Differential expression and bioinformatics analysis of circRNA in PDGF-BB-induced vascular smooth muscle cells

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Abstract

Background: Atherosclerosis is mediated by various factors and plays an important pathological foundation for cardiovascular and cerebrovascular diseases. Abnormal VSMC proliferation and migration have an essential role in atherosclerotic lesion formation. Circular RNAs have been widely detected in different species and are closely related to various diseases. However, the expression profiles and molecular regulatory mechanisms of circRNAs in VSMCs are still unknown. Methods: We used high-throughput RNA-seq as well as bioinformatics tools to systematically analyze circRNA expression profiles in samples from different VSMC phenotypes. PCR, Sanger sequencing, and qRT-PCR were performed for circRNA validation. Results: A total of 22191 circRNAs corresponding to 6273 genes (host genes) in the PS, NC or both groups, were detected, and 112 differentially expressed circRNAs were identified between the PS and NC groups, of which 59 were upregulated and 53 were downregulated. We selected 9 circRNAs for evaluation of specific head-to-tail splicing, and 10 differentially expressed circRNAs between the two groups for qRT-PCR validation. Gene Ontology and KEGG pathway enrichment analyses revealed that the parental genes of the circRNAs mainly participated in cardiac myofibril assembly and positive regulation of DNA-templated transcription, indicating that they might be involved in cardiovascular diseases. Finally, we constructed a circRNA-miRNA network based on the dysregulated circRNAs and VSMC-related miRNAs. Conclusion: The current study is the first to show the differential expression of circRNAs in PDGF-BB-induced vascular smooth muscle cells and may provide new ideas and targets for the prevention and therapy of vascular diseases.

Background

Atherosclerosis (AS) is the main pathological basis of cardiovascular and cerebrovascular
diseases and is mediated by various factors. With socioeconomic development, the morbidity and mortality of cardiovascular diseases are increasing worldwide; consequently, cardiovascular disease has become one of the important diseases threatening public health, and its causes and pathomechanism are not yet clear[1].

Vascular smooth muscle cells (VSMCs) are the major cellular components of the blood vessel wall, where they exist in a differentiated contractile phenotype to respond to arterial contraction and to produce extracellular matrix (ECM)[2]. Accumulating evidence shows that abnormal VSMC proliferation and migration have an essential role in atherosclerotic lesion formation. Genetic lineage tracing studies have illustrated that in atherosclerotic plaques, especially progressing plaques, extensive lipids are released by damaged or dying macrophages and VSMCs. Then, accumulating lipid infiltration appeared in the center of the plaque, forming the necrotic core. Moreover, VSMCs migrate and proliferate to the surrounding of the necrotic core and play an important role in creating a fibrous cap that stabilizes the atherosclerotic plaque[3, 4]. Moreover, VSMCs can differentiate into many other cell types found in the plaque core, suggesting that these cells might participate in multiple processes underlying atherosclerotic plaque stability[5–8].

An increasing body of evidence shows that contractile VSMC genes down regulate stemming from injury and inflammation, which may through effecting the reduced expression of MYOC, a key factor regulating the contractile VSMC state in the development of plaque[5]. In vitro, studies have demonstrated that SMCs, stimulated by growth factors, oxidative stress, and inflammatory cytokines, can phenotypically switch into proliferating and/or migrating cells. Among them, PDGF-BB is considered to be one of the most effective mitogens in the proliferation and migration of VSMC, which can initiate various biological effects by activating intracellular signal transduction pathways and play
a significant role in regulating the proliferation and migration of VSMC\[9-11\]. Consequently, it will be necessary to find a new target to inhibit PDGF-mediated VSMC proliferation and migration which will exert an important therapeutic intervention in atherosclerosis development.

