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RNA-seq analysis of extracellular vesicles from hyperphosphatemia-stimulated endothelial cells provides insight into the mechanism underlying vascular calcification

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Abstract

Background: Hyperphosphatemia (HP) is associated with vascular calcification (VC) in chronic kidney disease (CKD). However, relationship between HP-induced-endothelial extracellular vesicles (HP-EC-EVs) and VC is unclear, and miR expression in HP-EC-EVs has not been determined.

Methods: We isolated HP-EC-EVs from endothelial cells with HP and observed that HP-EC-EVs were up-taken by vascular smooth muscle cells (VSMCs). HP-EC-EVs inducing calcium deposition was characterized by Alizarin Red S, colourimetric analysis and ALP activity. To investigate the mechanism of HP-EC-EVs-induced VSMC calcification, RNA-sequencing for HP-EC-EVs was performed.

Results: We first demonstrated that HP-EC-EVs induced VSMC calcification in vitro. RNA-seq analysis of HP-EC-EVs illustrated that one known miR (hsa-miR-3182) was statistically up-regulated and twelve miRs were significantly down-regulated, which was verified by qRT-PCR. We predicted 58,209 and 74,469 target genes for those down- and up-regulated miRs respectively through miRDB, miRWalk and miRanda databases. GO terms showed that down- and up-regulated targets were mostly enriched in calcium-dependent cell–cell adhesion via plasma membrane cell-adhesion molecules (GO:0,016,338, BP) and cell adhesion (GO:0,007,155, BP), plasma membrane (GO:0,005,886, CC), and metal ion binding (GO:0,046,914, MF) and ATP binding (GO:0,005,524, MF) respectively. Top-20 pathways by KEGG analysis included calcium signaling pathway, cAMP signaling pathway, and ABC transporters, which were closely related to VC.

Conclusion: Our results indicated that those significantly altered miRs, which were packaged in HP-EC-EVs, may play an important role in VC by regulating related pathways. It may provide novel insight into the mechanism of CKD calcification.

Keywords: Hyperphosphatemia, Extracellular vesicles (EVs), Vascular calcification, High-throughput sequencing (HTS)

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Background

Vascular calcification (VC) is a pathological deposition of hydroxyapatite crystals in vascular wall, which is common in chronic kidney disease (CKD) [1]. As an independent predictor of cardiovascular events, VC is also significantly associated with mortality in CKD [2, 3]. There are multifactor (including metabolic disorders of serum phosphate and calcium, inflammatory cytokines, oxidative stress, and uremic toxins) accelerating VC in CKD patients, among which hyperphosphatemia (HP) is most strongly associated with VC [2]. It has been reported that endothelial extracellular vesicles (EC-EVs) can be released under HP circumstances [4]. However, there is rare information about the relationship between HP-EC-EVs and vascular smooth muscle cell (VSMC) calcification.

EC-EVs are formed from the outward budding of endothelial cells (EC) plasma membranes, and secreted to the extracellular chamber during stimuli such as HP [5, 6]. More and more researchers focus on the function of microRNAs (miRs) in EC-EVs. Several studies implicated that EC-EVs play an important role in inflammation, angiogenesis, and thrombosis by delivering miRs [7, 8]. There is a report showing that miR-126 and miR-26a in EC-EVs are significantly reduced in diabetic patients compared to non-diabetic patients [9]. Moreover, it has been demonstrated that miR-145-5p and miR-320a in EC-EVs could contribute to the progression of vasculitis [10]. Additionally, it has been reported that miR-29b, miR-133b, and miR-211 regulated VSMC calcification induced by HP [11]. Nevertheless, the different expression of miRs in HP-EC-EVs has not been determined.

In the current study, we firstly isolated HP-EC-EVs from endothelial cells treated with HP. We then observed the process of HP-EC-EVs being up-taken by VSMCs. The VSMC calcium deposition mediated by HP-EC-EVs was characterized by Alizarin Red S, quantified by colorimetric analysis and ALP activity. To better understand the mechanism of HP-EC-EVs-induced VSMC calcification in vitro, we detected the expression of miRs in HP-EC-EVs via high-throughput sequencing. Differentially expressed miRs (DEMs) from HP-EC-EVs, target genes of DEMs, and their related signaling pathway were performed by using bioinformatics analysis. The content of HP-EC-EVs may drive activation or down regulation of pathways related to vascular calcification.

Methods

Cell culture

The Human umbilical vein ECs (HUVECs) and VSMCs were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The HUVECs were maintained in F12 K (Hyclone, Logan,

Utah, USA) with 10% fetal bovine serum (Sigma, St. Louis, MA, USA) and 1% penicillin and streptomycin. To model hyperphosphatemia, NaH_2PO_4 was added to raise the [Pi] to 3.0 mM—a concentration that has been used extensively elsewhere [12, 13]. Pi concentration recorded was the concentration of the exogenous Pi. Medium harvested from experimental incubations was subjected to centrifugation as described below. The VSMCs were maintained in F12 (Hyclone, Logan, Utah, USA) with 10% fetal bovine serum and 1% penicillin and streptomycin. These cells were maintained under standard cell culture conditions of 37 °C, 5% CO_2 and 95% humidity. The VSMCs were used for all experiments between passages 4 and 9.

Isolation of EC-EVs

EC-EVs were isolated from the culture medium as previously described [4]. HP-EC-EVs were obtained by centrifugation of culture medium incubated with 3.0 mM Pi, while the control PBS-EVs were obtained with the same volume of PBS. Medium from cultures was centrifuged (step 1) at $1500 \times g$ at 20 °C for 20 min to remove detached cells and large particles/apoptotic bodies. The top 90% of the supernatant from step 1 was centrifuged (step 2) at $14,000 \times g$ at 20 °C for 30 min to pellet EVs. The top 90% of the supernatant from this step was aspirated, and the pellet was resuspended in the following 0.2-mm-filtered MP-buffer (145 mM NaCl, 2.7 mM KCl, and 10 mM HEPES, pH 7.4) and recentrifuged (step 3) as before to wash MPs before resuspending again in EV buffer and storing at -80 °C for additional analysis.

