Transcriptome-wide map of m6A circRNAs identified in a rat model of hypoxia mediated pulmonary hypertension

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Abstract

Background: Hypoxia mediated pulmonary hypertension (HPH) is a lethal disease and lacks effective therapy. CircRNAs play significant roles in physiological process. Recently, circRNAs are found to be m 6 A-modified. The abundance of circRNAs was influenced by m 6 A. Furthermore, the significance of m 6 A circRNAs has not been elucidated in HPH yet. Here we aim to investigate the transcriptome-wide map of m 6 A circRNAs in HPH. Results: Differentially expressed m 6 A abundance was detected in lungs of HPH rats. M 6 A abundance in circRNAs was significantly reduced in hypoxia in vitro. M 6 A circRNAs were mainly from protein-coding genes spanned single exons in control and HPH groups. Moreover, m 6 A influenced the circRNA–miRNA–mRNA co-expression network in hypoxia. M 6 A circXpo6 and m 6 A circTmtc3 were firstly identified to be downregulated in HPH. Conclusion: Our study firstly identified the transcriptome-wide map of m 6 A circRNAs in HPH. M 6 A can influence circRNA–miRNA–mRNA network. Furthermore, we firstly identified two HPH-associated m 6 A circRNAs: circXpo6 and circTmtc3. However, the clinical significance of m 6 A circRNAs for HPH should be further validated. Key words: m 6 A circRNAs; hypoxia mediated pulmonary hypertension; m 6 A circXpo6; m 6 A circTmtc3

Background

Pulmonary hypertension (PH) is a lethal disease and defined as an increase in the mean pulmonary arterial pressure ≥ 25 mmHg at rest, as measured by right heart catheterization [1]. Hypoxia mediated pulmonary hypertension (HPH) belongs to group III PH according to the comprehensive clinical classification of PH, normally accompanied by severe chronic obstructive pulmonary disease (COPD) and interstitial lung diseases [2]. HPH is a progressive disease induced by chronic hypoxia [1]. Chronic hypoxia triggers over-proliferation of pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs), and activation of quiescent fibroblasts, the hallmark of HPH [1, 3]. The pathological characteristics of HPH are pulmonary vascular remolding, pulmonary hypertension, and right ventricular hypertrophy (RVH) [4]. So far there is no effective therapy for HPH [2]. More effective therapeutic targets are needed to be discovered.

Circular RNAs (circRNAs) were firstly found abundant in eukaryotes using RNA-seq approach [5-7]. Pre-mRNA is spliced with the 5’ and 3’ ends, forming a ‘head-to-tail’ splice junction, then circRNAs are occurred [5]. According to the genome origin, circRNAs may be classified into four different subtypes: exonic circRNA, intronic circRNA, exon–intron circRNA and tRNA introns circRNA [5]. CircRNAs are reported to play crucial roles in miRNA binding, protein binding, regulation of transcription, and post-transcription [5, 8]. Recent reports indicated that circRNAs can translate to proteins [8, 9]. Moreover, circRNAs are widely expressed in human umbilical venous endothelial cells when stimulated by hypoxia [10, 11]. Up to date, only a few reports mentioned PH-associated circRNAs. CircRNAs expression profile is demonstrated in HPH and chronic thromboembolic pulmonary hypertension [12]. However, the post-transcript modification of circRNAs in HPH is still unknown.
N⁶-methyladenosine (m⁶A) is regarded as one part of “epitranscriptomics” and identified as the most universal modification on mRNAs and noncoding RNAs (ncRNAs) in eukaryotes [13, 14]. DRm⁶ACH (D denotes A, U or G; R denotes A, G; H denotes A, C, or U) is a consensus motif occurred in m⁶A modified RNAs [15-17]. M⁶A modification is mainly enriched around the stop codons, at 3’untranslated regions and within internal long exons [17-19]. Several catalyzed molecules act as “writers”, “readers”, and “erasers” to regulate the m⁶A modification status [14]. The methyltransferase complex is known as writers, including methyltransferase-like-3, -14 and -16 (METTL3/METTL14/METTL16), Wilms tumor 1-associated protein (WTAP), RNA binding motif protein 15 (RBM15), vir like m⁶A methyltransferase associated (KIAA1429) and zinc finger CCCH-type containing 13 (ZC3H13), appending m⁶A on DRACH [17, 20, 21]. METTL3 is regarded as the core catalytically active subunit, while METTL14 and WTAP play a structural role in METTL3’s catalytic activity [18, 22]. The “readers”, the YT521-B homology (YTH) domain-containing proteins family includes YTHDF (YTHDF1, YTHDF2, YTHDF3), YTHDC1, and YTHDC2, specifically recognizes m⁶A and regulates splicing, localization, degradation and translation of RNAs [14, 22, 23]. The YTHDF1 and YTHDF2 crystal structures forms an aromatic cage to recognize m⁶A sites in cytoplasm [24]. YTHDC1 is the nuclear reader and YTHDC2 binds m⁶A under specific circumstances or cell types [24]. Hypoxia may alter the balance of writers-erasers-readers and induce tumor growth, angiogenesis, and progression [25, 26].

