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Extracellular Vesicles Derived from Endothelial Progenitor Cells Protect Human Glomerular Endothelial Cells and Podocytes from Complement- and Cytokine-Mediated Injury

Davide Medica [1], Rossana Franzin [2], Alessandra Stasi [2], Giuseppe Castellano [3], Massimiliano Migliori [4], Vincenzo Panichi [4], Giovanni Camussi [5] and Vincenzo Cantaluppi [1,6,7,*]

[1] Department of Translational Medicine, University of Piemonte Orientale (UPO), Via Solaroli 17, 28100 Novara, Italy
[2] Nephrology, Dialysis and Transplantation Unit, Department of Emergency and Organ Transplantation, University of Bari, 70124 Bari, Italy
[3] Nephrology, Dialysis and Transplantation Unit Department of Medical and Surgical Sciences University of Foggia, 71122 Foggia, Italy
[4] Nephrology and Dialysis Unit, Versilia Hospital, 55041 Camaiore (LU), Italy
[5] Department of Medical Sciences, University of Torino, 10126 Torino, Italy
[6] Center for Autoimmune and Allergic Diseases (CAAD), University of Piemonte Orientale (UPO), Corso Trieste 15, 28100 Novara, Italy
[7] Nephrology and Kidney Transplantation Unit, "Maggiore della Carità" University Hospital, Corso Mazzini 18, 28100 Novara, Italy
* Correspondence: vincenzo.cantaluppi@med.uniupo.it

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Abstract: Glomerulonephritis are renal inflammatory processes characterized by increased permeability of the Glomerular Filtration Barrier (GFB) with consequent hematuria and proteinuria. Glomerular endothelial cells (GEC) and podocytes are part of the GFB and contribute to maintain its structural and functional integrity through the release of paracrine mediators. Activation of the complement cascade and pro-inflammatory cytokines (CK) such as TNF-α and IL-6 can alter GFB function, causing acute glomerular injury and progression toward chronic kidney disease. Endothelial Progenitor Cells (EPC) are bone-marrow-derived hematopoietic stem cells circulating in peripheral blood and able to repair injured endothelium by releasing paracrine mediators including Extracellular Vesicles (EVs), microparticles involved in intercellular communication by transferring proteins, lipids, and genetic material (mRNA, microRNA, lncRNA) to target cells. We have previously demonstrated that EPC-derived EVs activate an angiogenic program in quiescent endothelial cells and renoprotection in different experimental models. The aim of the present study was to evaluate in vitro the protective effect of EPC-derived EVs on GECs and podocytes cultured in detrimental conditions with CKs (TNF-α/IL-6) and the complement protein C5a. EVs were internalized both in GECs and podocytes mainly through an L-selectin-based mechanism. In GECs, EVs enhanced the formation of capillary-like structures and cell migration by modulating gene expression and inducing the release of growth factors such as VEGF and HGF. In the presence of CKs, EPC EVs protected GECs from apoptosis by decreasing oxidative stress and prevented leukocyte adhesion by inhibiting the expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin). On podocytes, EVs inhibited apoptosis and prevented nephrin shedding induced by CKs and C5a. In a co-culture model of GECs/podocytes that mimicked GFB, EPC EVs protected cell function and permeselectivity from inflammatory-mediated damage. Moreover, RNase pre-treatment of EVs abrogated their protective effects, suggesting the crucial role of RNA transfer from EVs to damaged glomerular cells. In conclusion, EPC-derived EVs preserved GFB integrity from complement- and cytokine-induced damage, suggesting their potential role as therapeutic agents for drug-resistant glomerulonephritis.

Keywords: endothelial progenitor cells; extracellular vesicles; glomerular endothelial cells; podocytes; glomerulonephritis; inflammation; angiogenesis; cytokines; complement cascade

1. Introduction

The glomerulus is a crew of capillaries implicated in the ultrafiltration processes of the kidney. The glomerular capillary wall is composed of 3 layers: a fenestrated endothelium of glomerular endothelial cells, a glycocalyx with a complex mesh of proteins called glomerular basement membrane (GBM), and a layer of visceral epithelial cells called podocytes [1]. GFB has a very high hydraulic permeability combined with a marked selective permeability that excludes macromolecules such as albumin. Therefore, GFB retains most of the plasma proteins, with only 0.06% of albumin getting across the GBM [2]. In particular, podocytes constitute the slit diaphragms between their interdigitating foot processes that prevent large molecules from reaching the urinary space [3]. The expression of nephrin in the podocyte slit diaphragm is crucial for maintaining GFB selectivity [4]. Injury to any of these three components can result in the development of proteinuria. In addition to external factors, several paracrine mediators released by resident glomerular or immune cells strictly regulate GFB integrity in precise cellular crosstalk [5,6]. In particular, the glomerular microenvironment maintains GEC function stimulating expression of endothelial receptors such as Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) and Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) [7]. On podocytes, this crosstalk preserves cell function by maintaining the expression of nephrin [8,9]. Following GEC damage, different growth factors stimulate the migration of surviving cells to the injured site to repair vessels triggering angiogenesis [10,11].

Glomerulonephritis are inflammatory diseases affecting renal glomeruli able to compromise their filtering capacity and leading to chronic renal failure due to progressive fibrotic damage [12]. Activation of the complement cascade is a key factor for glomerulonephritis development and progression [13]. Complement protein fragment C5a induces the synthesis of pro-inflammatory cytokines (CKs) such as Interleukin-6 (IL-6) and Tumor Necrosis Factor (TNF-α) in the kidney, thus amplifying tissue damage [14,15]. TNF-α also increases the production of reactive oxygen species (ROS) [16] that produce multiple biological effects on glomeruli including apoptosis or programmed cell death [17]; TNF-α induces in GECs an inflammatory phenotype by increasing interleukin-6 (IL-6) release and membrane expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin [18,19]: these biological changes increase vascular permeability and leukocyte migration [17,20,21]. Moreover, TNF-α induces cell injury and loss of nephrin expression on podocytes disrupting glomerular slit diaphragm [9,22,23]. IL-6 has an equally important role in glomerular cells, increasing inflammation, and the recruitment of leukocytes [24].

Several studies have suggested that bone marrow-derived stem cells can repair injured glomeruli in experimental glomerulonephritis models [25,26]. In this context, endothelial progenitor cells (EPCs) are adult stem cells circulating in the peripheral blood to localize within sites of endothelial injury, triggering a regenerative program [27]. EPCs express both stem cell (CD34, CD133) and endothelial (VEGFR2, CD31) markers but they do not express monocyte (CD14) and platelet (P-selectin, CD41, CD42b) proteins [28,29].

Injection of EPCs in experimental models of glomerulonephritis in rats with IgA nephropathy lowered disease progression by down-regulating the expression of inflammatory factors [30]; moreover, intra-renal injection of EPCs in the experimental rat model of Thy1.1 glomerulonephritis demonstrated a significant reduction of endothelial injury and complement-mediated mesangial cell activation [31].

The regenerative effect of EPCs is mainly ascribed to their ability to release paracrine mediators such as growth factors and extracellular vesicles (EVs) [32,33]. EVs have a critical role in intercellular communication by transferring proteins, lipids, and genetic information: EVs include different families such as exosomes and shedding vesicles that differ in size and intracellular formation [34,35].

We have previously demonstrated that EVs released from EPCs activated angiogenesis in quiescent endothelial cells through the horizontal transfer of mRNAs [33]; moreover, we also observed that EVs protected the kidney from acute ischaemic injury by delivering pro-angiogenic and anti-apoptotic microRNAs [36]. Last, in the anti-Thy1.1 glomerulonephritis experimental model, we found that EPC EVs localized within injured glomeruli and inhibited complement-mediated mesangiolysis [37].

In this study, we recreated in vitro an inflammatory glomerular micro-environment to study the protective effects of EPC-derived EVs on GECs and podocytes cultured with TNF-α, IL-6, and C5a, in an inflammatory microenvironment resembling that observed in glomerulonephritis.

2. Materials and Methods

2.1. Isolation and Characterization of Human EPCs and EPC-Derived EVs

EPCs were isolated by density centrifugation from peripheral blood mononuclear cells (PBMC) of healthy donors, characterized and maintained in culture on fibronectin-coated plates as previously described **[28].** EVs were obtained from EPC supernatants by ultracentrifugation (Beckman Coulter Optima L-90K ultracentrifuge; Beckman Coulter, Fullerton, CA) and characterized as previously described **[33,36]**. We resuspended EVs pellets in medium 199; we quantified protein content by the Bradford method (BioRad, Hercules, CA), and we evaluated EV concentration, shape, and size by transmission electron microscopy and Nanosight analysis **[36]**: we stored EVs at −80 °C until use. In selected experiments, EVs were labeled with the red fluorescent dye PKH26 (Sigma Aldrich, St. Louis, MO) or treated with 1 U/mL RNase (Ambion, Austin, TX) **[33]**.