Experimental procedures

Scanning electron microscopy, flow cytometry analysis of EC-EVs, EC-EVs labeling and uptake by VSMCs, in vitro calcification and quantification, Alizarin Red S, RNA extraction, miR sequencing library construction, high-throughput sequencing (HTS), bioinformatic analysis, and altered miR verification by qRT-PCR are described in detail in the [Supplementary Materials and Methods](#).

Statistical analysis

We examined the original data for normality and homogeneity of variance, and the data were expressed as the means \pm standard deviation (SD). We analyzed the changes in EV count, calcium deposition levels, miRs PCR expression, and statistical calculations with SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference. To analyze miR patterns in the PBS-EC-EVs and HP-EC-EVs groups, the miR counts were normalized using a modified global normalization method. Differential

expression analyses were performed with the EdgeR package (3.18.1).

Results

VSMC calcification mediated by HP-EC-EVs

We firstly isolated EC-EVs from the supernatants of HUVECs treated with 3 mM Pi (HP-EC-EVs). We showed the representative morphology of EC-EVs induced by HUVECs after 48 h treatment of 3 mM Pi (Fig. 1a) or PBS (Supplementary Fig. 1) detected by scanning electron microscopy. It showed that the mean diameter of the HP-EC-EVs was 282.6 nm. Our previous study showed that Pi stimulated EVs release from HUVECs for 48 h in a concentration-dependent manner [14]. Similar to the result, here, we detected a significant increase in HP-EC-EVs in comparison with PBS-EC-EVs by flow cytometry (Fig. 1b-c). Additionally, we observed that CM-Dil-labeled (red) HP-EC-EVs formed dots, rosettes, semicircle or circle around DAPI-labeled (blue) VSMCs (Fig. 1d). However, rare red could be found when PBS-EC-EVs were incubated with VSMCs (Supplementary Fig. 2).

To determine the ability of the HP-EC-EVs in regulating calcification, VSMCs were treated with HP-EC-EVs. After 7 days of the HP-EC-EVs (1000 µg/ml) exposure, VSMCs in HP-EC-EVs group exhibited more calcification deposition both macroscopically and microscopically by Alizarin Red S (Fig. 1e-g). Furthermore, calcium content was remarkably increased when VSMCs were exposed to the HP-EC-EVs (Fig. 1h).

Different expression of miRs in HP-EC-EVs

From the six samples (three HP-EC-EVs and three PBS-EC-EVs), we examined 13 known miRs and 34 novel miRs differently expressed in HP-EC-EVs by next generation sequencing. There were twelve known down-regulated miRs and one known up-regulated miRs. There were three novel down-regulated miRs and thirty-one up-regulated novel miRs. Table S2 showed the differentially expressed miRs. Figure 2a and b showed the volcano and clustering plots of the differentially expressed miRs.

Target gene prediction of differentially expressed miRs

We further analyzed the target gene prediction of the differentially expressed miRs via miRDB, miRWalk and miRanda databases. As the venn diagram showed in Fig. 3, there were 74,469 targets predicted in up-regulated miRs and 58,209 in down-regulated miRs totally. Our analysis showed 261 (miRDB database), 415 (miRWalk database), 74,279 (miRanda database), and 7 (common to the three databases) predicted target genes in up-regulated miRs, while 1705 (miRDB database), 12,473

(miRWalk database), 51,084 (miRanda database), and 450 (common to the three databases) predicted target genes in down-regulated miRs (Fig. 3a-b).

