Contribution of extracellular vesicles to human pulmonary arterial smooth muscle cell dysfunction in congenital heart disease-associated pulmonary arterial hypertension

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Research Article

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Posted Date: March 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1425524/v1

Contribution of extracellular vesicles to human pulmonary arterial smooth muscle cell dysfunction in congenital heart disease-associated pulmonary arterial hypertension

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Abstract

Background: Pulmonary arterial hypertension (PAH) is a common complication of congenital heart disease (CHD). Extracellular vesicles (EVs) are small endosome-derived membrane microvesicles that play a prominent role in intercellular communication by carrying signaling molecules, such as proteins, mRNAs, and microRNAs.

Methods: We obtained peripheral blood from irreversible CHD-PAH group and reversible CHD-PAH group and normal control group, and then isolated EVs from peripheral blood (PB-EVs) and monocytes/ macrophages (MC-EVs). We compared the effects of PB-EVs and MC-EVs from above three groups on the proliferation using Cell Counting Kit-8(CCK-8), migration using the transwell chamber assay, and phenotypic transformation of HPASMCs via detecting the expression of contractile phenotype markers and synthetic phenotype markers of HPASMCs by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunoblotting assay.

Results: In this study, PB-EVs or MC-EVs from the irreversible and reversible CHD-PAH groups each enhanced HPASMC proliferation and migration compared with PB- or MC-EVs from the normal control group, which was most pronounced for the irreversible CHD-PAH group. Besides, PB- or MC-EVs from the irreversible and reversible CHD-PAH groups showed increased expression of synthetic phenotype markers and migration-related molecules and decreased expression of contractile

phenotype markers compared with PB- or MC-EVs from the normal control group, especially in the case of the irreversible CHD-PAH group. Moreover, the level of phosphorylation of p38 in HPASMCs co-cultured with PB- or MC-EVs from the irreversible and reversible CHD-PAH groups were markedly higher than those in HPASMCs co-cultured with PB- or MC-EVs from the normal control group.

Conclusions: In summary, our results indicated that EVs from peripheral blood of CHD-PAH patients could promote switching of HPASMCs from a quiescent contractile to a proliferative, migratory, and synthetic phenotype.

Key words: congenital heart disease, pulmonary arterial hypertension, human pulmonary arterial smooth muscle cell, extracellular vesicles

Background

Pulmonary arterial hypertension (PAH) is a common complication of congenital heart disease (CHD) [1]. In China, 47.5% of CHD cases are complicated by development of PAH [2]. Despite considerable advances in the treatment of PAH, this devastating disease still result in a worse prognosis than many cancers, with three-year survival rates of 68-70% [3]. Thus, accurate evaluation and a good understanding of the pulmonary vascular lesion are of significant importance.

PAH is largely featured by sustained vasoconstriction and progressive obliteration of small resistance pulmonary arteries through intimal and medial thickening, and by the appearance of cells expressing smooth muscle-specific markers in pre-capillary arterioles (distal muscularization), resulting from the proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs), and, possibly, cellular trans-differentiation [4]. Although numerous vascular abnormalities are observed, the mechanism of PAH has not yet been widely elucidated. Reports have suggested that this disease is not likely to be resulted by an isolated event, but rather by a broad array of defects [5]. Indeed, pulmonary vascular remodeling observed in PAH involves complex interactions among endothelial cells, smooth muscle cells, adventitial fibroblasts, perivascular inflammatory cells, such as monocytes/macrophages, and others [6]. A new mechanism for cell-to-cell communication has emerged, which is based on the release of large quantities of extracellular vesicles (EVs) by vascular cells for intercellular interaction during pulmonary vascular remodeling in PAH [7]. The types of EVs produced from these cells include apoptotic bodies, microvesicles

(MVs), exosomes (EXOs), microparticles, and membrane particles, which are capable of carrying signaling molecules in the form of protein, mRNA, and microRNA, served as platforms for complex intercellular communication, and directly impact the gene expression profile and cellular phenotype of recipient cells [8, 9].

It has become increasingly evident that these circulating EVs rapidly increase in PAH patients, and their levels are correlated with pulmonary vascular resistance, functional impairment, and mortality [10, 11]. EVs obtained from the circulation or lungs of mice with monocrotaline -induced PAH (MCT-PAH) could elicit right ventricular hypertrophy and pulmonary vascular remodeling when injected into healthy mice, while the EXO fraction of EVs from mesenchymal stem cells (MSCs) could mitigate the development of both MCT-PAH and hypoxia-induced PAH [12-14]. Plasma EVs obtained from rats with hypoxia-induced PAH were shown to ameliorate the levels of endothelial nitric oxide synthase (eNOS), and thus nitric oxide production, in cultured endothelial cells *in vitro*, while leading to impaired endothelium-dependent relaxation in both the aorta and pulmonary arteries *in vivo* [15]. However, until now, the role of EVs in the pathophysiological development of CHD-PAH has not yet been clearly described. In this study, we hypothesized that EVs isolated from the circulation of CHD-PAH patients could potentially induce human pulmonary arterial smooth muscle cell (HPASMC) dysfunction. To test this hypothesis, EVs were isolated from human peripheral blood and peripheral blood monocyte/macrophages derived from individuals with CHD-PAH, and then co-cultured with HPASMCs. With this approach we further explored the effects and mechanisms of EVs on the proliferation, migration, and phenotypic transformation of HPASMCs.

