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Running headline: EPC microvesicles and kidney ischemia-reperfusion injury

ABSTRACT

Endothelial progenitor cells (EPCs) are known to reverse acute kidney injury (AKI) by paracrine mechanisms. We previously demonstrated that microvesicles (MVs) released from EPCs activate an angiogenic program in endothelial cells by mRNA transfer. The aim of the present study was to evaluate whether MVs derived from EPCs prevent AKI in a Wistar rat model of ischemia-reperfusion injury (IRI).

We characterized RNA content of MVs showing the enrichment in microRNAs (miRNAs) that modulate proliferation, angiogenesis and apoptosis. After i.v. injection in IRI rats, MVs localized within peritubular capillaries and tubular cells and conferred functional and morphologic protection from AKI by enhancing tubular proliferation and reducing apoptosis and leukocyte infiltration. MVs also protected by progression toward chronic kidney damage inhibiting capillary rarefaction, glomerulosclerosis and tubulo-interstitial fibrosis. Evidence for a role of MV-mediated transfer of RNA in the renoprotective effect of MVs was derived from the loss of MV activity after 1) their treatment with RNase, 2) unspecific miRNA-depletion of MVs by Dicer knock-down in EPCs and 3) MV depletion of the pro-angiogenic miRNAs miR-126 and miR-296 by EPC transfection with specific antagomiRs.

In conclusion, MVs derived from EPCs protected from ischemic AKI by delivering their RNA content. The miRNA cargo of MVs was shown to contribute to reprogramming hypoxic renal resident cells toward a regenerative program.

INTRODUCTION

Ischemia-reperfusion is one of the main causes of acute kidney injury (AKI) (1,2). Therapeutic strategies aimed to inhibit ischemia-reperfusion injury (IRI) may potentially limit AKI and the development of chronic kidney disease (CKD) (3). Several studies addressed the role of bone marrow-derived and tissue resident stem cells in the regeneration of ischemic kidneys (4-7). Endothelial progenitors (EPCs) are circulating bone marrow-derived precursors able to localize within sites of tissue damage inducing regeneration (8-9). EPCs are known to exert protective effects in experimental models of hindlimb ischemia, myocardial infarction and glomerular diseases (10-12). Moreover, it has been recently demonstrated that EPCs are recruited in the kidney after IRI and that they induce tissue repair via secretion of pro-angiogenic factors (13-15). EPC paucity and dysfunction have been proposed as mechanisms of accelerated vascular injury in CKD patients (16). The regenerative effects of EPCs on ischemic tissues have been ascribed to paracrine mechanisms including the release of growth factors and microvesicles (MVs) (17-18). MVs are small particles derived from the endosomal compartment known to play an important role in cell-to-cell communication through the transfer of proteins, bioactive lipids and RNA to target cells (19-22). We recently demonstrated that MVs released from EPCs are internalized into endothelial cells activating an angiogenic program by horizontal transfer of mRNAs (18).

The aim of the present study was to evaluate whether MVs released from EPCs exert a protective effect in an experimental model of acute renal IRI. Moreover, we studied *in vitro* the mechanisms of MV protection from hypoxia-induced endothelial and epithelial kidney cell injury.

RESULTS

Characterization of EPC-derived MVs

Transmission electron microscopy on EPCs revealed the shedding of MVs (Figure 1a-b) by a membrane sorting process (Figure 1c). Purified MVs showed a homogenous pattern of spheroid particles. About 90% of MVs showed a size ranging from 60-160 nm (Figure 1d), whereas a minority of them were larger with a size around 1□m. The purity and the size of EPC-MV preparations were confirmed by Nanosight analysis (Figure 1e). By FACS analysis, EPC-derived MVs expressed □4 and □1 integrin, CD154 (CD40-L), L-selectin, CD34 but not HLA class I and class II antigens and markers of platelets (P-selectin, CD42b) and monocytes (CD14) (Figure 1f). Bioanalyzer profile of EPC-derived MVs showed the presence of different subsets of RNAs and in particular an enrichment for small RNAs, including microRNAs (miRNAs) (Figure 1g): miRNA array analysis showed the presence of 131 miRNAs shared by EPCs and EPC-derived MVs and 26 miRNAs specifically concentrated in MVs (Figure 1h and Table 1 and 2). The presence in EPCs and EPC-derived MVs of several pro-angiogenic and anti-apoptotic miRNAs, including miR-126 and miR-296, was confirmed by qRT-PCR with specific primer pairs (Fig. 2). The expression of miR-126 and miR-296 seen by qRT-PCR was abrogated by RNAse treatment of MVs and was absent in MVs derived from Dicer-silenced or antagomiR-transfected EPCs (Fig. 2). MVs derived from fibroblasts were also characterized and used as negative experimental control (Figure 1 in Supplementary Information).