Cytoscape network analysis of differentially expressed miRs

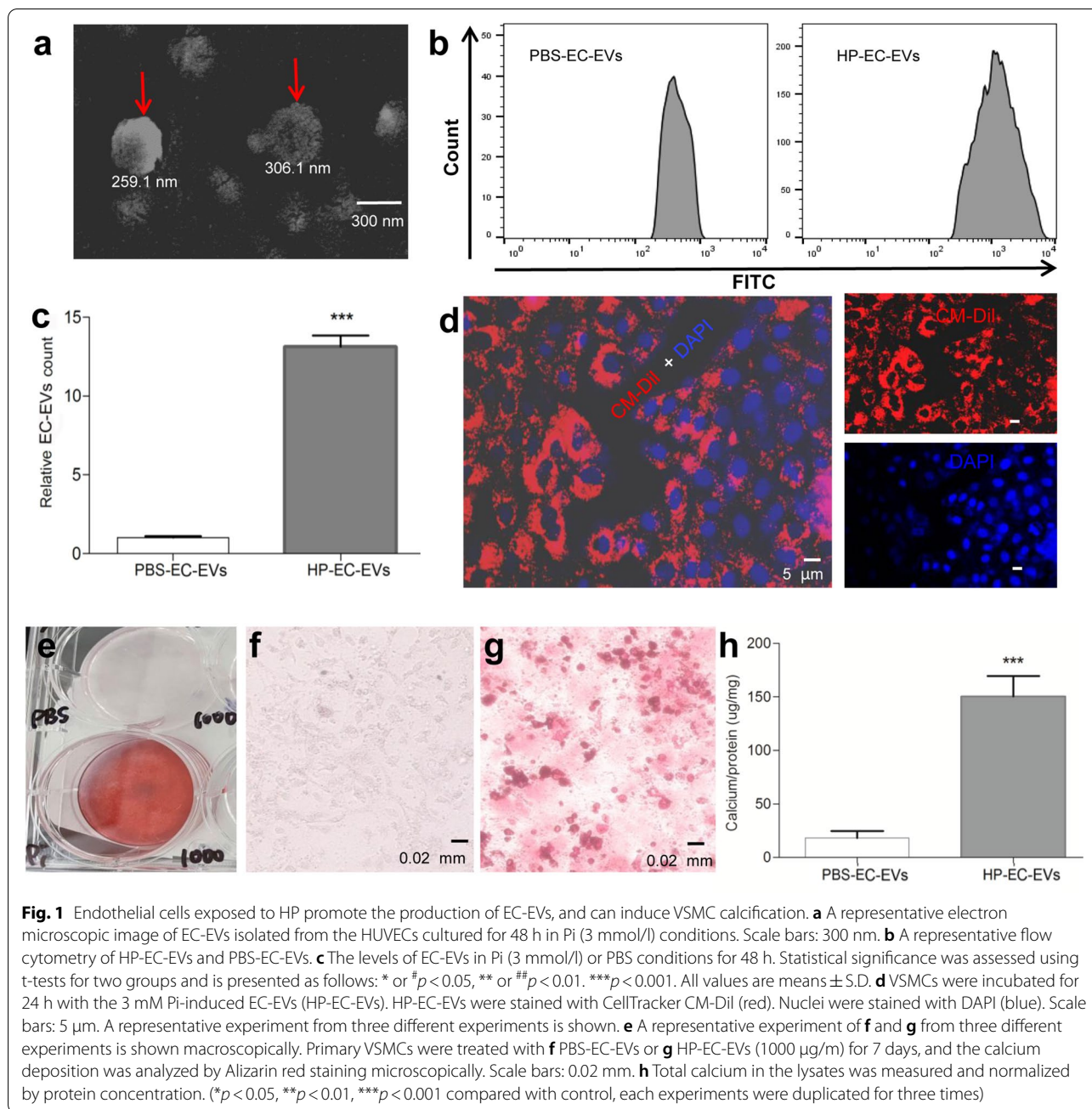
Figure 4 showed the cytoscape networks to illustrate the relationships between miRs differentially expressed in HP-EC-EVs and their target genes. Our analysis revealed that the miR-30c-2-3p, miR-7706, miR-365a-5p, miR-novel-Chr11_14,558, and miR-143-3p target genes were the most enriched and cross-linked networks in the down-regulated miRs (Fig. 4a). As for the up-regulated differentially expressed miRs, the most enriched and cross-linked networks focused on the miR-novel-Chr1_1205, miR-novel-Chr12_15,143, miR-novel-Chr2_3698, miR-novel-Chr8_10,922, miR-novel-Chr18_19,949, miR-novel-Chr6_9236, miR-novel-Chr1_1198, miR-3182, miR-novel-ChrX_24044, miR-novel-Chr10_13,624, miR-novel-Chr20_21,071, miR-novel-Chr7_10,732, and miR-novel-Chr5_7115 > miR-novel-Chr1_1380 target genes (Fig. 4b).

Gene ontology (GO) analysis

Clustering GO analyses were performed according to target gene function of differentially expressed miRs (DEMs) from HP-EC-EVs and PBS-EC-EVs. It revealed entries enriched in biological processes (BP), cellular component (CC) and molecular functions (MF) of DEMs in HP-EC-EVs. There were 4895 GO terms enriched in the target genes of these significantly altered miRs. Figure 5 shows the top-10 GO terms for up-regulated and down-regulated miRs of the 3 ontologies (BP, CC and MF). Our bioinformatics analysis demonstrated that the down-regulated miRs were mainly enriched in calcium-dependent cell-cell adhesion via plasma membrane cell-adhesion molecules (GO:0,016,338, BP), plasma membrane (GO:0,005,886, CC), and metal ion binding (GO:0,046,914, MF). As for the up-regulated miRs, they were enriched in cell adhesion (GO:0,007,155, BP), plasma membrane (GO:0,005,886, CC), and ATP binding (GO:0,005,524, MF).

KEGG pathway analysis

Our results showed that the down- and up-DEMs in the HP-EC-EVs groups participate in 320 and 321 pathways via KEGG pathway enrichment analysis. Supplementary Table S3 revealed the top-20 KEGG signaling pathways of total DEMs between HP-EC-EVs and PBS-EC-EVs groups. Autophagy signaling pathway (pathway ID: hsa04136) was the most significantly enriched pathways associated with total DEMs. However, ABC transporters (pathway ID: hsa02010) was a significantly enriched



pathways associated with the down-regulated DEMs between HP-EC-EVs and PBS-EC-EVs group (Fig. 6).

Novel miR prediction and verification of known miRs by qRT-PCR

There were 303 novel miRs in total in our current study. Among those novel miRs, we only found 3 down-regulated and 31 up-regulated differentially expressed novel miRs, based on the fold change (> 2 [up-regulated]

or < 0.5 [down-regulated], $P < 0.05$). Supplementary Figs. 3 and 4 showed the predicted secondary structure maps of the differentially expressed novel miRs.

We further confirmed the known differently expressed miRs by qRT-PCR. The results showed that those 13 known miRs (including hsa-miR-10a-5p, hsa-miR-10b-5p, hsa-miR-143-3p, hsa-miR-193b-5p, hsa-miR-30a-3p, hsa-miR-30a-5p, hsa-miR-30c-2-3p, hsa-miR-365a-5p, hsa-miR-486-5p, hsa-miR-7706, hsa-miR-941,