Noncoding RNAs (ncRNAs) are a group of biomolecules acting as pivotal regulators that play powerful and diverse roles in pathological and physiological processes [12]. Their gene expression patterns can also reveal changes in biological pathways that correlate with disease progression or even the risk of disease progression [13, 14]. Circular RNAs are an emerging group of ncRNAs that are ubiquitous, stable, and evolutionarily conserved in eukaryotes [15]. Though the phenomenon of RNA cyclization was first reported in the 1970s [16], circular RNAs were considered as byproducts of aberrant splicing during transcription and remained underappreciated. As RNA sequencing technologies evolve, accompanied by the development of computational algorithms, numerous circRNAs have been discovered[17]. Notably, circular RNAs have been found extensively in different species and are closely related to various diseases, including cardiopathy, which has a great impact on human health. [18–20]. Noncoding RNAs, especially microRNAs (miRNAs) and circRNAs, can function as competitive endogenous RNAs (ceRNAs) which can construt gene regulatory networks to regulate the expression of multiple genes with spatiotemporal specificity. Given the characteristics of ncRNAs, they could have great potential application in the treatment of diseases. However, the circRNA expression profiles and whether circRNAs participate in the regulatory of VSMCs still remain not clear.

In the present study, we aimed to perform high-throughput RNA sequencing in paired PDGF-BB-treated VSMCs and a normal control group to investigate VSMC-specific circRNA profiles, as well as potential functional characterization of the representative candidate circRNAs.
Methods

Cell cultures

Primary human aortic smooth muscle cells (HASMCs; ScienCell, USA) were cultured with Smooth Muscle Cell Medium (SMCM; ScienCell, USA) in a humidified incubator with 5% CO2 at 37 °C. HASMCs were inoculated at a density of $3 \times 10^5$ cells/well in 6-well culture plates overnight. Before the subsequent experiments, cells were made quiescent by starvation for 24 h and then treated with 10 ng/ml platelet-derived growth factor (PDGF-BB; Sigma-Aldrich) for 48 h. The blank control group did not receive PDGF-BB. Each group had three samples. Subsequently, morphological changes and the expression of specific genes, such as smooth muscle 22α (SM22α) and smooth muscle actin-α (α-SMA), were observed in the two groups.

RNA Library Construction And Sequencing

According to manufacturer's instructions, total RNA was isolated from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality, purity and integrity of total RNA are closely monitored. Then circRNA library was constructed. Firstly, approximately 5 µg RNA was subjected to ribosomal RNA depletion with the Ribo-Zero™ rRNA Removal Kit (Illumina, San Diego, USA). Secondly, linear RNAs were removed with RNase R (Epicentre Inc., Madison, WI, USA) to enrich circRNAs. Finally, the RNA fragmentation was obtained using divalent cations under high temperature for reverse-transcribed to generate first-stranded cDNA, then, the second-stranded DNAs were next synthesized with E. coli DNA polymerase I, RNase H and dUTP. To construct strand-specific cDNA, we added specificity terminal amino modification of the DNA fragment ends to prepare them for ligation to the adapters. After amplified by PCR, the library was purified and the average insert size was 300 bp (± 50 bp). Finally, paired-end were sequenced on an Illumina HiSeq 4000 (LC Bio,
Bioinformatics Analysis

Low-quality and undetermined bases was removed and sequence quality was verified using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then, we used Bowtie2 and Tophat2 to map reads to the reference genome. CIRCEExplorer and TopHat-fusion were utilized for de novo assembly of the mapped reads to circular RNAs and recognizing back splicing reads in unmapped reads. All samples generated unique circular RNAs. The differentially expressed circRNAs with log2 (fold change) > 1 or log2 (fold change) <-1 and with statistical significance (p value < 0.05) by R package-edgeR were selected for further studies.

CircRNA validation by PCR

Polymerase chain reaction (PCR) was used to validate the reliability of the high-throughput RNA sequencing data. A Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) was used for reverse transcription of circRNAs. cDNA and gDNA templates were PCR amplified for 35 cycles using Taq PCR MasterMix (Tiangen, China) following the manufacturer’s protocol, and PCR products were visualized using 2% GelRed-stained agarose gel. To confirm the PCR results, we further performed Sanger sequencing to directly examine the PCR product. To verify the accuracy of the differential expression of circRNAs, qRT-PCR was conducted using a FastStart Universal SYBR Green Master Kit (Roche, Germany) on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control, and PCR primers are listed in supplementary Table 1.