2.2. Isolation and Characterization of Human Renal Glomerular Cells

Primary cultures of human glomerular endothelial cells (GECs) and podocytes were isolated from glomeruli from the cortical segment of kidneys of patients undergoing surgery for renal carcinomas. Cells were characterized and immortalized to obtain cell lines as previously described **[38,39]**. We cultured GEC lines in vitro on gelatin-coated flasks on EBM medium containing endothelial growth factors (Lonza, Basel, Switzerland) and podocyte cell lines in DMEM (GIBCO). All mediums contained 10% Fetal Bovine Serum (FBS, Hyclone, Logan, UT, USA) and 2 mM glutamine (GIBCO): for experimental procedures, we plated all cell lines in multi-well plates (Falcon Labware, Oxnard, CA). In selected experiments, we incubated cells in an appropriate medium containing 20 ng/mL tumor necrosis factor (TNF)-α (Sigma Aldrich), 2.5 ng/mL IL-6, and 50 ng/mL human recombinant C5a protein (R&D Systems, Minneapolis, MN, USA) in the presence or absence of different concentrations of EPC-derived EVs assessed by Nanosight analysis.

2.3. Internalization of EPC-Derived EVs into Human GECs and Podocytes

We cultured GECs and podocytes on 6-well plates or chamber slides (Thermo Scientific, Waltham, MA, USA). We incubated cells with PKH26-labelled EVs for 1 h on cells seeded on chamber slides were fixed with paraformaldehyde (Sigma Aldrich), nuclei were counterstained in blue by 2.5 μg/mL Hoechst (Sigma Aldrich), evaluated by confocal microscopy (Zeiss LSM 5 PASCAL, Jena, Germany). Cells cultured on 6-well plates were detached by EDTA (Sigma) and analyzed by FACS (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). In selected experiments, PKH26-labelled EVs were pre-incubated with 1 μg/mL of different antibodies directed to block the binding to αVβ3-integrin (Biolegend, San Diego, CA, USA), α4-integrin, α6-integrin (Chemicon, Temecula, CA), CD29 or L-selectin (Becton Dickinson).

2.4. In vitro Studies on Human GECs and Podocytes

2.4.1. Angiogenesis

we studied the formation of capillary-like structures of GECs cultivated overnight on growth-factor reduced Matrigel (Becton Dickinson) on 24-well plates (5×10^4 for well). We observed GECs under an inverted microscope at ×100 magnification (Leica DM IRE2 HC, Leica Microsystem, Deerfield, IL, USA).

2.4.2. Proliferation

5×10^3 GECs for well were cultured on 96-well plates and incubated for 24 h with appropriate stimuli. GECs were then incubated for 24 h with 10 μM BrdU (Roche Diagnostics, Mannheim, Germany) and then analyzed in an automatized spectrophotometer at a wavelength of 405 nm, following the protocol of the manufacturer.

2.4.3. Migration

We studied GEC migration under an inverted microscope. We calculated the net migratory speed using the MicroImage software (Casti Imaging, Venice, Italy) based on the straight line distance between the starting and ending points divided by the time of observation **[40].**

2.4.4. Gene Array Analysis

We used the Human GEarray kit for the study of angiogenesis on GECs (SuperArray Inc., Bethesda, MD) to characterize the gene expression profile of cells cultured in the presence or absence of EVs. Microarray data archive: E-MEXP-3762, European Bioinformatics Institute: https://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-3762/*).*

2.4.5. Prediction of miRNAs- Target Genes Interaction

We used miRNet (https://www.mirnet.ca/), a bioinformatics software that gives information about miRNA-target interactions and displays the association in a visual network.**[41]** We predict miRNAs involved in down-regulation of the 16 genes identified by gene array analysis after searching in the miRNet human kidney database. We compared the suggested miRNAs by miRNet with previously identified miRNAs of EPC-derived EVs. (E-MEXP-2956, European Bioinformatics Institute: www.ebi.ac.uk/arrayexpress/).**[36]**

2.4.6. ELISA

We analyzed GEC supernatants for VEGF-A and HGF levels by ELISA (R&D Systems). We estimated their concentrations by generating a standard curve with appropriate controls according to the manufacturer.

2.4.7. Immunofluorescence Studies

After appropriate stimuli for 24 h, GECs cultured in chamber slides were fixed with ethanol-acetic acid 2:1 and stained for one h at 4 °C with a polyclonal antibody directed to anti-VEGF or anti-CD31 (Santa Cruz Biotech, Santa Cruz, CA, USA). After extensive washing, GECs were incubated for 1 h at 4 °C with appropriate anti-isotype Alexa fluor-conjugated antibodies (Life Technologies, Carlsbad, CA, USA). We fixed cells with paraformaldehyde, performed nuclei counterstaining with 1 μg/mL propidium iodide (Sigma Aldrich), and analyzed samples on fluorescence microscopy ×400 magnification **(**Leica DM LA, Leica Microsystem). We assessed fluorescence intensity in 10 different microscopic fields for each experimental point by the ImageJ program (NIH, Bethesda, MD).

In experiments on podocytes, after appropriate stimuli, we fixed cells in 4% paraformaldehyde for 15 min at 4 °C and incubated for 1 h at 4 °C with polyclonal antibody GP-N1 (Progen Biotechnik GmbH, Heidelberg, DE) to bind nephrin. After washing, we performed incubation for 40 min at 4 °C with Alexa Fluor-conjugated (Life Technologies) anti-guinea pig secondary antibodies. Finally, we performed nuclei counterstaining with 1 μg/mL propidium iodide (Sigma Aldrich), and we proceed

to analyze samples on fluorescence microscopy at ×400 magnification (Leica DM LA, Leica Microsystem).

2.4.8. PMN and PBMC Adhesion

After 12 h of stimulation in 24-well, we incubated GECs for 1 h with 5 × 10^4/well polymorphonuclear neutrophils (PMNs) or PBMCs isolated from healthy volunteers and labeled with 10 μm Vybrant cell tracer (Life Technologies). We fixed cells with paraformaldehyde, performed nuclei counterstaining with 1 μg/mL propidium iodide, and analyzed samples on fluorescence microscopy at ×400 magnification **(**Leica DM LA, Leica Microsystem). Samples were analyzed under a fluorescence microscope, counting green-stained cells in 10 different microscopic fields at ×200 magnification for each experimental point.

2.4.9. FACS Analysis

we seeded GECs on 6-well plates, and after appropriate stimuli for 24 h, cells were detached by EDTA and stained for 30 min at 4 °C with FITC- or PE-conjugated antibodies directed to bind ICAM-1, VCAM-1, E-Selectin (Beckton Dickinson). We used appropriate FITC- or PE-conjugated isotype antibodies as a negative control; FACS analysis was performed after fixation with paraformaldehyde 4% for 15 min at 4 °C. After appropriate stimuli, we detached cells by EDTA solution in experiments with podocytes, and we fixed cells with 4% paraformaldehyde solution for 15 min at 4 °C. We incubated cells with polyclonal antibody GP-N1, and after washing, we stained cells with FITC-(Sigma Aldrich) anti-guinea pig secondary antibodies incubation for 40 min at 4 °C before proceeding to FACS analysis.

2.4.10. Cytotoxicity Assay

5 × 10^4 GECs or podocytes were cultured on 24-well and incubated for 24 h in different experimental conditions; at the end of this period, we incubated cells with XTT (Trevigen, Gaithersburg, MD, USA) in a medium lacking phenol red. After one h, we analyzed samples in an automatized spectrophotometer at a wavelength of 450 nm.

2.4.11. Apoptosis

2 × 10^4 GECs or podocytes were cultured on 96-well plates, incubated for 24 h with different stimuli, and then subjected to TUNEL assay following instructions of the manufacturer (Apop-Tag; Oncor, Gaithersburg, MD, USA). Samples were analyzed under a fluorescence microscope, counting green-stained apoptotic cells in 10 different microscopic fields at ×100 magnification for each experimental point.

2.4.12. Reactive Oxygen Species (ROS) Detection Assay

After 12 h of stimulation, we added 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) to GECs following the instructions of the manufacture (Image-iT LIVE Green ROS Detection Kit, Life Technologies); after 30 min cells were analyzed by FACS and immunofluorescence studies on confocal microscopy, as previously reported **[42]**.

2.4.13. Co-Culture of GECs and Podocytes

We seeded GECs on 24-well plates and stimulated them for 24 h. Then we changed the medium and put them on collagen-coated transwells with podocyte monolayers for 24 h (Corning Costar Corp., Cambridge, MA). After stimulation, we put transwells in new plates, and we measured cytotoxicity, cell polarity, and permeability to albumin. For cytotoxicity, we put 250 μg/mL XTT (Sigma Aldrich) solution on podocytes. Supernatants and filtrates were collected after two h and analyzed at a wavelength of 450 nm. Cell polarity was analyzed by measuring transepithelial electrical resistance (TEER) with an epithelial volt-ohm meter (EVOM, World Precision Instruments,

Inc., Sarasota, FL, USA). We also evaluated permeability to albumin by diffusion of Trypan blue-albumin complexes across transwells. Aliquots of the medium from the upper and the lower wells were transferred to a 96-well plate and analyzed at the 590 nm wavelength (Model 680 Spectrophotometer, Biorad, Hercules, CA). Results are expressed as arbitrary units (upper medium O.D./lower medium O.D.).

2.5. Statistical Analysis

We express all data of different experimental procedures as average ± 1SD. We performed statistical analysis with ANOVA by Newmann-Keuls multi comparison test, and Student's t-test when indicated. For FACS data, we performed the Kolmogorov Smirnov nonparametric statistical test.