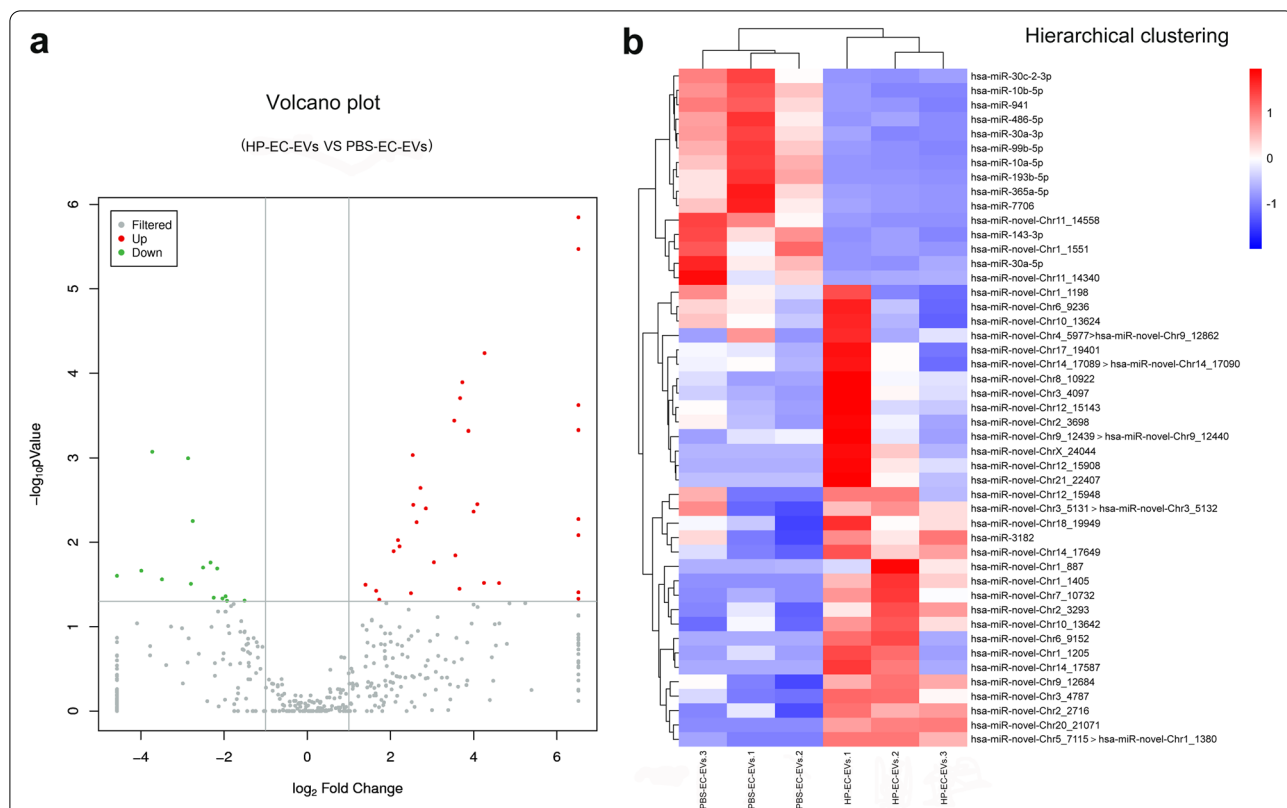


Fig. 2 Clustering of expression patterns of 47 differentially expressed miRs. The expression patterns of 47 differentially expressed miRs ($P < 0.05$) in the 6 samples libraries are displayed in the volcano plot and clustering plots. Volcano plot **a** and clustering **b** of miRs in the HP-EC-EVs group and PBS-EC-EVs group. **a** Volcano plot was constructed using P -values and fold-change of miRs, with $\log(P\text{-value})$ as the ordinate and $\log_2(\text{Fold change})$ for the abscissa. Red and green dots represent differentially up- or down-expressed miRs between HP-EC-EVs and PBS-EC-EVs groups ($P \leq 0.05$). **b** Hierarchical clustering was constructed according to the expression levels of miR, the six samples were classified into two groups. Blue represents low relative expression, and red represents high relative expression