Protective effect of EPC-derived MVs in experimental renal IRI

We evaluated the effects of EPC-derived MVs in an experimental model of acute renal IRI in Wistar rats (experimental plan in Figure 3). In comparison to sham-operated animals, rats subjected to kidney IRI showed a significant rise in serum creatinine (Figure 4a) and BUN (Figure 4b) that peaked at day 2 in association with histological signs of tubular injury such as formation of hyaline casts, vacuolization, widespread necrosis and denudation of basal membrane (Figure 4c and Table

3). When rats were treated with EPC-derived MVs, a significant reduction of tubular lesions in parallel with the decrease of serum creatinine and BUN was observed at day 2 (Figure 4a-c and Table 3). The specificity of EPC-derived MVs was indicated by the absence of protective effect exerted by MVs derived from human fibroblasts (Figure 4a-b and Table 3). EPC-derived MVs enhanced the proliferation rate of tubular cells after IRI as detected by BrdU (Figure 5a and c) and PCNA (Figure 5b and d) staining. Moreover, as shown by TUNEL assay (Figure 6a-b), MVs significantly reduced the number of apoptotic tubular cells. These renoprotective effects were significantly reduced when MVs were pre-treated with 1 U/ml RNase, (Figure 4a-c, Figure 5a-d, Figure 6a-c and Table 3). When MVs derived from Dicer knocked-down EPCs or MVs released from EPCs transfected with anti-miR126 and anti-miR-296 antagomiRs were used, a significant reduction of the functional and histological protective effects on ischemic kidneys was also observed (Figure 4a-c, Figure 5a-b, Figure 6a and Table 3). Moreover, in comparison to sham-operated animals, IRI induced a massive infiltration of granulocytes (Figure 6c) and monocytes (Figure 6d) within kidneys. A significant decrease of leukocyte infiltration was observed in rats subjected to IRI and injected with MVs but not with RNase-treated MVs (Figure 6c-d). Similar functional and histological renoprotective effects of MVs were observed also at day 7 after IRI (not shown).

Six months after IRI, animals treated with MVs showed reduced levels of serum creatinine (Figure 7a), tubulo-interstitial fibrosis and glomerulosclerosis (Figure 7b) and a preserved expression of RECA-1 antigen in the tubulo-interstitial structures (Figure 7c-d) and within the glomeruli (Figure 7e-f), suggesting an inhibition of microvascular rarefaction and of progression toward CKD.

In bio-distribution experiments, the accumulation of PKH26-labelled MVs was observed in the kidney 2 hr and 6 hr after IRI. After 2 hr, MVs were detectable within the endothelial cells of large vessels and within some peritubular capillaries and lumen of injured tubules (Figure 8a and b). After 6 hr, the amount of tubular cells containing MVs was markedly enhanced (Figure 8c). When injected in sham-operated control rats, the renal accumulation was significantly lower than in IRI,

and only a slight staining for MVs was detected within glomeruli and tubular cells (Figure 8d). MVs were also detected in the liver of sham-operated controls as well as in rats subjected to kidney IRI (Figure 8e).

