmentation and precocious puberty, it is known as McCune-Albright, which inferring FD is an endocrine relating disease [24]. Pain is a common clinical manifestation in FD and influences the health-related quality of life of FD patients [32]. Local inflammation may be the main cause of pain. Therefore, the four tRFs/tiRNAs may play important roles in FD.

Although the specific biological functions of tRFs/tiRNAs have not been fully elucidated, more and more evidences show that they have a variety of biological functions. Some studies have shown that tRFs/tiRNAs affect the stability of mRNA via binding protein [13, 33]. Other studies have observed that tRFs/tiRNAs perform a miRNA-like negative gene-regulation mechanism [34, 35]. It is also report that tRFs could form a double-stranded molecule with complementary RNA, acting as a primer binding site or base pairing with the target RNA in Argonaute [12, 35]. Because of the incomplete of tRNA database, the target prediction of tRFs/tiRNAs-related mRNAs is still based on miRNA-like methods. In the present study, we used two common miRNA databases to predict the targets of four differential expression tRFs/tiRNAs, and the roles of each tRFs/tiRNAs were inferred by the associated biological functions of the corresponding target mRNAs. We further established a PPI network for predicted target genes of the four tRFs/tiRNAs, and found that PPP2R5A, ADAMTS1, PPARA and POLR2C were the most frequently interacted proteins in target genes of these tRFs/tiRNAs.

Conclusion

In conclusion, our study provided a comprehensive analysis of tRFs/tiRNAs in FD, and the results indicated that tDR-006826, tDR-006049, tDR-001271 and tDR-001276 might play key regulatory roles in FD, which was expected to provide a theoretical basis and data support for clinical treatment.

Methods

Primary cell culture

FD tissues were obtained from bone lesions after surgical removal, and normal jaws were harvested from the implant surgery of patients at Department of Oral and Maxillofacial Surgery, Nanjing Medical University Affiliated Hospital of Stomatology. All experiments were performed under a protocol approved by the Ethics and Research Committee of Nanjing Medical University. Informed consent for all patients was obtained before specimen collection in this study. Cells were cultured in standard medium consisting of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, and 10% fetal bovine serum (ScienCell, Carlsbad, CA, USA) at 37 °C maintained in 5% CO_2. Culture medium was changed every 3 days until 70-80% confluence was achieved.

RNA extraction and quality control

Total RNA from cells was extracted using TRIzol reagent (Takara, Japan) according to the manufacturer's instruction. The integrity and quantity of RNA samples were checked using agarose gel electrophoresis and Nanodrop™ instrument. The optical density 260/280 absorbance ratio of total RNAs was between 1.8 and 2.0.

High-throughput sequencing

Total RNAs were pretreated to remove RNA modifications. Purified RNA was sent to KangChen Bio-tech (Shanghai, China) for small RNA-seq library construction. The libraries are qualified and absolutely quantified using Agilent BioAnalyzer 2100 (Agilent, California, CA). Small RNA sequencing analysis was performed using an Illumina NextSeq 500 system (Illumina, California, USA) according to the instructions of manufacturer.

Analysis of high-throughput sequencing data

The differential expression of tRFs/tiRNAs was analyzed using R package edgeR based on the high-throughput sequencing data. PCA, pie plots, Venn plots, hierarchical clustering, scatter plots, and volcano plots were analyzed in the R environment.

Real-time PCR and statistics

Total RNAs were reverse transcribed into cDNA using Bulge-loop^{TM} microRNA (miRNA) qRT-PCR Primer Sets (Ribobio, Guangzhou, China). Quantitative real-time PCR analyses were performed with U6 small nuclear RNA (snRNA) as an internal control, and reactions were detected using an Applied Biosystems 7900HT Fast Real-time PCR system (Applied Biosystems, Gaithersburg, CA, USA). The primers of tRFs were designed by Ribobio. The relative expression fold of tRFs in different samples was calculated using the $2^{-\Delta\Delta Ct}$ method. All reactions were performed in triplicate.