Methods

Human peripheral blood plasma collection

Human peripheral blood collection was conducted in accordance with the Helsinki Declaration and the Medical Ethics Committee of fuwai hospital. Written informed consent was obtained from all patients. Human peripheral blood was harvested from CHD-PAH patients [16]. One year later, CHD-PAH patients were divided into reversible (mean pulmonary arterial pressure (mPAP) $<$ 25 mmHg, n = 6) and irreversible (mPAP $\geq$ 25 mmHg, n = 4) PAH groups. Human peripheral blood obtained from four age- and sex-matched healthy volunteers served as normal

controls. Plasma was separated and collected from human peripheral blood, and then stored in liquid nitrogen in compliance with previous studies [17].

Human peripheral blood mononuclear cell (HPBMC) isolation

HPBMCs were isolated from human peripheral blood via Ficoll/Paque density gradient centrifugation, resuspended in α-MEM with 10% FBS, and cultured on plates in a 37 °C humidified incubator with 5% CO_2. On the next day, adherent cells were obtained and stored in liquid nitrogen.

Isolation of EVs

Monocytes were resuspended and seeded into a 6-well plate. After 48 h, the supernatant of the cells was collected. EVs from peripheral blood plasma (PB-EVs) and the culture medium of monocytes/macrophages (MC-EVs) were obtained using the ExoQuick™ kit (System Biosciences, USA) in compliance with previous studies [17, 18]. The protein concentration of EVs was detected using the BCA Protein Assay kit.

Cell Counting Kit-8 assay

HPASMCs (cat. no. 3110; ScienCell, USA) were seeded into 96-well microtiter plates, and cultured with EVs for the times indicated (cat. no. 3110; ScienCell, USA). Subsequently, 10 μL of CCK-8 (Dojindo, Japan) solution were added to each well. Proliferation was detected by assessing the absorbance at 450 nm using a microplate reader.

Transwell chamber assay

HPASMCs migration was assessed by a transwell chamber apparatus. HPASMCs were resuspended in serum-free SMCM, and added to the upper compartment of the chamber, while SMCM supplemented with 2% FBS and EVs was added to the bottom chamber. After incubation for 24 h, the membranes stained with crystal violet for 30 min, while the migrated cells were observed.

Immunofluorescence (IF) staining

HPASMCs were incubated with primary antibodies recognizing vimentin (VIM; cat. no. ab92547; Abcam) and proliferating cell nuclear antigen (PCNA; cat. no. ab92552;

Abcam). HPASMCs were then stained with Alexa Fluor 594-conjugated goat anti-rabbit IgG at 1:1,000 (cat.no. ab150080; Abcam), and the DNA was stained with DAPI. Finally, stained slices or cells were observed using a Leica TCS SP2 laser scanning confocal microscope.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

HPASMCs were co-cultured with EVs for 48 h. RNA was obtained from HPASMCs using the RNAiso Plus reagent (Takara Bio, Inc. Japan), and aliquots of 1 μg each were used for cDNA synthesis using the PrimeScript 1st Strand cDNA Synthesis kit (Takara Bio, Inc. Japan). The cDNA templates were subjected to RT-qPCR using the Power SYBR® Green PCR Master Mix kit (Ambion, USA) on a 7,500 qPCR system (Applied Biosystems, USA). Primers are showed in Table I. Amplification of the housekeeping gene β-actin was used to standardize the amount of sample RNA.

Immunoblotting assay

HPASMCs were co-cultured with EVs for 48 h. Cells were lysed in RIPA lysis and extraction buffer (Roche, Basel, Switzerland) for 30 min on ice. Immunoblotting assays were performed using SDS-PAGE followed by protein transfer to a PVDF membrane. The membranes were probed with primary antibodies against extracellular signal-regulated protein kinase (ERK; cat. no. 4695; CST.), phosphorylated (p)-ERK (p-ERK; no.4376; CST), c-Jun N-terminal kinase (JNK, cat. no. 9252; CST), phosphorylated (p)-JNK (p-JNK; cat. no. 4668; CST), p38 mitogen-activated protein kinase (p38; cat. no. 9212; CST), phosphorylated (p)-p38(p-p38; cat. no. 4511; CST), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat. no. 5174; CST), matrix metalloprotein-2 (MMP-2; cat. no. ab37150; Abcam), alpha smooth muscle actin (α-SMA; cat. no. ab32575; Abcam), Calponin (CALP; cat. no. ab46794; Abcam), MMP-9, PCNA and VIM overnight at 4 ˚C. The levels of protein are calculated as fold change in comparison to GAPDH levels, while the expression of phosphorylated proteins was normalized to total protein.