In vitro effects of EPC-derived MVs on hypoxic peri-Tubular Endothelial Cells (TEnCs) and Tubular Epithelial Cells (TEpCs)

In consideration of the in vivo localization of MVs in peritubular capillaries and tubular cells, we evaluated the role of adhesion molecules in the internalization of MVs in isolated human TEnCs and TEpCs. Hypoxia significantly enhanced the internalization of EPC-derived MVs in both TEnCs (Figure 8h) and TEpCs (Figure 8i). Experiments performed with blocking antibodies revealed that L-selectin was the main mediator of MV internalization in hypoxic cells (Figure 8h-i). Internalization was not altered in RNase-treated MVs (not shown). Control fibroblast-derived MVs showed a reduced internalization in normoxic as well as in hypoxic TEnCs and TEpCs (Figure 2 in Supplementary Information).

Internalization of MVs within hypoxic TEnCs was followed by reduced apoptosis (Figure 9a) and enhanced angiogenesis on Matrigel-coated surfaces (Figure 9b). The anti-apoptotic and pro-angiogenic effects of MVs on hypoxic TEnCs was almost completely abrogated by RNase pre-treatment or by using MVs released by EPCs engineered to knock-down Dicer or by EPCs transfected with the selective anti-miR-126 and anti-miR-296 antagomiRs (Figure 9a-b). Gene array analysis revealed that MVs restored in TEnCs the expression of pro-angiogenic and anti-apoptotic genes that were down-regulated by hypoxia (Figure 9c).

Internalization of MVs within TEpC was followed by a significant inhibition of hypoxia-induced apoptosis. Indeed, as shown by TUNEL assay (Figure 10a) and ELISA for caspase-3, -8 and -9 activity (Figure 10b), MVs significantly reduced apoptosis of hypoxic TEpCs. The anti-apoptotic effect of MVs was inhibited by RNase pre-treatment or by using MVs released by EPCs engineered to knock-down Dicer or by EPCs transfected with anti-miR-126 and anti-miR-296 antagomiRs. Gene array analysis revealed that MV stimulation of hypoxic TEpCs reduced the expression of

inflammatory and pro-apoptotic caspases (Figure 10c) and of genes involved in both mitochondrial and death receptor pathways of apoptosis (Figure 10d).

DISCUSSION

In this study, we demonstrated that MVs derived from EPCs exert a protective effect on experimental acute renal IRI as detected by the significant decrease of serum creatinine/BUN levels and by the improvement of histological signs of microvascular and tubular injury.

EPCs were shown to induce angiogenesis and tissue repair in experimental models of acute glomerular and tubular injury (12-13, 23-25). The origin of EPCs is still matter of debate. Some studies suggested that contamination with monocytes and platelet-derived products of EPCs derived from circulation may account for their pro-angiogenic potential (26,27). To avoid such contamination, we purified MVs from EPCs after 3-5 passages in culture. The cells used and the derived MVs expressed the CD34 stem cell marker and markers of endothelium, but not of monocytes and platelets. Previous studies suggested that EPCs do not act via a direct trans-differentiation into mature endothelial cells, but rather by paracrine mechanisms (10,28). We demonstrated that MVs act as a paracrine mediator as they may enter the target cells through specific receptor-ligand interaction and deliver selected patterns of mRNAs and miRNAs (20, 29-31). Moreover, MVs released from mesenchymal stem cells were shown to favour recovery from toxic and ischemic AKI (32,33).

Herein, we demonstrated that EPC-derived MVs protected kidney from IRI–induced functional impairment and morphologic injury. Indeed, the administration of MVs significantly decreased serum creatinine and BUN levels, renal cell apoptosis and leukocyte infiltration. Moreover, MVs enhanced tubular cell proliferation and angiogenesis. These renoprotective effects were specific for EPC-derived MVs, since MVs obtained from human fibroblasts were ineffective.

In vivo, MVs were detected both in endothelial cells and in tubular epithelial cells. The in vitro studies on isolated hypoxic TEnCs and TEpCs demonstrated that L-selectin was instrumental in MV internalization, probably through the binding to fucosylated residues or other oligosaccharide ligands known to be up-regulated after IRI (34,35).