Target gene prediction of tRFs/tiRNAs

The tRNA database applied to map the reads was based on the Genomic tRNA Database (http://gtrnadb.ucsc.edu/). Because of the miRNA-like functions of tRFs/tiRNAs, TargetScan and miRanda databases were used to predict tRFs/tiRNAs target genes.

GO, KEGG, and PPI network analyses

To determine the biological function of the target genes, GO was performed to analyze the target genes. The KEGG database (http://www.kegg.jp/) was used to identify significantly enriched pathways of differential expression tRFs/tiRNAs for pathway analyses. STRING database (https://string-db.org/cgi) was used to construct a PPI network among the predicted target genes with confidence ≥ 0.4 as the threshold condition. The network was constructed by cytoscape and cytoHubba plug-in was used to obtain core genes.

Statistical analysis

Statistical analysis was conducted via GraphPad Prism 8 software and performed using unpaired Student's *t*-test. The statistical significance was considered at $P<0.05$. All experiments were repeated independently at least three times.

Abbreviations

FD: Fibrous dysplasia

tRFs: tRNA-derived RNA fragments

tiRNAs: tRNA halves

qRT-PCR: Quantitative real-time polymerase chain reaction

tDRs: tRNA-derived small RNAs

GO: gene ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

BMSCs: bone marrow stromal cells

cAMP: cyclic adenosine monophosphate

ncRNAs: Non-coding RNAs

mRNA: messenger RNA

tRNA: transfer RNA

tRF-5: 5' derived tRF

tRF-3: 3' derived tRF

PCA: principal component analysis

BP: biological process

CC: cellular components

MF: molecular function

Gsα: guanine nucleotide binding protein G protein α subunit

PPI: protein-protein interaction

Declarations

Ethics approval and consent to participate

All experiments were performed under a protocol approved by the Ethics and Research Committee of Nanjing Medical University. All procedures in this study complied with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards, when human participants were involved in. Informed consent for all patients was obtained before specimen collection in this study.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Z.L. designed and performed research, analyzed and interpreted data, wrote manuscript; Y.F. performed research, analyzed and interpreted data, wrote manuscript; Y.L. analyzed and interpreted data; H.X. collected and assembled data; T.X. collected and assembled data; H.J. participated in conception and provided study material or patients. All authors gave final approval and agree to be accountable for all aspects of the work.