Statistical analysis

Statistical analyses were performed using SPSS 23.0 software (IBM, USA). Data are

showed as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was applied to compare data among groups, while Bonferroni's test was employed for post hoc comparisons. A *P*-value < 0.05 was regarded as statistically significant.

Results

Effects of EVs on HPASMC proliferation and migration

As shown in Fig.1A, PB- or MC-EVs derived from irreversible and reversible CHD-PAH groups led to enhanced HPASMC proliferation compared with those from normal controls, being most pronounced in the irreversible CHD-PAH group. The transwell chamber assay results were similar with respect to the capacity of PB-EVs or MC-EVs from the irreversible and reversible CHD-PAH groups to promote HPASMC migration (Fig.1B).

Effects of EVs on the expression of phenotypic markers of HPASMCs

Compared with the normal control group, PB- or MC-EVs from the irreversible and reversible CHD-PAH groups were all found to enhance synthetic phenotype markers (PCNA and VIM) and migration-related molecules (MMP-2 and -9) at both the mRNA and protein levels, which was accompanied by decreased expression of contractile phenotype markers (α-SMA and CALP; Fig.2A and 2B). PB-EVs from the irreversible group were more potent in increasing the mRNA level of PCNA, as well as the protein levels of PCNA and VIM (Fig.2A and 2B) than PB-EVs from the reversible group. Moreover, PB-EVs from the irreversible group were more effective in decreasing the mRNA level of α-SMA, as well as the protein levels of α-SMA and CALP than PB-EVs from the reversible group. MC-EVs from the irreversible group were more likely to increase the mRNA levels of PCNA and MMP-2, as well as the protein levels of PCNA and VIM (Fig.2A and 2B) than PB-EVs from the reversible group. IF staining further confirmed that PB-EVs or MC-EVs from the irreversible and reversible CHD-PAH groups each enhanced the expression of PCNA and VIM compared with those from normal controls, in which it was more pronounced in the irreversible CHD-PAH group (Fig.2C).

Effects of EVs on mitogen-activated protein kinase (MAPK) activation in HPASMCs

The results presented indicate that PB- or MC-EVs derived from the irreversible and reversible CHD-PAH groups could more significantly promote the switch from the quiescent contractile to proliferative, migratory, and synthetic phenotypes than those from normal controls. It is widely known that MAPK signaling pathways participate in cell proliferation, migration, and phenotypic transformation in HPASMCs [19, 20]. To address the possible involvement of MAPKs in the observed HPASMC dysfunction mediated by PB- and MC-EVs, we initially compared total and phosphorylated levels of MAPKs in HPASMCs. As expected, the level of p-p38 in HPASMCs co-cultured with PB- or MC-EVs from the irreversible and reversible CHD-PAH groups were obviously higher than those in HPASMCs co-cultured with those from the normal control group, especially in the case of irreversible CHD-PAH group-derived EVs, whereas the levels of p-JNK and p-ERK were not affected (Fig.3A). Treatment with the p38 inhibitor SB203580 (15 μM, Sigma-Aldrich) significantly inhibited p38 activation and effectively restored the effects of PB- or MC-EVs on HPASMC migration (Fig.3B).

Discussion

A lot of researches recently confirmed that vascular system-associated cells, comprising endothelial cells, smooth muscle cells, and inflammatory cells, release EVs that play biological and/or pathological roles in vascular lesions [21]. Interestingly, we showed that the EVs from the irreversible and reversible CHD-PAH groups could more significantly influence HPASMCs to switch from the quiescent contractile phenotype to synthetic phenotype than those from the normal control group, as evidenced by increased expression of synthetic phenotype markers (PCNA and VIM) and migration-related molecules (MMP-2 and -9), and decreased expression of contractile marker molecules (α-SMA and CALP), especially in the irreversible CHD-PAH group. In addition, we also found that the level of phosphorylation of p38 in HPASMCs co-cultured with PB- or MC-EVs from the

irreversible and reversible CHD-PAH groups were markedly higher than those in HPASMCs co-cultured with PB-EVs or MC-EVs from the normal control group. Treatment with the p38 inhibitor SB203580 effectively restored the effects of PB- or MC-EVs on HPASMC migration.

