Integrated Analysis of tRNA-Derived Small RNAs in Proliferative Human Aortic Smooth Muscle Cells

Jian-Zhi Zhao
Hebei Medical University
Jia-Jie Lin
Hebei Medical University
Miao Gong
Hebei Medical University
Yu Wang
Hebei Medical University
Ke-Xin Liu
Hebei Medical University
Mei-Yang Du
Hebei Medical University
Huan-Huan Li
Hebei Medical University
Si-Fan Wang
Hebei Medical University
Li-Yun Yang
Hebei Medical University
Bo Sun
Hebei Medical University
Peng Kong
Hebei Medical University
Kun Liu
Hebei Medical University
Bin Li
Hebei Medical University
Shao-Guang Sun (sunshaoguang00@163.com)
Hebei Medical University https://orcid.org/0000-0002-2827-0948

Research

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Abstract

Background

Abnormal proliferation of vascular smooth muscle cells (VSMCs) contributes to vascular remodeling diseases. Recently, it has been discovered that tRNA-derived small RNAs (tsRNAs), a new type of non-coding RNAs, are related to the proliferation and migration of VSMCs. tsRNAs regulate target gene expression through miRNA-like function. This study aims to explore the potential of tsRNAs in human aortic smooth muscle cell (HASMC) proliferation.

Methods

High-throughput sequencing was performed to analyze the tsRNA expression profile of proliferative and quiescent HASMCs. Quantitative real-time PCR (qRT-PCR) was performed to validate sequence results and subcellular distribution of AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076. Based on microRNA-like functions of tsRNAs, we predicted target promoters, mRNAs, and circular RNAs (circRNAs), constructed tsRNA-promoter, tsRNA-mRNA, and circRNA-tsRNA interaction networks. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed to reveal the function of target genes. Western blot and EdU incorporation assays were utilized to detect the effect of tsRNAs on HASMC proliferation.

Results

Compared with quiescent HASMCs, 887 up-regulated and 951 down-regulated tsRNAs in proliferative HASMCs were identified (fold change > 2 or < -2, p-value < 0.05). AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 were up-regulated in proliferative HASMCs and were mainly located in the nucleus. Bioinformatics analysis suggested that they involve a variety of terms and pathways related to VSMC proliferation. Knockdown of AS-tDR-000067 promoted the expression of target gene p53 and inhibited HASMC proliferation. Knockdown of AS-tDR-000076 promoted the expression of target gene mitofusin 2 (MFN2) and inhibited HASMC proliferation.

Conclusion

During HASMC proliferation, the expression levels of many tsRNAs are altered. AS-tDR-000067 and AS-tDR-000076 may become a new therapeutic target for vascular remodeling diseases.

Introduction

The death toll of cardiovascular diseases increases year by year, among which ischemic heart disease and cerebrovascular disease account for more than 80% of the deaths [1]. Vascular smooth muscle cells

(VSMCs), one of the cell types in blood vessels, mainly contract and relax to maintain blood pressure and flow [2]. Abnormal proliferation of VSMCs is a vital process in the pathogenesis of numerous vascular remodeling diseases, such as atherosclerosis [3], hypertension [4], vascular stenosis [5], and diabetic vascular complications [6]. Therefore, exploration of VSMC proliferation will contribute to the treatment of vascular proliferative diseases.

With the development of deep sequencing technology, tRNA-derived small RNAs (tsRNAs), a new class of small non-coding RNAs derived from tRNAs, have been discovered in various organisms [7]. tsRNAs function on cell phenotype, especially cell proliferation [8–13]. tRF^{GlnCTG}, which is highly expressed in the rat common carotid artery (CCA) intimal hyperplasia model, targets FAS cell surface death receptor (FAS) to promote the proliferation and migration of rat VSMCs [14]. In the mouse model of ischemia, tsRNAs derived from $tRNA^{Val}$ and $tRNA^{Gly}$ are significantly up-regulated in endothelial cells, thereby inhibiting their proliferation, migration, and tube formation [8]. CU1276, a microRNA (miRNA)-like tsRNA in human mature B cells, is down-regulated in lymphoma cells and attenuates its inhibition of target gene replication protein A1 (RPA1), thereby promoting cell proliferation [9]. In breast cancer cells under hypoxia, a class of tsRNAs derived from $tRNA^{Glu}$, $tRNA^{Asp}$, $tRNA^{Gly}$, and $tRNA^{Tyr}$ destabilize multiple oncogenic transcripts through competitive RNA-binding protein Y-box binding protein 1 (YBX1) displacement, thereby inhibiting cell proliferation, invasion, and migration [10]. Three 5'-sex hormone-dependent tRNA-derived RNAs (5'-SHOT-RNAs), 5'-SHOT-RNA^{LysCUU}, 5'-SHOT-RNA^{AspGUC}, and 5'-SHOT-RNA^{HisGUG}, accelerate prostate cancer cell proliferation [11]. In addition, cell proliferation-related tsRNAs have also been revealed in other studies [12, 13].

Previous studies have shown that tsRNAs regulate various diseases, including cancer [15], alcoholic fatty liver disease [16], viral infection [17], and neurodegenerative diseases [18], mainly by interacting with RNAs or proteins. So far, most studies on tsRNAs have focused on cancer; however, regulation of tsRNAs on VSMC proliferation has not been clearly elucidated yet.

This study focuses on screening tsRNAs related to human aortic smooth muscle cell (HASMC) proliferation. Four differentially expressed tsRNAs (DEtsRNAs) (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) were selected for qRT-PCR validation. tsRNA-promoter, tsRNA-mRNA, and circular RNA (circRNA)-tsRNA interaction networks were constructed to facilitate the analysis of four DEtsRNAs. Our data showed that the target genes of four DEtsRNAs were enriched in Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to cell proliferation. Knockdown of AS-tDR-000067 and AS-tDR-000076 inhibited HASMC proliferation. Our study innovatively elucidates tsRNAs related to HASMC proliferation, which provides novel clues to explore vascular remodeling diseases.

Methods

Cell culture

Proliferative HASMCs (ScienCell, California, USA) were induced by smooth muscle cell medium (SMCM, ScienCell) containing 2% fetal bovine serum (FBS, ScienCell), 1% 100 × smooth muscle cell growth supplement (SMCGS, ScienCell), and 1% 100 × penicillin/streptomycin solution (ScienCell). Quiescent HASMCs were induced by FBS and SMCGS starvation for 24 h. Cells were cultured at 37°C in an incubator containing 5% CO_2.

tsRNA-seq libraries preparation and sequencing

To screen tsRNAs related to HASMC proliferation, we performed high-throughput RNA sequencing of three sets of proliferative and quiescent HASMCs. Total RNA of proliferative and quiescent HASMCs was extracted by RNA Simple Total RNA Kit (TIANGEN, Beijing, China). Total RNA samples were pretreated to eliminate the interference of RNA modification in the construction of small RNA-seq libraries: 1) 3-aminoacyl (charged) was deacylated to 3'-OH for 3' adaptor ligation; 2) 3'-cP (2', 3'-cyclic phosphate) was removed to 3'-OH for 3' adaptor ligation; 3) 5'-OH (hydroxyl group) was phosphorylated to 5'-P for 5'-adaptor ligation; 4) m1A and m3C were demethylated for effective reverse transcription. Subsequently, tsRNA-seq libraries were constructed using the commercial kit for tsRNA sequencing library preparation (Illumina, California, USA). The kit includes 3'-adapter and 5'-adapter ligation adaptor ligation, cDNA synthesis, and library PCR amplification. PCR amplified fragments with a size of 135 ~ 160 bp (corresponding to the size range of 15 ~ 40 nt small RNA) were selected as tsRNA-seq libraries. Finally, the prepared tsRNA-seq libraries were quantified using Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) and then sequenced using Illumina NextSeq 500 (Illumina). Sequencing was performed by Kangchen Bio-tech (Shanghai, China).

Sequencing data analysis

Image analysis and base calling were performed by Solexa pipeline v1.8 (Off-Line Base Caller software, v1.8). Valid sequences were preserved by alignment statistical analysis for subsequent tsRNA expression profile analysis and differential expression analysis. Sequencing quality was tested by FastQC software, and NovoAlign software (v2.07.11) was applied to align the trimmed reads (with 5', 3'-adaptor bases removed) with the mature tRNA and pre-tRNA sequences of GtRNAdbb: Genomic tRNA Database (http://gtrnadb.ucsc.edu/). Remaining reads were aligned to the transcriptome including mRNA/rRNA/snRNA/piRNA/snoRNA/miRNA biotypes. Expression profile and differential expression of tsRNAs were calculated based on standardized TPM (Transcripts Per Million).

Quantitative real-time PCR (qRT-PCR) validation

Total small RNAs (smRNAs) of proliferative and quiescent HASMCs were isolated by MiRcute miRNA Isolation Kit (TIANGEN). Quantity and integrity of total smRNAs were measured by NanoDrop ND-1000 (Thermo Fisher Science, Massachusetts, USA) and 1.2% agarose gel electrophoresis. Total smRNAs were reverse transcribed by miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN). qRT-PCR was performed using SYBR Green analysis of miRcute Plus miRNA qPCR Kit (TIANGEN). Reaction conditions of all samples were: Initial denaturation at 95°C for 10 min, heat denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 10 s, with 40 cycles. All samples were normalized using U6 as an

internal control. $2^{-\Delta\Delta Ct}$ method was applied to calculate the fold change of expression of tsRNAs; Student's *t*-test was performed for statistical significance. Primers of AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, AS-tDR-000076, and U6 were shown in **Supplementary Table 1**.