3. Results

3.1. Internalization of EPC-Derived EVs in Human Glomerular Cells

As shown by confocal microscopy studies (Figure S1A), we observed that PKH26 red-labeled EVs were efficiently internalized in vitro in GECs as well as in podocytes. FACS analysis showed that EVs stained both glomerular cell lines in a dose-dependent manner (Figure S1B). These results confirmed our previous in vivo findings on EV cell internalization in experimental Thy1.1 glomerulonephritis [37]. In selected experiments, we pre-incubated EVs with specific blocking antibodies (Ab), observing that Ab directed to L-selectin significantly inhibited EV internalization in both cell lines. By contrast, Abs directed to α4, α4, β1, and $\alpha V\beta$3 integrins did not affect EV internalization (Figure S1C).

3.2. EPC-Derived EVs Triggered GEC Angiogenesis

In comparison to vehicle alone, EPC-derived EVs significantly promoted the formation of capillary-like structures on Matrigel-coated plates (Figure S2A-B), proliferation (Figure S2C), and migration of GECs in vitro (Figure S2D). Pre-treatment of EPC-derived EVs with RNase abrogated all these effects (Figure S2).

As demonstrated by PCR array, EPC-derived EVs modulated the expression of different genes involved in GEC angiogenesis (Figure 1). In particular, EPC-derived EVs increased the expression of the following genes: ANGPT1, ANPEP, CDH5, COL18A1, CXCL10, CXCL9, EFNA3, EGF, ENG, EREG, FGFR3, FLT1, HAND2, HGF, ID1, IFNA1, IFNB1, IFNG, IGF1, IL1B, ITGB3, JAG1, KDR, LECT1, LEP, MMP9, NOTCH4, PDGFA, PECAM1, PF4, PGF, PLAU, TGF-β1. We also observed 14 genes involved in GEC angiogenesis down-regulated by EVs: ANGPTL3, BAI1, COL4A3, CXCL1, CXCL6, S1PR1, EPHB4, FGF1, FGF2, FIGF, HPSE, ITGAV, LAMA5, NRP2, TGF-β2, THBS1. Next, we analyzed the potential interacting miRNAs with these inhibited genes through the miRNet bioinformatic platform. Among the different suggested miRNAs, we identified 16 miRNAs carried by EPC-derived EVs that we had previously **[36]**: miR-137; miR-142-3p; miR-142-5p; miR-17-3p; miR-17-5p; miR-18a; miR-19a; miR-30a-3p; miR-30e-3p; miR-30a-5p; miR-30e-5p; miR-324-5p; miR-425-5p; miR-484; miR-650 (Figure 2A). Each of these molecules interacts with one or more target genes downregulated by EPC-derived EVs (Figure 2B).

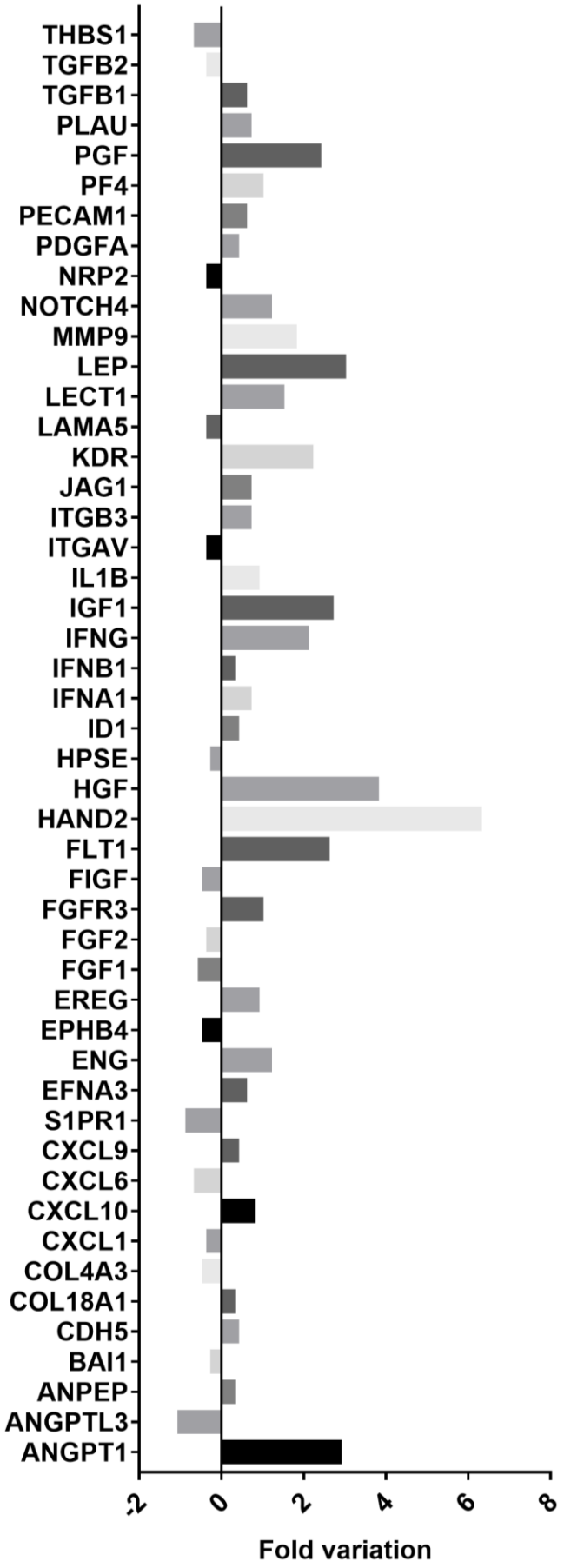
THBS1
TGFB2
TGFB1
PLAU
PGF
PF4
PECAM1
PDGFA
NRP2
NOTCH4
MMP9
LEP
LECT1
LAMA5
KDR
JAG1
ITGB3
ITGAV
IL1B
IGF1
IFNG
IFNB1
IFNA1
ID1
HPSE
HGF
HAND2
FLT1
FIGF
FGFR3
FGF2
FGF1
EREG
EPHB4
ENG
EFNA3
S1PR1
CXCL9
CXCL6
CXCL10
CXCL1
COL4A3
COL18A1
CDH5
BAI1
ANPEP
ANGPTL3
ANGPT1
-2
0
2
4
6
8
Fold variation

Figure 1. Intracellular pathways involved in GEC angiogenesis induced by EPC-derived EVs. RT-PCR array analysis of GEC incubated with EPC-derived EV vs. vehicle alone (angiogenesis-related genes). The graph shows the fold variation of angiogenesis-related genes between cells stimulated with EV vs. vehicle alone. We normalized Samples for the signals found in housekeeping genes (actin, Hypoxanthine phosphoribosyltransferase 1, Ribosomal protein, large P0, GAPDH, β2-microglobulin). We performed three different experiments with similar results. Gene table: ANGPT1: angiopoietin 1; ANGPTL3: Angiopoietin-like 3; ANPEP: Alanyl (membrane) aminopeptidase; BAI1: Brain-specific angiogenesis inhibitor 1; CDH5: Cadherin 5, type 2 (vascular endothelium); COL18A1: Collagen, type XVIII, α1; COL4A3: Collagen, type IV, α3 (Goodpasture antigen); CXCL1: Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, α); CXCL10: Chemokine (C-X-C motif) ligand 10; CXCL6: Chemokine (C-X-C motif) ligand 6; CXCL9: Chemokine (C-X-C motif) ligand 9; S1PR1: Sphingosine-1-phosphate receptor 1; EFNA3: Ephrin-A1; EGF: Epidermal growth factor; ENG: endoglin/CD105; EPHB4: EPH receptor B4; EREG: Epiregulin; FGF1: Fibroblast growth factor 1 (acidic); FGF2: Fibroblast growth factor 2 (basic); FGFR3: Fibroblast growth factor receptor 3; FIGF: C-fos induced growth factor (vascular endothelial growth factor D); FLT1: vascular endothelial growth factor type 1/vascular permeability factor receptor; HAND2: hearth and neural crest derivatives expressed; HGF: hepatocyte growth factor; HPSE: Heparanase; ID1: Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein; IFNA1: Interferon, α1; IFNB1: Interferon, β1; IFNG: Interferon, γ; IGF1: Insulin-like growth factor 1 (somatomedin C); IL1B: Interleukin, 1β; ITGAV: Integrin, αV (vitronectin receptor, α polypeptide, antigen CD51); ITGB3: Integrin, β3 (platelet glycoprotein IIIa, antigen CD61); JAG1: Jagged 1; KDR: (FLK-1) vascular endothelial growth factor receptor type 2; LAMA5: Laminin Subunit α5; LECT1: Leukocyte cell derived chemotaxin 1; LEP: Leptin; MMP9: matrix-metal protease 9; NOTCH4; NRP2: Neuropilin 2; PDGFA: Platelet-derived growth factor α polypeptide; PECAM1: platelet/endothelial cell adhesion molecule (CD31 antigen); PF4: Platelet factor 4; PGF: placental growth factor; PLAU: Plasminogen activator, urokinase; TGFB1: Transforming growth factor, β1; TGFB2: Transforming growth factor, β2; THBS1: Thrombospondin 1.