Interestingly, circRNAs can be m⁶A-modified. M⁶A circRNAs displayed cell-type-specific methylation patterns in human embryonic stem cells and HeLa cells [14]. CircRNAs contained m⁶A modifications are likely to promote protein translation in a cap-independent pattern [9]. However, m⁶A circRNAs has not been elucidated in HPH yet. Here we are the first to identify the expression profiling of m⁶A circRNAs in HPH.

Results

M⁶A level of circRNAs was reduced in HPH rats and most circRNAs contained one m⁶A peak

3 weeks treatment by hypoxia resulted in right ventricular systolic pressure (RVSP) elevating to 42.23 ± 1.96 mmHg compared with 27.73 ± 1.71 mmHg in the control ($P < 0.001$, Figure 1A and 1B). The ratio of the right ventricle (RV), left ventricular plus ventricular septum (LV + S) [RV/ (LV + S)] was used as an index of RVH. RVH was indicated by the increase of RV/ (LV + S) compared with the control (0.25 ± 0.03 vs. 0.44 ± 0.04, $P = 0.001$, Figure 1C). The medial wall of the pulmonary small arteries was also significantly thickened (19.28 ± 2.19% vs. 39.26 ± 5.83%, $P < 0.001$, Figure 1D and 1E). Moreover, in the normoxia group, 53.82 ± 3.27% of the arterioles were non-muscularized (NM) vessels, and 25.13 ± 1.83% were fully muscularized (FM) vessels. In contrast, partially muscularized vessels (PM) and FM vessels showed a greater proportion (32.88 ± 3.15% and 41.41 ± 3.35%) in HPH rats, while NM vessels occupied a lower proportion (25.71 ± 2.55%) (Figure 1F). Figure 1G displayed the heatmap of m⁶A circRNAs
expression profiling in N and HPH. $\text{m}^6\text{A}$ abundance in 166 circRNAs was significantly upregulated. Meanwhile, $\text{m}^6\text{A}$ abundance in 191 circRNAs was significantly downregulated (Additional file 1: Data S1, filtered by fold change $\geq 4$ and $P \leq 0.00001$). Lungs of N and HPH rats were selected to measure $\text{m}^6\text{A}$ abundance in purified circRNAs. The $\text{m}^6\text{A}$ level in total circRNAs isolated from lungs of HPH rats was lower than that from controls (Figure 1H). Moreover, over 50% circRNAs contained only one $\text{m}^6\text{A}$ peak either in lungs of N or HPH rats (Figure 1I).

**$\text{m}^6\text{A}$ circRNAs were mainly from protein-coding genes spanned single exons in N and HPH groups**

We analyzed the distribution of the parent genes of total circRNAs, $\text{m}^6\text{A}$-circRNAs, and non-$\text{m}^6\text{A}$ circRNAs in N and HPH, respectively. N and HPH groups showed a similar genomic distribution of $\text{m}^6\text{A}$ circRNAs and non-$\text{m}^6\text{A}$ circRNAs (Figure 2A and 2B). Moreover, about 80% of $\text{m}^6\text{A}$ circRNAs and non-$\text{m}^6\text{A}$ circRNAs were derived from protein-coding genes in both groups. A previous report indicated that most circRNAs originated from protein-coding genes spanned two or three exons [14]. While in our study, over 50% and 40% of total circRNAs from protein-coding genes spanned one exon in N and HPH groups, respectively (Figure 2C and 2D). Similarly, $\text{m}^6\text{A}$ circRNAs and non-$\text{m}^6\text{A}$ circRNAs were mostly encoded by single exons. Therefore, it was indicated that $\text{m}^6\text{A}$ methylation was abundant in circRNAs originated from single exons in N and HPH groups.

**The distribution and functional analysis for host genes of circRNAs with differentially expressed $\text{m}^6\text{A}$ peaks**

The length of differentially-expressed $\text{m}^6\text{A}$ circRNAs was mostly enriched in 1-10000 bps (Figure 3A). The host genes of upregulated $\text{m}^6\text{A}$ circRNAs were located in chromosome 1, 2 and 10, while the downregulated parts were mostly located in chromosome 1, 2 and 14 (Figure 3B).

Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed to explore the host genes of circRNAs with differentially-expressed $\text{m}^6\text{A}$ peaks. In the GO analysis (Figure 3C, left), the parent genes of circRNAs with upregulated $\text{m}^6\text{A}$ peaks were enriched in the protein modification by small protein conjugation or removal and macromolecule modification process in the biological process (BP). Organelle and membrane-bounded organelle were also the two largest parts in the cellular component (CC) analysis. Binding and ion binding were the two main molecular functions (MF) analysis. The top 10 pathways from KEGG pathway analysis were selected in the bubble chart (Figure 3C, right). Among them, the oxytocin signaling pathway, protein processing in endoplasmic reticulum and cGMP-PKG signaling pathway were the top 3 pathways involved. In addition, vascular smooth muscle contraction pathway was the most associated pathway in PH progression [27].
In Figure 3D left, the parent genes of circRNAs with downregulated m$^6$A peaks were mainly enriched in the cellular protein modification process and protein modification process in BP. Organelle and membrane-bounded organelle made up the largest proportion in the CC classification. The MF analysis was focused on receptor signaling protein activity and protein binding. The parent genes of circRNAs with decreased m$^6$A peaks were mainly involved in the tight junction and lysine degradation in the KEGG pathway analysis (Figure 3D, right).

Hypoxia can influence the m$^6$A level of circRNAs and circRNAs abundance

360 m$^6$A circRNAs were shared in N and HPH groups. 49% of m$^6$A circRNAs detected in N group were not detected in HPH group, and 54% of m$^6$A circRNAs detected in HPH group were not detected in N group (Figure 4A). To explore whether m$^6$A methylation would influence circRNAs expression level, expression of the 360 common m$^6$A circRNAs were identified. More circRNAs tended to decrease in HPH compared to N (Figure 4B). Moreover, expression of m$^6$A circRNAs was significantly downregulated compared with non-m$^6$A circRNAs in hypoxia, suggesting that m$^6$A may downregulate the expression of circRNAs in hypoxia (Figure 4C, $P = 0.0465$).

Construction of a circRNA–miRNA–mRNA co-expression network in HPH

We found 76 upregulated circRNAs with increased m$^6$A abundance, and 107 downregulated circRNAs with decreased m$^6$A abundance (Figure 5A, Additional file 2: Data S2). As known, circRNAs were mostly regarded as a sponge for miRNAs and regulated the expression of corresponding target genes of miRNAs [28]. To explore whether circRNAs with differentially-expressed m$^6$A abundance influence the availability of miRNAs to target genes, we selected differentially-expressed circRNAs with increased or decreased m$^6$A abundance. GO enrichment analysis and KEGG pathway analysis were also performed to analyze target mRNAs. Target mRNAs displayed similar GO enrichment in the two groups (Figure 5B and 5C). Two main functions were determined in BP analysis: positive regulation of biological process and localization. Intracellular and intracellular parts make up the largest proportion in CC part. Target mRNAs were mostly involved in protein binding and binding in MF part. In the KEGG pathway analysis, the top 10 most enriched pathways were selected (Figure 5D and 5E). Wnt and FoxO signaling pathways were reported to be involved in PH progression [29-31]. Then, we analyzed the target genes involved in these two pathways. SMAD4 was associated with PH and involved in Wnt signaling pathways. MAPK3, SMAD4, TGFBR1, and CDKN1B were involved in FoxO signaling pathways. To explore the influence of circRNA-miRNA regulation on PH-associated genes expression, we constructed a circRNA-miRNA-mRNA network, integrating matched expression profiles of circRNAs, miRNAs and mRNAs (Figure 5F and 5G). MicroRNAs sponged by the target genes of interest were analyzed. MiR-125a-3p, miR-23a-5p, miR-98-5p, let-7b-5p, let-7a-5p, let-7g-5p, and miR-205 were analyzed because they were reported to be associated with PH [32,
We filtered the key mRNAs and miRNAs, and founded that the two circRNAs were the most enriched, which were originated from chr1:204520403-204533534- (Xpo6) and chr7:40223440-40237400- (Tmtc3).

