Published in final edited form as:

Proteomics Clin Appl. 2018 September ; 12(5): e1700097. doi:10.1002/prca.201700097.

Proteomic Profiling Exosomes from Vascular Smooth Muscle Cell

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Abstract

Purpose: Vascular smooth muscle cells (VSMC) and endothelial cells (EC) communicate mutually to coordinate vascular development and homeostasis. Exosomes are emerging as one type of the mediators involved in this communication. Characterizing proteins in the exosomes is the critical first step in understanding how the VSMC-EC crosstalk is mediated by exosomes.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

The authors declare no conflict of interest.

Experimental design: The proteins in the human VSMC-derived exosomes are profiled using nanoLC-MS/MS based proteomics. The identified proteins are subjected to gene ontology analysis. The VSMC-derived exosomes are also assessed for proangiogenic activity in vivo.

Results: Four hundred and fifty-nine proteins are identified in the VSMC-derived exosomes. Gene ontology analysis revealed that the exosome proteins are involved in 179 cellular components, 120 molecular functions, and 337 biological processes, with cell–cell adhesion and platelet activation/coagulation ranked at the top. VSMC-derived exosomes do not display a proangiogenic activity in the in vivo angiogenesis assay, suggesting that the major function of VSMC-derived exosomes is to maintain vessel homeostasis.

Conclusion and clinical relevance: The analyses obtained a systematic view of proteins in the VSMC-derived exosomes, revealed the potential regulatory functions of the exosome in VSMC-EC communication, and suggest that dysregulation of VSMC-derived exosome-mediated functions may disturb vessel homeostasis thereby contributing to vascular diseases.

Keywords

angiogenesis; cross-talk; exosomes; nanoLC-MS/MS; vascular smooth muscle cells

1. Introduction

Vascular smooth muscle cells (VSMC) and endothelial cells (EC) are essential components of blood vessels. The mutual communication between VSMC and EC coordinates vascular development and maintains blood vessel homeostasis.^[1,2] Disorder of this communication leads to various vascular diseases such as atherosclerosis, vascular calcification, aneurysm, vascular hyperpermeability, and neointimal hyperplasia.^[1,3,4] Recent studies have elucidated that several signaling pathways, such as the PDGFB/PDGFR β , TGF β , and Slit-Robo pathways, critically regulate the VSMC-EC crosstalk during vascular development and in maintaining vessel homeostasis in adults.^[1,2,5,6] However, our understanding of the mechanistic basis of the complex communication between the two cell types and the effect of such communication on vascular function remain largely unknown.

Exosomes are cell-secreted extracellular vesicles (EVs) with size ranging from 30 to 150 nm. They were first described in the reticulocyte in 1983,^[7] and have lately been found in other cell types such as dendritic cells, tumor cells, EC, and mesenchymal stem cells.^[7,8] It has been recognized that exosomes are signaling transmitters for intercellular communication.^[9] In vasculature, the miRNAs in exosomes have been reported to mediate VSMC-EC communication.^[3] In current studies, we aim to identify the proteins that are loaded into VSMC-derived exosomes and to explore their related biological functions.

2. Results and Discussion

We examined a widely used human VSMC cell line, T/G-HAVSMC, as the cell source.^[10] In our initial studies, we noticed that T/G-HA-VSMCs underwent apoptosis in the serum free condition. Under such conditions, apoptotic blebs were produced with sizes in the same range as exosomes. To avoid this shortcoming, we cultured the T/G-HA-VSMCs with medium supplemented with exosome-free serum that elicited healthy cell growth and

production of exosomes. To isolate the exosomes, T/G-HA-VSMCs at 90% confluence were cultured in medium supplemented with exosome-free serum for 24 h. The conditional medium was collected and subjected to serial centrifugations: $300 \times g$ for 10 min, $2000 \times g$ for 10 min, $10\,000 \times g$ for 30 min, and $100\,000 \times g$ for 2 h. The sediment at $100\,000 \times g$ was washed with cold PBS, re-centrifuged at $100\,000 \times g$ for 2 h, collected, and processed for the following characterizations. In transmission electron microscopy (TEM) analysis, our isolated EVs showed a cup-shape morphology (Figure 1A), exhibiting a morphology typical for exosomes.^[7] We also calculated the sizes of the EVs from the TEM images. We determined the size of the isolated EVs is 54.72 ± 0.91 nm (Figure 1B), within the range of defined exosome size of 30–150 nm. We also assessed the size of the isolated EVs by dynamic light scattering and determined the size of the EVs to be 116 nm, which is also in the size range of exosomes. Therefore, these morphology and size analyses confirmed that our isolated EVs were exosomes.