Nuclear and cytoplasmic RNA extraction

Nuclear and cytoplasmic components of proliferative HASMCs were isolated using the Nuclear/Cytosol Fractionation Kit (BioVision, California, USA) following the manufacturer's instructions. Extraction, quantification, and integrity detection of smRNAs were consistent with the above. Reaction condition of qRT-PCR was consistent with the above. GAPDH and U6 were applied as positive controls for the cytoplasm and nucleus, respectively. Primers were listed in **Supplementary Table 1.**

Construction of tsRNA-promoter interaction networks

Based on seed region (tsRNA nucleotides 2–8), downstream target promoters of four DEtsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) were predicted by RNAhybrid [19] and MiRanda [20]. To further screen proliferation-related genes containing target promoters, Venn analysis was performed on predicted genes containing target promoters by means of differentially expressed mRNAs (DEmRNAs) in proliferative HASMCs (GSE77279) (fold change > 2 or < -2, *p*-value < 0.05). Finally, tsRNA-promoter interaction networks were constructed using Cytoscape_v3.7.1.

Construction of tsRNA-mRNA interaction networks

Downstream target genes of four DEtsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) were predicted by TargetScan [21] and MiRanda [20]. Venn analysis between predicted target genes and DEmRNAs in the proliferative HASMCs (GSE77279) (fold change < -1.5, *p*-value < 0.05) was performed to further obtain target DEmRNAs. Finally, tsRNA-mRNA interaction networks were constructed using Cytoscape_v3.7.1.

Construction of circRNA-tsRNA interaction networks

Based on the miRNA-like function of tsRNAs, upstream target circRNAs of AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 were predicted using RNAhybrid [19]. Venn analysis between predicted target circRNAs and differentially expressed circRNAs (DEcircRNAs) in the proliferative HASMCs (GSE77278) (fold change > 2 or < -2, *p*-value < 0.05) was utilized to further enrich target DEcircRNAs. Finally, circRNA-tsRNA interaction networks were constructed using Cytoscape_v3.7.1.

GO and pathway analyses

GO and KEGG pathway analyses of the proliferation-related gene sets containing target promoters and proliferation-related target DEmRNA sets were performed to explore the potential of AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 in HASMC proliferation using the DAVID database [22].

Antisense oligonucleotide transfection

Antisense oligonucleotide (ASO) of AS-tDR-000076 and negative control (NC) were designed and synthesized by GENEWIZ (Beijing, China). According to the procedures of Lipofectamine 3000® Transfection Reagent (Invitrogen, California, USA), Lipofectamine 3000 was used to transfect 5,000 ng ASO into HASMCs cultured in a 25 cm^2 cell culture flask. After 7 h of incubation in a 37°C and 5% CO_2 humidified incubator, the transfection medium was replaced with fresh medium and cultured to the appropriate time point. Sequences of NC and ASO were listed in Supplementary Table 2.

Western blot analysis

Total protein in HASMCs transfected with ASO or NC for 48 h was extracted using RIPA buffer (Solarbio, Beijing, China) and 1 mM PMSF (Solarbio). Protein concentration was detected by the Bradford method (Solarbio). Equal amounts of protein were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck, Darmstadt, Germany). At 37°C, 5% skimmed milk was used to block the membrane for 2 h, followed by washing with Tris-buffered saline (TBS), and then incubated overnight at 4°C with the following specific primary antibodies: MFN2 (dilution at a 1: 500, Abcam), GAPDH (dilution at a 1: 1000, Wanleibio). After washing three times in Tris-buffered saline within Tween 20 (TBST) for 5 min, the membrane was incubated in goat anti-rabbit secondary antibody (dilution at a 1: 20,000, Sino Biological) for 1 h at room temperature. Subsequently, ChemiDoc™ MP Imaging System (BIO-RAD) visualized the protein signals and quantified band strength.

Cell proliferation analysis

BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, Shanghai, China) was used for the 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay. Proliferative HASMCs were seeded into 24-well plates, 420 ng ASO or NC were transfected into each well. Proliferative HASMCs were incubated with SMCM containing 2% FBS, 1% 100 × SMCGS, and 1% 100 × penicillin/streptomycin solution for 48 h in a humidified chamber containing 5% CO_2 and 95% O_2 at 37°C. Representative images were obtained through OLYMPUS IX71 (OLYMPUS, Tokyo, Japan).

Statistical analysis

All data are from at least three independent experiments, expressed in the form of mean ± standard deviation (SD). Student's *t*-test was performed to compare the differences between the two groups. When p-value < 0.05, the difference was statistically significant.

Results

Analysis of tsRNA expression profiling

To screen for tsRNAs related to HASMC proliferation, we performed high-throughput RNA sequencing of three sets of proliferative and quiescent HASMCs. A total of 3,891 tsRNAs were identified by sequencing, of which 887 and 951 tsRNAs were up-regulated and down-regulated in proliferative HASMCs compared to quiescent HASMCs, respectively (fold change > 2 or < -2, p-value < 0.05). Scatter plots (Fig. 1a), volcano

plots (Fig. 1b), and hierarchical clustering (Fig. 1c) were performed to describe the tsRNA expression profiling. According to the fold change and expression abundance, four DEtsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) were selected for qRT-PCR. Consistent with the sequencing, AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 were up-regulated in proliferative HASMCs (Fig. 1d). To evaluate the subcellular localization of tsRNA, the nuclear and cytoplasmic components of proliferative HASMCs were separated. Four DEtsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) were mainly located in the nucleus, as confirmed by qRT-PCR (Fig. 1e).

Construction of tsRNA-promoter interaction networks

Function of tsRNAs binding to Argonaute (AGO) protein is similar to miRNAs [23]. Binding of nuclear miRNAs to promoters leads to activate [24–26] or silence [27–29] the transcription of target genes. Therefore, we speculate that tsRNAs are similar to miRNAs and play a regulatory role through targeting promoters. Four DEtsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) were selected to construct tsRNA-promoter interaction networks (Fig. 2). From the networks, AS-TDR-001370, AS-TDR-000067, AS-TDR-009512, and AS-TDR-000076 might target 243, 855, 202, and 120 proliferation-related genes in a promoter targeted manner, respectively (Fig. 2).

Functional annotation of genes containing target promoters of tsRNAs

GO and KEGG pathway analyses of target promoters were performed to explore the potential of AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 in HASMC proliferation using DAVID database [22]. GO analysis revealed that four molecules were enriched in the GO terms related to cell proliferation. Cell proliferation-related genes containing target promoters of AS-tDR-001370, such as cyclin D1 (CCND1) [30], sprouty RTK signaling antagonist 2 (SPRY2) [31], and bone morphogenetic protein receptor type 2 (BMPR2) [32] were enriched in histone deacetylase binding (ontology: molecular function, GO: 0042826), peptidyl-threonine phosphorylation (ontology: biological process, GO:0018107), and regulation of developmental growth (ontology: biological process, GO: 0048638), respectively (Fig. 3a). Cell proliferation-related genes containing target promoters of AS-tDR-000067, such as Yes1 associated transcriptional regulator (YAP1) [33], high mobility group AT-hook 2 (HMGA2) [34], cyclin dependent kinase 6 (CDK6) [35], and autophagy related 4B cysteine peptidase (ATG4B) [36] were enriched in hippo signaling (ontology: biological process, GO: 0035329), regulation of stem cell population maintenance (ontology: biological process, GO: 2000036), cyclin-dependent protein serine/threonine kinase activity (ontology: molecular function, GO: 0004693), and cysteine-type endopeptidase activity (ontology: molecular function, GO: 0004197), respectively (Fig. 3c). Cell proliferation-related genes containing target promoters of AS-tDR-009512, such as nuclear receptor subfamily 2 group F member 1 (NR2F1) [37], angiogenic factor with G-patch and FHA domains 1 (AGGF1) [38], caldesmon 1 (CALD1) [39], and reticulon 4 (RTN4) [40] were enriched in negative regulation of kinase activity (ontology: biological process, GO: 0033673), regulation of angiogenesis (ontology: biological process, GO: 0045765), actin cytoskeleton (ontology: cellular component, GO: 0015629), and regulation of cell death

(ontology: biological process, GO: 0010941), respectively (Fig. 3e). Cell proliferation-related genes containing target promoters of AS-tDR-000076, such as CCND1 [41], secreted frizzled related protein 1 (SFRP1) [42], and transforming growth factor beta 1 (TGFB1) [43] were enriched in regulation of cell cycle arrest (ontology: biological process, GO: 0071156), negative regulation of cell growth (ontology: biological process, GO: 0030308), and Notch signaling pathway (ontology: biological process, GO: 0007219), respectively (Fig. 3g).

KEGG analysis revealed that four molecules were enriched in cell proliferation-related pathways. Cell proliferation-related genes containing target promoters of AS-tDR-001370, such as RELA proto-oncogene (RELA) [44], fibroblast growth factor 9 (FGF9) [45], and calcium voltage-gated channel subunit alpha1 G (CACNA1G) [46] were enriched in MAPK signaling pathway (hsa04010) (Fig. 3b). Cell proliferation-related genes containing target promoters of AS-tDR-000067, such as cyclin D3 (CCND3) [47], p53 [48], CDK6 [35], and cyclin dependent kinase 2 (CDK2)[49] were enriched in p53 signaling pathway (hsa04115) (Fig. 3d). Cell proliferation-related genes containing target promoters of AS-tDR-009512, such as CALD1 [39] and inositol 1,4,5-trisphosphate receptor type 1 (ITPR1) [50] were enriched in vascular smooth muscle contraction (hsa04270) (Fig. 3f). Cell proliferation-related genes containing target promoters of AS-tDR-000076, such as calcium/calmodulin dependent protein kinase II delta (CAMK2D) [51], cyclin D2 (CCND2) [52], and mitogen-activated protein kinase 9 (MAPK9) [53] were enriched in Wnt signaling pathway (hsa04310) (Fig. 3h).