GO and KEGG pathway analyses

The differentially expressed circRNA-host gene data were analyzed by the DAVID tool.
(V6.8)[21] with its GO function enrichment and KEGG pathway analyses. An enrichment gene count ≥ 2 and hypergeometric test significance threshold P value < 0.05 were considered to indicate significant enrichment.

Interaction between circRNA and miRNA

VSMC-associated miRNAs were selected from disease-miRNA interactions validated in previous studies [22, 23]. A detailed list of the miRNAs is provided in Table 2. For the obtained VSMC-related miRNAs, we predicted whether there was a regulatory relationship between them and the selected differentially expressed circRNAs. We used miRanda and TargetScan to predict the relationships between the VSMC-related miRNAs and the differentially expressed circRNAs, and the Cytoscape tool was used to construct a network map of target miRNAs and circRNAs.

Table 2
Detailed list of the VSMC-associated miRNAs

| miRNA     | Target(s)                             | Role and function in SMC dynamics | References |
|-----------|---------------------------------------|-----------------------------------|------------|
| miRNA-214 | NCK associated protein 1 (NCKAP1)     | Migration, Proliferation, and Neointima Hyperplasia | [37]       |
| miRNA-130a| MEOX1                                 | Proliferation, migration           | [38]       |
| miRNA-675 | PTEN                                  | proliferation                      | [39]       |
| miR-221-222| CDKN1B, CDKN1C                        | Proliferation, migration, and anti-apoptotic effects | [40, 41] |
| miRNA-22-3p| High mobility group box-1 (HMGB1)    | Proliferation and Migration and Neointimal Hyperplasia | [42]       |
| miRNA-23b | The transcription factor forkhead box O4 (FoxO4) | phenotypic switching | [43]       |
| miRNA-195 | The Cdc42, cyclin D1, and fibroblast growth factor 1 (FGF1) genes | regulate cell phenotype and prevents neointimal formation. | [44]       |
| miR-206   | ARF6, SLC8A1                          | Differentiation                    | [45]       |
| miRNA-34a | Neurogenic locus notch homolog protein-1 (Notch1) | proliferation and migration | [46]       |
| miR-146a  | KLF4/5                                | Differentiation, proliferation     | [47]       |