**M^6^A circXpo6 and m^6^A circTmtc3 were downregulated in PASMCs and PAECs in hypoxia**

M^6^A abundance was significantly reduced in PASMCs and PAECs when exposed to hypoxia (0.107% ± 0.007 vs. 0.054% ± 0.118, \( P = 0.023 \) in PASMCs; 0.114% ± 0.011 vs. 0.059% ± 0.008, \( P = 0.031 \) in PAECs, **Figure 6A**). M^6^A abundance in circRNAs was lower than it in mRNAs (0.1–0.4%) \([17, 18] \). Next, we confirmed the back-splicing of circXpo6 and circTmtc3 by CIRI software. The sequence of linear Xpo6 and Tmtc3 mRNA was analyzed. Then we identified that circXpo6 was spliced form exon 7, 8, and 9 of Xpo6. CircTmtc3 was spliced form exon 8, 9, 10, and 11 (**Figure 6B**). Using cDNA and genomic DNA (gDNA) from PASMCs and PAECs as templates, circXpo6 and circTmtc3 were only amplified by divergent primers in cDNA, while no product was detected in gDNA (**Figure 6C**). To identify whether circXpo6 and circTmtc3 were modified by m^6^A, we performed M^6^A RNA Immunoprecipitation (MeRIP)-RT-PCR and MeRIP-quantitative RT-PCR (MeRIP-qRT-PCR) to detect the expression of circXpo6 and circTmtc3 (**Figure 6D and 6E**). m^6^A circXpo6 and m^6^A circTmtc3 were significantly decreased in PASMCs and PAECs when exposed to hypoxia (\( P = 0.002, \) and \( P = 0.015 \) in PASMCs and \( P = 0.02, \) and \( P = 0.047 \) in PAECs).

**Discussion**

In this study, we identified the transcriptome-wide map of m^6^A circRNAs in hypoxia mediated pulmonary hypertension. On the whole, we found that m^6^A level in circRNAs was reduced in lungs when exposed to hypoxia. M^6^A circRNAs were mainly derived from single exons of protein-coding genes in N and HPH. M^6^A abundance in circRNAs was downregulated in hypoxia in vitro. M^6^A influenced the circRNA–miRNA–mRNA co-expression network in hypoxia. Moreover, circXpo6 and circTmtc3 were the novel identified circRNAs modified by m^6^A in hypoxia mediated pulmonary hypertension.

M^6^A plays important roles in various biological processes. M^6^A is associated with cancer progression, promoting the proliferation of cancer cells and contributing to the cancer stem cell self-renewal \([18, 21] \). Lipid accumulation was reduced in hepatic cells when m^6^A abundance in peroxisome proliferator-activator (PPaR) was decreased \([34] \). Enhanced m^6^A level of mRNA contributed to compensated cardiac hypertrophy \([35] \). Also, m^6^A modification of large intergenic noncoding RNA 1281 was necessary for mouse embryonic stem cells differentiation \([36] \).

Although it has been reported that m^6^A mRNAs were influenced by hypoxia, there is no report about m^6^A circRNAs in HPH yet. Up to now, no consistent conclusion was reached about the link between m^6^A and hypoxia. Previous reports found that the m^6^A abundance in mRNA was increased under hypoxia stress in HEK293T cells and cardiomyocytes \([37, 38] \). The increased m^6^A level stabilized the mRNA of Glucose
Transporter 1 (Glut1), Myc proto-oncogene bHLH transcription factor (Myc), Dual Specificity Protein Phosphatase 1 (Dusp1), Hairy and Enhancer of Split 1 (Hes1), and Jun Proto-Oncogene AP-1 Transcription Factor Subunit (Jun) without influencing their protein level [37]. In contrast, another reported that m^6^A level of total mRNA was decreased when human breast cancer cell lines were exposed to 1% O_2 [26]. Hypoxia increased demethylation by stimulating hypoxia-inducible factor (HIF)-1α- and HIF-2α–dependent over-expression of ALKBH5 [26]. In addition, transcription factor EB activates the transcription of ALKBH5 and downregulates the stability of METTL3 mRNA in hypoxia/reoxygenation-induced autophagy in ischemic diseases [38]. Our study found that m^6^A abundance in total circRNAs was decreased in hypoxia exposure. Moreover, our study indicated that circXpo6 and circTmtc3 were the novel identified circRNAs modified by m^6^A in HPH. M^6^A abundance in circXpo6 and circTmtc3 was decreased in hypoxia. It is probably because of HIF-dependent and ALKBH5-mediated m^6^A demethylation [26].

