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High-resolution mass spectrometric analysis of the secretome from mouse lung endothelial progenitor cells

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Abstract

Recently we isolated and characterized resident endothelial progenitor cells from the lungs of adult mice. These cells have a high proliferation potential, are not transformed and can differentiate into blood- and lymph-vascular endothelial cells under *in vitro* and *in vivo* conditions. Here we studied the secretome of these cells by nanoflow liquid chromatographic mass spectrometry (LC-MS). For analysis, 3-day conditioned serum-free media were used. We found 133 proteins belonging to the categories of membrane-bound or secreted proteins. Thereby, several of the membrane-bound proteins also existed as released variants. 35 proteins from this group are well known as endothelial cell- or angiogenesis-related proteins. The MS analysis of the secretome was supplemented and confirmed by fluorescence activated cell sorting analyses, ELISA measurements and immunocytological studies of selected proteins. The secretome data presented in this study provides a platform for the in-depth analysis of endothelial progenitor cells and characterizes **potential** cellular markers and signaling components in hem- and lymphangiogenesis.

Keywords endothelial progenitor cells, cell growth, secretome, angiogenic proteins,

Introduction

Vascular endothelial cells form the inner lining of all blood and lymph vessels and play an important role in cardiovascular diseases as well as in inflammation- and tumour-induced formation of new vessels (tumor angiogenesis). In order to characterize new regulators of angiogenesis, proteomic approaches have been used for rat artery endothelium and human umbilical vein and microvessel endothelial cells [1, 2, 3]. In all studies, secreted proteins in the conditioned media have been analyzed. Compared to intracellular proteins, secreted proteins more likely play a direct role in the control of angiogenic processes. Like the vascular endothelial growth factor (VEGF) -family they may be directly involved in growth, differentiation and maturation of new blood and lymph vessels.

The concept that circulating precursor cells of the endothelial lineage may contribute to vascular development via postnatal vasculogenesis was initially proposed by Asahara et al. [4]. These so-called endothelial progenitor cells (EPCs) were isolated from peripheral blood of humans and mice on the basis of cell surface antigen expression [5]. These cells turned out to be a heterogeneous group of cells, characterized by varying expression of markers such as KDR/VEGFR-2, CD34 and CD133 as well as their binding to different lectins [6]. EPCs probably exist in a variety of hierarchical stages, ranging from the hemangioblast to the fully differentiated endothelial cell. Each subset of these cells expresses different surface markers, but they all share common stem cell properties like the potential of clonal expansion, high proliferation rates and the potency to differentiate into different endothelial cell subtypes. Like other progenitor cells, they remain in a quiescent state within their niche until specific signals induce them to expand and differentiate [7].

According to a very recent theory, endothelial cells establish a vascular niche, which may promote tumour growth [8]. This may be accomplished not only by the delivery of nutrients and oxygen, but also by the production of “angiocrine factors” (known as growth factors and cytokines) that act on stem and progenitor cells. Therefore, the identification of EPC-derived angiocrine molecules is of great interest for tumour biology and tissue repair.

In this study, we have characterized the secretome of EPCs isolated from the lungs of adult mice. The cells represent a type of resident, organ-specific progenitor cells [9]. They have an exceptionally high proliferation capacity, are non-transformed and can differentiate *in vitro* into lymph and blood endothelial cells. They also possess the potential for *de novo* vessel formation *in vivo*. A similar cell type has previously been isolated from rat lungs [10]. Using three-day conditioned medium from mouse EPCs, we were able to detect approximately 528 proteins of which ~130 belong to the categories of membrane-bound proteins or secreted proteins. Within these two groups we identified 35 proteins, which are already known to be expressed in endothelial cells or are related to both angiogenesis or anti-angiogenesis. Interestingly, several of the membrane-bound proteins were also detected as soluble forms. The presence of a substantial part of these proteins could be confirmed by FACS analysis, immunofluorescence and ELISA measurements.

Material and Methods

Cells

Mouse lung endothelial progenitor cells (MLEPCs) were isolated as described recently from Balb/c mice [9]. Single-cell subclone A4 was used here, which had been generated by limiting dilution assay.