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Not applicable

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Figures

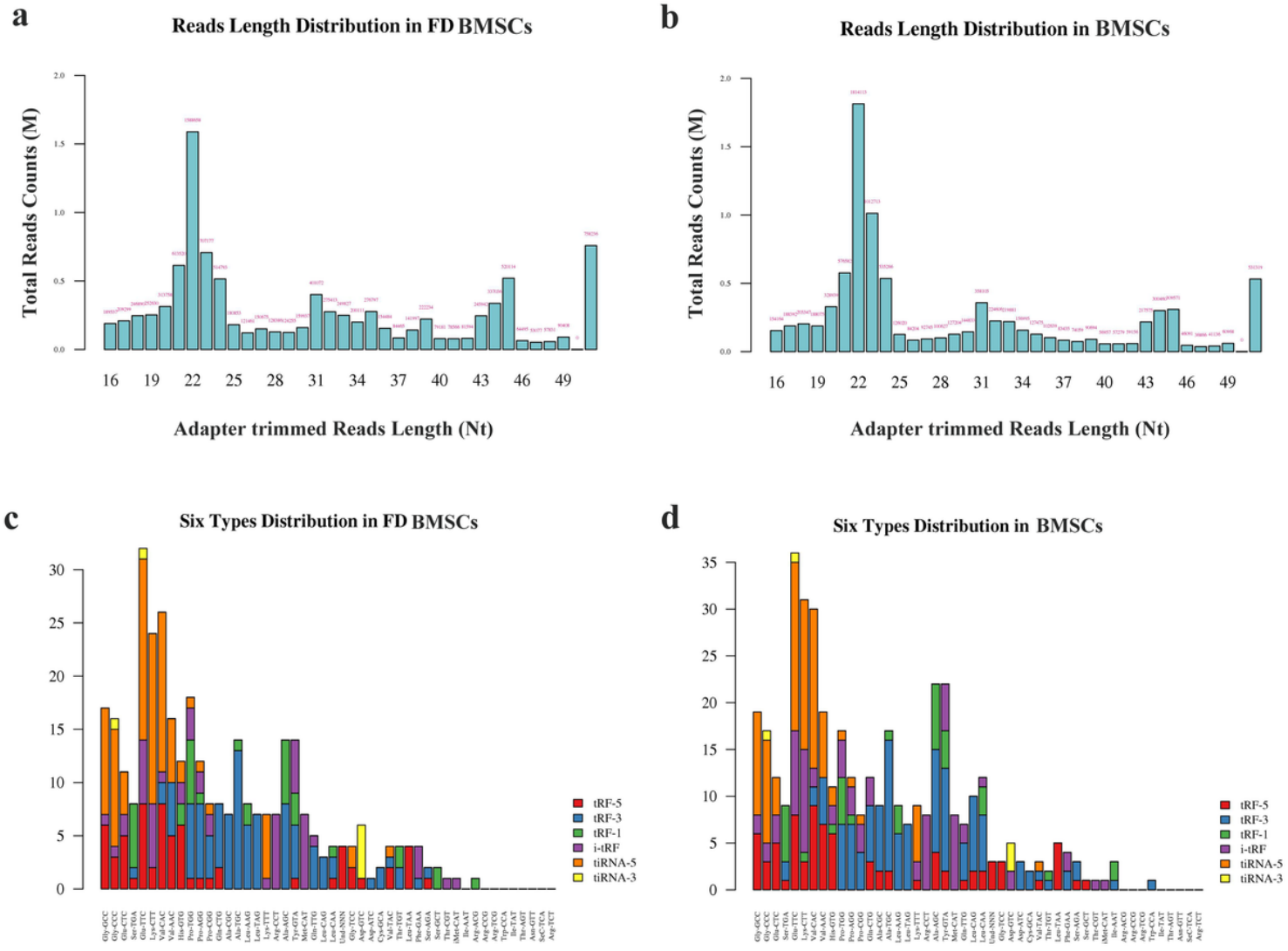

Figure 1

Expression profiles of tRFs/tiRNAs in FD BMSCs and normal BMSCs. a, b Total read counts against the read lengths for the complete adapter-trimmed read set. c, d Stacked plot for all subtypes of tRFs/tiRNAs of each group clustered by the anticodon of the tRNAs.