Over the past years, research has unraveled many mechanisms that occur during the pathogenesis of PAH, indicating that intercellular communication between vascular cells. EVs have attracted increasing interest from researchers in term of their newly identified function as mediators of cell-to-cell communication, and for providing the advantage of being relatively stable and not easily damaged by the external environment [22]. EVs are able to transport complex cargo molecules composed of proteins, lipids, and nucleic acids, and transmit them to the target cells they come across, which may finally reprogram recipient cells distal from their sites of release [23]. Elevated levels of EVs are correlated with diverse diseases, such as thrombosis (platelet EVs), congestive heart failure (endothelial EVs), breast cancer (leukocyte EVs), and women with preeclampsia (syncytiotrophoblast EVs) [24]. Recent studies have demonstrated the crucial roles of endotheliocyte- and mesenchymal stem cell-derived EVs in PAH [12, 14]. In addition, studies have shown that EVs from peripheral blood of MCT-induced mice contribute to the development of MCT-induced pulmonary hypertension [13, 14]. In the present study, PB-EVs from the irreversible and reversible CHD-PAH groups could more evidently accelerate the proliferation, migration, and phenotypic transformation of HPASMCs than those from the normal control group, especially in the case of EVS derived from the irreversible CHD-PAH group, indicating that PB-EVs play a central role in HPASMCs by determining whether pulmonary vascular remodeling is reversible or not.

PB-EVs can be obtained from peripheral blood cells [24]. Nevertheless, due to a lack of knowledge about the characteristics of EVs, there is no effective method to separate leukocyte-, monocyte-, and macrophage-derived EVs from a pool of EVs isolated from an *in vivo* source until now. We used a classical cell model that

mimicked peripheral blood monocyte/macrophage-derived EVs in CHD-PAH. Monocytes/ macrophages and vascular smooth muscle cells (VSMCs) are crucial players in the progress of PAH [25]. Countless data showed that pulmonary inflammation regulaed by lung perivascular macrophages is a prominent pathogenic driver of pulmonary vascular remodeling in PAH [26, 27]. VSMCs often reside in vascular lesions close to macrophage clusters, and are believed to be influenced by factors released from proinflammatory cells [28]. Many studies have demonstrated that macrophages can induce arterial smooth muscle cell proliferation and matrix metallo-proteinase (MMP) production by secreting platelet-derived growth factor (PDGF), interleukin 6 (IL-6), and/or tumor necrosis factor-α (TNF-α), which provided critical evidence for a potential cross-talk between macrophages and VSMCs [29-31]. In addition, monocyte-derived EVs could promote inflammatory and functional alterations in human bronchial smooth muscle cells [32]. Recently, it has been reported that macrophage-derived EVs could induce VSMC migration, adhesion, and phenotypic transformation [33]. In our present study, experiments on the effects of MC-EVs on HPASMCs yielded the same results as the PB-EVs trials, suggesting that the most important mediators of EV-induced HPASMCs were common to both types of EVs, and the effects of PB-EVs on HPASMCs might partly be attributable to MC-EVs.

While EVs induce the proliferation, migration, and phenotypic transformation of HPASMCs, the variety of downstream pathways leading to the induction of these effects remain to be identified. MAPKs are recognized as canonical signaling molecules, and have been widely demonstrated to be involved in growth responses to a number of HPASMC growth effectors [19, 20]. Moreover, increased MAPK activation has been correlated with pulmonary vascular remodeling in PAH rat models [34]. It has also been reported that macrophage foam cell-derived EVs have a considerable effect on VSMC migration and adhesion by activating ERK and protein kinase B (AKT) [33]. In our study, considering the rapid activation of p38 in HPASMCs co-cultured with PB- or MC-EVs from irreversible and reversible

CHD-PAH groups, it is highly likely that EVs enhanced the effects previously described for HPASMCs via activation of p38.

However, several problems need to be noted in this study. First, we used ultra-centrifugation at 100,000 × g to obtain EVs for our experiments, which did not allow us to distinguish between microparticles and exosomes. Establishing a more perfect flow cytometric analysis method to characterize and quantify EVs based on their sizes, heterogeneous cellular origins, and the different types of EVs, is extremely urgent. In addition, we explored the effects of PB- and MC-EVs on HPASMCs. It is worthy to further investigate the roles and mechanisms of leukocyte- and endothelial-derived EVs in HPASMCs. Considering that EVs play important roles during proliferation, migration, and phenotypic transformation of HPASMCs, further animal studies are needed to investigate whether the EVs from mice with PAH induced by high pulmonary blood flow could induce pulmonary vascular remodeling and PAH in healthy mice. With the exception of these, our results showed that PB or MC-EVs from the irreversible CHD-PAH group are more likely to increase the expression of synthetic phenotype markers (PCNA and VIM), and to decrease the expression of contractile marker molecules (α-SMA and CALP) than the reversible CHD-PAH group, indicating that several components may help determine whether pulmonary vascular remodeling is reversible. The present study focused only on the effects of EVs on HPASMCs, but did not take into account the protein composition, or the mRNA and microRNA species contained in EVs, which play a predominant role in the pathogenesis of PAH. In particular, circulating miRNAs can be stably detected in plasma or in serum, which are partly protected by EVs. For instance, the expression of miR-509-3p has been found to be decreased in the serum of patients with CHD-PAH, and its diagnostic value in CHD-PAH was close to that obtained by echocardiography [35]. Circulating miR-19a has been shown to be significantly higher in CHD-PAH than in normal controls, and it has proven satisfactory for clinical applications [36]. Thus, the genetic information contained in EVs may be interesting and worthy of further study.