Construction of tsRNA-mRNA interaction networks

Many studies have shown that tsRNAs possess miRNA-like functions (that is, down-regulate the expression of target genes in a sequence-dependent manner by binding to AGO) [9, 54, 55]. To explore whether tsRNAs also play a biological role in HASMCs through this mechanism, four DEtsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) were selected to construct tsRNA-mRNA interaction networks (Fig. 4). From the interaction networks, AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 might target 46, 69, 184, and 306 DEmRNAs, respectively (Fig. 4).

Functional annotation of target DEmRNAs of tsRNAs

GO and KEGG pathway analyses of four target DEmRNAs sets were performed to explore the potential of AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 in HASMC proliferation [22]. GO analysis revealed that four molecules were involved in cell proliferation-related GO terms. Cell proliferation-related target DEmRNA prohibitin 2 (PHB2) [56] of AS-tDR-001370 was enriched in regulation of complement activation (ontology: biological process, GO: 0030449) (Fig. 5a). Cell proliferation-related target DEmRNAs ataxin 3 (ATXN3)[57] and PYD and CARD domain containing (PYCARD) [58] of AS-tDR-000067 were enriched in actin cytoskeleton organization (ontology: biological process, GO: 0030036) and regulation of tumor necrosis factor-mediated signaling pathway (ontology: biological process, GO: 0010803), respectively (Fig. 5c). Cell proliferation-related target DEmRNAs protein tyrosine phosphatase receptor type J (PTPRJ) [59], transmembrane protein with EGF like and two follistatin like domains 2 (TMEFF2) [60], T-box transcription factor 5 (TBX5) [61], tropomyosin 1 (TPM1) [62], and Cbp/p300

interacting transactivator with Glu/Asp rich carboxy-terminal domain 2 (CITED2) [63] of AS-tDR-009512 were enriched in negative regulation of cell migration (ontology: biological process, GO: 0030336) (Fig. 5e). Cell proliferation-related target DEmRNAs mitofusin 2 (MFN2) [64], osteoglycin (OGN) [65], and splicing factor 1 (SF1) [66] of AS-tDR-000076 were enriched in negative regulation of smooth muscle cell proliferation (ontology: biological process, GO: 0048662) (Fig. 5g).

KEGG analysis revealed that four molecules were enriched in cell proliferation-related pathways. Cell proliferation-related target DEmRNA NUMB like endocytic adaptor protein (NUMBL) [67] of AS-tDR-001370 was enriched in Notch signaling pathway (hsa04330) (Fig. 5b). Cell proliferation-related target DEmRNA protein kinase C gamma (PRKCG) [68] of AS-tDR-000067 was enriched in VEGF signaling pathway (hsa04370) (Fig. 5d). Cell proliferation-related target mRNAs coenzyme cytochrome P450 family 2 subfamily C member 8 (CYP2C8) [69] and glutathione peroxidase 7 (GPX7) [70] of AS-tDR-009512 were enriched in Arachidonic acid metabolism (hsa 00590) (Fig. 5f). Target DEmRNAs cell division cycle 42 (CDC42) [71] and PRKCG [72] of AS-tDR-000076 were enriched in VEGF signaling pathway (hsa04370) (Fig. 5h).

Construction of circRNA-tsRNA interaction networks

CircRNAs interact with miRNAs and act in the form of competing endogenous RNAs (ceRNAs) [73]. Our previous study revealed that circRNAs regulate HASMC proliferation through sponging miRNAs [74]. Based on the miRNA-like function of tsRNAs, four DEtsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) were selected to construct circRNA-tsRNA interaction networks (Fig. 6). From the interaction networks, AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 might target 21, 71, 37, and 53 DEcircRNAs (Fig. 6).

Knockdown of AS-tDR-000067 inhibits the proliferation of HASMCs

In the AS-tDR-000067-promoter interaction network, we found an important apoptosis-related target gene p53 [75] (Fig. 2b). Through RNAhybrid [76], it was found that AS-tDR-000067 contains two binding sites on the p53 promoter (Fig. 7a). ASO was used to specifically knockdown AS-tDR-000067 in proliferative HASMCs, and the knockdown efficiency of AS-tDR-000067 was as high as 60% (Fig. S1). Western blot confirmed that knockdown of AS-tDR-000067 promoted the expression of p53 (Fig. 7b, c). Subsequently, EdU was executed to test the proliferation rate of HASMCs, and AS-tDR-000067 suppression resulted in a reduction of up to 80% in EdU positive cells (Fig. 7d, e). Therefore, we speculate that AS-tDR-000067 may promote HASMC proliferation by targeting the p53 promoter to inhibit its transcription.

Knockdown of AS-tDR-000076 inhibits the proliferation of HASMCs

AS-tDR-000076 was selected for further analysis because GO analysis showed that the target DEmRNAs of AS-tDR-000076 were involved in negative regulation of smooth muscle cell proliferation (ontology: biological process, GO: 0048662) (Fig. 5g). Another important reason is that MFN2, an important

proliferation inhibitor [77], is one of the target DEmRNAs of AS-tDR-000076 (Fig. 4d). Through RNAhybrid [76], it was found that the binding site of AS-tDR-000076 was located in CDS (coding sequence) and 3'-UTR (untranslated region) of MFN2 (Fig. 8a). ASO was utilized to specifically knockdown AS-tDR-000076 in proliferative HASMCs, and the knockdown efficiency of AS-tDR-000076 was as high as 60% (Fig. S2). Western blot confirmed that knockdown of AS-tDR-000076 promoted the expression of MFN2 (Fig. 8b, c). Subsequently, EdU was executed to test the proliferation rat of HASMCs, and AS-tDR-000076 suppression resulted in a reduction of up to 80% in EdU positive cells (Fig. 8d, e). Therefore, we speculate that AS-tDR-000076 may promote cell proliferation by targeting MFN2.

Discussion

tsRNAs, as a new type of non-coding RNA derived from tRNA, are receiving more and more attention. tsRNAs are rich in content, evolutionarily conservative, and widely present in all areas of life. These characteristics suggest that they are not the by-product of tRNA production or degradation, and they may be involved in the regulation of the body [23, 78]. There are two hot spots in the mechanism research of tsRNAs. One is that it interacts with various proteins [54, 79–81]. The other is its miRNA-like function to inhibit the expression of target genes [23, 82]. Through the above mechanisms, tsRNAs can regulate cell proliferation [83], migration [84], apoptosis [85], and other biological processes [86]. The research focus of tsRNAs is cancer, which is involved in regulating the pathogenesis of various cancers [87], and multiple databases of tsRNAs in cancer have been constructed [88, 89]. However, research on tsRNAs is in its infancy, and its role in cardiovascular disease is still not fully understood. Huang et al. reported that miR-1280 is a tsRNA derived from $tRNA^{Leu}$, which binds to the 3'-UTR of JAG2 and silences its expression, thereby inhibiting the proliferation, migration, and self-renewal of colorectal cancer cells mediated by the Notch signaling pathway [83]. This provides a new idea for exploring the role of tsRNAs in vascular diseases, that is, tsRNAs can regulate abnormal proliferation-related vascular diseases by inhibiting the expression of target genes.

The purpose of this paper is to screen and identify tsRNAs related to HASMC proliferation. Here, we performed high-throughput RNA sequencing and screened out 1,838 DEtsRNAs. QRT-PCR confirmed that AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 were up-regulated in proliferative HASMCs, and mainly located in the nucleus. Then, we predicted and screened their target genes respectively to construct tsRNA-promoter, tsRNA-mRNA, and circRNA-tsRNA interaction networks. Bioinformatics analysis showed that target genes of 4 tsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) are involved in various proliferation-related terms and pathways. In tsRNA-promoter interaction networks, we found that AS-tDR-001370 can target cell proliferation-related proteins CCND1 [90], SPRY2 [91], and BMPR2 [92]. The expression of CCND1 is increased in proliferative VSMCs and can promote its proliferation [90]. Thus, AS-tDR-001370 may regulate VSMC proliferation by promoting CCND1 transcription. Besides, AS-tDR-000076 can also target CCND1, indicating that AS-tDR-000076 can coordinate with AS-tDR-001370 to regulate VSMC proliferation. SPRY2 can inhibit the VSMC proliferation and migration, thereby reducing neointimal growth after vascular injury [91]. Loss of