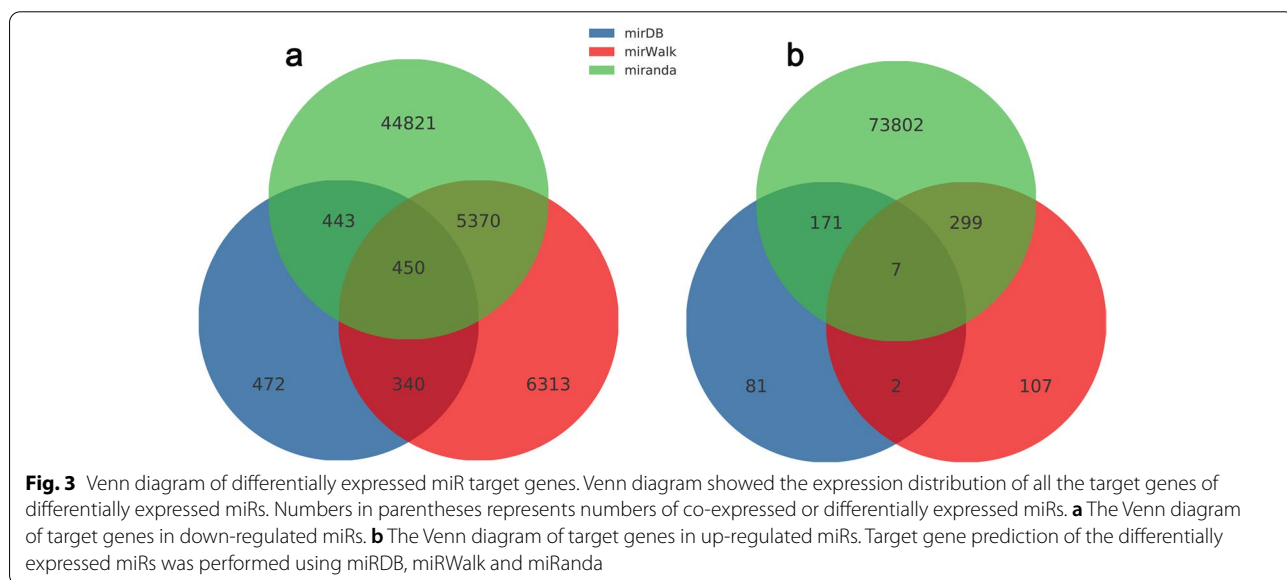
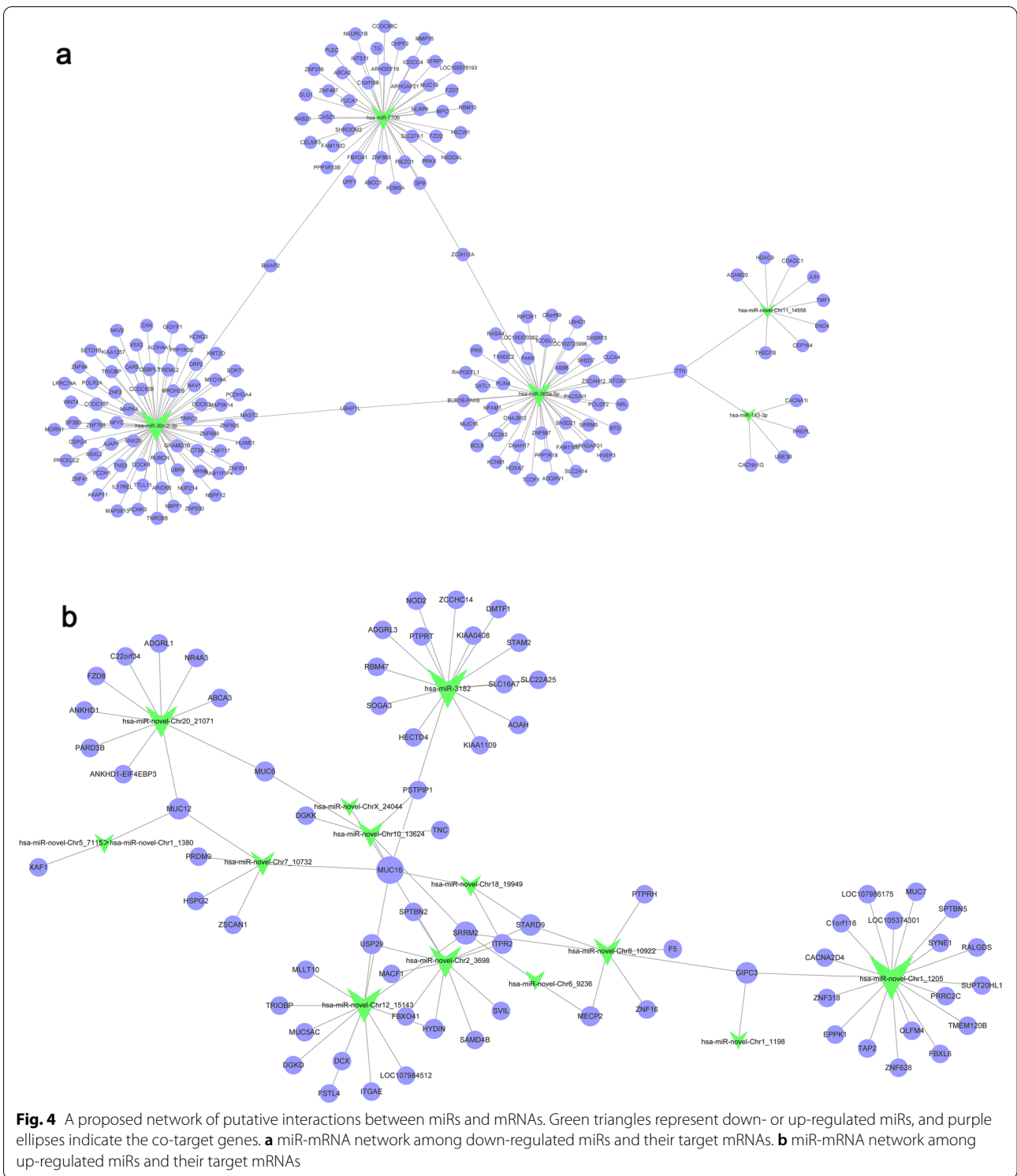