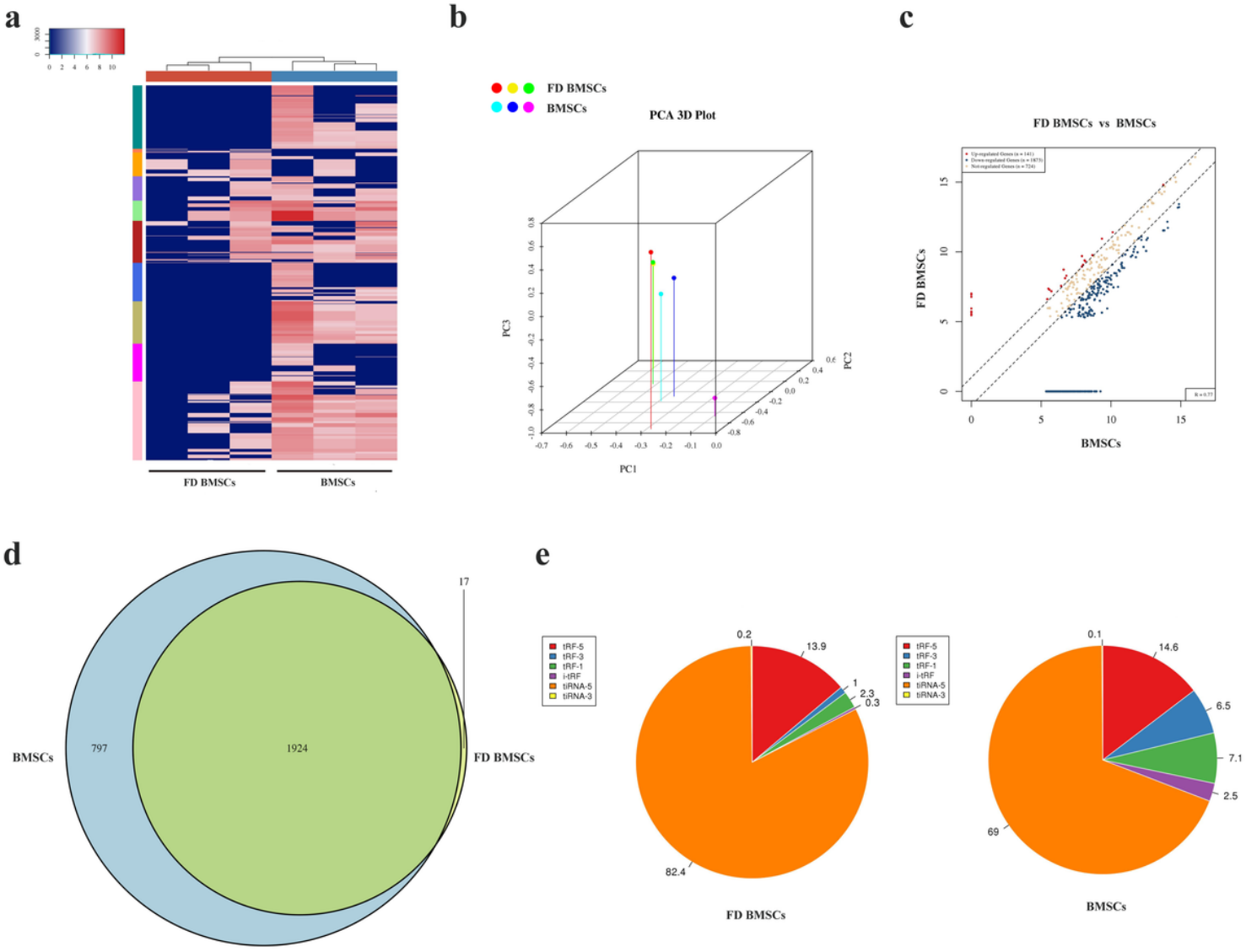

Figure 2

Differential expression of tRFs/tiRNAs in BMSCs and FD BMSCs. a Heatmap of differentially expressed in BMSCs and FD BMSCs. b The principal component analysis (PCA) plot of tRFs/tiRNAs expressed in BMSCs and FD BMSCs. The colored points represented the 6 samples, and the location showed the main character of the sample. c The scatter plot of differentially expressed genes in BMSCs and FD BMSCs. Transcripts per million (TPM) values of all tRFs/tiRNAs are plotted. tRFs/tiRNAs above the top line (red dots, upregulation) or below the bottom-line (green dots, downregulation) indicated greater than 2.0-fold change between BMSCs and FD BMSCs. Gray dots indicated tRFs/tiRNAs without differentially expression. d Venn diagram showed the specifically expressed tRFs/tiRNAs in BMSCs and FD BMSCs. e The pie charts showed the expression level of each subtype of tRFs/tiRNAs in FD BMSCs and BMSCs.