Conclusions

In summary, EVs from the peripheral blood of CHD-PAH patients could exaggerate proliferation, migration, and phenotypic transformation of HPASMCs, depending on the source of EVs from monocytes/macrophages, while the EV-related effects might have been due to the activation of p38, implying that EVs may serve as potential biomarkers and therapeutic targets in CHD-PAH patients.

Declarations

Ethics approval and consent to participate

This study was conducted in compliance with the Helsinki Declaration. The study was approved by the Medical Ethics Committee of fuwai Hospital.

Consent for publication

All participants have approved publication of the data in this manuscript.

Availability of data and materials

The datasets collected and analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors report no conflicts of interests and have no relevant disclosures.

Funding

Not applicable.

Authors' contributions

ZJJ conceived and designed all experiments. Besides, ZJJ wrote the manuscript and approved the final version.

Acknowledgements

We wish to thank all the patients and the staff members of the medical staff for their cooperation.

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Figure legends

Figure 1. Effects of extracellular vesicles (EVs) on human pulmonary arterial smooth muscle cell (HPASMC) proliferation and migration. (A) Proliferation of HPASMCs induced by peripheral blood-derived and monocyte/macrophage- derived EVs (PB-EVs and MC-EV, respectively) from CHD-PAH patients and normal control groups was evaluated using the CCK-8 test. (B) Migration of HPASMCs induced by PB-EVs or MC-EVs from the CHD-PAH patients and normal control groups was evaluated using a transwell chamber assay. Scale bars, 200 μm. The data are represented as mean ± SEM. *reversible or irreversible CHD-PAH group vs. normal control group, $^{*}P < 0.05$; $^{\#}$reversible vs. irreversible CHD-PAH group, $^{\#}P < 0.05$

Figure 2. Effects of extracellular vesicles (EVs) on the expression of phenotypic markers of human pulmonary arterial smooth muscle cells (HPASMCs). (A) The mRNA expression of phenotypic markers in HPASMCs co-cultured with peripheral

blood-derived and monocyte/macrophage-derived EVs (PB-EVs and MC-EV, respectively) from CHD-PAH patients and normal control groups was evaluated using RT-qPCR. (B)The protein expression of phenotypic markers in HPASMCs co-cultured with PB-EVs or MC-EVs from CHD-PAH patients and normal control groups was evaluated using immunoblotting assay (L1: PB-EVs from normal control; L2: PB-EVs from reversible CHD-PAH group; and L3: PB-EVs from irreversible CHD-PAH group. L4: MC-EVs from normal control; L5: MC-EVs from reversible CHD-PAH group; and L6: MC-EVs from irreversible CHD-PAH group). (C) Expression of proliferating cell nuclear antigen (PCNA) and vimentin (VIM) in HPASMCs co-cultured with PB-EVs or MC-EVs from CHD-PAH patients and normal control groups was evaluated using IF staining. Scale bars, 25 μm. The data are represented as mean ± SEM. *reversible or irreversible CHD-PAH group vs. normal control group, $^{*}P < 0.05$, $^{**}P < 0.01$; $^{\#}$reversible vs. irreversible CHD-PAH group, $^{\#}P < 0.05$

Figure 3. Effects of extracellular vesicles (EVs) on mitogen-activated protein kinase (MAPK) activation in human pulmonary arterial smooth muscle cells (HPASMCs). (A) Phosphorylation levels of MAPKs in HPASMCs co-cultured with peripheral blood-derived EVs (PB-EVs) or monocyte/macrophage-derived EVs (MC-EVs) from CHD-PAH patients and normal control groups was evaluated using an immunoblotting assay (L1: PB-EVs from normal control; L2: PB-EVs from reversible CHD-PAH group; and L3: PB-EVs from irreversible CHD-PAH group. L4: MC-EVs from normal control; L5: MC-EVs from reversible CHD-PAH group; and L6: MC-EVs from irreversible CHD-PAH group). (C) Inhibition of p38 activity by SB203580 in HPASMCs co-cultured with PB-EVs or MC-EVs from CHD-PAH patients and normal control groups was evaluated using an immunoblotting assay. Suppression of p38 activation inhibited PB-EV- or MC-EV-induced HPASMC migration, as evaulated by transwell chamber assays. Scale bars, 200 μm. The data are represented as mean ± SEM. *without SB203580 treatment vs. with SB203580 treatment, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figures