Previous reports indicated that m^6^A methylation close to 3'UTR and stop codon of mRNA is inversely correlated with gene expression [14, 39]. Low m^6^A level is negatively associated with circRNAs expression, while high m^6^A level is not linked to circRNAs expression in human embryonic stem cells and HeLa cells [14]. Consistent with the previous reports [14, 39], our study found that m^6^A reduced the total circRNAs abundance in hypoxia. The association between m^6^A level and specific gene abundance is remained as an open question. Some previous reports indicated that m^6^A level was positively associated with long non-coding RNA (lncRNA) or mRNA expression [40, 41]. M^6^A was positively associated with RP11-138 J23.1 (RP11) expression when ALKBH5 was overexpressed in colorectal cancer [40]. mRNAs were downregulated after METTL14 deletion in b-cells [41]. On the contrary, another reports insisted that m^6^A level was negatively associated with mRNA expression [42-44]. the mRNA lifetime of Family with Sequence Similarity 134, Member B (FAM134B) was prolonged when the m^6^A site was mutant [42]. The decreased m^6^A level resulted in the increased expression of N-methyl-D-aspartate receptor 1 (NMDAR1) in Parkinson's disease [43]. Forkhead Box protein M1 (FOXM1) abundance was increased when ALKBH5 was upregulated in glioblastoma [44]. Our study indicated that the expression of circXpo6 and circTmtc3 was decreased with the downregulated m^6^A level. The association between m^6^A level and circRNAs abundance was not determined yet. We suspected that m^6^A may influence the expression of circXpo6 and circTmtc3 through similar manners as before [40, 41]. But it needs further validation.

Competing endogenous RNA (CeRNA) mechanism was proposed that mRNAs, pseudogenes, lncRNAs and circRNAs interact with each other by competitive binding to miRNA response elements (MREs) [45, 46]. M^6^A acts as a post-transcript regulation of circRNAs and influences circRNAs expression, thus we suggested that m^6^A could also regulate the circRNA–miRNA–mRNA co-expression network. When the circRNAs were classified, we found that these downstream targets regulated by circRNA–miRNA of interest were mostly enriched in PH-associated Wnt and FoxO signaling pathways [30, 31]. The Wnt/β-catenin (bC) pathway and Wnt/ planar cell polarity (PCP) pathway are the two most critical Wnt signaling pathways in PH [30]. As known, the two important cells associated with HPH are PASMCs and PAECs [1, 3]. The growth of PASMCs was increased when Wnt/bC and Wnt/PCP pathways were activated by
platelet derived growth factor beta polypeptide b (PDGF-BB) [30, 47]. In addition, the proliferation of PAECs was enhanced when Wnt/bC and Wnt/PCP pathways were activated by bone morphogenetic protein 2 (BMP2). Furthermore, the FoxO signaling pathway is associated with the apoptosis-resistant and hyper-proliferative phenotype of PASMCs [31]. Reactive oxygen species is increased by hypoxia and activates AMPK-dependent regulation of FoxO1 expression, resulting in increased expression of catalase in PASMCs [48]. Our study firstly uncovered that m$^6$A influenced the stability of circRNAs, thus affecting the binding of circRNAs and miRNA, resulting in the activation of Wnt and FoxO signaling pathways.

**Conclusion**

In conclusion, our study firstly identified the transcriptome-wide map of m$^6$A circRNAs in HPH. M$^6$A level in circRNAs was decreased in lungs of HPH and in PASMCs and PAECs exposed to hypoxia. M$^6$A level influenced circRNA–miRNA–mRNA co-expression network in HPH. Moreover, we firstly identified two downregulated m$^6$A circRNAs in HPH: circXpo6 and circTmtc3. CircRNAs may be used as biomarkers because it is differentially enriched in specific cell types or tissues and not easily degraded [6]. Also, the aberrant m$^6$A methylation may contribute to tumor formation and m$^6$A RNAs may be a potential therapy target for tumor [17].

Limitations still exist in the study. First, we did not analyze the m$^6$A level between circRNAs and the host genes. Second, the exact mechanism of hypoxia influences m$^6$A was not demonstrated. Thirdly, the function of circXpo6 and circTmtc3 in HPH was not elaborated. Lastly, besides hypoxia mediated pulmonary hypertension, many other significant PH models should also be noted, such as monocrotaline mediated PH, monocrotaline + pneumonectomy mediated PH, and so on. It is insufficient that we explored the expression profiling of m$^6$A circRNAs only in hypoxia mediated pulmonary hypertension. We plan to explore the expression profiling of m$^6$A circRNAs in monocrotaline-induced PH and other PH models. Moreover, the clinical significance of m$^6$A circRNAs for HPH should be further validated.