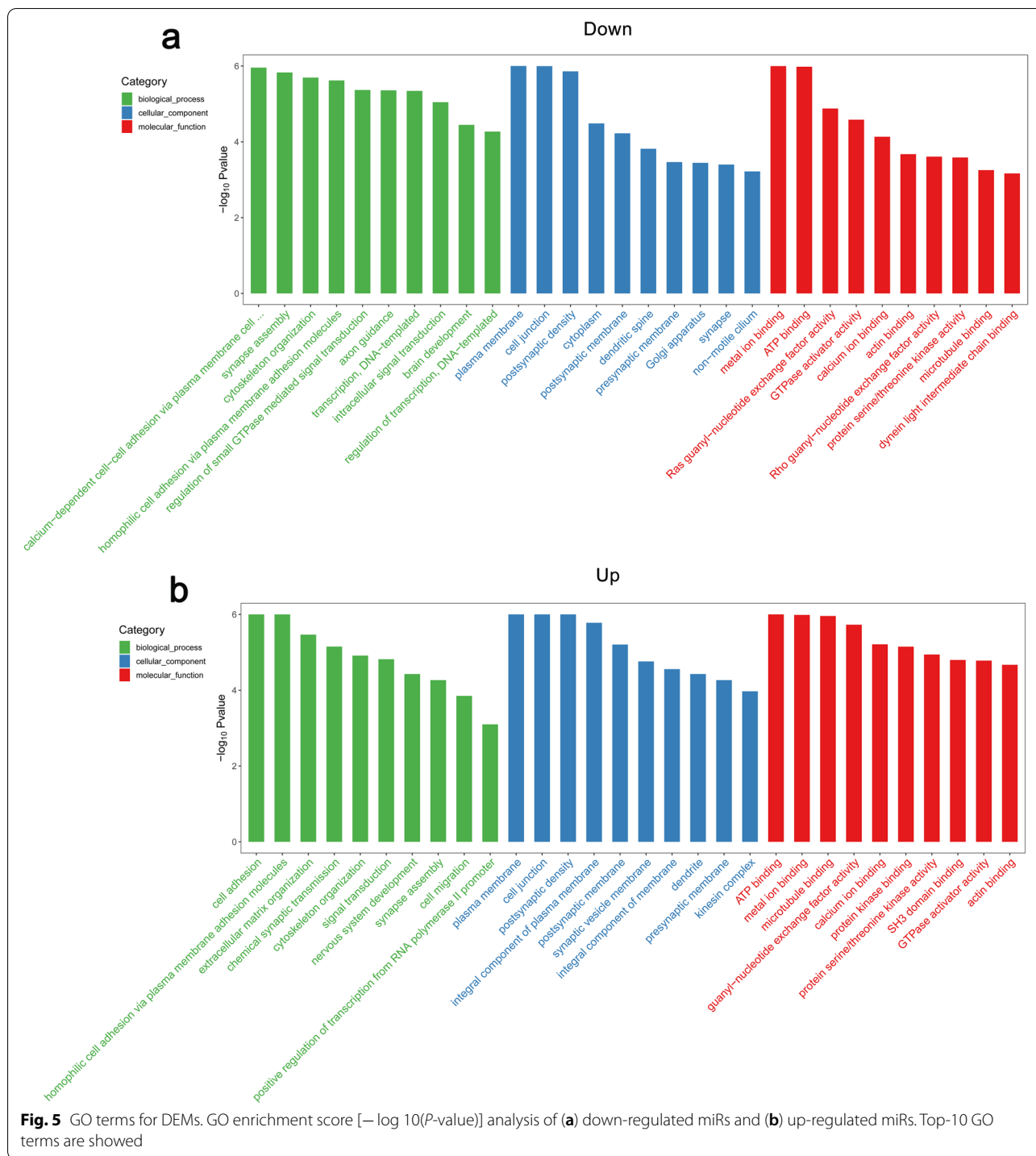
Fig. 3 Venn diagram of differentially expressed miR target genes. Venn diagram showed the expression distribution of all the target genes of differentially expressed miRs. Numbers in parentheses represents numbers of co-expressed or differentially expressed miRs. **a** The Venn diagram of target genes in down-regulated miRs. **b** The Venn diagram of target genes in up-regulated miRs. Target gene prediction of the differentially expressed miRs was performed using miRDB, miRWalk and miRanda



and hsa-miR-99b-5p) had relative lower levels in HP-EC-EVs group than PBS-EC-EVs group ($P < 0.05$). As for has-miR-3182, it had higher level in HP-EC-EVs group in comparison to that in PBS-EC-EVs ($P < 0.05$, Fig. 7).

Discussion

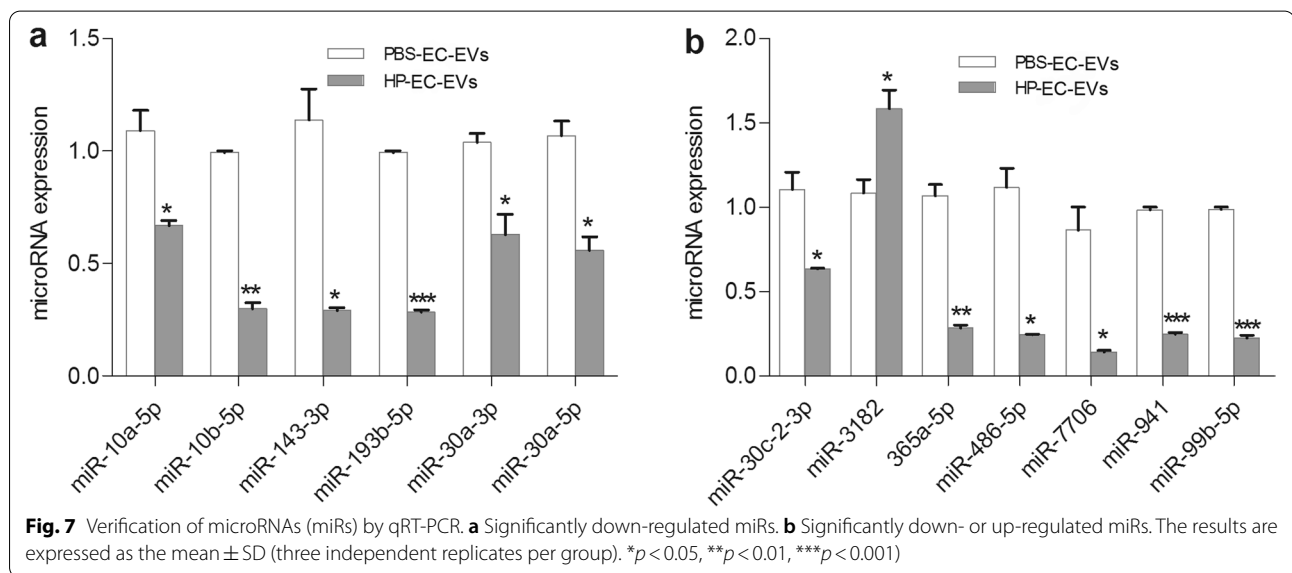
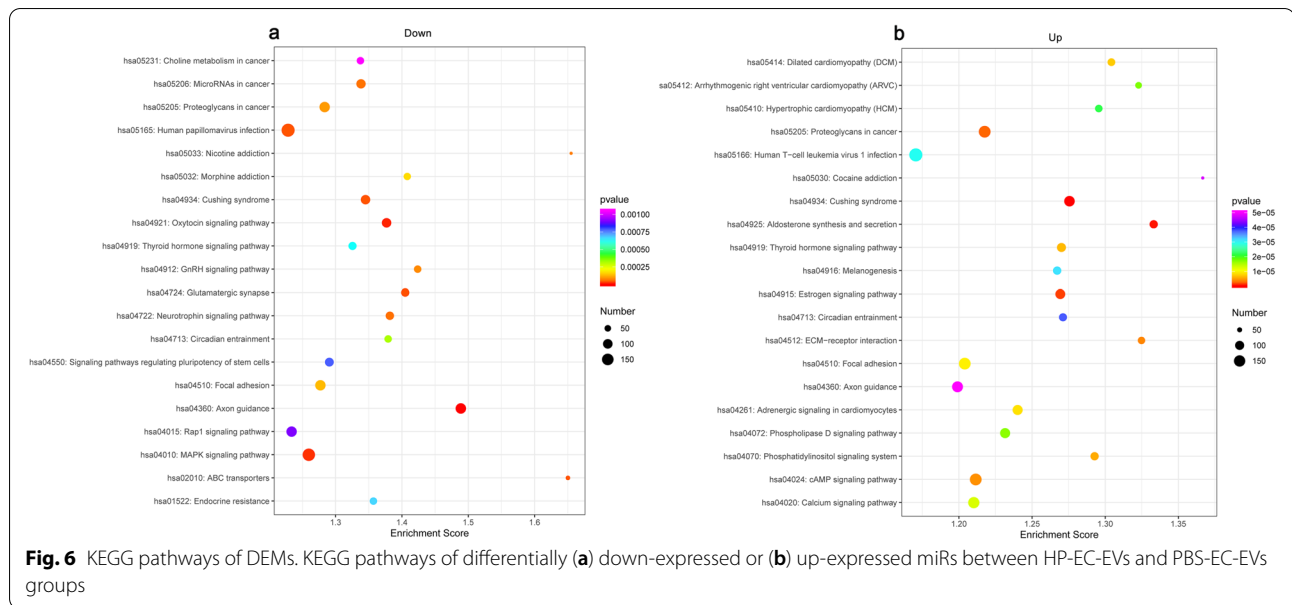
There is increasing evidence that miRs play an important role in vascular calcification, and several researches have investigated the association among hyperphosphatemia,