Name                     | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|-------------------------|---------------------------|---------------------------|
| GAPDH                    | AAGGTCGGAGTCAACCGGA       | ATGGAAATTGCCATGGGT        |
| circRNA4452              | GTGGCCAGTACAAAGACA        | AGTATCTGCTGTCACCT         |
| circRNA13360             | CGAAAGTTCTTCAAAGG         | CTCGGAGCCTACATCTTT        |
| circRNA1698              | GCTCTCTCAGAACCAGAAC       | AGTGAATCTTCTTATGTGG       |
| circRNA8979              | GAGATCGTGAGTGTGA          | AGGGAAGAAGTGGCGT          |
| circRNA14411             | TCTCTCACAGCCATTCCA        | CTGGCAATGTTCCATCTC        |
| circRNA3041 | GATAAAAACCTTAAGGTTACATAAATCTG GCCATTTCCATTCTTTGCA |
| circRNA1848 | TGTTTCTTCTTCAATGACAATTTATTTTCA TGCCCTCTCCACTGAC |
| circRNA5780 | TGGCAGTGAAGAAGCGGAAAAGGCC TGAAGATGGGATGTCATG |
| circRNA536  | GCACGGAACACCAAGGATGTAATGTTATGTA CTTACGACACAGTG |
| circRNA2637 | TGCTTTTCTTCTTCAATGACAATTTATTTTCA TGCCCTCTCCACTGAC |
| circRNA2637 | CGACACCACTGCAATGAGGAAAGGCTC TACCTGACCTTTCAACCA |
| circRNA4624 | AGGATAATGCCCTGCTGAACACTCTGTACG TCTTGGAAATGATGGAG |
| circRNA4624 | GAATCTCTTCTTTGGATGACACCTGCCTCCTTCCACTGAC |
| circRNA4487 | CCACAACTGCATCAAAGACAGAA TCTGACCGGAAGGGATGTCATG |
| circRNA4487 | GAGCAAGTGTCAGCAAAAGTGAGTATGAAC TTCTTGGAAATGATGGAG |
| circRNA3875 | AAGTTCAGCAGTTCCTACCTCCA TGCGGTGATGACCGAC |
| circRNA3875 | ACAATGATGATGACCGAC |
| circRNA4209 | CAATGGTGGGTTTAACCTCCACTT |
| circRNA4209 | GTGCAACTCTGGCATG |
| circRNA5591 | CAATGGTGGGTTTAACCTCCACTT |
| circRNA5591 | CAGTGCAATTCAAGGAAAGTGAAGAAAGGTG |
| circRNA5550 | GCCAGATTTGCATAAGGC |
| circRNA5550 | CTGTCCCGGGAAGGGATCTTGGCAATGCT |
| circRNA5550 | CATTCGCAATGGTGGCAGT |
| circRNA5223 | GACACCCCTCTACAGTGACCTTGGCAATGCT |
| circRNA5223 | CATCACAAGGACTGGAGATG |
| RPL13A(◄►) | AAGCCAAGATGCTGGAGTGAAGCAGG |
| RPL13A(◄►) | AGAATGTGCAAGCAGCCTTGGT |

**Supplementary Table 1: Primers used in RT-PCR**

**Statistical analysis**

Data were analyzed and visualized with SPSS 22.0 (IBM Corporation, Somers, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Data are presented as the mean ± standard deviation. Wilcoxon rank-sum test, Student's t-test and fold change were used to analyze the significant differences between the sequencing data of samples. A t test was applied to compare qRT-PCR results. Differences with p < 0.05 were considered statistically significant.
Results

CircRNA expression profiles in PDGF-BB-treated VSMCs

Cells were treated with 10 ng/ml PDGF-BB (PS), and the blank control group did not receive PDGF-BB (NC). After stimulation for 48 h, morphological changes and the expression levels of SM22α and α-SMA in the two groups were detected and are shown in supplementary Fig. 1. As expected, VSMCs tended to phenotypically switch to function as proliferative and/or migratory cells in response to stimulation by PDGF-BB. The cell morphology became spindle-shaped and elongated, and the expression of differentiation-associated genes was decreased, suggesting that the cells had a stronger capacity for proliferation. To investigate the effects of PDGF-BB on circRNA expression in VSMCs, two groups of HASMCs were prepared for high-throughput sequencing using an Illumina HiSeq 4000 (LC Bio, China). We used CIRCExplorer to de novo assembly of the mapped reads and to identify back splicing reads. The following criteria were restricted for circRNA identification: 1) mismatch ≤ 2; 2) back-spliced junction reads ≥ 1; and 3) two splice sites ≥ 100 kb apart on the genome. Accordingly, a total of 22191 circRNAs, corresponding to 6273 genes (host genes) in the PS, NC or both groups, were detected, of which 7322 and 7870 circRNAs were specifically expressed in the NC and PS groups, respectively. A total of 6999 circRNAs were identified in both the PS and NC groups (Fig. 1A). Further analysis revealed that three categories of circRNAs were represented: exons (94.06%), introns (5.43%) and intergenic regions (0.50%) (Fig. 1B). The results showed that the majority of the circRNAs originated from protein-coding exons. By analyzing the sequencing data, we identified 5794 circRNAs recorded in circBase (http://circrna.org/) and 16397 novel circRNAs that were discovered in this study. The chromosomal distribution of all circRNAs showed that these circRNAs were distributed on almost all
human chromosomes (Fig. 1C).