**Methods**

**Hypoxia mediated PH rat model and measurement of RVSP and RVH**

Sprague-Dawley rats (SPF, male, 180-200 g, 4 weeks) were obtained from the Animal Experimental Center of Zhejiang University, China. Rats were maintained in a normobaric normoxia (FiO$_2$ 21%, n = 6) or hypoxic chamber (FiO$_2$ 10%, n = 6) for 3 weeks [3, 49]. Rats were then anesthetized with pentobarbital sodium (130 mg/kg, ip) [50]. Then, rats were fixed in supine position on the board. All of the operations were performed after rats were anesthetized and became unconscious. RVSP was measured as below. Right ventricle catheterization was performed through the right jugular using a pressure-volume loop catheter (Millar) as the previous reports [49, 51]. After measurement of RVSP, rats were sacrificed and heart tissues were removed and segregated. The ratio of [RV/ (LV + S)] was used as an index of RVH. Lung were removed and immediately frozen at liquid nitrogen or fixed in 4% buffered paraformaldehyde
solution. All experimental procedures were conducted in line with the principles approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Histological analysis

Lung tissues were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (H&E) and a-smooth muscle actin (a-SMA, 1:100, ab124964, Abcam, USA). The ratio of pulmonary small artery wall thickness and muscularization were calculated [3].

Isolation and hypoxia-treatment of PASMCs and PAECs

PASMCs and PAECs were isolated using the methods according to previous reports [32, 50, 52]. PASMCs and PAECs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and 20% FBS for 48h, respectively [32, 53]. The cells were incubated in a 37°C, 21% O₂ or 1% O₂–5% CO₂ humidified incubator. PASMCs at 70–80% confluence in 4 to 7 passages were used in experiments. PAECs at 80–90% confluence in 4 to 5 passages were used in experiments [54].

RNA isolation and RNA-seq analysis of circRNAs

Total RNA (10 mg) was obtained using TRlzol reagent (Invitrogen, Carlsbad, CA, USA) from lungs (1 g) of control and HPH rats. The extracted RNAs were purified with Rnase R (RNR07250, Epicentre) digestion to remove linear transcripts. Paired-end reads were harvested from Illumina Hiseq Sequence after quality filtering. The reads were aligned to the reference genome (UCSC RN5) with STAR software. CircRNAs were detected and annotated with CIRI software [55]. Raw junction reads were normalized to per million number of reads mapped to the genome with log2 scaled.

MeRIP and Library Preparation

Total RNA was extracted as the methods described above. Then, rRNA was depleted following DNase I treatment. RNase R treatment (5 units/mg) was performed in duplicate with 5 mg of rRNA-depleted RNA input. High-throughput m⁶A and circRNAs sequencing were performed by Cloudseq Biotech Inc. (Shanghai, China). Fragmented RNA was incubated with anti-m⁶A polyclonal antibody (Synaptic Systems, 202003) in IPP buffer for 2 hours at 4°C. The mixture was then incubated with protein A/G magnetic beads (88802, Thermo Fisher) at 4°C for an additional 2 hours. Then, bound RNA was eluted from the beads with N⁶-methyladenosine (PR3732, BERRY & ASSOCIATES) in IPP buffer and extracted
with Trizol reagent (15596026, Thermo Fisher). NEBNext® Ultra™ RNA Library Prep Kit (E7530L, NEB) was used to construct RNA-seq library from immunoprecipitated RNA and input RNA. The m^6^A-IP and input samples were subjected to 150 bp paired-end sequencing on Illumina HiSeq sequencer. Methylated sites on circRNAs were identified by MetPeak software.

**Construction of circRNA–miRNA–mRNA co-expression network**

The circRNA–miRNA–mRNA co-expression network was based on the ceRNA theory that circRNA and mRNA shared the same MREs \[45, 46\]. Cytoscape was used to visualize the circRNA–miRNA–mRNA interactions based on the RNA-seq data. The circRNA-miRNA interaction and miRNA–mRNA interaction of interest were predicted by TargetScan and miRanda.

**Measurement of Total m^6^A, MeRIP-RT-PCR and MeRIP-qRT-PCR**

Total m^6^A content was measured in 200 ng aliquots of total RNA extracted from PASMCs and PAECs exposed to 21% O_2_ and 1% O_2_ for 48 h using an m^6^A RNA methylation quantification kit (P-9005, Epigentek). MeRIP (17-701, Millipore) was performed according to the manufacturer's instruction. A 1.5 g aliquot of anti-m^6^A antibody (ABE572, Millipore) or anti-IgG (PP64B, Millipore) was conjugated to protein A/G magnetic beads overnight at 4°C. A 100 ng aliquot of total RNA was then incubated with the antibody in IP buffer supplemented with RNase inhibitor and protease inhibitor. The RNA complexes were isolated through phenol-chloroform extraction (P1025, Solarbio) and analyzed via RT-PCR or qRT-PCR assays. Primers sequences are listed in Table 1.