Conditioned medium preparation

Confluent cells were washed twice with PBS and once with basal medium (DMEM). Cells were incubated for 48 - 72 h with defined medium w/o serum but in the presence of ECGF (endothelial cell growth supplement), - a bovine brain extract. After two to three days, the conditioned medium was harvested and centrifuged in order to remove cells and debris. **Supernatant samples were collected from two cultivation approaches. Concurrently, for each cultivation an identical volume of fresh medium was provided as a control to define proteins that are available in the supernatant of EPCs but originally are derived from the medium ingredients. In addition, each of the four samples was divided into two equal parts to finally identify EPC supernatant proteins and medium proteins from two biological and two technical replicates. The eight supernatants were stored at -20°C until protein precipitation and further sample preparation.**

Precipitation of proteins

The chloroform methanol precipitation method according to Wessel and Flugge [11] was used **to concentrate proteins from each of the eight samples**. Briefly, one part of conditioned medium (2 ml) was mixed with 4 parts of methanol, one part of chloroform and 3 parts of distilled water. Centrifugation was performed for 10 min. at 5000 g and 4°C. The upper phase (H₂O) was removed and the remaining interphase/organic phase was mixed with three parts of methanol. After centrifugation, the supernatant was removed and the **resulting** pellet was air dried. Dried protein **pellets from all eight samples (typically ~ 200 µg each)** were used for further analysis

Sample preparation for LC-MS/MS analysis

The purified pellets were resolved in 200µl 0.2M triethylammonium bicarbonate at pH8.3 (Fluka). Proteins were digested with sequencing grade modified trypsin (Promega) as recommended in a ratio of 1:100 at 37°C overnight. The resulting peptide solutions were vacuum-dried, peptides resolved in 0.2% TFA (trifluoroacetic acid) in water, desalted with µZipTip C18 RP pipette tips (Millipore) according to the manufacturer's protocol and eluted with 0.2%TFA/60%ACN (acetonitrile) in water. Finally, peptides were again vacuum-dried and stored at -20°C until MS/MS analysis.

HPLC-MS/MS analysis

Each peptide sample was separated by online reverse phase nano-liquid chromatography and analyzed by electrospray MS/MS using Acquity ultraperformance LC system (Waters Corp.) connected to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Peptides were separated on an analytical column (1.7-µm

BEH130, 75 μm $\times$ 250 mm, Waters Corp.) with UPLC buffer A (0.1% formic acid in water) and UPLC buffer B (0.1% formic acid in acetonitrile) via linear 120-min (first **technical** replica each experiment) or 180-min (second **technical** replica each experiment) gradients at a flow rate of 300 nl/min controlled with AcquityUPLC software

V1.30. Eluting peptides were ionized using PicoTip emitter needles (New Objective Inc.) and voltages of ~ 1800

V. Data-dependent acquisition was performed on the LTQ-Orbitrap Velos using LTQ Orbitrap Velos software 2.6.0 and Xcalibur software 2.1.0 in the positive ion mode. Survey full scan MS spectra (from m/z 300 to 2000) were acquired in the FT-Orbitrap with a resolution of 60,000 at m/z 400. A maximum of ten peptides were sequentially isolated for fragmentation in the linear ion trap using collision-induced dissociation (CID). CID spectra were generated with a normalized collision energy of 35%, an isolation width of 4 mass units, an activation Q of 0.250 and an activation time of 10ms. Dynamic exclusion of fragmented precursor ions was used to reduce fragmentation redundancy (repeat: 1, repeat duration: 17sec, exclusion duration: 60sec).

Data processing and analysis

Mascot Daemon 2.2.2 was used to convert raw data file format into .dta file format and to carry out database searches against the UniProtKB/Swiss-Prot database (release 57.0 of March 24, 2009 with 495,880 entries; taxonomy, *mus musculus* with 16,169 entries). Search parameter settings were as follows: enzyme: trypsin, maximum missed cleavages: 1; no fixed modification; variable modification: oxidation (Met); peptide tolerance: 10 ppm; MS/MS tolerance: 0.4 Da. Decoy searches were performed in a reversed UniProtKB/Swiss-Prot database with search parameters identical to those described above. False discovery rates (FDR) were usually $\sim 2\%$ for matches above identity threshold. Scaffold 3.0 (Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at more than 95.0% probability as specified by the Peptide Prophet algorithm [12]. Protein identifications were accepted if they could be established at more than 99.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm [13]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Raw data information of all experiments will be provided at request as Scaffold data file.