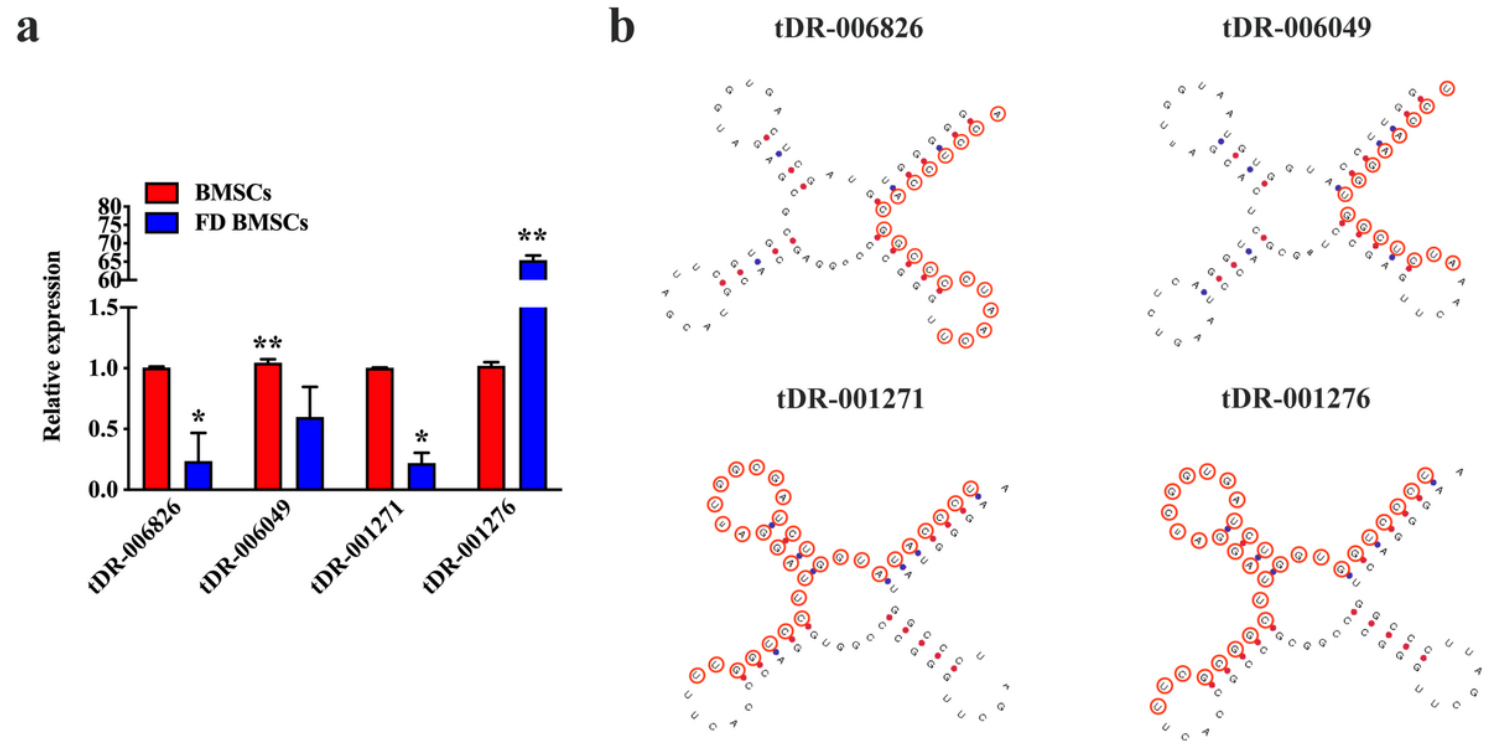

Figure 3

Validation of differential expression tRFs/tiRNAs in BMSCs and FD BMSCs. a Quantitative real-time PCR analysis was used to detect tDR-006826, tDR-006049, tDR-001271 and tDR-001276 expression in FD BMSCs and BMSCs. b The position of tDR-006826, tDR-006049, tDR-001271 and tDR-001276 on cloverleaf secondary structure. The data are presented as the mean ± S.E.M. values ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$.