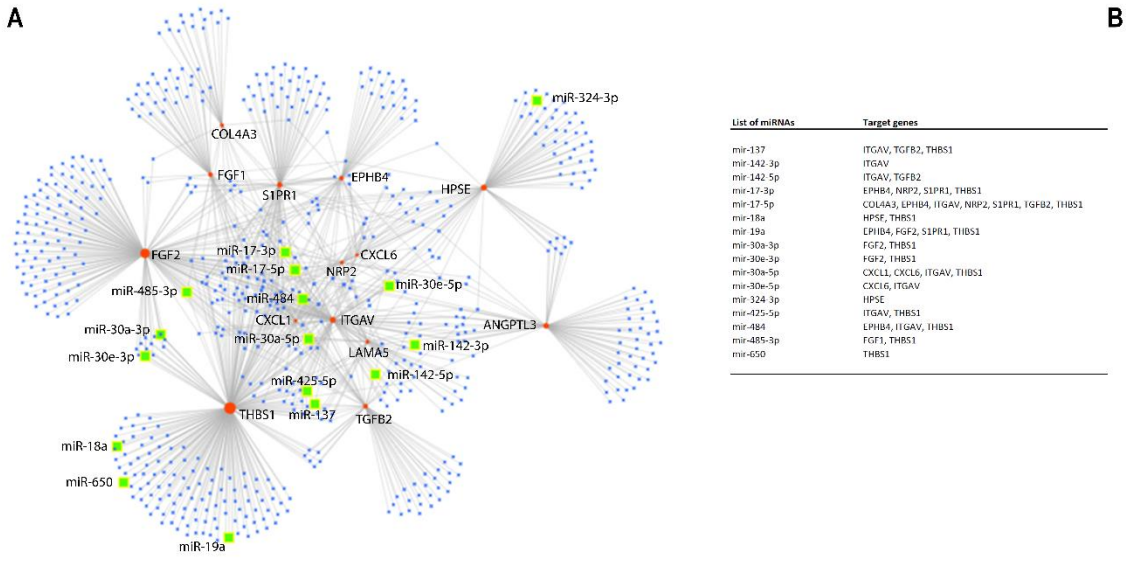

Figure 2. Network of predicted miRNAs-target genes interaction. (A) We observed 1038 potential interactions between the miRNAs (blue spots) predicted by miRNet to inhibit the expression of genes (orange spots). Between the miRNAs we observed that 16 of them, there were 16 miRNAs carried by EPC-derived EVs (green spots): miR-137; miR-142-3p; miR-142-5p; miR-17-3p; miR-17-5p; miR-18a; miR-19a; miR-30a-3p; miR-30e-3p; miR-30a-5p; miR-30e-5p; miR-324-5p; miR-425-5p; miR-484; miR-650. (B) Table showing the miRNAs present in EPC-derived EVs and their target genes. ANGPTL3: Angiopoietin-like 3; BAI1: Brain-specific angiogenesis inhibitor 1; COL4A3: Collagen, type IV, α3 (Goodpasture antigen); CXCL1: Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, α); CXCL6: Chemokine (C-X-C motif) ligand 6; S1PR1: Sphingosine-1-phosphate receptor 1;

EPHB4: EPH receptor B4; FGF1: Fibroblast growth factor 1 (acidic); FGF2: Fibroblast growth factor 2 (basic); FIGF: C-fos induced growth factor (vascular endothelial growth factor D); HPSE: Heparanase; ITGAV: Integrin, αV (vitronectin receptor, α polypeptide, antigen CD51); LAMA5: Laminin Subunit α5; NRP2: Neuropilin 2; TGFB2: Transforming growth factor, β2; THBS1: Thrombospondin 1.

Immunofluorescence studies (Figure 3A) and FACS analysis (Figure 3B) showed that EPC-derived EVs significantly up-regulated GEC expression of PECAM-1 and VEGF-A in comparison to vehicle; moreover, EPC-derived EVs increased the release of VEGF-A (Figure 3C) and HGF (Figure 3D) in GEC supernatants as detected by ELISA. These effects were abrogated after incubation of GECs with EPC-derived EVs pre-treated with RNase (Figure 3).

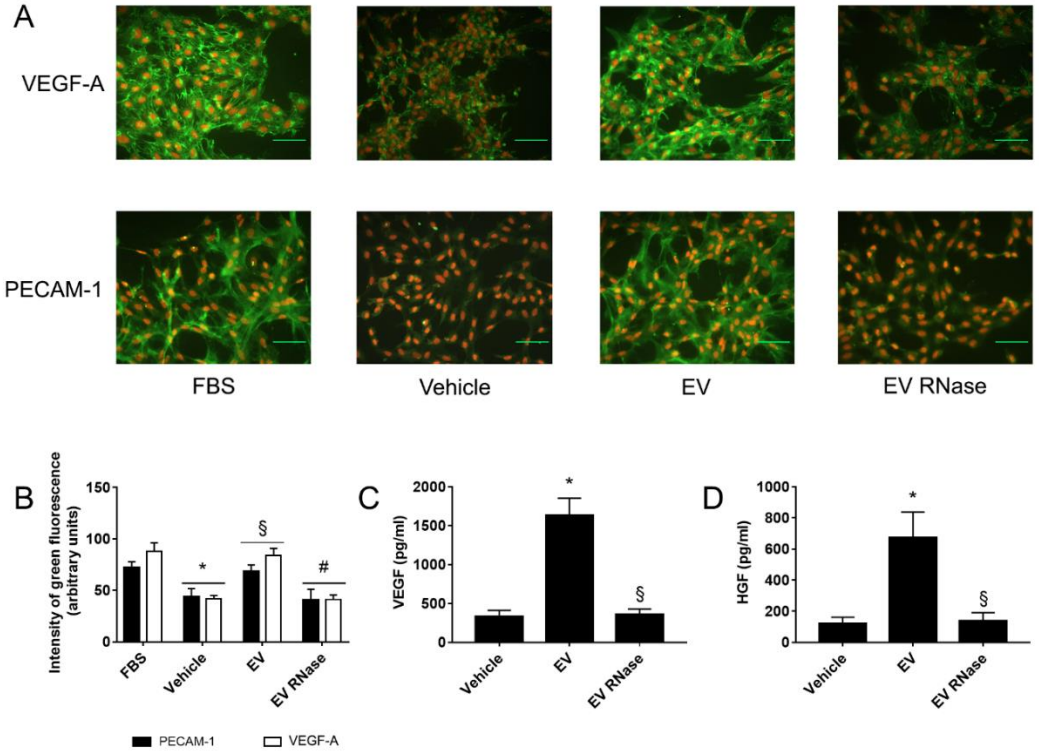

Figure 3. EPC-derived EVs induced GEC angiogenesis through release of pro-angiogenic factors. (A, B) Representative immunofluorescence micrographs (A) and quantification on fluorescence intensity of PECAM-1 and VEGF-A expression (B, green staining) in GECs. Nuclei were counterstained with 1 μg/mL propidium iodide; original magnification x100. We expressed data as mean arbitrary units ± 1SD of three different experiments for quantification on fluorescence intensity. We performed three experiments with similar results; we performed the statistical analysis by ANOVA with Newmann-Keuls multiple comparison tests and Student's t-test. In comparison to normal culture conditions with fetal bovine serum (FBS), serum deprivation (Vehicle) inhibited the expression of PECAM-1 and VEGF-A. EPC-derived EVs significantly increased both protein expression (§$p < 0.05$ EV vs. Vehicle), which was abrogated by RNase pre-treatment of EVs (#$p < 0.05$ EV RNase vs. EV). (C, D) ELISA for VEGF (C) and HGF (D) on supernatants of GECs incubated with different culture conditions. We expressed results as mean pg/mL ± 1SD of three different experiments. We performed statistical analysis was performed by ANOVA with Newman–Keuls multiple comparison test and Student's t-test. EPC-derived EVs significantly increased the release of both growth factors by GECs (*$p < 0.05$ EV vs. Vehicle). By contrast, RNase pre-treatment of EVs abrogated the release of VEGF and HGF (§$p < 0.05$ EV RNase vs. EV).

3.3. EPC-Derived EVs Protect GECs and Podocytes from Complement- and Cytokine-Mediated Injury

Incubation of GECs with EPC-derived EVs significantly increased viability (Figure 4A), resistance to apoptosis (Figure 4B), and inhibited expression of reactive oxygen species (ROS) in comparison to cells treated with C5a and inflammatory cytokines TNF- α and IL-6 (CKs) (Figure 4C-

D). EPC-derived EVs also inhibited PBMC and PMN (Figure 5A) adhesion to GEC monolayers cultured in an inflammatory micro-environment. In addition, EPC-derived EVs significantly down-regulated the protein expression of ICAM-1, VCAM-1, and E-selectin (Figure 5B). Of note, the pre-treatment of EVs with RNase abrogated their protective effects (Figure 4; Figure 5).