Definition of EPC culture supernatant specific proteins (secretome)

To define proteins that are specifically detectable in the supernatant samples from EPC cultures the protein identification lists from the four supernatant samples were compared with the identification lists from the four analyses of the fresh medium samples as follows: the Mascot search result files from the mouse database searches from all eight samples (chapter “data processing and analysis”) were loaded into Scaffold 3.0 (Proteome Software Inc.). Scaffold 3.0 calculated the quantitative value number by normalizing spectral counts (number of MS/MS spectra that were used to identify a protein) across the eight protein identification lists and then multiplying the spectrum counts in each biosample by the average divided by the individual biosample. The spectral counting approach was used to visualize the abundance of proteins across the eight samples as shown in figure 1. Proteins that were identified in at least one of the fresh medium derived samples by using the mouse database were not considered as EPC secretome specific because those proteins are already available within the

fresh medium, most likely due to homology between murine and bovine proteins. Thus, proteins originating from the fresh medium were rigorously subtracted from the list of identified proteins from the culture supernatant without making use of any quantitative information from the spectral counting approach. This strategy assured the most reliable basis for the identification of secretome specific proteins.

Confirmation of detected proteins by immunological methods

Confirmation of protein expression by FACS analysis

For fluorescence activated cell analysis (FACS) cells were treated with accutase (PAA, Austria) in order to detach them. For each measurement 1×10^5 cells per tube in 200 ml cold PBS/2% FCS were used. Cells were incubated for 30 min on ice and then rinsed with cold PBS/2% FCS and incubated with the primary antibody or isotype control antibody for 30 min on ice. Cells were then rinsed with cold PBS/2% FCS and then incubated with the corresponding fluorescent dye-conjugated secondary antibody in the dark. Finally, cells were washed again and transferred to flow cytometric tubes (Micronic, The Netherlands). Flow analysis was performed with FACS Calibur (BD) and the following primary antibodies: CD31/Pecam1 (rat; BD), CD102/Icam2 (rat; BD), Kdr/VEGFR-2 and Flt4 /VEGFR-3 (rat, U.D), CD144/Cadherin-5 (rat; D.V.), Lyve1 (rabbit; Reliatech), CD109 (mouse, BioLegend), ESAM (rat; D.V.), Emilin1 (mouse; A.C.) and Stabilin/CLEVER1 (rat; S.J.).

ELISA measurement of soluble VEGF receptors

Mouse endothelial growth factor receptor 1 + 2 duo-set ELISA kits were purchased from R&D Systems (Rüsselheim, Germany). ELISAs for the two receptors were performed according to the manufacturer's instructions. Briefly, the supernatant was diluted 1:3 in dilution buffer and incubated in 96-well plates precoated with the capture antibody directed against the extracellular domain of the two receptors for 1-2 hours. The wells were then washed three times (PBS with 0.1% Tween) and incubated with the biotinylated detector antibodies for 1-2 hrs. The plates were then washed three times and incubated with streptavidin-horseradish peroxidase for 30-60 min and, after washing, incubated with tetramethyl benzidine (TNB Plus from KEMENTEC Diagnostic, Denmark). All assays were performed in duplicate and the protein concentrations were calculated using a standard curve derived from the known concentrations of the two soluble receptor proteins.

Immunofluorescence

For immunofluorescence-staining, mouse endothelial progenitor cells were seeded in cell culture dishes or in Lab-Tek II chamber slides (Nunc) coated with gelatine or lysine. Almost confluent cultures were fixed 5 min with 4% paraformaldehyde. They were incubated for 15-30 min at room temperature with blocking buffer (1% FBS in PBS) and permeabilization (if necessary) was achieved by incubation with 0.1% Triton X-100 for one minute. Cells were incubated 60 min with primary antibodies and 45 min with the secondary antibodies. Nuclei were counter-stained with 0.5 mg/ml 4', 6-Diamidino-2-phenylindole (DAPI; Sigma). The following primary anti-mouse antibodies were used in this study for single- or double-staining: Pecam1/CD31 (rat-anti-mouse; BD, San Diego, CA), Lyve1 (rabbit-anti-mouse dil. 1:200; Reliatech, Wolfenbuettel, Germany), Icam2/Cd102 (rat-anti-mouse dil. 1:100; BD San Diego, CA), Stabilin-1/CLEVER1 (rat-anti-mouse/human dil. 1:200; S. J.), Cadherin-5/VE cadherin (rat-anti-mouse dil. 1:100; BD San Diego, CA), Esam (rat-anti mouse; D.V.) and