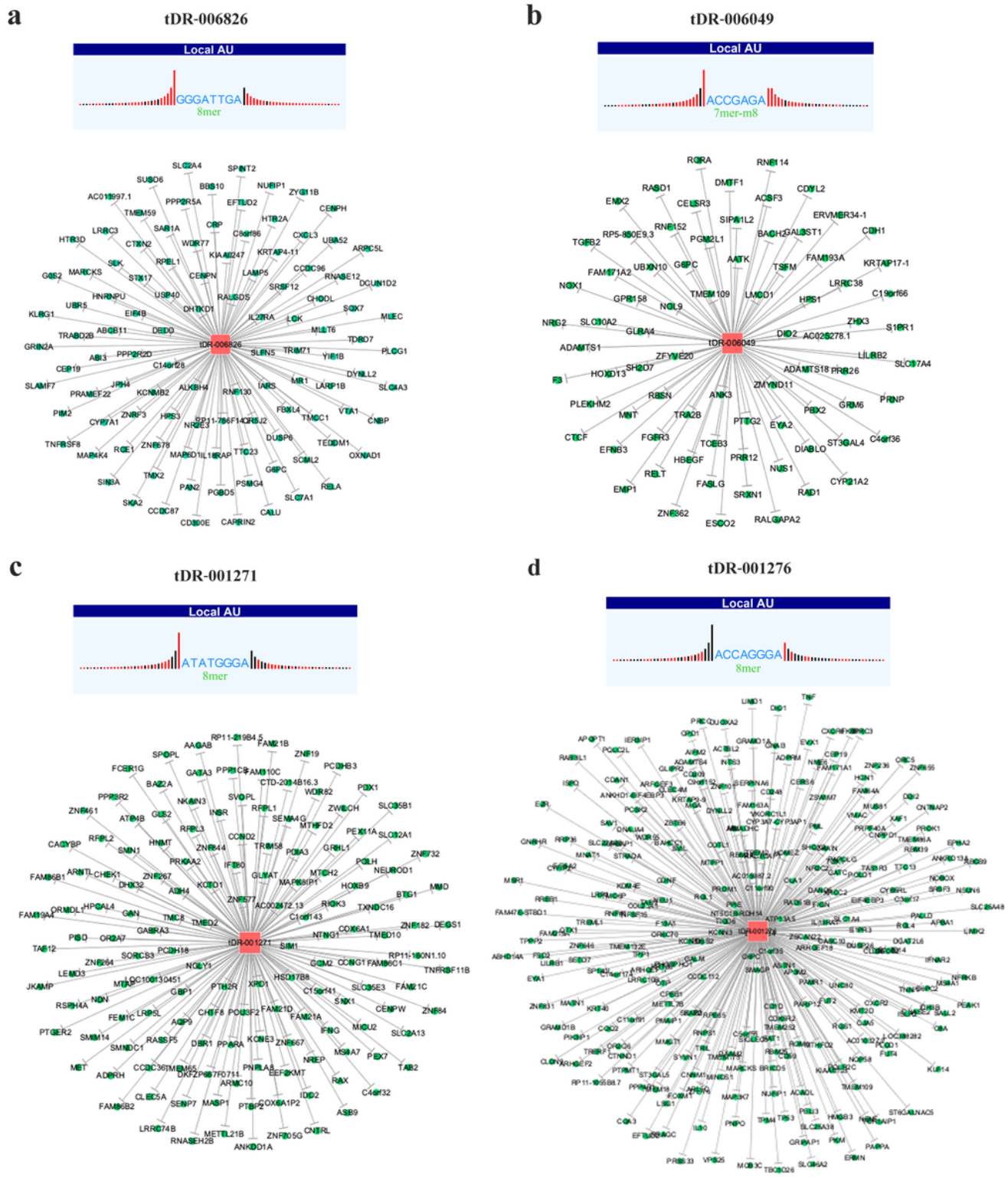

Figure 4

Prediction of tRFs/tiRNAs target genes. a The potential targets components and prediction target genes of tDR-006826. b The potential targets components and prediction target genes of tDR-006049. c The potential targets components and prediction target genes of tDR-001271. d The potential targets components and prediction target genes of tDR-006826.