function of BMPR2 is often found in patients with pulmonary arterial hypertension (PAH) induced by abnormal proliferation of VSMC, which has the effect of inhibiting the VSMC proliferation [92]. Thus, AS-tDR-001370 may promote the proliferation of VSMC by targeting SPRY2 and BMPR2 promoters and inhibiting their transcription. Cell proliferation-related proteins YAP1 [93], CDK6 [94], ATG4B [95], and p53 [48] are the target genes of AS-tDR-000067. YAP1 promotes VSMC proliferation by interacting with TEA domain transcription factor 1 (TEAD1) on the enhancer of platelet-derived growth factor receptor beta (PDGFRB) [93]. Thus, AS-tDR-000067 may regulate VSMC proliferation by regulating PDGFRB downstream pathway. CDK6, like CCND1, is an important cell cycle regulator and can promote VSMC proliferation [94]. ATG4B can promote the proliferation, invasion, migration [95], and autophagy [96] of cancer cells. Thus, AS-tDR-000067 may regulate VSMC proliferation by promoting CDK6 and ATG4B transcription. p53 is an important tumour suppressor gene, and the transcription factor it encodes is essential for the regulation of the cell cycle and apoptosis [75]. A large number of studies have confirmed that p53 can prevent atherosclerosis [97], hypertension [98], vascular stenosis [99], and other vascular remodelling diseases by inhibiting the proliferation, invasion, and migration of VSMCs and inducing their apoptosis. Our research has confirmed that knocking down AS-tDR-000067 can promote the expression of p53, indicating that AS-tDR-000067 may promote HASMC proliferation by inhibiting the transcription of p53 (Fig. 7). However, this mechanism is not explained in this article, and further experimental verification is needed. Cell proliferation-related proteins ITPR1 [100], CALD1 [101], and RTN4 [102] are the target genes of AS-tDR-009512. ITPR1 is also called IP3R1, and its regulated Ca^{+} signal is essential for VSMC proliferation [100]. CALD1 is related to the contractile function of VSMC [101]. RTN4 is also called Nogo, which can inhibit VSMC proliferation and migration [102]. Therefore, AS-tDR-009512 can regulate VSMC proliferation by targeting ITPR1, CALD1, and RTN4 promoters. Cell proliferation-related proteins TGFB1 [103], MAPK9 [104], and SFRP1 [105] are the target genes of AS-tDR-000076. TGFB1 can inhibit VSMC proliferation and promote its apoptosis by regulating long noncoding RNA MEG3 [103]. Bioinformatics analysis showed that MAPK9 was related to the Wnt signaling pathway, an important VSMC proliferation-related pathway [106]. Besides, SFRP1 was an important inhibitor of the Wnt signaling pathway, suggesting that AS-tDR-000076 may regulate VSMC proliferation through it [107].

In tsRNA-mRNA interaction networks, AS-tDR-001370's target mRNA NUMBL is involved in the inhibition of the Notch signaling pathway [67, 108], an important VSMC proliferation-related pathway [109]. It is suggested that AS-tDR-001370 can promote VSMC proliferation by reducing the effect of NUMBL. Also, the target genes PHB2 [58] and CLMN [59] of AS-tDR-001370 can inhibit cell proliferation, suggesting AS-tDR-001370 may also promote proliferation through these two ways. AS-tDR-000067's target mRNA PYCARD is a pro-apoptotic molecule [54]. Thus, AS-tDR-000067 may inhibit the process of apoptosis by targeting PYCARD. As the downstream target genes of AS-tDR-009512, TPM1 [110], TMEFF2 [111], and PTPRJ [112, 113] are involved in cell proliferation and metastasis. It has been reported that TPM1 is involved in the inhibition of VSMC proliferation and metastasis [110]. MicroRNA-21 regulates VSMC function of lower extremity arteriosclerosis obliterans by targeting TPM1 [62]. Therefore, AS-tDR-009512 may drive VSMC proliferation by downregulating TPM1. As a target of many molecules, TMEFF2 participates in the inhibition of tumour cell proliferation, migration, and invasion by inhibiting the MAPK

signaling pathway [111]. The involvement of the MAPK signaling pathway in VSMC proliferation has been confirmed [114]. Here, we propose that AS-tDR-009512 participates in VSMC proliferation through the MAPK signaling pathway. Similarly, PTPRJ (also known as DEP-1 or CD148) can inhibit the proliferation and migration of multiple cells through the ERK [112] or PI3K signaling pathways [113] and is closely related to cytoskeletal rearrangements [59]. TMEFF2 and PTPRJ also serve as target genes of AS-tDR-000076, indicating that AS-tDR-009512 and AS-tDR-000076 may coordinate the proliferation of VSMC through the MAPK and PI3K signaling pathways. MFN2 [77], SF1 [66, 115], and OGN [65, 116] are target genes of AS-tDR-000076, which are down-regulated in proliferative HASMCs. It has been reported that MFN2 can inhibit the proliferation and promote the apoptosis of VSMCs by inhibiting the Ras-Raf-ERK1/2 pathway [77] and PI3K-Akt pathway [117], respectively. Therefore, AS-tDR-000076 may promote the VSMC proliferation by inhibiting the MFN2-Ras-Raf-ERK1/2 pathway. Wnt signaling pathway is an important proliferation-related pathway, which plays an important role in the VSMC proliferation [118]. SF1 is a downstream molecule of the Wnt signaling pathway [119] and can inhibit the VSMC proliferation [66, 115]. Hence, AS-tDR-000076 may regulate the VSMC proliferation by silencing SF1. Similarly, OGN is also involved in the inhibition of proliferation. It can negatively regulate the epidermal growth factor receptor (EGFR) signaling pathway [116] and vascular endothelial growth factor receptor 2 (VEGF2) signaling pathway [65], but there is no study on its regulation of VSMC proliferation. It has been reported that OGN inhibits the proliferation and migration of cancer cells through the PI3K/Akt/mTOR signaling pathway [120]. Therefore, whether AS-tDR-000076 also regulates the VSMC proliferation through ONG/PI3K/Akt/mTOR signaling pathway is also worthy of further discussion. In addition to the VSMC proliferation, AS-tDR-000076 is also involved in the regulation of cardiomyocyte function related molecules, such as the transcription factor Nkx2.5 that regulates cardiomyocyte contraction [121], EH domain containing 3 (EHD3) that maintains the excitability and physiological function of myocardial cell membranes [122], cardiac conduction related SCN10A (sodium voltage-gated channel alpha subunit 10) [123], their insufficient expression are involved in the occurrence of diseases such as abnormal cardiac conduction, ventricular fibrillation, and heart failure. Therefore, further research on the function of AS-tDR-000076 is helpful to understand the mechanism of heart disease and may provide a new therapeutic target.

The purpose of this article is to screen the tsRNAs involved in the HASMC proliferation and explore their miRNA-like functions. The tsRNA-promoter interaction networks show that AS-tDR-000067 can regulate more than 800 target genes, of which p53 is involved in VSMC proliferation [48]. EdU fluorescent stain and western blot confirmed that AS-tDR-000067 may promote HASMC proliferation by targeting p53 promoter. The tsRNA-mRNA interaction networks show that AS-tDR-000076 can regulate more than 300 target genes, of which MFN2 is involved in VSMC proliferation [77]. Studies have reported that miRNA can promote cell proliferation by down-regulating MFN2, such as MiR-93 [124] and MicroRNA-497 [125]. Therefore, we speculate that AS-tDR-000076 promotes proliferation by inhibiting the expression of MFN2. EdU fluorescent stain and western blot confirmed that AS-tDR-000076 may promote HASMC proliferation by targeting MFN2. However, the specific regulatory mechanism of AS-tDR-000067 and AS-tDR-000076 on target gene was not covered in this article, and experiments are needed to further explore.

Conclusions

In this article, we conducted a comprehensive analysis of the tsRNA expression profiles of proliferative and quiescent HASMCs. Subsequently, qRT-PCR was performed to confirm the sequencing results. The expression of AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 was up-regulated in proliferative HASMCs and mainly located in the cytoplasm. We constructed their tsRNA-promoter, tsRNA-mRNA, and circRNA-tsRNA interaction networks. DAVID database was used to analyze the function of target genes, and pointed out that AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076 may positively regulate VSMC proliferation. Finally, it was confirmed that AS-tDR-000067 and AS-tDR-000076 may participate in the proliferation of HASMC through down-regulation of p53 and MFN2, respectively, indicating that they may become new targets for the treatment of vascular diseases. This paper reports the role of tsRNAs in human vascular disease for the first time, emphasizing the extensive regulation of tsRNAs, and providing new insights into the mechanism of vascular disease.

Abbreviations

VSMC	vascular smooth muscle cell
tsRNA	tRNA-derived small RNA
HASMC	human aortic smooth muscle cell
qRT-PCR	quantitative real-time PCR
circRNA	circular RNA
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
MFN2	mitofusin 2
CCA	rat common carotid artery
FAS	FAS cell surface death receptor
miRNA	microRNA
YBX1	Y-box binding protein 1
5'-SHOT-RNA	5'-sex hormone-dependent tRNA-derived RNA
DEtsRNA	differentially expressed tsRNA
SMCM	smooth muscle cell medium
FBS	fetal bovine serum
SMCGS	smooth muscle cell growth supplement
smRNA	small RNA
DEmRNA	differentially expressed mRNA
DEcircRNA	differentially expressed circRNA
ASO	antisense oligonucleotide
NC	negative control
PVDF	polyvinylidene fluoride
TBS	Tris-buffered saline
EdU	5-ethynyl-2'-deoxyuridine
SD	standard deviation
AGO	Argonaute
CCND1	cyclin D1
SPRY2	sprouty RTK signaling antagonist 2