By the criteria of log2 (fold change) > 1 or log2 (fold change) < -1 and p value < 0.05, we identified 112 differentially expressed circRNAs between the PS and NC groups, of which 59 were upregulated and 53 were downregulated. The DE circRNAs in the samples of the two groups are displayed with a Volcano plot, bar graph and heatmap. Additionally, the chromosome distributions of the DE circRNAs are shown in the bar graph. (Fig. 2A-D). The top ten upregulated and downregulated circRNAs are listed in Table 1.

| CircRNA Name | Gene symbol | Regulation | Annotation | Chromosome | Fold change (log2) | P value |
|--------------|-------------|------------|------------|-------------|-------------------|--------|
| circRNA12134 | AC004922.1  | up         | exon       | chr7        | 5.312             | 0.011  |
| circRNA6181  | SSH1        | up         | exon       | chr12       | 4.847             | 0.007  |
| circRNA7019  | APIG1       | up         | exon       | chr16       | 4.691             | 0.013  |
| circRNA7000  | SNTB2       | up         | exon       | chr16       | 4.646             | 0.016  |
| circRNA8379  | HMGCR       | up         | exon       | chr5        | 4.605             | 0.016  |
| circRNA10248 | CDR1        | up         | exon       | chrX        | 4.509             | 0.014  |
| circRNA10737 | HINFP       | up         | exon       | chr11       | 3.905             | 0.016  |
| circRNA13693 | SOX13       | up         | exon       | chr1        | 3.238             | 0.028  |
| circRNA13360 | GIGYF2      | up         | exon       | chr2        | 3.222             | 0.030  |
| circRNA8979  | HPCAL1      | up         | exon       | chr2        | 3.111             | 0.028  |
| circRNA5297  | TBCE        | down       | exon       | chr1        | -5.159            | 0.049  |
| circRNA876   | CCNY        | down       | exon       | chr10       | -4.855            | 0.000  |
| circRNA198   | PCCA        | down       | exon       | chr13       | -4.714            | 0.049  |
| circRNA5218  | NEK7        | down       | exon       | chr1        | -4.629            | 0.039  |
| circRNA3568  | PRELID2     | down       | exon       | chr5        | -4.602            | 0.019  |
| circRNA3679  | AFAP1       | down       | exon       | chr4        | -4.283            | 0.033  |
| circRNA1611  | IST1        | down       | exon       | chr16       | -4.270            | 0.009  |
| circRNA46    | SRF         | down       | intron     | chr6        | -4.130            | 0.004  |
| circRNA4487  | MTD1        | down       | exon       | chr2        | -4.052            | 0.008  |
| circRNA793   | ZMYND11     | down       | exon       | chr10       | -4.046            | 0.015  |

Validation of VSMC-enriched circRNAs

To verify the accuracy of the RNA-seq data, 9 circRNAs (circRNA2637, circRNA4624, circRNA4487, circRNA3875, circRNA4209, circRNA5591, circRNA5550, circRNA5497, and circRNA5223) were selected for validation experiments. We used polymerase chain reaction (PCR) to evaluate specific head-to-tail splicing. First, divergent (circular) and convergent (linear) circRNA-specific primers were designed for RT-PCR to verify that the selected candidate RNAs are indeed circRNAs. The results showed that the divergent primers produced amplicons from RNA-derived samples and not from genomic DNA. The
PCR products were visualized using 2% GelRed-stained agarose gel (Figure 3A). Then, Sanger sequencing was performed to validate the PCR product, and the head-to-tail splice junctions were identified, unambiguously demonstrating that the selected candidates were circRNAs (Figure 3B). Finally, ten circRNAs that were differentially expressed in the two groups were selected for qRT-PCR validation, and three biological replicates were performed. The results showed that the expression levels of circRNA-4452, circRNA-13360, circRNA-1698, circRNA-8979, and circRNA-14411 were significantly upregulated, and the expression levels of circRNA-3041, circRNA-5780, circRNA-1848, and circRNA-3875 were significantly downregulated. The qRT-PCR assay results were consistent with our RNA-seq assay results, confirming the accuracy of sequencing. However, circRNA-536 was not consistently and/or significantly differentially expressed between the two groups (Figure 3C).