Emilin1 (mouse; A.C.). See Acknowledgement for abbreviations.

Results

We have recently described the isolation of endothelial progenitor cells from the lung of adult mice in detail. These cells express markers of endothelial progenitor cells (EPCs) as well as markers for native blood and lymphovascular endothelial cells [9]. The secretome of mouse endothelial progenitor cell has not been analyzed so far. We initiated this study because we expected to find new proteins and signaling molecules in the context of endothelial cell development. The serum was removed from the cells by two washes with buffer and another wash with basal medium without any proteins. For the generation of 3-day conditioned medium of almost completely confluent cells they were cultured without serum to reduce artificial protein background aggravating peptide analysis by mass spectrometry. However, the basal medium used during conditioning contained ECGF, which is a bovine brain extract and contains important factors for the maintenance of endothelial cells in culture [14]. FACS analysis before and after the starvation period indicated, that the amount of dead cells almost doubled (not shown). Since proteins from murine and bovine origin certainly share peptide sequences and have a distinct degree of homology the analyses of culture supernatants of EPCs alone is not sufficient to identify EPC specific proteins. Thus, a reliable identification of proteins specifically derived from mouse EPCs should strictly exclude proteins identified in fresh medium containing ECGF. Therefore, we analyzed EPC culture supernatant and equal amounts of fresh medium, each in two biological and two technical replicates, as described in the method chapter. Protein identification was carried out against the murine section of the Uniprot/Swiss-Prot database to define proteins present in the fresh medium and proteins present in the EPC culture supernatant. In total, we identified 1085 proteins that met the criteria of 99% protein identification probability and 95% peptide identification probability. 531 out of these were abundant in both specimen (conditioned medium and fresh medium) and 26 proteins were only identified in the fresh medium. We conservatively excluded proteins from the secretome specific subset that were at least identified with one peptide in one of the four mass spectrometric analyses. Thus, we subtracted the accumulated number of 557 proteins from the complete list of identified proteins and yielded a subset of 528 proteins that were specifically identified in the four secretome samples and never detected in the four fresh medium derived samples. Online Resource 1 shows the molecular weight (in kDa), the protein scores, the number of unique peptides and the sequence coverage in percent. Concerning the subset of 528 secretome specific proteins, 53% (278) of them were mass spectrometrically identified in all four EPC supernatant derived samples, 80% (417) were at least identified in three of four analyses and 93% (485) of proteins were identified in at least two of four proteins, indicating a high degree of reproducibility.

From the identified 528 proteins approx. 130 (~25%) belonged to the categories of membrane-bound or secreted extracellular proteins (Online Resource 2). Further analyses by literature searches (e.g. PubMed) revealed that as many as 35 proteins were already well known in the context of endothelial cells, angiogenesis or anti-

angiogenesis (Tab. 1). 29 (83%) of these proteins were identified in all four mass spectrometry analyses, the remainder was identified in three (5 proteins, 14%) or in at least two (1 protein, 3%) of four screens (Fig. 1) confirming the reproducibility of our experimental approach. With 528 mass spectrometric identifications the number of proteins we found in the secretome was unexpected high. Similar approaches with rat and human endothelial cells had resulted in much lower numbers [1,2]. The proteins detected here (Tab. 1) belonged mainly to the categories of cytokines and growth factors, growth factor receptors and co-receptors, as well as adhesion molecules and cell-type-specific membrane proteins. Many of the membrane and secreted proteins identified with our approach have not yet been reported in proteomic analyses of conditioned media from EPCs or endothelial cells.