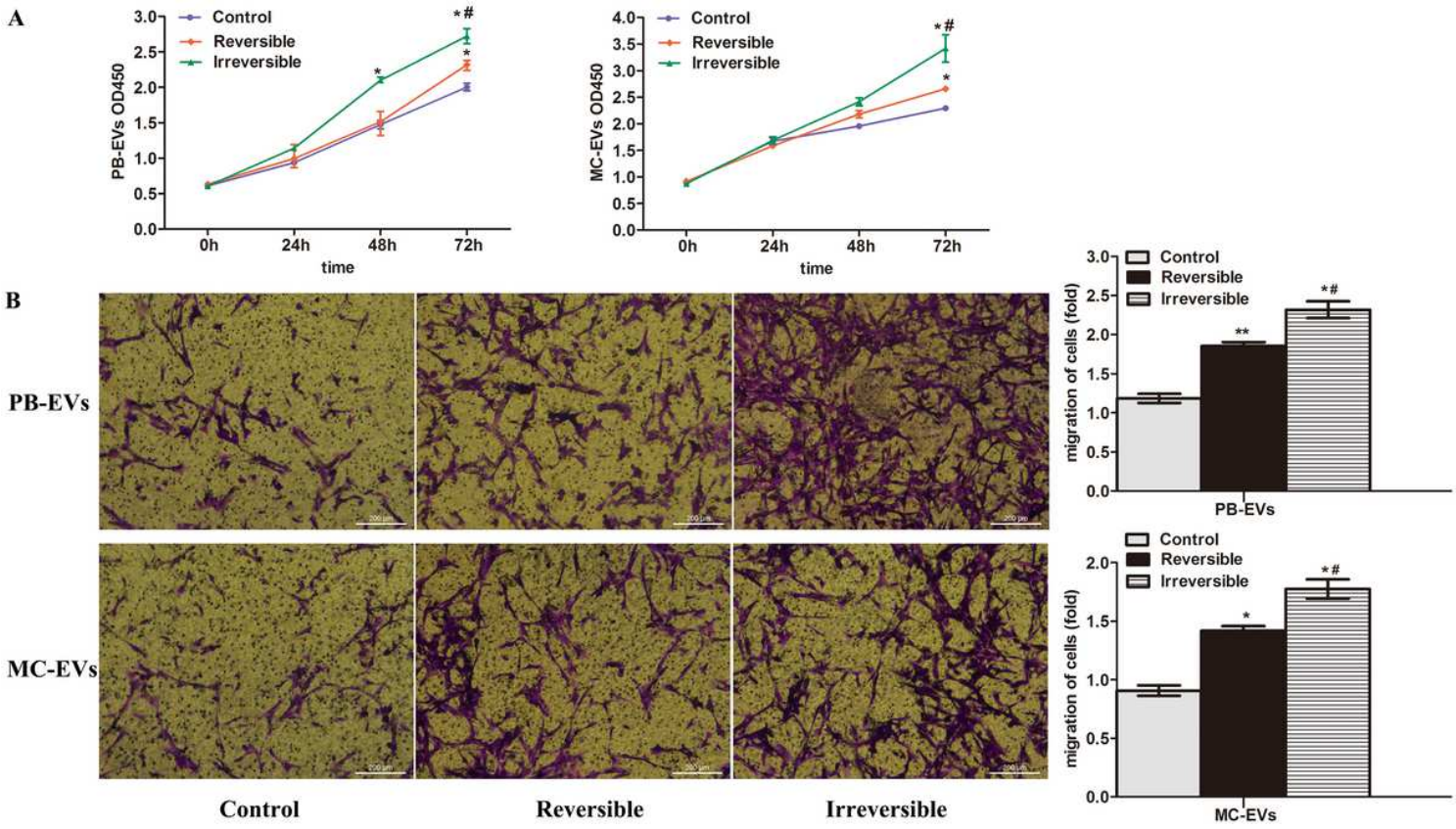

Figure 1

Effects of extracellular vesicles (EVs) on human pulmonary arterial smooth muscle cell (HPASMC) proliferation and migration. (A) Proliferation of HPASMCs induced by peripheral blood-derived and monocyte/macrophage- derived EVs (PB-EVs and MC-EV, respectively) from CHD-PAH patients and normal control groups was evaluated using the CCK-8 test. (B) Migration of HPASMCs induced by PB-EVs or MC-EVs from the CHD-PAH patients and normal control groups was evaluated using a transwell chamber assay. Scale bars, 200 μm. The data are represented as mean ± SEM. * reversible or irreversible CHD-PAH group vs. normal control group, *P < 0.05; # reversible vs. irreversible CHD-PAH group, #P < 0.05