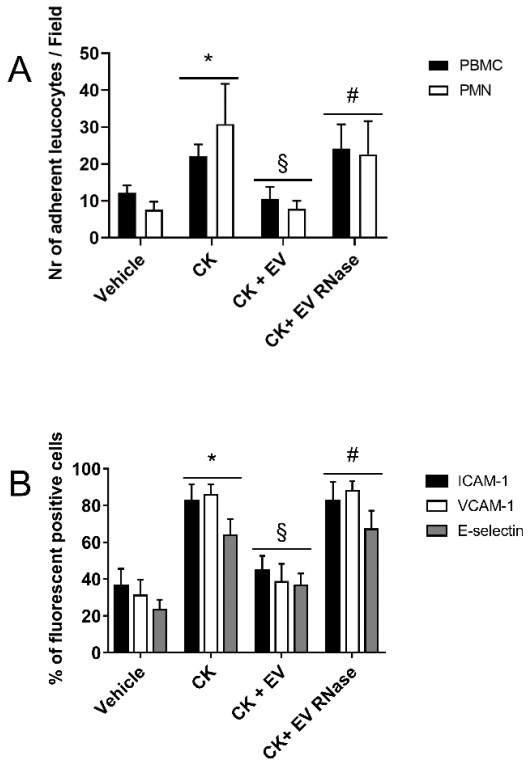

Figure 4. EPC-derived EVs inhibited leukocyte adhesion to GECs. (A) Graphs showing the count of adherent PBMCs (black columns) and PMNs (white columns) to GECs. After 24h incubation in different culture conditions, GEC monolayers were washed and incubated for 2h with FITC-labelled PBMC or PMN. We counted adherent FITC-leukocytes in 10 fields/well at x200 magnification under a UV light microscope after fixation. Data are representative of the average number of adherent cells/field ± 1SD. We performed three experiments with similar results and statistical analysis by ANOVA with Newmann-Keuls multiple comparison test and Student's t-test. In comparison to vehicle alone, CKs increased the number of adherent FITC-labelled PBMCs or PMNs significantly to GEC monolayers (*$p<0.05$ CK vs. Vehicle). The addiction of 25 μg/mL EVs to medium with CKs, decreased the number of adherent PBMs and PMNs to GECs (§$p<0.05$ CK + EV vs. CK) Pre-treatment of EPC-derived EVs with 1 U/mL RNase abrogated this effect (#$p<0.05$ CK + EV RNase vs. CK + EV). (B) FACS analysis of ICAM-1, VCAM-1, and E-selectin in GECs. We expressed the results as the mean of the percentage of positive cells ± 1SD. We performed the statistical analysis by ANOVA with Newman–Keuls multiple comparison test and Kolmogorov-Smirnov test. In comparison to serum deprivation (Vehicle), CKs induced a significant increase ICAM-1, VCAM-1, and E-selectin expression in GECs (D, *$p < 0.05$ CK vs. Vehicle). EVs significantly decreased the expression on the GEC surface of ICAM-1, VCAM-1, and E-selectin (D, §$p<0.05$ CK + EV vs. CK). By contrast, RNase treatment abrogated these effects induced by EVs (#$p<0.05$ CK + EV RNase vs. CK + EV). We performed the statistical analysis by ANOVA with Newman–Keuls multiple comparison test and Student's t-test.

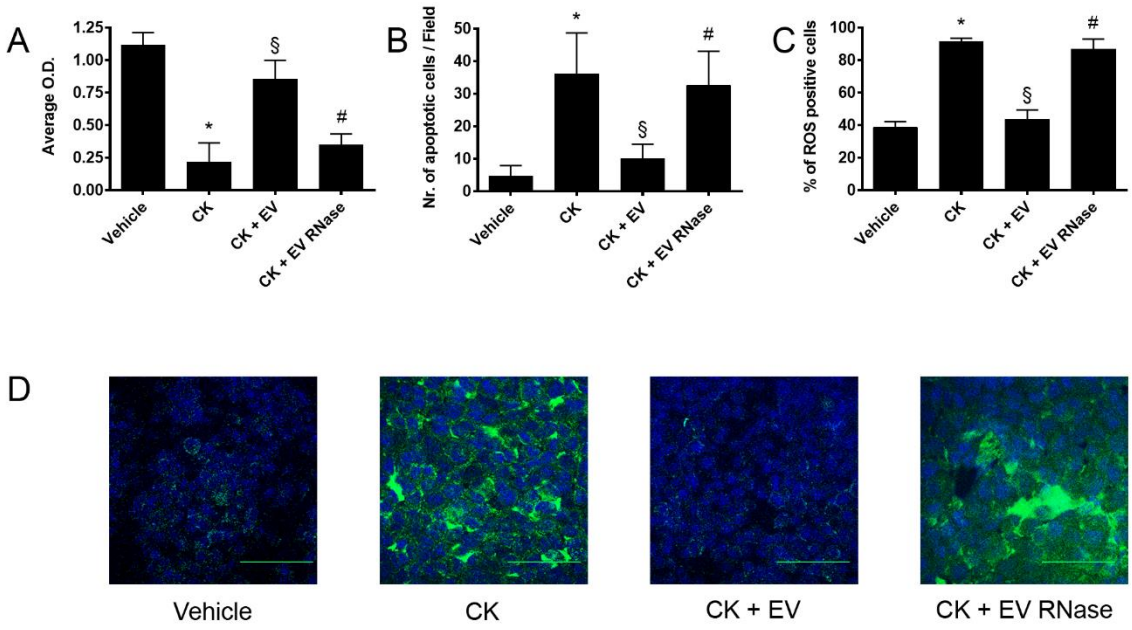

Figure 5. EPC-derived EVs protected GECs from complement- and cytokine-induced damage (A, B). Graphs showing GEC cytotoxicity by XTT assay (A) and apoptosis by TUNEL assay (B). For XTT assays, data are expressed as average OD intensity ± 1SD, whereas we expressed TUNEL assays data as the average number of green fluorescent apoptotic cells ± 1SD. We performed three experiments with similar results for all the assays and the statistical analysis by ANOVA with Newmann-Keuls multiple comparison test and Student's t-test. (C, D) FACS analysis (C) and representative micrographs (D) of ROS expression of GEC (green staining) by confocal microscopy studies (magnification x400). Nuclei were counterstained in blue by 2.5 μg/mL Hoechst. We performed three experiments with similar results for all the assays, and we performed the statistical analysis by ANOVA with Newmann-Keuls multiple comparison test and the Kolmogorov-Smirnov test. Incubation with CKs significantly increased GEC vitality (A), inhibited resistance to apoptosis (B), and increased oxidative stress (C) in comparison to treatment with vehicle alone (Vehicle, *$p<0.05$ CK vs. Vehicle). EV stimulation significantly inhibited these effects (§$p<0.05$ CK + EV vs. CK), but not EV were pre-treated with 1 U/mL RNase (#$p<0.05$ CK + EV RNase vs. CK + EV).

We observed similar results on podocytes: in comparison to vehicle alone, cells treated with pro-inflammatory C5a and CKs showed a significant decrease of cell viability (Figure 6A), resistance to apoptosis (Figure 6B), and nephrin expression on the cell surface (Figure 6C). EPC-derived EVs, but not RNase-pre-treated EVs, prevented all these biological effects on podocytes (Figure 6).