GO and KEGG Analyses of the Dysregulated circRNA Parental Genes

Previous studies have demonstrated that circRNAs are closely related to their parental genes and have the ability to regulate their parental genes[24, 25]. Thus, to further investigate the panorama of circRNA functions and interactions in VSMCs, we performed GO function enrichment analysis and KEGG pathway enrichment analysis based on the significantly differentially expressed circRNA host genes. The results of GO enrichment analysis showed that 782 GO BP (biological process), 198 GO CC (cellular component) and 241 GO MF (molecular function) terms were enriched. The top 25 (GO BP), top 15 (GO CC) and top 10 (GO MF) are displayed in Figure 4A. GO enrichment showed that 66 genes were enriched in protein binding, 53 genes were expressed in the cytoplasm, and 16 genes were mainly associated with regulation of transcription, DNA-templated. In the GO category “cellular component”, the most significant terms were actin cytoskeleton, clathrin-coated vesicle and flotillin complex, while in the GO category “molecular
function”, the main molecular functions were actin binding, clathrin heavy chain binding, coenzyme binding, transcription factor activity, and RNA polymerase II transcription factor binding. In the GO category “biological process”, neural crest cell migration, positive regulation of transcription via serum response element binding, cardiac myofibril assembly, and positive regulation of DNA-templated transcription were the most representative significant terms. The top 20 GO terms are displayed in a scatter plot (Figure 4B). KEGG pathway enrichment analysis suggested that there were 81 signaling pathways related to the differentially expressed genes, among which the “RNA degradation” and the “phototransduction” signaling pathways were the most significant pathways. The top 20 pathways are shown in Figure 4C. The two most significant pathways are displayed in Figure 5.

Construction of a circRNA and disease-related miRNA network

To further explore the regulatory mechanism of the DE circRNAs and investigate the relationships between DE circRNAs and the function of VSMCs, we compared VSMC-associated miRNAs in validated disease-associated miRNA. Based on the ceRNA regulatory mechanism, TargetScan and miRanda software were used to analyze the miRNAs binding sites for DE circRNA. Finally, we constructed a circRNA-miRNA interaction network, 73 nodes (23 dysregulated circRNAs and 50 miRNAs) and 83 edges were identified in the circRNA–miRNA network (Figure 6). According to the network, we predicted that 12 down- and 11 upregulated circRNAs have miRNA binding sites that could act as ceRNAs participating in the regulation of posttranscriptional gene expression. Notably, we found that circRNA160 (hsa_circ_0008776) showed the highest degree of connectivity in the network, with up to 11 miRNA binding sites, followed by circRNA13360 (hsa_circ_0003341) and circRNA7637 (hsa_circ_0001222), indicating their potential important function in VSMCs and cardiovascular disease.
Discussion

AS is a complex pathological process characterized by endothelial dysfunction, lipid infiltration, oxidative stress, inflammation, cell proliferation and apoptosis[26, 27] During the development of atherosclerosis, the arterial wall is stimulated by multiple harmful conditions, such as hyperlipidemia, hypertension, diabetes, smoking, homocysteinemia, and other agents that may respond to multiple signaling molecules that interact with the lining of the endothelium, altering the homeostatic condition of the arterial wall and resulting in the migration and proliferation of VSMCs within the lesions[28]. It has been considered that phenotypic transformation of VSMCs is an important contributor to vascular disease development, including the pathologic process of atherosclerotic plaque development[29]. In recent years, through the combination of high-throughput sequencing and bioinformatics analysis, an increasing number of RNA categories and important potential targets for gene therapy have been discovered[30, 31]. A variety of studies confirmed a strongly association between circRNAs and cardiovascular disease[32]. Lesca M. et al. demonstrated that circANRIL could induce cell apoptosis and inhibit cell proliferation through inducing nucleolar stress and p53 activation[19]. Huang et al. found that circRNA Nfix regulated by superenhancers (SEs) acts as a pivotal element in regulating cardiac regeneration[20]. However, the expression profiles of circRNAs in different VSMC phenotypes are not yet known.