VSMC	vascular smooth muscle cell
BMPR2	bone morphogenetic protein receptor type 2
YAP1	Yes1 associated transcriptional regulator
HMGA2	high mobility group AT-hook 2
CDK6	cyclin dependent kinase 6
ATG4B	autophagy related 4B cysteine peptidase
NR2F1	nuclear receptor subfamily 2 group F member 1
AGGF1	angiogenic factor with G-patch and FHA domains 1
CALD1	caldesmon 1
RTN4	reticulon 4
SFRP1	secreted frizzled related protein 1
TGFB1	transforming growth factor beta 1
RELA	RELA proto-oncogene
FGF9	fibroblast growth factor 9
CACNA1G	calcium voltage-gated channel subunit alpha1 G
CCND3	cyclin D3
CDK2	cyclin dependent kinase 2
CAMK2D	calcium/calmodulin dependent protein kinase II delta
CCND2	cyclin D2
MAPK9	mitogen-activated protein kinase 9
PHB2	prohibitin 2
ATXN3	ataxin 3
PYCARD	PYD and CARD domain containing
PTPRJ	protein tyrosine phosphatase receptor type J
TMEFF2	transmembrane protein with EGF like and two follistatin like domains 2
TBX5	T-box transcription factor 5
TPM1	tropomyosin 1
CITED2	Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2
OGN	osteoglycin

VSMC	vascular smooth muscle cell
SF1	splicing factor 1
NUMBL	NUMB like endocytic adaptor protein
PRKCG	protein kinase C gamma
CYP2C8	coenzyme cytochrome P450 family 2 subfamily C member 8
GPX7	glutathione peroxidase 7
CDC42	cell division cycle 42
CDS	coding sequence
3'-UTR	untranslated region
PAH	pulmonary arterial hypertension
TEAD1	TEA domain transcription factor 1
PDGFRB	platelet-derived growth factor receptor beta
EHD3	EH domain containing 3
SCN10A	sodium voltage-gated channel alpha subunit 10

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Availability of data and materials

The original data discussed in this study have been deposited in NCBI Gene Expression Omnibus and are accessible with the GEO Series accession number GSE164540 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164540).

Competing interests

The authors declare no competing interests.

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Authors' contributions

S.G.S. and B.L. designed and supervised the study. J.Z.Z., J.J.L., M.G., Y.W., M.Y.D, H.H.L., B.S., P.K., and K.L. performed the bioinformatics analysis. J.Z.Z., J.J.L., M.G., K.X.L, L.Y.Y., and S.F.W. performed the experiments. J.Z.Z., J.J.L., and M.G. wrote the manuscript. All authors read and approved the final manuscript.

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Authors' information

*Correspondence: Shao-Guang Sun (email: sunshaoguang00@163.com) and Bin Li (email: libin_cj@163.com)

† Jian-Zhi Zhao, Jia-Jie Lin and Miao Gong contribute equally to this work

Department of Biochemistry and Molecular Biology, Key Laboratory of Medical Biotechnology of Hebei Province, Cardiovascular Medical Science Center, Hebei Medical University, Shijiazhuang 050017, China.

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Figures

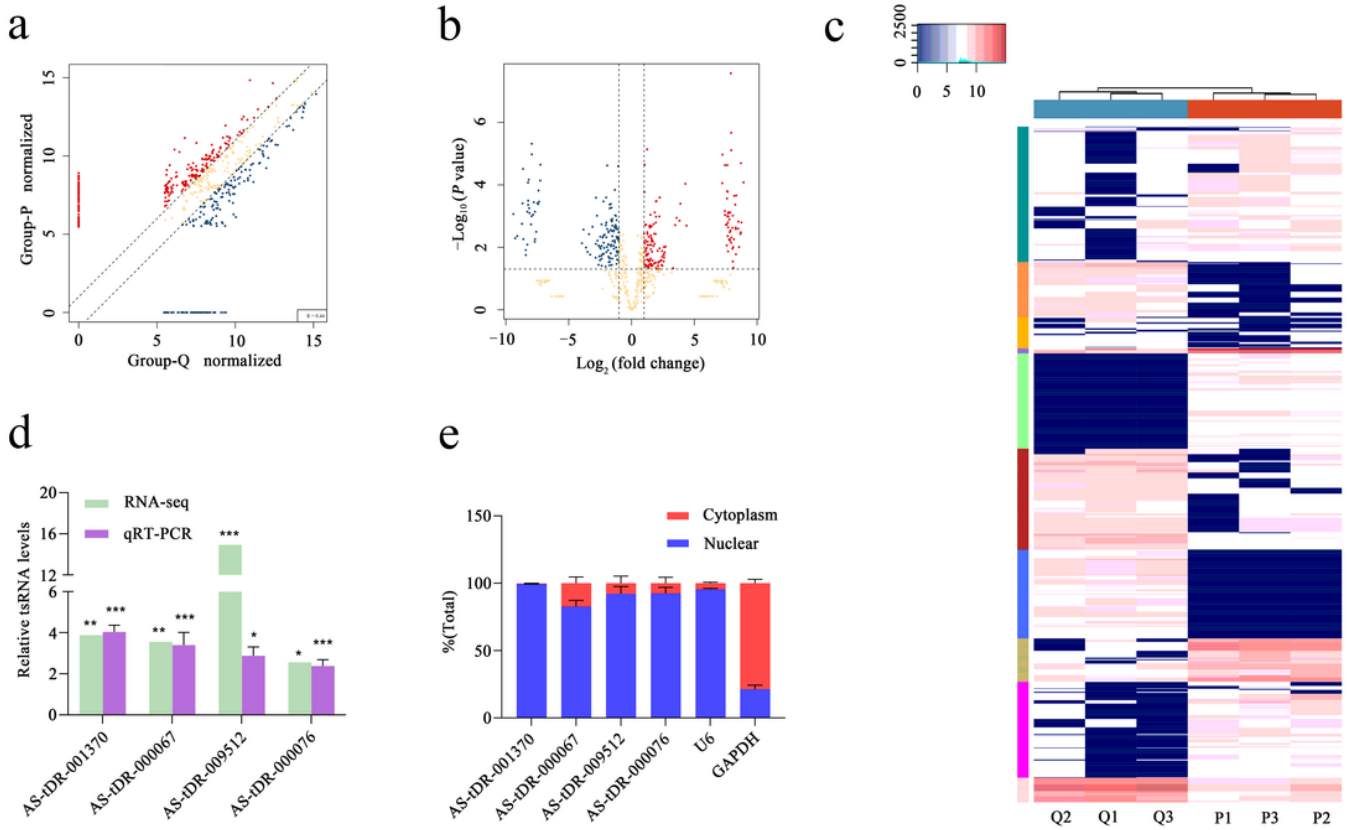

Figure 1

Expression profile of DEtsRNAs in proliferative and quiescent HASMCs. a Scatter plot shows the DEtsRNAs between proliferative and quiescent groups. X-axis and Y-axis values in the scatter plot are the average TPM (Transcripts Per Million) values (log2 scaled) of each group. Genes above the top line (red dots, up-regulation) or below the bottom line (green dots, down-regulation) indicate more than 2.0 fold change (default fold change value is 2.0) between two compared groups. Gray dots indicate tsRNAs without differential expression. b Volcano plot describes the DEtsRNAs between proliferative and quiescent groups. Values of the X-axis and Y-axis are fold change (log2-transformed) and p-value (-log10-transformed) between two groups. DEtsRNAs with statistical difference and more than 2.0 fold change are indicated by the red dots (up-regulation) or green dots (down-regulation); tsRNAs without statistical difference are indicated by the gray dots. c Hierarchical clustering was performed to analyze the expression of DEtsRNAs between proliferative and quiescent groups. Samples were arranged based on the coefficient of variation (CV) of tsRNAs on the TPM count, which facilitated the assumption of the relationship between them. The color in the panel represents the relative expression level (log2-transformed). Color scale is shown above: blue represents an expression level below the mean; red represents an expression level above the mean. P1, P2, and P3 represent the proliferative group; Q4, Q5, and Q6 represent the quiescent group. d Expression levels of DEtsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) in proliferative and quiescent HASMCs were validated by qRT-PCR, and results were consistent with sequencing. Control primers: hsa-U6. e DEtsRNAs (AS-tDR-001370, AS-tDR-000067, AS-tDR-009512, and AS-tDR-000076) are mostly located in the nucleus, which was confirmed by nuclear and cytoplasmic tsRNAs Fractionation experiment. U6 and GAPDH were applied as positive controls for the nucleus and cytoplasm, respectively. The results presented are representative of three independent experiments; bars: SD. *P < 0.05, **P < 0.01, ***P < 0.001.