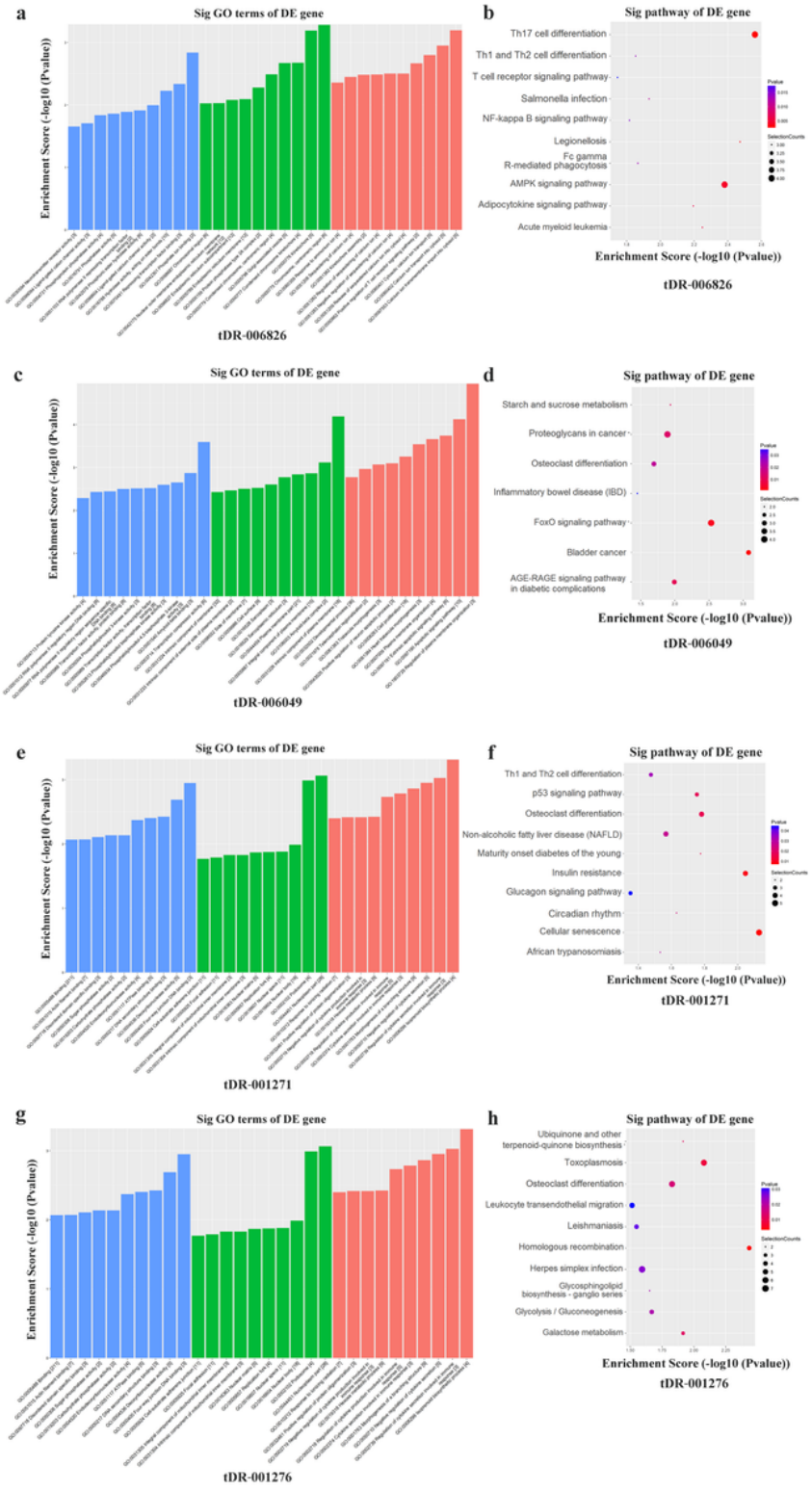

Figure 5

GO enrichment and KEGG analyses of tRFs/tiRNAs. a GO enrichment and KEGG pathway analysis of tDR-006826. b GO enrichment and KEGG pathway analysis of tDR-006049. c GO enrichment and KEGG pathway analysis of tDR-001271. d GO enrichment and KEGG pathway analysis of tDR-006826.