**Data analysis**

3’ adaptor-trimming and low quality reads were removed by cutadapt software (v1.9.3). Differentially methylated sites were identified by the R MeTDiff package. The read alignments on genome could be visualized using the tool IGV. Differentially expressed circRNAs were identified by Student’s t-test. GO and KEGG pathway enrichment analysis were performed for the corresponding parental mRNAs of the DE circRNAs. GO enrichment analysis was performed using the R topGO package. KEGG pathway enrichment analysis was performed according to a previous report [56]. GO analysis included BP analysis, CC analysis, and MF analysis. MicroRNAs sponged by the target genes were predicted by TargetScan and microRNA websites. \( P \) values are calculated by DAVID tool for GO and KEGG pathway analysis. The rest statistical analyses were performed with SPSS 19.0 (Chicago, IL, USA) and GraphPad Prism 5 software (La Jolla, CA). N refers to number of samples in figure legends. The statistical significance was determined by Student’s t-test (two-tailed) or two-sided Wilcoxon-Mann-Whitney test. \( P \)
< 0.05 was considered statistically significant. All experiments were independently repeated at least three times.

**Abbreviations**

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<tr>
<th>Abbreviation</th>
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<td>PH</td>
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<td>COPD</td>
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CC  Cellular component analysis
MF  Molecular functions analysis
MeRIP  M\textsuperscript{6}A RNA Immunoprecipitation
PPaR  Peroxisome proliferator-activator
Glut1  Glucose Transporter 1
Myc  Myc proto-oncogene bHLH transcription factor
Dusp1  Dual Specificity Protein Phosphatase 1
Hes1  Hairy and Enhancer of Split 1
Jun  Jun Proto-Oncogene AP-1 Transcription Factor Subunit
HIF-1\textalpha, HIF-2\textalpha  Hypoxia-inducible factor-1\textalpha,-2\textalpha
LncRNA  Long non-coding RNA
RP11  RP11-138 J23.1
FAM134B  Family with Sequence Similarity 134, Member B
NMDAR1  N-methyl-D-aspartate receptor 1
FOXM1  Forkhead Box protein M1
CeRNA  Competing endogenous RNA
MREs  MiRNA response elements
PDGF-BB  Platelet derived growth factor beta polypeptide b
BMP2  Bone morphogenetic protein 2
MeRIP-qRT-PCR  MeRIP-quantitative RT-PCR

**Declarations**

**Ethics approval and consent to participate**

The protocol of this research was submitted to and approved by the Institutional Animal Care and Use Committee of Zhejiang University, China (permit number: SYXX 2017-0006).
Consent to publish

Not Applicable.

Availability of data and materials


Competing interests

The authors declare that they have no competing interests.

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Authors’ Contributions

HS performed the experiments, analyzed the data, and made the figures; GWW, LFW and XQM analyzed the results; KJY and RFZ designed the study, analyzed the data, explained the findings and wrote the paper. All authors read and approved the final manuscript.

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References


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**Table**

**Table 1. Primers for RT-PCR or qRT-PCR**

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<tr>
<th>Name</th>
<th>Sequence</th>
<th>Product size(bp)</th>
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<td>linear Xpo6</td>
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<tr>
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<td>Antisense: 5’ATCGAGTCTCTCTAGCCTGC3’</td>
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<tr>
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<tr>
<td></td>
<td>Antisense: 5’GAAGCCAAGCATTACAGGA3’</td>
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**Additional File Legends**

Additional file 1:

**Data S1.** Differentially expressed m⁶A abundance in circRNAs.

Additional file 2:
**Data S2.** Differentially expressed m$^6$A abundance linked with differentially expressed circRNAs abundance.

**Additional file 3:**

Differentially expressed m$^6$A circRNAs in the lungs of HPH rat model.

**Additional file 4:**

Differentially expressed circRNAs in the lungs of HPH rat model.

**Figures**
Figure 1

M6A level of circRNAs in HPH rats and the number of m6A peak in circRNAs. Rats were maintained in a normobaric normoxic (N, FiO2 21%) or hypoxic (HPH, FiO2 10%) chamber for 3 weeks, then RVSP was detected (A, B). (C) The ratio of RV/ (LV+S). (D) H&E staining and immunohistochemical staining of α-SMA were performed in the lung sections. Representative images of pulmonary small arteries. Scale bar = 50 μm. Quantification of wall thickness (E) and vessel muscularization (F). (G) Heatmap depicting
hierarchical clustering of altered m6A circRNAs in lungs of N and HPH rats. Red represents higher expression and yellow represents lower expression level. (H) Box-plot for m6A peaks enrichment in circRNAs in N and HPH. (I) Distribution of the number of circRNAs (y axis) was plotted based on the number of m6A peaks in circRNAs (x axis) in N and HPH. Values are presented as means ± SD (n = 6 in each group). Only vessels with diameter between 30 and 90 μm were analyzed. NM, nonmuscularized vessels; PM, partially muscularized vessels; FM, fully muscularized vessels. **0.001 ≤ P < 0.009 (different from N); ***P < 0.001 (different from N).
Figure 2