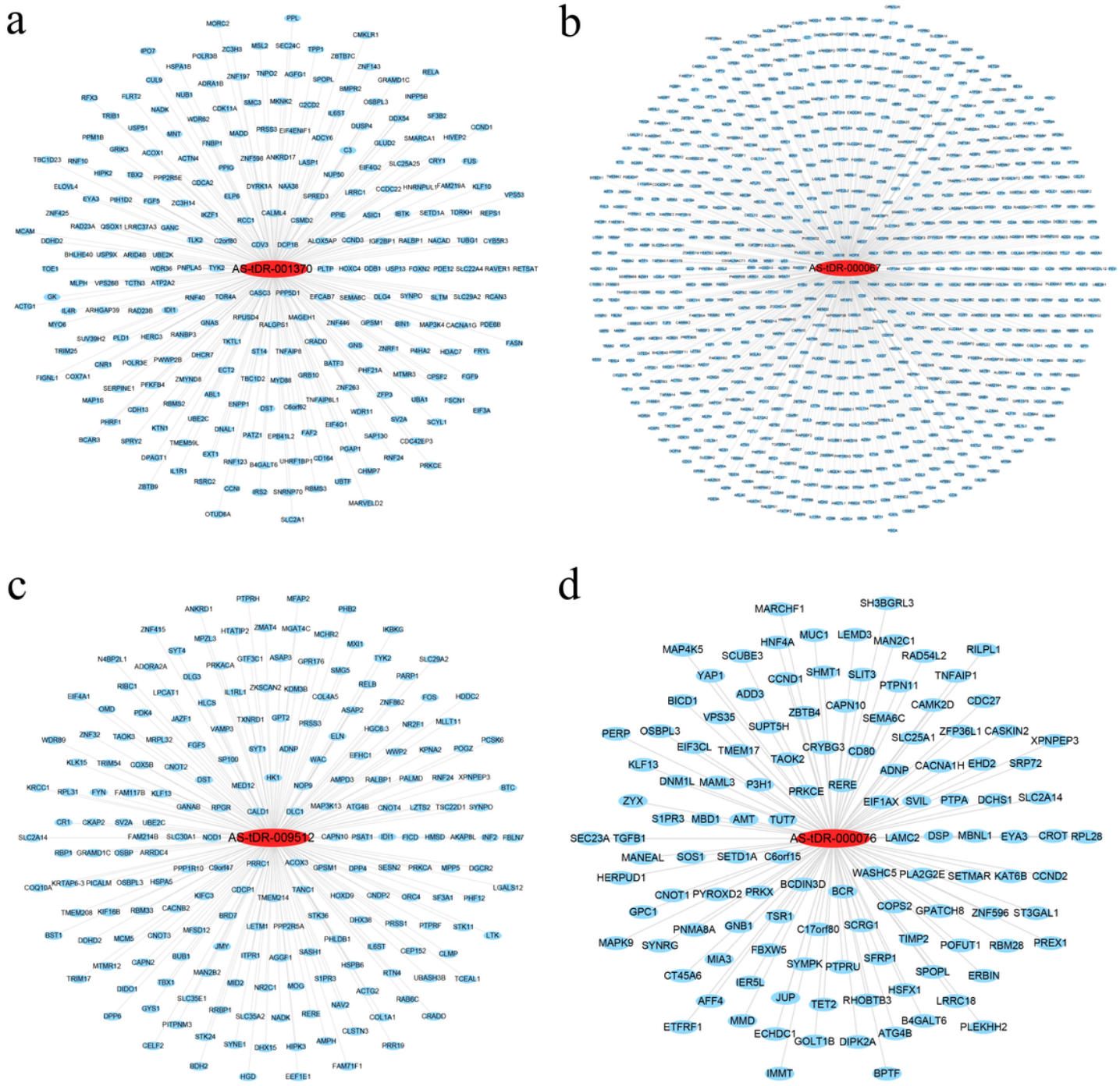

Figure 2

Interaction networks between DEtsRNAs and related genes containing target promoters in proliferative HASMCs. Subnetworks of (a) AS-tDR-001370, (b) AS-tDR-000067, (c) AS-tDR-009512, and (d) AS-tDR-000076. Red nodes: DEtsRNAs; blue nodes: genes containing target promoters.

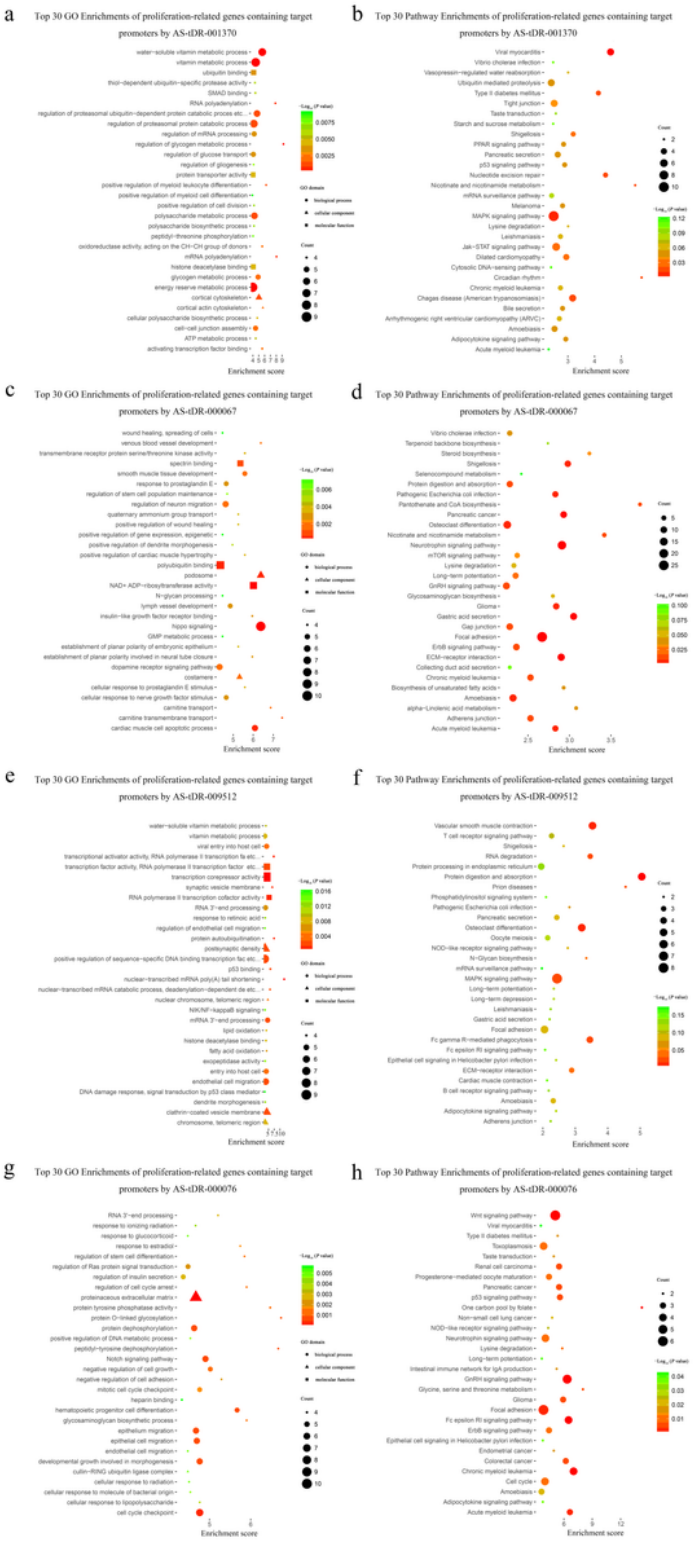

Figure 3

GO and KEGG analyses of genes containing target promoters of DEtsRNAs. Top 30 GO terms of genes containing target promoters of (a) AS-tDR-001370, (c) AS-tDR-000067, (e) AS-tDR-009512, and (g) AS-tDR-000076. Top 30 pathways of genes containing target promoters of (b) AS-tDR-001370, (d) AS-tDR-000067, (f) AS-tDR-009512, and (h) AS-tDR-000076.