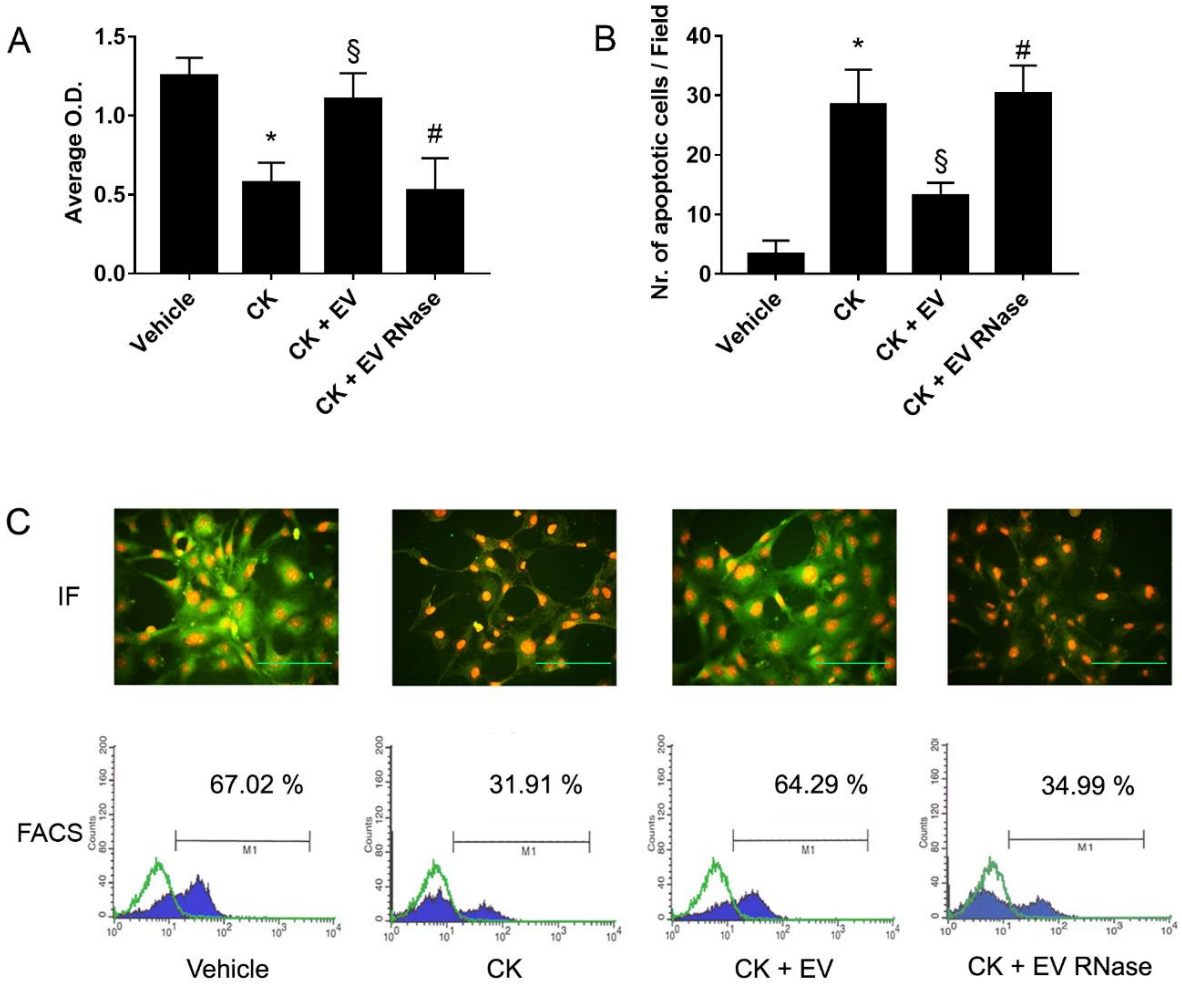

Figure 6. EPC-derived EVs protected podocytes from complement- and cytokine-mediated damage (A, B). Analysis of podocyte apoptosis by TUNEL assay (A) and cytotoxicity by XTT assay (B). We performed three experiments with similar results for all the assays and the statistical analysis by ANOVA with Newmann-Keuls multiple comparison test and Student's t-test. For TUNEL assays, data are expressed as the average number of green fluorescent apoptotic cells ± 1SD, whereas for XTT assays, we report data as average OD intensity ± 1SD. Incubation with CKs significantly increased podocyte apoptosis and inhibited cell viability compared to treatment with vehicle alone (*$p<0.05$ CK vs. Vehicle). EV stimulation significantly inhibited these effects (§$p<0.05$ CK + EV vs. CK), but not EV were pre-treated with 1 U/mL RNase (#$p<0.05$ CK + EV RNase vs. CK + EV). (C) Representative micrographs of nephrin expression in podocytes through immunofluorescence studies (IF) and FACS analysis (FACS). We stained nephrin in green for microscope analysis, and we counterstained nuclei with 1 μg/mL propidium iodide (400x). For FACS analysis, we compared the staining of nephrin (blue-filled curves) to internal control (green-line curve) represented by appropriate secondary isotype incubation. Stimulation with CKs for 1h significantly decreased nephrin expression on the cell surface compared to incubation with the vehicle. Podocytes cultured with EPC-derived EVs maintained nephrin expression by inhibiting shedding; RNase pre-treatment of EVs abrogated this effect. For FACS experiments, we performed the Kolmogorov-Smirnov statistical analysis.

3.4. Protective Role of EPC-Derived EVs in GEC-Podocyte Co-Culture Mimicking GFB

After pre-treatment of GECs with C5a and CKs, we used them in a co-culture model with podocytes that showed significantly decreased vitality (Figure 7A) and functional alterations such as loss of cell polarity assessed by TEER (Figure 7B) and increased permeability to albumin (Figure 7C). These indirect effects on podocytes were significantly decreased by pre-stimulation of GECs with

EPC-derived EVs, but not with RNase-treated EVs (Figure 7). These results suggest a potential positive effect of EPC EV in the mechanisms of GEC-podocyte interaction in GFB.

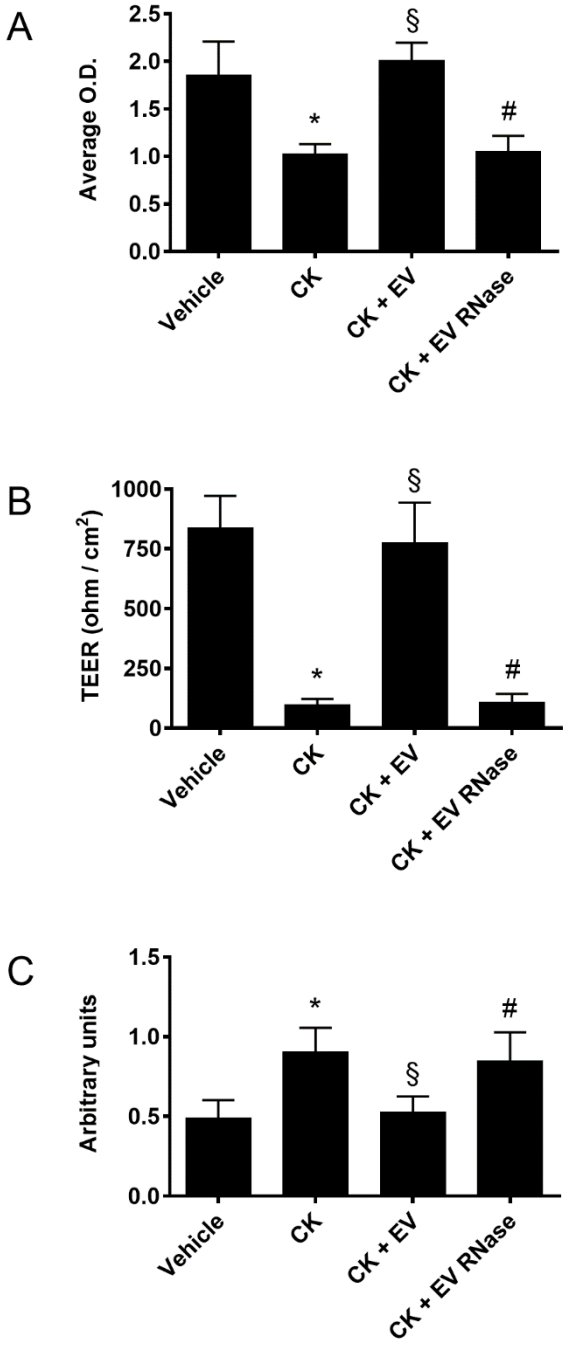

Figure 7. Co-culture model of GECs and podocytes. Analysis of podocytes cultivated in transwells over GECs in a co-culture model of cytotoxicity by XTT assay (A), cell polarity by Trans-Epithelial Electrical Resistance (B, TEER), and permeability to Trypan blue-albumin (C). We express the data XTT assays as average optical density (OD) intensity ± 1SD, for TEER results as average ohm/cm^2 ± 1SD, and permeability to Trypan blue-albumin as average arbitrary units ± 1SD. We performed the statistical analysis by ANOVA with Newmann-Keuls multiple comparison test and Student's T-test. If GECs were pre-treated with CKs, podocytes cultivated in transwells in contact with GEC supernatants showed significant loss of vitality (A), loss of cell polarity (B), and become permeability to Trypan blue-albumin (C, *$p<0.05$ CK vs. Vehicle). The addition of 25 μg/mL EPC-derived EVs to GECs significantly protected podocytes from these detrimental effects induced by CKs (§$p<0.05$ CK + EV vs. CK). We observed no protective effect of EPC-derived EVs pre-treated with 1 U/mL RNase (#$p<0.05$ CK + EV RNase vs. CK + EV). We performed three experiments for all the assays with similar results.

4. Discussion

In this study, we demonstrated that EVs derived from EPC exerted a protective effect on glomerular endothelial and epithelial cells by modulating their interaction and biological behavior in the presence of an inflammatory microenvironment resembling that observed during glomerulonephritis. In particular, EPC-derived EVs are internalized in both GECs and podocytes in an L-selectin-dependent manner, a mechanism similar to that adopted by the cells of origin to reach injured vascular tissues [28]. EVs limited inflammation sustained GEC angiogenesis and inhibited the detrimental activity of the complement fraction C5a and of inflammatory cytokines TNF- α and IL-6 on both GEC and podocytes. All these effects were significantly reduced by EV pre-treatment with RNase, suggesting the pivotal role of RNA transfer from progenitor-derived EVs to injured glomerular cells.

Highly specialized fenestrated endothelial cells internally cover glomerular capillaries: plasma solutes can pass across these endothelial fenestrations, reaching the basement membrane first and then the slit diaphragm of podocytes. These different layers constitute the glomerular filtration barrier (GFB) and orchestrate the filtration of different molecules in the pre-urine according to their molecular weight and electrical charge [3]. For these reasons, the integrity of these cells is fundamental to maintain GFB function. We know that glomerular endothelial cells (GECs) and podocytes exchange mediators for maintaining normal GFB function with precise intercellular crosstalk [5,6]: this interaction within the glomeruli is mediated by different growth factors and soluble mediators that preserve cellular differentiation and functionality [6,43].