The genomic origins of m6A circRNAs The distribution of genomic origins of total circRNAs (input, left), m6A circRNAs (eluate, center), and non-m6A circRNAs (supernatant, right) in N (A) and HPH (B). The percentage of circRNAs (y axis) was calculated according to the number of exons (x axis) spanned by each circRNA for the input circRNAs (left), m6A-circRNAs (red, right) and non-m6A circRNAs (blue, right) in N (C) and HPH (D). Up to seven exons are shown.
Figure 3

The distribution and functional analysis for host genes of circRNAs with differentially expressed (DE) m6A peaks (A) Length of DE m6A circRNAs. (B) The chromosomes origins for host genes of DE m6A circRNAs. GO enrichment and KEGG signaling pathway analysis for host genes of upregulated (C) and downregulated (D) m6A circRNAs. GO enrichment analysis include biological process (BP) analysis, cellular component (CC) analysis, and molecular function (MF) analysis. P values are calculated by DAVID tool.

Figure 4
The relationship of m6A level and circRNAs abundance in hypoxia (A) Venn diagram depicting the overlap of m6A circRNAs between N and HPH. (B) Two-dimensional histograms comparing the expression of m6A circRNAs in lungs of N and HPH rats. It showed that m6A circRNAs levels for all shared circRNAs in both groups. CircRNAs counts were indicated on the scale to the right. (C) Cumulative distribution of circRNAs expression between N and HPH for m6A circRNAs (red) and non-m6A circRNAs (blue). P value was calculated using two-sided Wilcoxon-Mann-Whitney test.
Construction of a circRNA–miRNA–mRNA co-expression network in HPH (A) Comparison of the relationship between m6A level and expression of circRNAs between N and HPH. The fold-change ≥2.0 was considered to be significant, which was the m6A abundance of HPH relative to N. Red dots represents circRNAs with upregulated m6A level and blue dots represents circRNAs with downregulated m6A level. IP/Input referred to the m6A abundance in circRNAs detected in MeRIP-Seq (IP) normalized to that detected in input. (B and C) GO enrichment analysis includes BP analysis, CC analysis, and MF analysis. P values are calculated by DAVID tool. (D and E) KEGG signaling pathway analysis for the down-stream mRNAs which was predicted to be ceRNA of DE cirRNAs. Methy. down & exp. down represents downregulated cirRNAs with decreased m6A level. Methy. up & exp. up represents upregulated cirRNAs with increased m6A level. (F and G) CeRNA analysis for DE circRNAs. Network map of circRNA-miRNA-mRNA interactions. Green V type node: miRNA; yellow circular node: DE circRNAs; blue hexagon node: target genes of miRNAs; red hexagon node: PH-related genes.
The expression profiling of m6A circXpo6 and m6A circTmtc3 in pulmonary arterial smooth muscle cells (PASMCs) and pulmonary artery endothelial cells (PAECs) in hypoxia (A) M6A levels of total circRNAs were determined based on colorimetric method in vitro. PASMCs and PAECs were exposed to 21% O2 and 1% O2 for 48 h, respectively. Total RNA was extracted and treated by RNase R. M6A levels were determined as a percentage of total circRNAs. (B) Schematic representation of exons of the Xpo6 and Tmtc3 genes.
Tmtc3 circularization forming circXpo6 and circTmtc3 (black arrow). (C) RT-PCR validation of circXpo6 and circTmtc3 in PASMCs and PAECs exposed to 21% O2. Divergent primers amplified circRNAs in cDNA, but not in genomic DNA (gDNA). The size of the DNA marker is indicated on the left of the gel. (D and E) RT-PCR and qRT-PCR was performed after m6A RIP in PASMCs and PAECs exposed to 21% (N) and 1% O2 (H) for 48 h, respectively. Input was used as a control (D). IgG was used as a negative control (D and E). Values are presented as means ± SD. *P < 0.05 (different from 21% O2 or the N-anti-m6A); **0.001 ≤ P ≤ 0.009 (different from the N-anti-m6A), (n = 3 each).

### Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.xlsx
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- Additionalfile3.xlsx
- Additionalfile5.xlsx
- Additionalfile2.xlsx