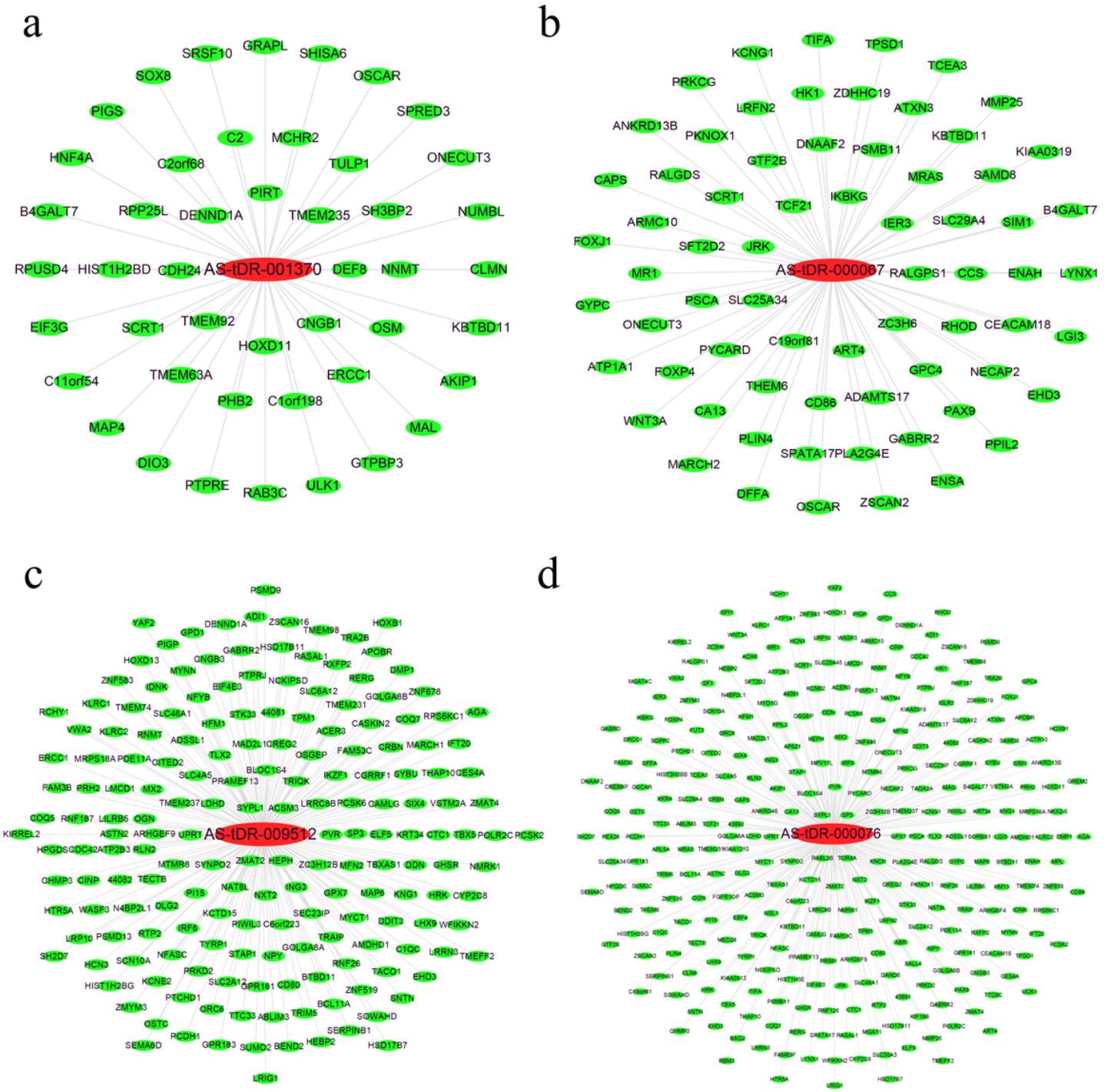

Figure 4

Interaction networks between DEtsRNAs and related target DEmRNAs in proliferative HASMCs. Subnetworks of (a) AS-tDR-001370, (b) AS-tDR-000067, (c) AS-tDR-009512, and (d) AS-tDR-000076. Red nodes: up-regulated DEtsRNAs; green nodes: down-regulated target DEmRNAs.

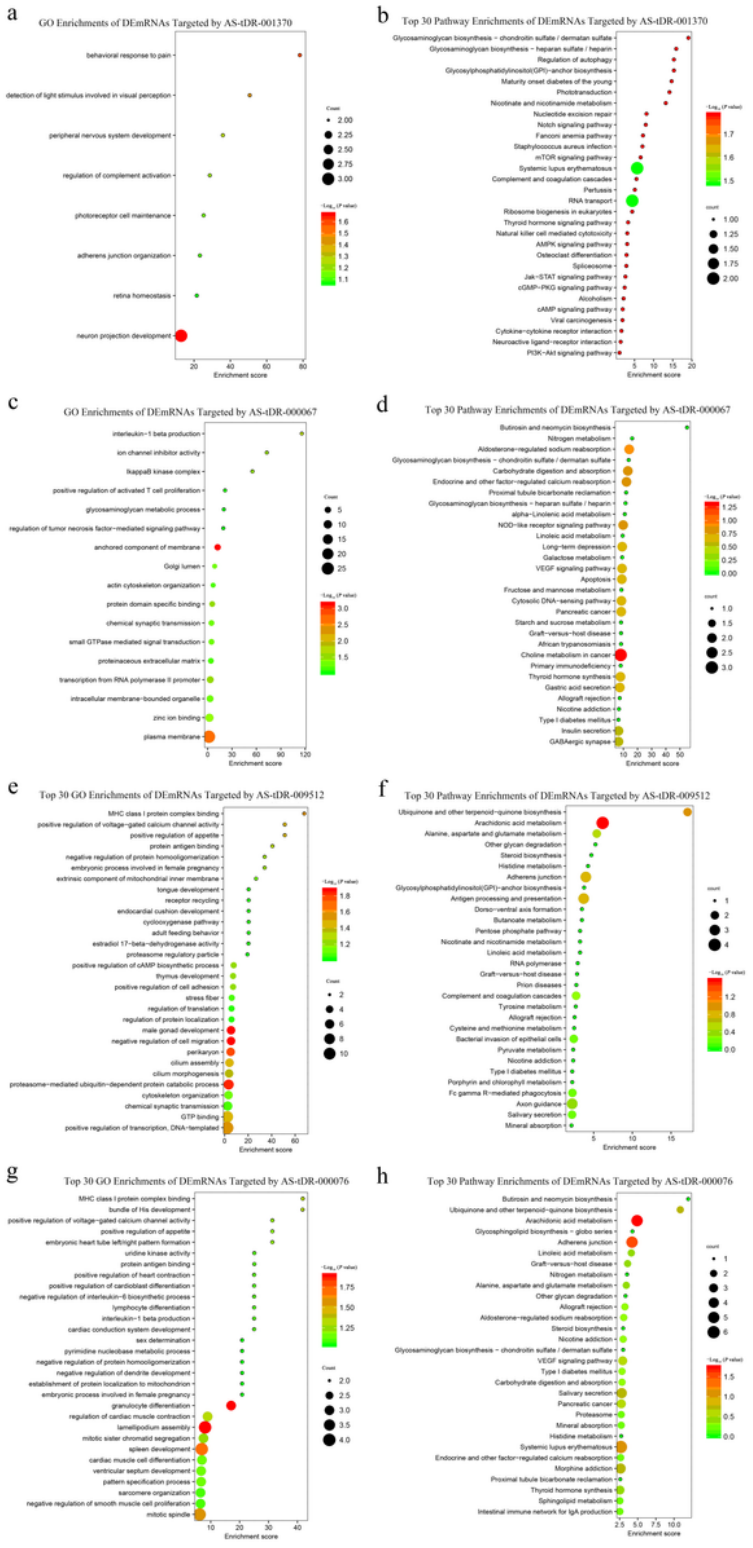

Figure 5

GO and KEGG analyses of target DEmRNAs of DEtsRNAs. GO terms of target DEmRNAs of (a) AS-tDR-001370, (c) AS-tDR-000067, (e) AS-tDR-009512, and (g) AS-tDR-000076. Pathways of target mRNAs of (b) AS-tDR-001370, (d) AS-tDR-000067, (f) AS-tDR-009512, and (h) AS-tDR-000076.

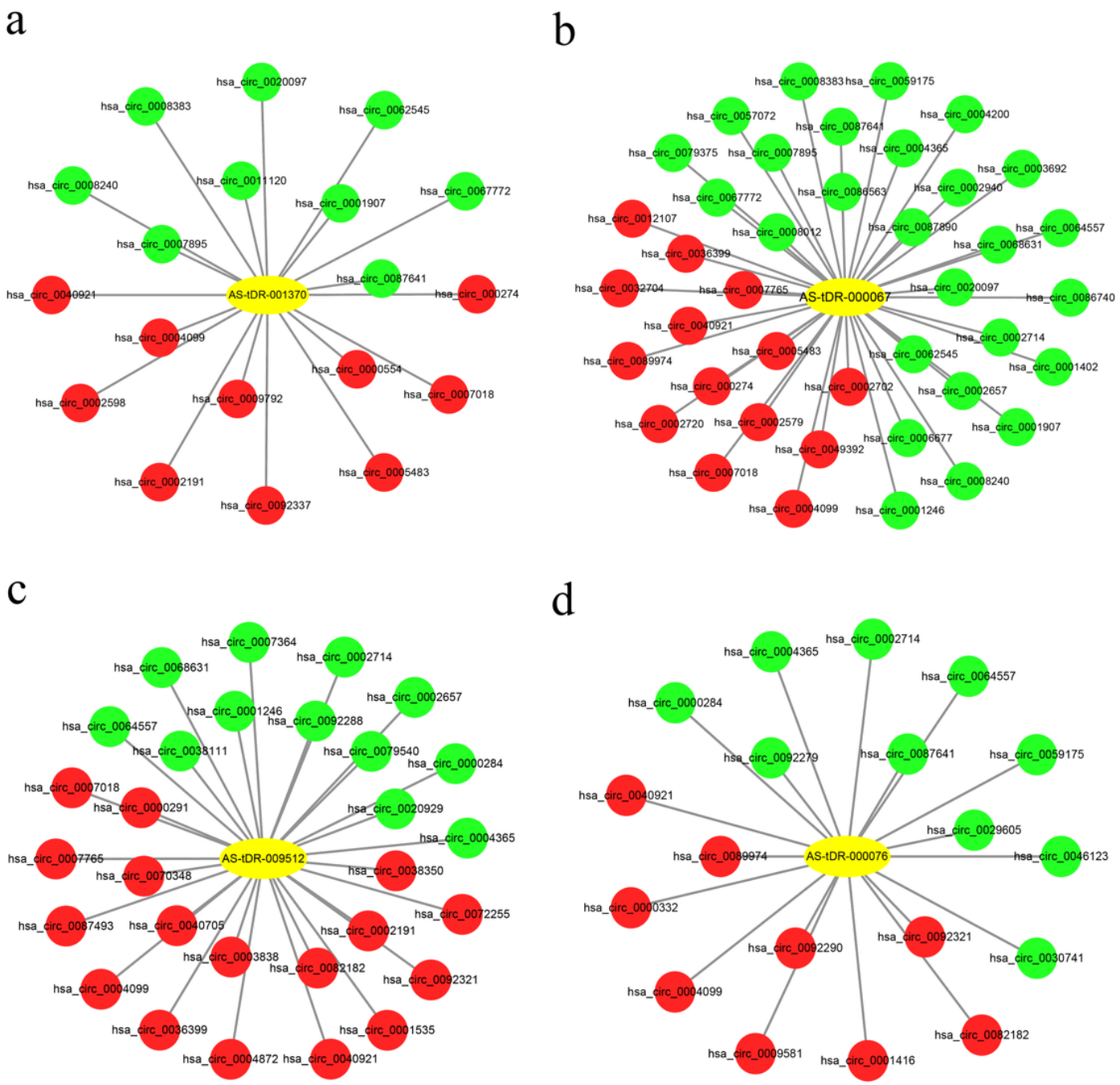

Figure 6

Interaction networks between DEtsRNAs and related target DEcircRNAs in proliferative HASMCs. Subnetworks of (a) AS-tDR-001370, (b) AS-tDR-000067, (c) AS-tDR-009512, and (d) AS-tDR-000076. Yellow nodes: up-regulated DEtsRNAs; red nodes: up-regulated target DEcircRNAs; green nodes: down-regulated target DEcircRNAs.