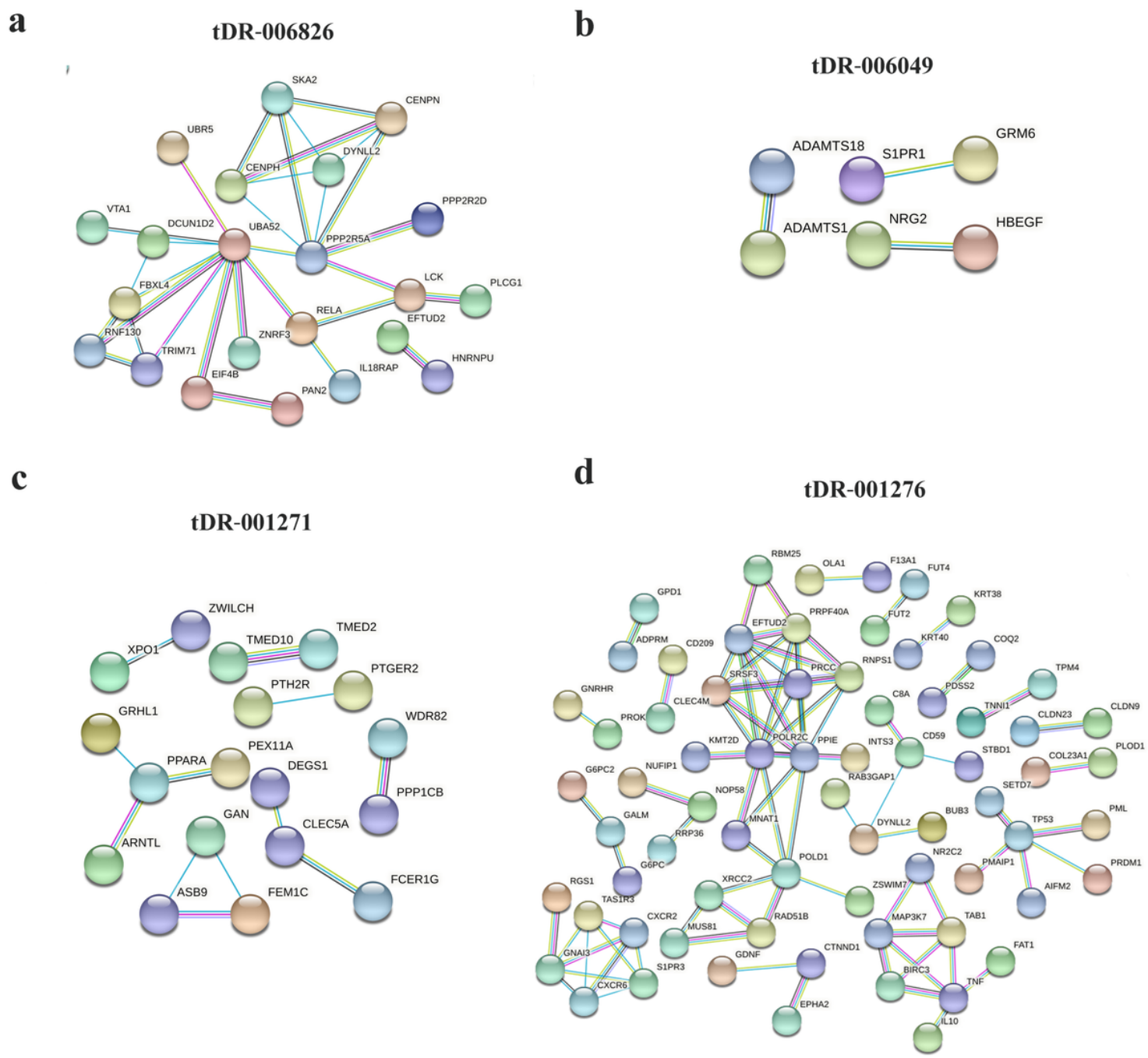

Figure 6

Protein-protein interaction networks. The networks of target genes of tDR-006826 (a), tDR-006049 (b), tDR-001271 (c), and tDR-001276 (d).

Supplementary Files

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- Additionalfile1.docx