In this study, circRNA profiles in two groups of VSMCs were screened to identify the dynamically changed circRNAs in order to discover pivotal biomarkers for vascular biology. A total of 22191 circRNAs were identified in both the treatment and control groups. Currently, five classes of circRNAs have been detected, circular RNA genomes (viroid and hepatitis delta virus circles), circular RNA from introns, circular RNA intermediates in RNA processing reactions, circular RNAs from exons and circular RNAs in archaea with snRNP
Further analysis of the present study revealed that three categories of circRNAs were represented, among which exon-based circRNAs were the overwhelming majority (94.06%). After filtering with $|\log_2(\text{fold change})| > 1$ and $p < 0.05$, 112 circRNAs were found to be differentially expressed, of which 59 were upregulated and 53 were downregulated. Cluster analysis was used to display the differences of circRNA expression between the PDGF-treated and control groups. From the analysis, we found that the expression of circRNAs showed a significant difference between the treatment group compared to the control group. The results showed that different circRNAs are turned off and on in different cell states at different times and are involved in numerous metabolic processes. Such circRNAs may become novel prognostic markers for diseases. Then, we used qRT-PCR assay to verify the validity of the RNA sequencing findings. Ten of the DE circRNAs were selected for the analysis, and the results were quite consistent with the sequencing results, confirming the high reliability of the high-throughput sequencing data.

GO and KEGG pathway enrichment analyses of the host genes we detected showed that the parental genes of the circRNAs mainly participate in cardiac myofibril assembly and positive regulation of DNA-templated transcription, indicating that they might be involved in cardiovascular diseases. Moreover, according to the BP, CC, and MF terms with substantial enrichment, the genes were mainly associated with gene expression at the transcriptional or posttranscription level in the cytoplasm. The “RNA degradation” pathway is an important signaling pathway associated with many biological processes in eukaryotes.

A variety of studies have revealed that circRNAs can function as sponges for related miRNAs, thus playing a vital regulatory role in influencing physiological processes as well as various diseases, including cardiovascular disease, of which miRNAs act as indispensable bridges joining various RNAs[34, 35]. The circRNA-miRNA network has been
proven to be a widely accepted mechanism of gene expression regulation. Hence, a circRNA-miRNA network was constructed based on the dysregulated circRNAs and VSMC-related miRNAs. Among the altered circRNAs in the network, we found that circRNA160 (hsa_circ_0008776) showed the highest degree of connectivity throughout the network, with up to 11 miRNA binding sites, followed by circRNA13360 (hsa_circ_0003341) and circRNA7637 (hsa_circ_0001222). Additional analyses revealed that circRNA160, encoded by the parental gene THSD1 (thrombospondin type I domain 1), was significantly downregulated in PDGF-BB-treated VSMCs. Although the regulatory roles of circRNA160 in the proliferation or migration of VSMCs remain largely unknown, THSD1 is a novel regulator during vascular development and functions to protect the intraplaque microvasculature and prevent hemorrhaging in advanced atherosclerotic lesions [36]. The expression of THSD1 could be regulated, depending on activation by multiple microenvironmental factors. However, studies that elucidate the regulation of THSD1 are still lacking, and whether circRNA160 can regulate the transcription of its parental genes remains to be further studied.