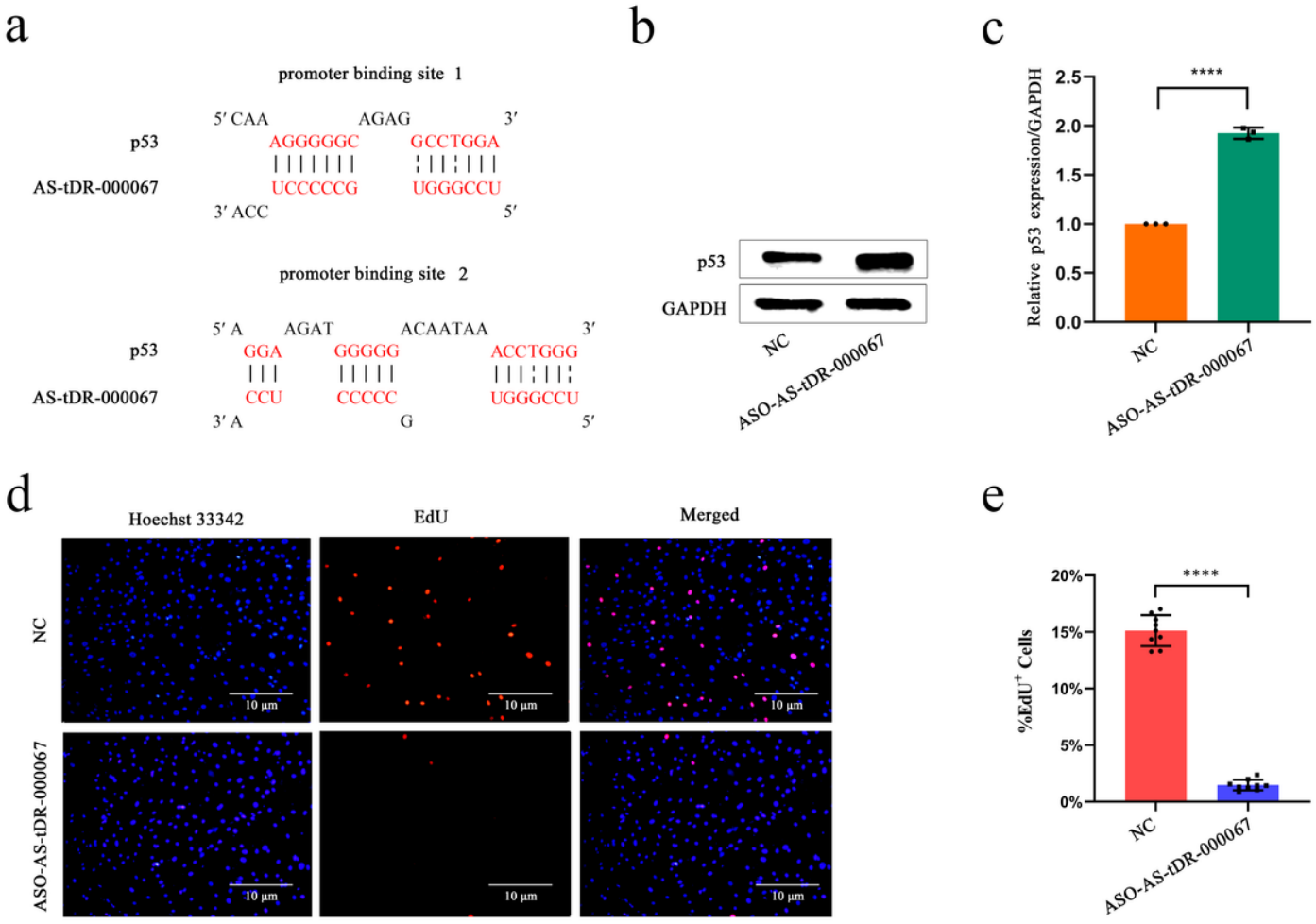

Figure 7

Knockdown of AS-tDR-000067 inhibits the proliferation of HASMCs. a The combination of AS-tDR-000067 with the promoter of p53 depends on 14 and/or 15 bp base pairing. b By western blot analysis, the expression of p53 was up-regulated in proliferative HASMCs transfected with ASO-AS-tDR-000067, comparing with NC. c Based on the NC group, the protein expression level of p53 detected by Western blot was analyzed. d EdU was used to detect HASMC proliferation viability. Red dots indicate EdU positive cells, blue dots indicate live cells stained with Hoechst 33342. e The percentage of EdU positive cells is in (d). The results presented are representative of three independent experiments; bars: SD. *P < 0.05, **P < 0.01, ***P < 0.001.

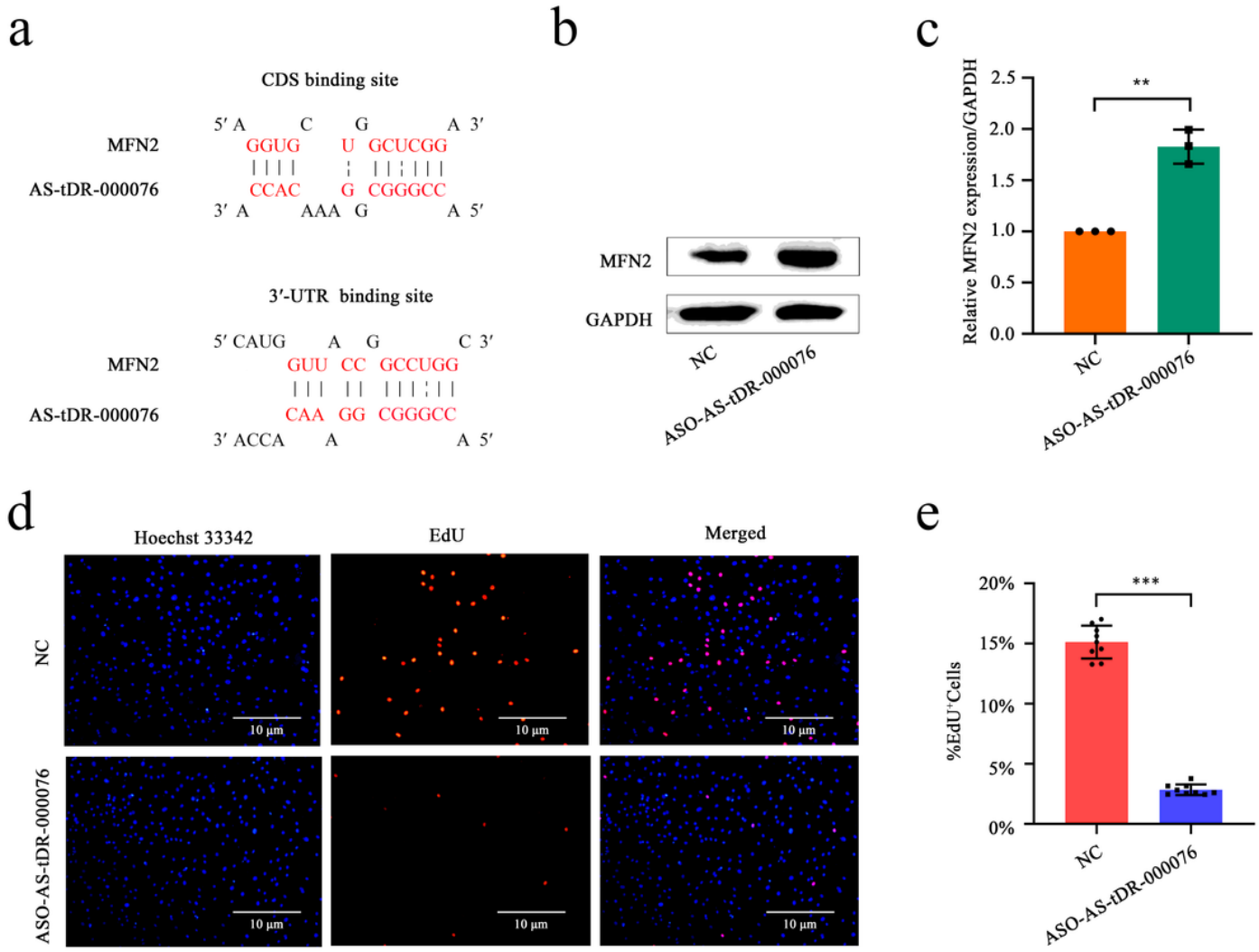

Figure 8

Knockdown of AS-tDR-000076 inhibits the proliferation of HASMCs. a The combination of AS-tDR-000076 with CDS and 3'-UTR of MFN2 depends on 11 bp base pairing. b By western blot analysis, the expression of MFN2 was up-regulated in proliferative HASMCs transfected with ASO-AS-tDR-000076, comparing with NC. c Based on the NC group, the protein expression level of MFN2 detected by Western blot was analyzed. d EdU was used to detect HASMC proliferation viability. Red dots indicate EdU positive cells, blue dots indicate live cells stained with Hoechst 33342. e The percentage of EdU positive cells is in (d). The results presented are representative of three independent experiments; bars: SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary Files

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