miRs in VSMC or its vesicles, and vascular calcification [11, 15]. However, there is rare information about the miR expression in HP-EC-EVs.

There are report that endothelial dysfunction can cause vascular calcification through BMP activation [16]. Hyperphosphatemia is a well-known factor that induces

vascular calcification in CKD [17]. In the current study, our results indicated that HP-EC-EVs from injured endothelial cells might have the ability to induce VSMC calcification, and we observed the capture of HP-EC-EVs by VSMCs. Those captured HP-EC-EVs packaging miRs, might lead VSMCs calcification. Our results implicated



that HP-EC-EVs and its cargos such as miRs potentially provide a link between the communication of activated endothelial cells and VSMCs to induce vascular calcification under hyperphosphatemia circumstances. Thus, we decided to explore the miR expression of HP-EC-EVs to better understand the CKD calcification.

RNA-seq and bioinformatics analysis were carried out to understand the different expression of miRs between HP-EC-EVs group and PBS-EC-EVs group. Those miRs (including hsa-miR-143-3p, hsa-miR-30c-2-3p, hsa-miR-30a-3p, hsa-miR-30a-5p, hsa-miR-486-5p, and hsa-miR-193b-5p) were associated with

vascular calcification, and statistically down-regulated in HP-EC-EVs group. miR-143 is a potential biomarker of vascular calcification and cardiovascular disease associated with CKD, and it was down-regulated in VSMCs during the time course of Pi-induced vascular calcification [18–20]. Our current results showed that miR-143-3p was remarkably down-regulated in HP-EC-EVs. In consistent with our results, miR-143 expression in extracellular vesicles (EV) derived from urea and indoxyl sulphate-stimulated EC (EV^{UR}) were also down-regulated, and mimicking of miR-143 in EV^{UR} blocked the pro-calcifying effects of EV^{UR}

[21]. Among those miRs, the miR-30 family has been reported to play an important role in osteogenesis [22]. Our analysis revealed low level of miR-30c-2-3p in HP-EC-EVs. The published research that BMP-2 down-regulation miR-30c to increase Runx2 expression in human coronary artery SMCs and promoting mineralization may partly explain our result [23]. Additionally, the expression of miR-30a was significantly higher in VSMCs during vascular calcification [24–26]. However, miR-30a-3p and miR-30a-5p was significantly reduced in the HP-EC-EVs compared to controls in this study. This discrepancy may be due to miR different expression in VSMCs and HP-EC-EVs. Three novel miR (hsa-miR-novel-Chr11_14558, hsa-miR-novel-Chr11_14340, hsa-miR-novel-Chr1_1551) were also significantly down-regulated in HP-EC-EVs, but its structure and function remain elusive. Therefore, HP-EMP-induced VSMC calcification may be associated with the down-regulation of the abovementioned miRs.

In contrast, hsa-miR-3182 was markedly up-regulated in HP-EC-EVs. A recent research showed that hsa-miR-3182 and its host coding genes are genetically associated with cardiovascular disease [27], which supported our findings. Our analysis demonstrated that those target mRNAs for hsa-miR-3182 included NOD2, ZCCHC14, DMTF1, STAM2, SLC22A25, AOA, KIAA1109, HECTD4, SOGA3, RBM47, ADGRL3, PTPRT, KIAA0408, SLC16A7, PSTPIP1. In consistent with our results, several lines of evidence demonstrated that NOD2 deficiency enhanced pulmonary VSMC proliferation, and exacerbated plaque necrosis in advanced atherosclerotic lesions [28, 29]. Furthermore, Sanneke et al. observed that SLC22A25 gene had strong signals associated with plaque morphology [30]. Additionally, RBM47 was identified to be associated with blood pressure or hypertension [31]. Taken together, target genes of hsa-miR-3182 may be associated with vascular calcification through regulation of blood pressure, VSMC proliferation, plaque morphology and necrosis. Surprisingly, only one up-regulated known miR was found while others were all belong to novel miRs. This remains us that we lack the understanding for those miRs, and further work about those differentially expressed miRs, especially those novel miRs, in HP-EC-EVs is still needed in the future.