During glomerulonephritis, inflammatory cytokines (CKs) can alter glomerular architecture and function [15]. Tumor Necrosis Factor-α (TNF-α) is a pro-inflammatory molecule able to alter the permeability of GFB [44]. TNF-α plays a crucial role in the pathogenesis of several types of glomerulonephritis [45]: in recent years, the use of monoclonal antibodies and molecules blocking its biological action has come into clinical practice [46]. Moreover, the persistence of these inflammatory molecules in the tissue stimulates fibrosis and chronic loss of renal function [47–49]. Furthermore, several studies demonstrated that the glomerular inflammatory environment could be enhanced by the activation of the complement cascade: complement fraction C5a increases expression of Interleukin-6 (IL-6) and TNF-α at the glomerular level [14].

Recently, extracellular vesicles (EVs) have been described as paracrine mediators released by resident glomerular cells [5]. EVs are a heterogeneous population of micro-organoid bodies that include exosomes and microvesicles that present different size, antigenic composition, and functional properties for the cargo of proteins and RNA subsets [34]. Of interest, stem cell-derived EVs have the property of repairing damaged tissues [50]: indeed, the regenerative properties of stem cells are mediated by the release of paracrine factors such as EVs, rather than by replacing cells lost after tissue injury.

We have previously observed the effects of EVs derived from different adult stem cells as paracrine mediators, particularly the biological effect of EPC-derived EVs in kidney damage [33,51]. EPCs are bone marrow-derived stem cells circulating in the peripheral blood that can localize within endothelial injury sites [27]. EPCs express both stem cells (CD34, CD133) and endothelial (VEGFR2, CD31) markers but they do not express monocyte (CD14) and platelet (P-selectin, CD41, CD42b) proteins [28,29]. EPCs can trigger a regenerative program by revascularizing damaged tissues favoring angiogenesis by secreting growth factors and other paracrine mediators [32,52,53].

The first aim of this study was to evaluate the biological activities of EPC-derived EVs on human renal glomerular cells *in vitro*. We observed that EPC-derived EVs internalized efficiently in human GECs, and podocytes through a mechanism mainly mediated by L-selectin, confirming the data previously observed in other experimental models [37]: antibodies directed to several integrins (α4, α6, β1, and αVβ3) inhibited less efficiently the internalization of EPC-derived EVs. These results suggest that L-selectin is the key molecule expressed on the EPC surface essential for homing on sites of vascular injury [28] and for internalization of EPC-derived EV in human renal glomerular cells.

Since we have previously demonstrated that EVs released from EPCs triggered an angiogenic program in quiescent endothelial cells by a horizontal transfer of mRNA [33] and protected the

kidney from acute ischemic injury by delivering their RNA content inducing hypoxic resident renal cells to a regenerative program [36], the second purpose of the present study was to investigate whether EPC-derived EVs induced specific biological effects also on GECs. We observed that EVs triggered angiogenesis in GECs by increasing the formation of capillary-like structures, proliferation, and migration *in vitro*. These effects were abrogated by pre-treatment of EVs with RNase, suggesting that horizontal RNA transfer is fundamental for EV-induced biological activity. These data confirmed previous findings found on endothelial cells of different tissue origin [33,36,54].

We then investigated the angiogenic pathways induced by EPC-derived EVs in GECs. EVs increased mRNA expression of endothelial cell receptors endoglin (ENG/CD105), platelet/endothelial cell adhesion molecule (PECAM-1), and vascular endothelial growth factor receptor type 2 (VEGFR-2): these data were confirmed at the protein level. Furthermore, EVs up-regulated adhesion molecules involved in endothelium integrity such as CDH5, but down-regulated ITGAV and integrin β3 gene expression. We also observed an increased expression of pro-angiogenetic genes involved in glomerular cell crosstalk [5,6]: angiopoietin-1, FLT1, HGF, PDGF-α, TGF-β1. To confirm intraglomerular pathways modulated by EPC-derived EVs, we found that GECs released high levels of HGF and VEGF-A in supernatants. EVs also increased the expression of other growth factors such as IGF1, PGF, EREG, but decreased FGF-1 and FGF-2 and FIGF gene expression. Moreover, we found the up-regulation of genes of trans-membrane receptors such as NOTCH4, FGFR3, and the down-regulation of NRP2, EPHB4, S1PR1. LEP, a molecule associated with endothelial cell differentiation during angiogenesis [14], was increased in GECs after EV treatment. EPC-derived EVs also decreased the expression of anti-angiogenic genes such as THBS1, BAI1, and pro-fibrotic genes [55], including TGF-β2. EVs modulated gene expression of proteins of glomerular basement membrane by up-regulating MMP9, COL4A3, and down-regulating LAMA5 and COL18A1. Moreover, the expression of other exoenzymes involved in angiogenesis was modulated by EVs (PLAU, ANPEP, and HPSE). Interestingly, EVs inhibited GEC expression of angiopoietin-like 3, a molecule that increases endothelial cell barrier permeability in glomeruli, [10] and PF4, a negative regulator of mesangial cell proliferation [56], thus suggesting other further potential protective mechanisms on GFB integrity. Moreover, we found in GECs an increased mRNA expression of EFNA3, ID1, and JAG1, all genes involved in proliferation and migration of endothelial cells [57] as well as EPCs [58,59]. The most EV-induced gene was HAND2, a molecule that belongs to the Twist family, involved in the development of different organs [60]: however, its biological function in kidney glomerulus is still unknown. Another inexplicable up-regulated gene by EPC-derived EVs was LECT1, a protein known to promote chondrocyte growth, inhibit angiogenesis, but with an unknown function in the kidney.

In previous studies, we identified more than 150 miRNAs carried by EPC-derived EVs [36]. We have herein identified 16 microRNAs among those carried by EVs able to down-regulate anti-angiogenic genes. In fact, 12 of these miRNAs interact with THBS1, an important negative regulator of angiogenesis. Interestingly, miR-17-5p is potentially able to bind other 6 mRNAs in addition to THBS1: COL4A3, EPHB4, ITGAV, NRP2, S1PR1, TGF-β2. In addition, 12 further miRNAs are potentially able to interact with 2 or more target mRNAs: on this basis, we could speculate that EV-induced GEC angiogenesis is mediated by the concomitant action of different miRNAs able to interact with several mRNAs within target cells.

Another relevant aim of this study was to evaluate whether EPC-derived EVs could protect renal glomerular cells cultured in detrimental inflammatory conditions. For this purpose, we decided to re-create an inflammatory microenvironment by using a mix of CKs (TNF-α, IL-6) and the complement fraction C5a on glomerular cells in vitro and to study the effect of EPC-derived EVs in this setting. CKs induced activation in GECs of a pro-inflammatory phenotype, increasing the expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin [17,20,21] and leading to an enhanced recruitment of PBMCs and PMNs. All these events are mediated by reactive oxygen species (ROS) [16] that in the presence of prolonged inflammatory stimuli increase GEC cytotoxicity and death by apoptosis [16]. On podocytes, CKs induced cell injury and triggered apoptosis, but the early observed effects are the rapid loss of nephrin expression by shedding [9,22,23]. EPC-derived EV inhibited all these effects of GECs and podocytes treated with CKs and C5a. By acting on different

mechanisms such as inflammation or the activation of regenerative mechanisms on glomerular cells, EVs are one of the potential treatments for redoubtable and unpredictable diseases such as glomerulonephritis.

We have previously described that EVs derived from EPC exert a protective effect in Thy1.1 glomerulonephritis on rats by inhibiting antibody- and complement-mediated injury of mesangial cells [31]. EVs protected glomeruli by fibrotic processes that lead inexorably to chronic renal failure. Moreover, in the same experimental model, EV treatment preserved endothelial- (RECA-1) and podocyte-markers (synaptopodin) expression suggesting a role of EVs in protecting these glomerular cells. In the light of the data herein described, we could speculate that EPC-derived EVs not only can antagonize complement cascade but also inflammatory injury mediated by CKs.

On this basis, we set-up a co-culture model of GECs and podocytes: we observed that pre-stimulation of GECs with EPC-derived EVs maintained podocyte viability, trans-epithelial electrical resistance, and inhibited loss of permeability to albumin, all established markers of GFB integrity. All these effects on podocytes might be related to the pre-angiogenic phenotype induced by EPC EV on GECs and mediated by the release of growth factors such as HGF and VEGF-A.

RNase pre-treatment abrogated all the effects mediated by EPC-derived EVs, confirming once again that mRNA and microRNA transfer play a critical role in these biological effects as previously described in other experimental models [33,36,37,54].

5. Conclusions

EPC-derived EVs may preserve GFB integrity from complement- and cytokine-induced damage: this protective effect on glomerular cells seem to be mainly ascribed to RNA transfer from progenitor-derived EVs to injured GECs and podocytes. Basing on previously published data in experimental glomerulonephritis models and on the results of the present study, EPC-derived EV could represent an attractive alternative in patients resistant to classical therapeutic agents. Moreover, EVs can induce immunomodulation and glomerular healing without the potential adverse effects of stem cell therapy, including maldifferentiation and tumorigenesis.