To profile the proteins in the VSMC-derived exosomes, biological duplicates of exosome preparations were processed in in-gel digestion and the peptide samples were analyzed by LC/MS/MS. We identified 3220 distinct peptides that correspond to 459 proteins (Table S1, Supporting Information; <https://www.proteomicsdb.org/proteomicsdb/#projects/4265/6189?accessCode=d7d6efbbc4145ab9fb62c42b8bce754dc486eca5bf133dff67490c358e06e0f1>). Among them, exosome marker proteins such as syntenin-1, CD81, CD9, and HSPC71 were in high abundance. In addition, a Western blotting assay detected the expression of Alix, another exosome marker, in the isolated EVs (Figure 1D). Together, these characterizations profiled the proteins in the VSMC-derived EVs and also further confirmed that our isolated EVs were exosomes.

In literature, two studies have reported the protein profiles in exosomes isolated from VSMCs.^[11,12] The first report identified 64 proteins in the exosomes from coronary smooth muscle cells that were derived from the myocardium of 8-month-old domestic crossbred pigs (*Sus scrofa domestica*).^[12] The second report identified 345 proteins in exosomes that were derived from primary VSMC isolated from patient aortic explants.^[11,13] In these two reports, 387 proteins were identified in total. Our analysis identified 459 proteins in the T/G HA VSMC-derived exosomes (Figure 2A; Table S2, Supporting Information). Compared to the 345 proteins reported in human primary VSMC-derived exosomes, there are 184 proteins in common, and our study identified 275 novel proteins in the VSMC-derived exosomes (Figure 2A and Table S2, Supporting Information). When compared to the proteins pooled from the two reports, our study identified 261 new proteins, gaining a much more comprehensive view of proteins found in the VSMC-derived exosomes (Figure 2A and Table S2, Supporting Information).

Many studies found in the literature have focused on cancer cell-derived exosomes. We were interested in determining if the proteins in VSMC-derived exosomes are different from the ones in cancer cell-derived exosomes. To address this issue, we retrieved proteins identified in bladder cancer cell exosome (372 protein, Vesiclepedia_76) and colorectal cancer cell exosome (110 proteins, Vesiclepedia_2) from VesiclePedia and compared them with our data. We found that proteins in our VSMC-derived exosomes are quite different from the proteins in the cancer cell-derived exosomes. There are only 60 proteins in bladder cancer

cell-derived exosomes and 24 proteins in colorectal cancer cell exosomes that are in common with our identified proteins in T/G HS VSMC-derived exosomes (Figure 2B and Table S3, Supporting Information), thus highlighting that VSMC-derived exosomes play unique roles in VSMC-EC communication in vessels.

To reveal the biological function of the identified exosome proteins, we performed GO analysis using the online DAVID software (version 6.8).^[14] We found that the identified proteins are involved in 120 molecular functions (Figure 2C, Table S4, Supporting Information), 337 biological processes (Figure 2E, Table S6, Supporting Information), and are part of 179 cellular components (Figure 2D, Table S5, Supporting Information). The most enriched cellular component is the extracellular exosome. This further confirmed that the extracellular vesicles we isolated were exosomes. The top molecular functions include cadherin binding involved in cell–cell adhesion, protein binding, and GTPase activity. The top biological processes include cell–cell adhesion, cell–matrix adhesion, extracellular matrix organization, and angiogenesis. Interestingly, blood coagulation, platelet aggregation, platelet activation, and platelet degranulation are also ranked as top biological processes. VSMCs are known to rapidly express tissue factor in response to arterial injury, thereby playing an important role in promoting arterial thrombosis.^[15] Our study suggested that VSMCs regulate platelet behaviors via secreted exosomes to modulate hemostasis and thrombosis in vasculature.

VSMCs actively communicate with ECs during vascular development and in maintaining vessel homeostasis.^[1,2] Our GO analysis determined cell–cell adhesion as the top biological process of the identified proteins, which suggests that the VSMC-derived exosomes participate in the communication between VSMC and EC. This is supported by our detection of 16 angiogenesis-related proteins including transforming growth factor beta induced (TGF β 1), fibronectin, HSPG2, integrin α 5, α V, annexin A2, and caveolin in the VSMC-derived exosome (Table S7, Supporting Information). We further examined the effect of VSMC-derived exosomes in angiogenesis in an in vivo matrigel plug neovascularization assay that determines the responses of ECs to form new blood vessels under external stimuli. Considering that low level of exosome secretion occurs in normal vasculature,^[11] we tested the exosome at low concentration (1 μ g mL⁻¹) trying to differentiate its function in angiogenesis and vascular hemostasis in normal vasculature. We mixed 500 ng exosomes with 500 μ L BD matrigel and subcutaneously injected the mixed gel into the ventral region of 8- to 10-week-old female mice as we reported previously.^[6,16] The matrigel mixed with 75 ng VEGF165 or PBS was injected as positive or blank control, respectively. After 14 days, the injected matrigel plugs were assessed for neovascularization. As expected, the VEGF165-supplemented plugs were reddish, indicative of potent neovascularization, and the PBS group was pale, indicative of a lack of neovascularization. The exosome-supplemented plugs were slightly reddish (Figure 3B). However, quantitation of the newly formed vasculature did not show a significant proangiogenic activity for VSMC-derived exosomes (Figure 3C). In combination with the aforementioned bioinformatics analyses, this in vivo observation suggests that the major function of VSMC-derived exosomes may maintain vessel homeostasis via mediating VSMC-EC adhesion, verifying the concept that VSMC interacts with ECs to stabilize blood vessels in normal vasculature. Upon vascular injury,