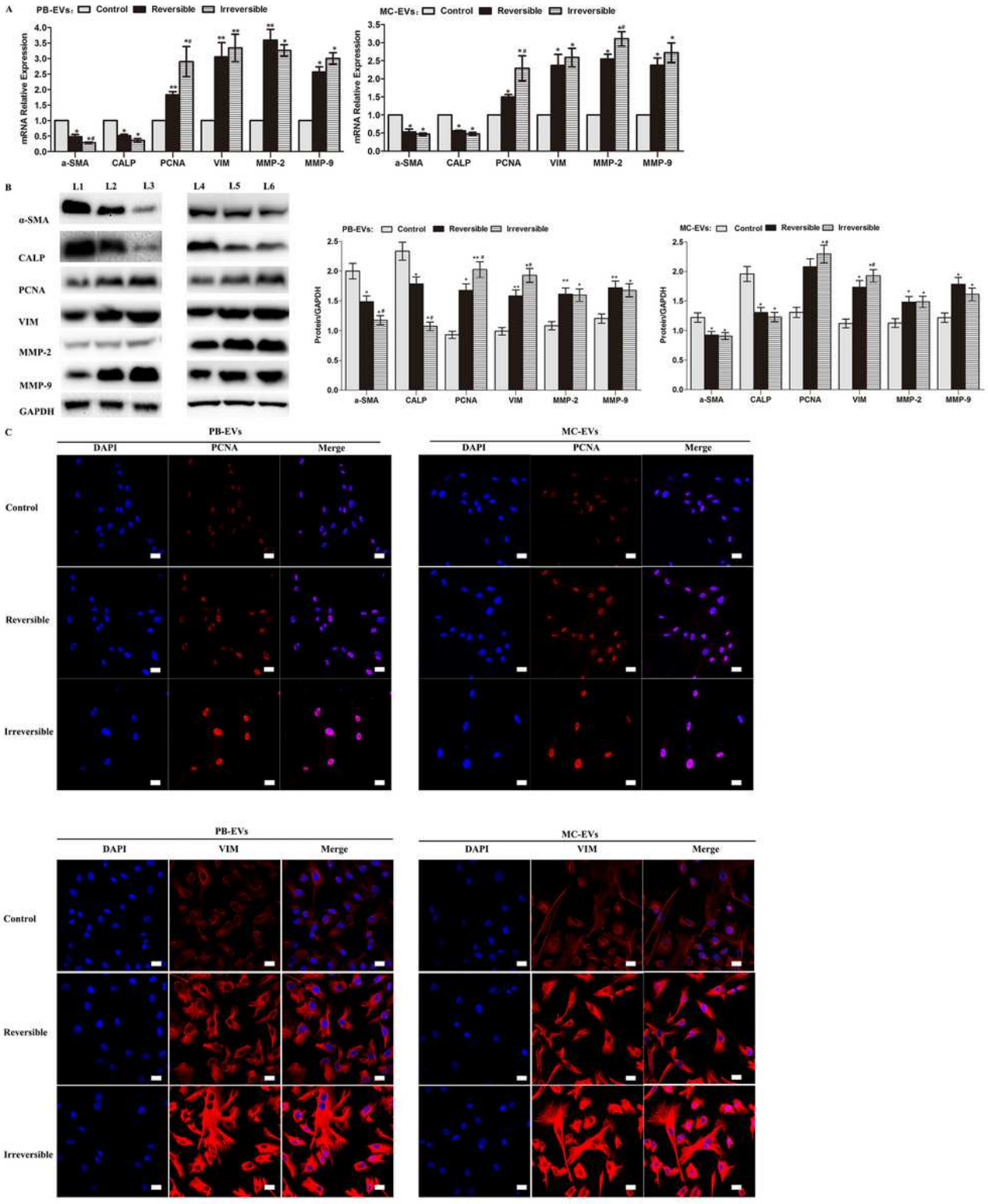

Figure 2

Effects of extracellular vesicles (EVs) on the expression of phenotypic markers of human pulmonary arterial smooth muscle cells (HPASMCs). (A) The mRNA expression of phenotypic markers in HPASMCs co-cultured with peripheral 17 blood-derived and monocyte/macrophage-derived EVs (PB-EVs and MC-EV, respectively) from CHD-PAH patients and normal control groups was evaluated using RT-qPCR. (B)The protein expression of phenotypic markers in HPASMCs co-cultured with PB-EVs or MC-EVs from CHD-PAH

patients and normal control groups was evaluated using immunoblotting assay (L1: PB-EVs from normal control; L2: PB-EVs from reversible CHD-PAH group; and L3: PB-EVs from irreversible CHD-PAH group. L4: MC-EVs from normal control; L5: MC-EVs from reversible CHD-PAH group; and L6: MC-EVs from irreversible CHD-PAH group). (C) Expression of proliferating cell nuclear antigen (PCNA) and vimentin (VIM) in HPASMCs co-cultured with PB-EVs or MC-EVs from CHD-PAH patients and normal control groups was evaluated using IF staining. Scale bars, 25 μm. The data are represented as mean ± SEM. * reversible or irreversible CHD-PAH group vs. normal control group, *P < 0.05, **P < 0.01; # reversible vs. irreversible CHD-PAH group, #P < 0.05