Author Contributions: D.M. conceived the experiments, performed the experiments, wrote the article, analyzed data, and interpreted data. R.F. wrote the article and analyzed data. A.S. wrote the article and analyzed data. G.Cas. contributed to the study design and conceived the experiments. M.M. contributed to the study design and reagents/materials/analysis tools. V.P. contributed to the study design and reagents/materials/analysis tools. G.Cam. conceived the experiments, reviewed the article, analyzed and interpreted data. V.C. conceived the experiments, wrote the article, analyzed and interpreted data. All authors read and approved the final article.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix

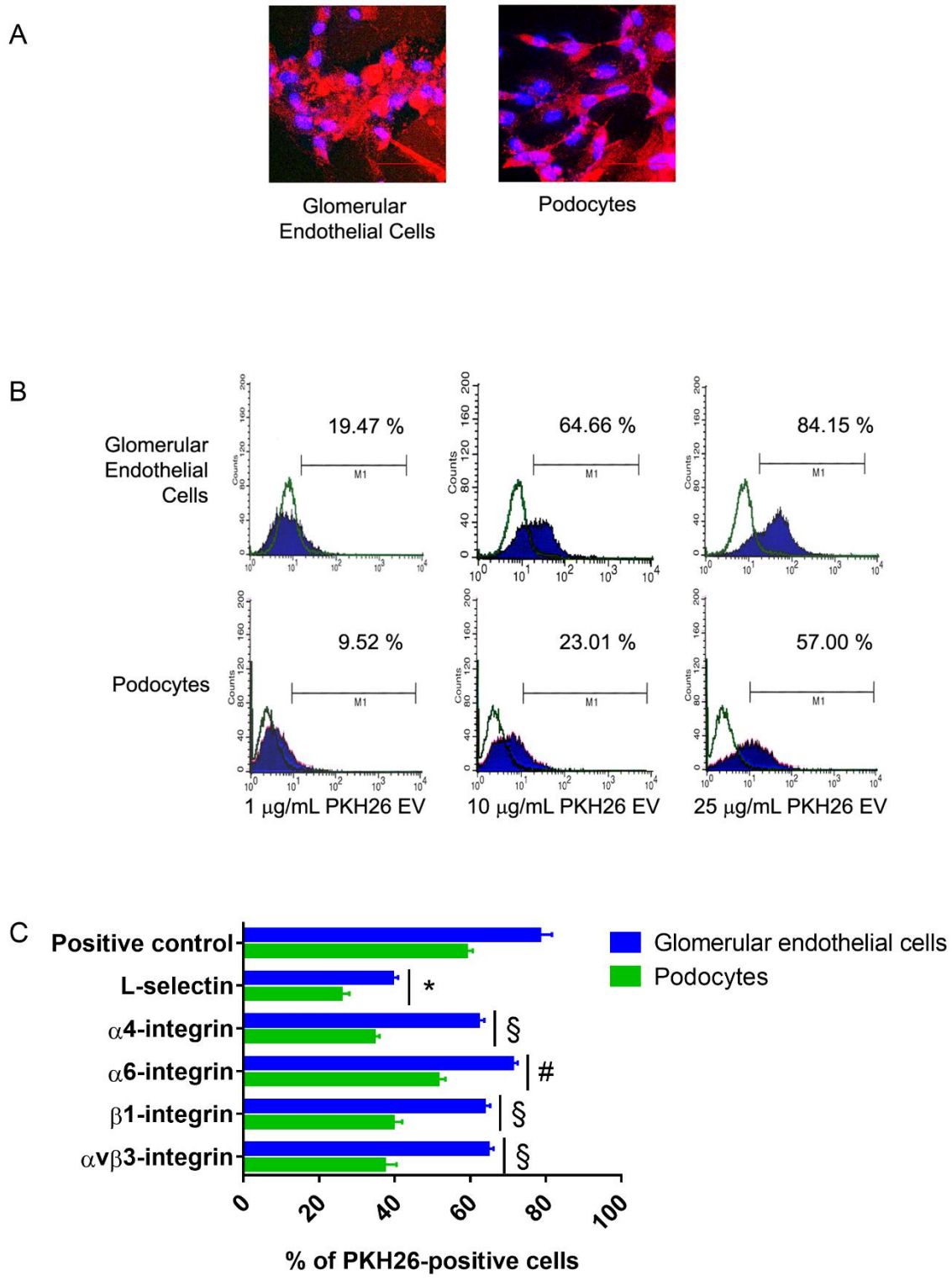

Figure 1. S: Internalization of EPC-derived EVs in human kidney glomerular cells *in vitro*. (A) Representative confocal microscopy micrographs showing the internalization of 25 µg/mL PKH26 red dye-labeled EPC derived EVs in human GECs and podocytes. Nuclei were counterstained in blue by 2.5 µg/mL Hoechst (magnification x400). (B) Representative FACS analysis of dose-response PKH26-labelled EV internalization in human GECs and podocytes. (C) graph showing FACS analysis of PKH26-labelled EV internalization in GECs and podocytes. We expressed results as the mean of the percentage of positive cells ± 1SD. We performed the statistical analysis was performed by ANOVA with Newmann-Keuls's multiple comparison test and the Kolmogorov-Smirnov test. Pre-incubation of all cell lines with PKH26-labelled EVs with 1 µg/mL blocking mAb directed to L-selectin significantly inhibited EV internalization compared to a positive control (*$p<0.05$ L-selectin vs.

Positive Control). The inhibition of internalization was significantly weaker when PKH26-labeled EVs were pre-incubated with blocking mAb directed to α4, β1, αVβ3 integrin (§p<0.05 α4, β1 or αVβ3 vs. L-selectin; *p<0.05 α4, β1 or αvβ3 vs. Positive Control), in particular with the mAb directed to α6 integrin (#p<0.05 α6 vs. α4, β1 or αVβ3; §p<0.05 α6 vs. L-selectin; *p<0.05 α6 vs. Positive Control). We performed three experiments.

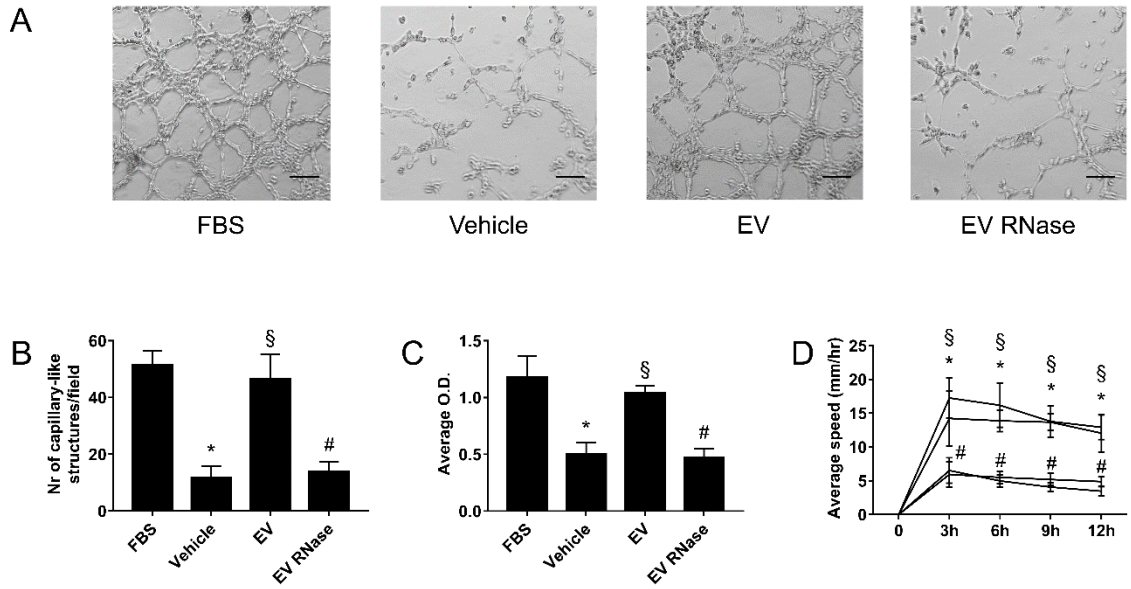

Figure 2. S. EPC-derived EVs induced GEC angiogenesis by stimulating proliferation and migration. (A, B) Representative light microscopy images (A) and count (B) of GECs cultured on Matrigel in vitro in different experimental conditions. Original magnification x100. We expressed data as the average number of capillary-like structures/field ± 1SD. (C, D) Analysis of GEC proliferation by BrdU assay (C) and migration test on six-well plates after 3h, 6h, 9h, and 12h (D). For BrdU assay, we expressed data as average OD intensity ± 1SD; for migration test, we express the data like average speed (mm/hr) ± 1SD. For all the assays, we performed three experiments with similar results; we performed the statistical analysis by ANOVA with Newmann-Keuls multiple comparison tests and Student's t-test. In comparison to standard culture conditions with fetal bovine serum (FBS), serum deprivation (Vehicle) inhibited the formation of the number of capillary-like structures per field (B), proliferation (C), migration (D, *p < 0.05 Vehicle vs. FBS). EPC-derived EVs significantly increased these effects (§p < 0.05 EV vs. Vehicle), that were abrogated by RNase pre-treatment of EVs (#p < 0.05 EV RNase vs. EV). We performed the statistical analysis by ANOVA with Newman–Keuls multiple comparison test and Student's t-test.

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