high exosome secretion was reported,^[11] it will be very interesting to determine the function of exosomes at high concentration in vascular repair.

3. Conclusions

Our MS-based proteomics identified 459 proteins in the VSMC-derived exosomes, including 261 new proteins that have not as yet been reported. Bioinformatics analyses revealed that the identified proteins are involved in many different biological processes, with “cell–cell adhesion” ranked at the top. In vivo studies demonstrated that the VSMC-derived exosomes possess no significant proangiogenic activity in the matrigel plug neovascularization assay. This suggests that a major function for the VSMC-derived exosome is to maintain vascular homeostasis, which is in good agreement with a known major function of VSMC-EC interaction is to stabilize blood vessels. In addition, coagulation and platelet behavior are two of the top biological processes in our GO analysis. This reveals VSMC-derived exosomes are a novel critical regulator of hemostasis and thrombosis in vasculature, which is in good agreement with a recent study that reported VSMC-derived exosomes induce thrombogenesis.^[17] Further studies on the enriched biological functions revealed by GO analysis with finely designed biological functional assays are expected to lead to a better understanding of the regulatory role of exosomes in vasculature under physiological and pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Karen Howard for language editing and Mary B. Ard for electron microscopy analysis on the exosomes. The work was supported by NIH grants HL-093339 (L.W.), GM103390 (L.W.), U01CA225784 (L.W.), and R21HL131553 (L.W.), American Heart Association postdoctoral fellowship 15POST21260001 (H.Q.), American Heart Association Scientist Development Grant 17SDG33660550 (H.Q.), and Overseas Visiting Scholar Program for Middle-aged and Young Teachers in Shanghai Municipal Universities (S.W. and S.S.).

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Clinical Relevance

Vascular smooth muscle cells (VSMC) and endothelial cells (EC) are essential components of blood vessels. They mutually communicate to coordinate vascular development and to maintain blood vessel homeostasis. Disorders of this communication lead to various vascular diseases such as atherosclerosis, vascular calcification, aneurysm, vascular hyper-permeability, and neointimal hyperplasia. Currently, the molecular mechanisms underlying the mutual communication remain elusive. In this study, we profiled proteins in VSMC-derived exosomes, a type of extracellular vesicle that has been shown to play critical roles in cell–cell communication. Our analysis gained an overall view of the proteins loaded into the VSMC-derived exosomes and revealed the potential regulatory functions of the exosome in VSMC-EC communication in vasculature. These profiling and activity data are expected to be timely and very informative to researchers, including clinicians, with interests in physiological and pathological angiogenesis as well as related vascular diseases.

Figure 1. Characterization of VSMC-derived exosomes. A) Transmission electron microscopy image of the exosomes. B) Size distribution of exosomes measured by transmission electron microscopy. C) The size distribution determined by dynamic light scattering. D) Alix expression determined by Western blotting.

Figure 2. Bioinformatics analysis of the proteins identified in VSMC-derived exosomes. A) Comparison of proteins identified from human primary VSMC (HPV VSMC), pig VSMC, and T/G HA VSMC. B) Comparison between proteins identified in T/G HA VSMC-derived exosomes and in cancer cells derived exosomes. C–E). Gene ontology analysis of the identified proteins from T/G HA VSMC-derived exosomes in cellular components (C), molecular functions(D), and biological processes (E). Here only the ranked top 45 are shown.

Figure 3. Assessing the activity of VSMC-derived exosomes in matrigel plug angiogenesis assay. A) Procedure of the in vivo matrigel plug assay. B,C) The matrigel plugs dissected out 14 days after implantation. The plugs supplemented with VSMC-derived exosomes are somewhat more reddish compared to those from the PBS group, indicating VSMC-derived exosomes possess a weak proangiogenic activity (B). However, the visual difference was not statistically significant (C). The VEGF165 served as positive control. Statistics was performed by Student's *t*-test.