Conclusions

In summary, the proliferation and migration of vascular smooth muscle cells are important contributing factors to vascular disease development, including the pathologic process of atherosclerotic plaque progression. In this study, we identified the differential expression of circRNA in PDGF-BB-induced vascular smooth muscle cells. A circRNA-miRNA network was constructed, and bioinformatics analysis suggested that circRNAs may play vital roles in the pathology of AS, especially at the posttranscriptional level. This is only the beginning towards a better understanding of the roles of circRNAs in VSMCs, and more functional experiments are still needed to confirm the precise molecular regulatory mechanisms of circRNA functions.
Abbreviations
AS: Atherosclerosis; VSMCs: Vascular smooth muscle cells; ECM: extracellular matrix; ncRNAs: noncoding RNAs; miRNA: microRNA; circRNA: circular RNA; ceRNAs: competitive endogenous RNAs; SM22α: smooth muscle 22α; α-SMA: smooth muscle actin-α; DE circRNAs: differentially expressed circRNAs

Declarations

Authors’ contributions
Jiangtian Tian and Yahong Fu performed bioinformatics analyses and wrote the paper; Qi Li and Ying Xu designed the primers and conducted qRT-PCR assays; Xiangwen Xi and Yuqi Zheng performed cell experiments; Li Yu and Zhongzhuo Wang analyzed the data; Jinwei Tian and Bo Yu conceived of, designed, and supervised the study. All authors read and approved the final manuscript.

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Not applicable

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Supplemental Figure

Not provided with this version of the manuscript.

**Supplementary Figure 1: VSMCs were treated with PDGF-BB or the control. (A)** and **(B)** show the morphological changes and the expression of SM22α and α-SMA in the two groups. VSMCs tended to phenotypically switch to function as proliferative and/or migratory cells in response to stimulation by PDGF-BB. The cell morphology became spindle-shaped and elongated, and the expression of differentiation-associated genes was decreased, indicating that the cells had a stronger capacity for proliferation.

Figures
Overview of the identified circular RNAs (circRNAs) in PDGF-BB-treated VSMCs (PS) and controls (NC). (A) The Venn diagram shows the number of unique and common circRNAs in PS and NC. (B) The pie chart displays the ratio of circRNAs originating from exonic, intronic, and intergenic regions. (C) Chromosomal distribution of all identified circRNAs.
Differentially expressed (DE) circRNAs in PS and NC. (A) and (B) display the volcano plot and bar graph for the DE circRNAs in the samples of the two groups. (C) The chromosome distributions of differentially expressed circRNAs. (D) The DE circRNAs and samples are coclustered by hierarchical clustering analysis. The upper maps are based on DE circRNAs with log2 (fold change) >1 or log2 (fold change) <-1 and p value < 0.05 for the comparisons of PS vs NC.
Validation of VSMC-enriched circRNAs. (A) Nine circRNAs were selected for validation experiments. RT-PCR with divergent (circular) and convergent (linear)
primers was used to confirm the candidate circRNAs. Divergent (circular) primers (↔) successfully amplified a single fragment at the expected sizes from cDNA but not from genomic DNA (gDNA). Convergent (linear) primers (►◄) could amplify from both cDNA and gDNA. (B) Sanger sequencing of the selected candidate circRNAs shows the back-splice junctions. (C-D) The relative expression levels of 10 DE circRNAs were determine by qRT-PCR. The data are presented as the mean ± SD, n=3. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 4

GO and KEGG pathway analysis of the parental genes of the identified circRNAs.

(A) The top 25 GO BP, top 15 GO CC and top 10 GO MF are displayed. (B) The top 20 most represented significant GO terms are displayed in a scatter plot. (C) The top 20 enriched KEGG pathways of the circRNA parental genes.
Figure 5

Genes mapped to KEGG pathways by pathway analysis. (A) and (B) show the signaling pathways “RNA degradation” and “phototransduction”, which were the most significant pathways.
The generated network consists of 73 nodes (23 dysregulated circRNAs and 50 miRNAs) and 83 edges. According to the network, we predicted that 12 down- and 11 upregulated circRNAs have miRNA binding sites and could act as ceRNAs to regulate posttranscriptional gene expression.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

supplementary Table1.docx