To validate the proteome analyses, we applied immunological methods, mostly against antigens located on the surface of the cells. By FACS analyses we found e.g. *Pecam1/CD31*, *Icam2*, *Lyve1* and *Esam*. Selectively, we were able to confirm the presence of 10 membrane molecules by FACS analyses (Fig. 2). With the exception of *Flt-4*, all proteins were detected at relative high abundance. An interesting result is the detection of soluble forms of all three VEGF receptors (Tab. 1). The existence of two of them, *sVEGFR-1* and *sVEGFR-2* was confirmed by FACS analysis or by sandwich ELISA (Fig.2, Tab. 2). Quantification showed that the amounts of *sVEGFR-1+2* were high enough to block the free corresponding ligands by complex formation, which is of importance for the physiological regulation of both, hemangiogenesis and lymphangiogenesis (Tab. 2).

Immunocytological studies of mouse lung EPCs

The proteome analyses revealed expression of many proteins, which are associated with endothelial cells and angiogenesis, including membrane proteins such as adhesion molecules and growth factor receptors. By immunocytology, we investigated the EPCs and could validate their endothelial phenotype as well as the results of the proteome analyses. We stained confluent cells for typical endothelial markers like *CD31 (PECAM-1)* in combination with other antibodies. We confirmed expression of the intercellular adhesion molecule 2 (*ICAM2*), the endothelial specific adhesion molecule (*ESAM*) and *Stabilin-1 (Clever1)*, a protein involved in lymphocyte transmigration through blood and lymphatic endothelium (Fig. 3). EPCs were also positive for *Cadherin-5*, a molecule with control functions in intercellular junctions of endothelial cells, and *Emilin1*, which is responsible for regulation of vessel assembly. Furthermore, a subset of cells express also *Lyve1*, a hyaluronan receptor and specific marker for lymphatic endothelial cells. *Lyve1* was one of the proteins also found in the secretome. In cell culture, co-expression of *ICAM2*, *Stabilin-1* or *Cadherin-5* with *Lyve1*, it could be observed in many of the cells and proved the lymphendothelial character (Fig. 3A-C). In contrast, expression of the markers without *Lyve1* argues for a blood endothelial phenotype. The same holds true for *Emilin1* (Fig. 3D).

Discussion

The secretome of microvascular endothelial cells is *a priori* of particular interest, because new marker proteins or signalling molecules may be discovered, which are important for diseases like cancer, arteriosclerosis or

other cardiovascular diseases. The knowledge of these proteins may be important for the identification of potential new markers for diagnosis and disease prognosis, or surrogate markers for anti-vascular therapies. We used a subclone of our recently identified mouse lung endothelial progenitor cells (EPCs), which have a high proliferative capacity [9], to study the secretome and to identify new molecules, which may be involved in angiogenesis or tissue regeneration. Endothelial progenitor cells are of great importance, because in many pathological and physiological processes they are involved in tissue repair and regeneration. The secretome of EPCs that have the potency to form blood and lymphovascular endothelial cells has not been studied yet. Proteins secreted or released from vascular endothelial cells are difficult to study *in vitro*, because the cells usually need serum for their growth. Serum proteins then contaminate the secretome, which is an important aspect when using MS analyses. To circumvent this problem, cells were grown until confluence in serum-containing medium and were then washed three times with buffer. The medium used during the following 3-day period was basal medium with ECGF, which is a bovine brain extract containing different growth factors for the maintenance of primary endothelial cells in culture. The proteins identified by mass spectroscopy in the ECGF-medium were subtracted from the total number of identified proteins. Using this approach we were able to reduce the concentration of total (serum) proteins by 120-fold. However, culture of cells in serum-free medium has probably altered the secretome or reduced the amount of secreted proteins. For example, soluble Flt-1 was reduced about 3-fold in the serum-free conditioned medium as compared to normal medium (Tab. 3).