In order to explore the regulatory mechanisms of differentially expressed miRs between HP-EC-EVs and PBS-EC-EVs, we annotated biological functions of miRs, predicted their targets and constructed regulation networks. This is the first study to identify a number of putative miR-mRNA interactions for DEMs in HP-EC-EVs (Fig. 4). GO enrichment analysis of DEMs in HP-EC-EVs

is useful to describe BP, CC, and MF in relation to predicted target gene candidates.

In the current study, our top-20 KEGG pathway analysis revealed that calcium signaling pathway, cAMP signaling pathway, and ABC transporters were closely related to vascular calcification [32]. Early studies have suggested that roles for cellular calcium signaling involved in the regulation of calcification [33]. Integrative genomic study confirmed calcium signaling pathway genes RUNX2 and CACNA1C are associated with calcific disease [34]. Previous study has demonstrated that cAMP pathway promotes in vitro vascular calcification by enhancing osteoblast-like differentiation of calcifying vascular cells [35]. Later, Prosdocimo et al. reported that increased cAMP signaling and elevated extracellular inorganic phosphate (Pi) act synergistically to induce calcification of VSMC [36]. Thus, it is reasonable to understand that increased calcium signaling pathway or cAMP signaling pathway could be involved in the HP-EMP-induced VSMC calcification in vitro. ABC transporters represent a large family of ATP-driven transmembrane transporters involved in uni- or bidirectional transfer of substrates [37]. ABCC6, a unidirectional exporter protein, has been reported that its deficiency could alter ABC transporter gene expression and cause the ectopic mineralization disorder, characterized by calcification [37, 38]. Taken together, up-regulation of calcium signaling pathway or cAMP signaling pathway, and down-regulation of ABC transporters may be important in HP-EMP-induced VSMC calcification.

Surprisingly, except the vascular calcification-related pathways mentioned above, signaling pathways associated with cancer (such as Choline metabolism in cancer, MicroRNAs in cancer, and proteoglycans in cancer), endocrine system (such as Cushing syndrome, thyroid hormone signaling pathway, GnRH signaling pathway and endocrine resistance), nervous system (such as Rap1 signaling pathway, Axon guidance, signaling pathway regulating pluripotency of stem cells, and neurotrophin signaling pathway), and cardiomyopathy (such as dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, and hypertrophic cardiomyopathy) were also found in the HP-EC-EVs. Actually, EC-EVs have been reported to be a potentially useful biomarker of endothelial dysfunction in heart failure risk stratification [39]. However, there is rare information about the role of HP-EC-EVs in CKD or uremia patients. Our current study revealed that HP-EC-EVs may not only play important role in vascular calcification, but also in cardiomyopathy, nervous disease such as uremic encephalopathy, cancer, and endocrine disease in ESRD patients. This shed new light on the possible mechanism of several diseases in uremia patients, and further study about the

potential role of HP-EC-EVs in those diseases especially for the cardio cerebrovascular disease leading high morbidity in CKD, should be explored in the future.

There are some limitations in this study. Our current study only focused on EVs induced by HP-ECs *in vitro*, which are not an arterial model. Although HP is important in VC, it is not the only factor and Pi binders is disappointed in the management of vascular calcification in CKD [40]. Thus, it is not clear whether these findings would apply to human disease. Considering the limitation of this study, further researches are absolutely needed to evaluate these findings, especially *in vivo*.

Conclusion

This is the first study to perform miR-seq analysis in HP-EC-EVs. We identified 12 known down-regulated miRs (hsa-miR-10a-5p, hsa-miR-10b-5p, hsa-miR-143-3p, hsa-miR-193b-5p, hsa-miR-30c-2-3p, hsa-miR-30a-3p, hsa-miR-30a-5p, hsa-miR-365a-5p, hsa-miR-486-5p, hsa-miR-7706, hsa-miR-941 and hsa-miR-99b-5p) and 1 (hsa-miR-3182) known up-regulated miR. We also constructed miR-mRNA network, and performed GO term and related pathway analysis of the targets to predict the biological function of the altered miRs. We find that calcium signaling pathway, cAMP signaling pathway, and ABC transporters may have their special role in regulating vascular calcification. Our findings indicated that HP-EC-EVs might induce VSMC calcification and they do activate several pathways in the VSMCs. The differentially expressed miRs packaged in HP-EC-EVs might shed light on the mechanism of vascular calcification.

Abbreviations

CKD: Chronic kidney disease; VSMCs: Vascular smooth muscle cells; Pi: Inorganic phosphate; HP: Hyperphosphatemia; EC-EVs: Endothelial extracellular vesicles; HP-EC-EVs: HP-induced-EC-EVs; ECs: Endothelial cell; HUVECs: Human umbilical vein ECs; VC: Vascular calcification; miRs: MicroRNAs; DEMs: Differentially expressed miRs.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12882-022-02823-6>.

Additional file 1.

Additional file 2: Table S1. The primer used in QPCR. **Table S2.** Down-regulated and up-regulated miRNAs. **Table S3.** KEGG pathways of total differentially expressed miRNAs between HP-EMPs and PBS-EMPs groups.

Additional file 3.

Additional file 4.

Additional file 5.

Additional file 6.

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Authors' contributions

Study concept (ZH); study design (ZH, ZP, YD); experiment and data acquisition (ZP, YD, SZ, JC, JL, ZH); statistical analysis (ZH, ZP, YD, JC); data interpretation and manuscript drafting (ZH, PZ, YD). All authors reviewed the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are provided in the [Supplementary Materials and Methods](#).

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The First Affiliated Hospital of University of South China. As this study was not a clinical study with intervention, informed consent was exempted by the Ethics Committee.

Consent for publication

Not applicable.

Competing interests

There is no conflict of interest to report here.

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