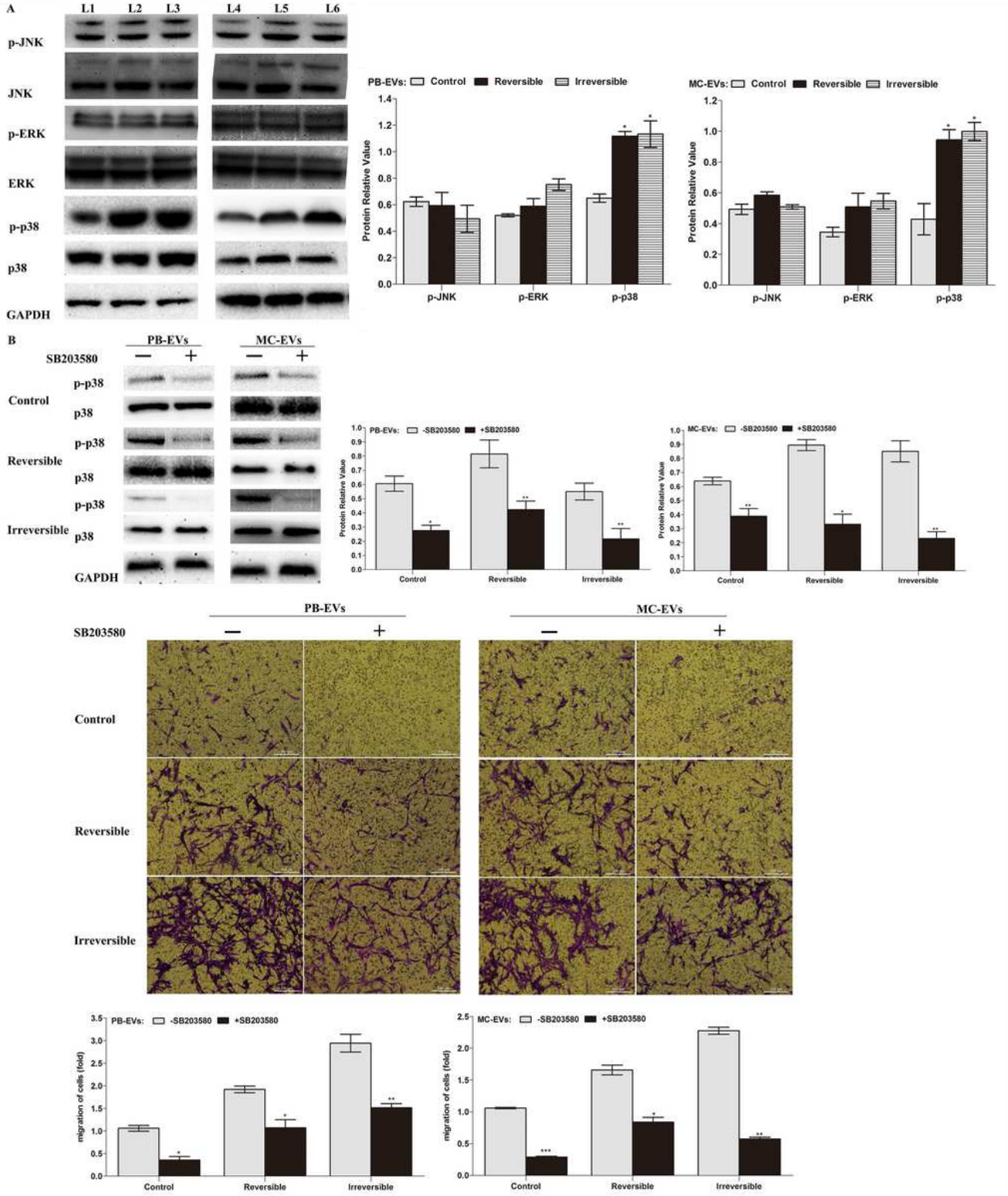

Figure 3

Effects of extracellular vesicles (EVs) on mitogen-activated protein kinase (MAPK) activation in human pulmonary arterial smooth muscle cells (HPASMCs). (A) Phosphorylation levels of MAPKs in HPASMCs co-cultured with peripheral blood-derived EVs (PB-EVs) or monocyte/macrophage-derived EVs (MC-EVs) from CHD-PAH patients and normal control groups was evaluated using an immunoblotting assay (L1: PB-EVs from normal control; L2: PB-EVs from reversible CHD-PAH group; and L3: PB-EVs from irreversible

CHD-PAH group. L4: MC-EVs from normal control; L5: MC-EVs from reversible CHD-PAH group; and L6: MC-EVs from irreversible CHD-PAH group). (C) Inhibition of p38 activity by SB203580 in HPASMCs co-cultured with PB-EVs or MC-EVs from CHD-PAH patients and normal control groups was evaluated using an immunoblotting assay. Suppression of p38 activation inhibited PB-EV- or MC-EV-induced HPASMC migration, as evaulated by transwell chamber assays. Scale bars, 200 μm. The data are represented as mean ± SEM. *without SB203580 treatment vs. with SB203580 treatment, *P < 0.05, **P < 0.01, ***P < 0.001.