One of the most important results may be the detection of all three VEGF receptors, obviously all of them as soluble forms. The two soluble VEGFR receptors-1 and -2 are generated by differential splicing and are well known as natural occurring endogenous inhibitors of VEGF-A and VEGF-C bioavailability [15, 16]. By immunodetection and quantification we found amounts of soluble VEGFR 1+2, which would be sufficiently high to block free VEGF-A or VEGF-C, respectively, by complex formation for the physiological regulation of angiogenesis. Hemangiogenesis and lymphangiogenesis are governed by a precise balance of positive and negative regulators, but the regulation of this balance is still unknown. Soluble VEGFR-1 (sVEGFR-1) generated by differential splicing acts as potent naturally occurring inhibitor of hemangiogenesis by regulation the bioavailability of the blood-endothelial mitogen VEGF-A [17]. A splice variant of VEGFR-2 (sVEGFR-2) has recently been reported to block developmental and regenerative lymphangiogenesis by binding VEGF-C [16]. So, both of the naturally occurring soluble receptors can act as signal uncouplers for the development and maintenance of blood and lymph vessels. The possibility of the generation of soluble VEGFR-3 (sVEGFR-3) by differential splicing in humans or mice has not been reported yet. However, the extracellular part of VEGFR-3 protein can be detected and measured in sera from tumour patients [18]. It is not known yet if the proteins in the sera are generated by differential splicing or by shedding of membrane proteins. This will attract more attention in the future.

We observed other well known endogenous inhibitors of lymph vessel and blood vessel formation in the conditioned media of our progenitor cells [19,20]. These are semaphorin 3F and transforming growth factor- β (TGF- β), both detected by LC-MS/MS. Early lymph vessel development by mouse embryonic stem cells in the presence of VEGF-A and -C is repressed by TGF- β [20]. The authors suggest that TGF- β plays an important role in the down-regulation of lymphangiogenesis *in vivo*. However, we were able to show that mouse EPCs

implanted in Matrigel can form blood and lymph vessels *in vivo* [9]. It will be interesting to study if the cells still produce TGF- β when engrafted *in vivo*. We also detected CD109, a GPI-anchored protein belonging to the complement family. It is expressed on hematopoietic stem cells, megakaryocyte-precursors and vascular endothelial cells [21]. CD109 binds TGF- β receptors I and II, resulting in inhibition of signaling. It is likely that TGF- β signaling is not active in our mouse lung EPCs.

Semaphorins have been shown to inhibit endothelial cell migration but may also be inhibitors of metastatic tumor cells. Cells expressing Sema-3F are chemorepulsive for vascular and lymphatic endothelial cells expressing neuropilins, the corresponding high affinity receptors for semaphorins [19]. Additionally, neuropilin-1 (NRP-1) and NRP-2 are low affinity receptors for VEGF-A (only heparin-binding isoforms) and facilitate the binding of VEGF₁₆₅ to its high affinity receptor KDR/VEGFR-2 [5]. We NRP-1 and NRP-2 in the supernatant of EPCs, as well as the two semaphorins, sema-3F and sema-3A. Class 3 semaphorins are secreted proteins and many of them are involved in axonal guidance. It remains to be studied if the semaphorins and their receptors are involved in angiogenesis or may have functions during vascular development or axonal patterning *in vivo*.

We detected members of the roundabout (robo) family of proteins (robo-1 and -4) in the supernatants of the progenitor cells. Robo proteins are best known for their roles as receptors for the slit family of repellent axonal guidance cues. The robo family members are a group of highly conserved transmembrane glycoproteins that make up a small subgroup of the Ig superfamily. In endothelial cells, slit inhibits blood vessel growth by signaling through endothelial robo-4 and down-regulates VEGF/VEGFR-2 signaling [22]. Loss of robo-1 does not affect blood vessel growth. *In vitro*, both robo-1 and robo-4 knockdown by siRNA inhibits endothelial cell movement and disrupts tube formation on Matrigel [23]. This group could also show that robo-1 forms a heterodimeric complex with robo-4 and that robo-1 has an effect of robo-4-mediated filopodium formation. Other receptors with important endothelial functions identified in our study are EphB4 and Tie2.

In the supernatant we also found several cellular adhesion molecules related to endothelial cells such as ICAM-2, VE cadherin, protocadherin-12, PECAM and ESAM. These adhesion molecules are a very characteristic group for vascular endothelial cells and some of them, like ESAM, are expressed early in hematopoietic differentiation [24]. Others are used as classical markers for vascular endothelial cells, like CD31/PECAM-1 and VE cadherin.

In recent studies we have shown that the EPCs used here differentiate *in vitro* and *in vivo* into blood and lymph endothelial cells [9]. A high percentage of them expressed specific lymphatic endothelial markers such as Prox1, Lyve1 and podoplanin. The particular release of lymphatic markers could be confirmed by the detection of Lyve1, Emilin1 and Stabilin-1/CLEVER1 in the supernatant. Emilin1 knockout mice display an abnormal lymphatic phenotype. Emilin1 is an extracellular matrix molecule and the results show that the molecule is an important regulator of lymphangiogenesis [25]. The Stabilin-1/CLEVER-1 molecule is present on lymphatic and sinusoidal endothelium and seems to mediate migration of lymphocytes to the draining lymph nodes *in vivo* [26].

In conclusion, the results presented in this study show for the first time, the proteins that are released from mouse lung endothelial progenitor cells. More than 500 proteins were identified, 35 belonging to a group of well known proteins related to endothelial cell biology and angiogenesis. Other less well characterized proteins may be

useful for functional studies concerning their role in angiogenesis or as potential novel markers for progenitor cell or mouse endothelial cells.

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Figure legends

Fig. 1: Mass spectrometric analysis of culture supernatants reveals mouse lung EPC specific proteins. The normalized spectrum-counts-bar-chart represents the relative abundance of each protein across the different secretome (S) and background (B) samples. The height of each bar depicts the relative number of spectrum-counts. The 35 exemplified proteins were exclusively present in the secretome samples of EPCs. Selected non-specific proteins that were present in both specimens or only abundant in the background sample are shown in the line.

Bars from left to right indicate (each for B and S): Experiment 1/Replica 1, Experiment 1/Replica 2, Experiment 2/Replica 1, Experiment 2/Replica 2. Spectrum-counting was done automatically by Scaffold V3.0.

Fig. 2: Characteristic proteins detected in mouse EPCs conditioned media by MS/MS analysis and further

confirmed by FACS analysis. Mouse endothelial cells were grown to confluence, disaggregated and used for FACS analysis. The presence of the following cell surface proteins could be confirmed: Pecam1/Platelet endothelial cell adhesion molecule (CD31); Icam2/Intracellular adhesion molecule 2 (CD102); Lyve1/Lymphatic vessel endothelial hyaluronin acid receptor 1; Cd109/CD109 antigen; Kdr/Vascular endothelial growth factor receptor 2; Cdh5/Cadherin-5 (VE-cadherin, CD144); Esam/Endothelial cell-selective adhesion molecule; Stab1/Stabilin-1(CLEVER1); Flt4/Vascular endothelial growth factor receptor 3; Emilin1/Elastin microfibril interfacier 1.

Fig. 3: Immunofluorescence staining of mouse EPCs. A-C) Cells double stained with antibodies against Lyve1 (red) and A) Stabilin-1 (green) , B) Icam2 (green), and C) Cadherin-5 (green). Anti-Emilin1 (red) staining is shown in D). Anti-CD31 (E) and anti-ESAM (F) staining is shown in green. Protein expression of immunohistological specimens is in accordance with the results of proteome analyses of the conditioned media. Several cells co-express two of the markers (arrows in A-C). This argues for a lymphendothelial cell fate because Lyve1 is specific for lymphatic endothelium. In contrast, single expression of Stabilin-1, Icam2, Cadherin-5 or Emilin1 suggests a blood endothelial phenotype (arrowheads). Nuclei are stained with Dapi (blue). Scale bar = 20 μ m.

Table 1: Subsecretome of mouse lung EPCs indicates proteins related to endothelial cells and angiogenesis.

Table 2: Concentration of soluble Flt-1 (VEGFR-1) and soluble Kdr (VEGFR-2) in supernatants of the EPCs.

The subclon A4 was cultured in the presence and absence of serum and supernatants were measured by sandwich ELISA. Note that the two receptors are significantly higher when the cells are cultured with serum. For secretome analysis the medium w/o serum was used. Indicated are the mean values of two measurements. n.d.= not detectable (detection limit was ~ 30 pg/ml for sFlt-1 and ~80 pg/ml for sKdr).