It is known that IRI induces both microvascular and tubular injury and that TEnC dysfunction is associated with an extension phase of AKI (36,37). Moreover, the rarefaction of renal microvascular density in the presence of sustained hypoxia is associated with an accelerated progression toward CKD (38). On this basis, we observed the effects of EPC-derived MVs on kidneys 6 months after IRI, finding that MVs significantly reduced glomerulosclerosis, tubulo-interstitial fibrosis and microvascular rarefaction, thus preserving renal function.

The results of the present study suggest that the protective effects of EPC-derived MVs in experimental renal IRI seem to be associated with the triggering of angiogenesis in TEnCs and by the inhibition of apoptosis in TEpCs. Indeed, the detrimental effects induced by hypoxia on TEnCs were limited by MVs. Of interest, gene array analysis of MV-stimulated hypoxic TEnCs revealed the up-regulation of molecules involved in cell proliferation, angiogenesis and inhibition of apoptosis. After an ischemic damage, TEpCs are subjected to loss of polarity with mislocalization of proteins located at the apical or at the basolateral membrane and finally to necrosis and/or apoptosis (39,40). Herein, we showed that MVs protected TEpCs from hypoxia-induced apoptosis through the down-regulation of inflammatory and pro-apoptotic caspases and by modulation of molecules involved in the mitochondrial as well as in the death receptor pathways (41,42).

We observed that RNAse treatment induced the loss of the protective effect of MVs on functional and morphological alterations induced by IRI in vivo and on hypoxia-induced TEnC and TEpC injury in vitro. The significant reduction of MV biological activities after treatment with RNase suggests a putative horizontal transfer of RNAs from MVs to injured renal cells. It is known that MVs protect RNAs from physiological concentrations of RNase. However, as seen in previous studies (18,32,33), the treatment of MVs with high concentrations of RNase inactivate the RNAs.

We previously demonstrated that MVs released from EPCs shuttle mRNAs involved in angiogenic pathways such as eNOS and Akt (18). We now identified in EPC-derived MVs several miRNAs typical of hematopoietic stem cells and of endothelium which are associated with cell proliferation, angiogenesis and inhibition of apoptosis (43,44). In particular, MVs carried the angiomiRs miR-126

and miR-296 (45). The role of miRNAs shuttled by MVs in renal cell regeneration in vivo and in vitro was confirmed by experiments with MVs derived from EPCs previously subjected to the knock-down of Dicer, the intracellular enzyme essential for miRNA production (46,47). These results suggest that miRNAs shuttled by MVs contribute to their regenerative potential. Moreover, miR-126 and miR-296 were identified to play a key role in MV-associated renoprotective effects, since MVs derived from EPCs transfected with specific antagomiRs anti-miR-126 and anti-miR-296 were less effective.

In conclusion, MVs released from EPCs exert a RNA-mediated protective effect in experimental acute renal IRI overcoming the cross-species barrier. The protective effect of MVs released from EPCs in hypoxic tissues may find therapeutic application in AKI, CKD, vascular diseases and IRI after solid organ transplantation without the potential risks of stem cell therapy such as maldifferentiation and tumorigenesis.

METHODS

Isolation and characterization of EPCs and EPC-derived MVs

EPCs were isolated from PBMCs of healthy donors by density centrifugation and characterized as previously described (9,18). EPCs from 3-5 passages were used to avoid monocyte and platelet contamination. By FACS and western blot analysis, EPCs expressed the CD34 stem cell marker and markers of endothelial cells such as CD31, KDR, CD105 and vWF. Moreover, EPCs were able to uptake acetylated-LDL (9). In selected experiments, EPCs were engineered to knock-down Dicer by specific siRNA (Santa Cruz Biotech., Santa Cruz, CA) or transfected with anti-miR-126 and anti-miR-296 antagomiRs (Ambion, Austin, TX). Western blot for Dicer expression was performed by using an anti-Dicer polyclonal antibody (Abcam, Cambridge, UK). MVs were obtained from supernatants of EPCs by ultracentrifugation as previously described (18). MV shape and size were evaluated by transmission electron microscopy and by Nanosight technology (Nanosight, London, UK). Antigen expression on MVs was studied by FACS using antibodies directed to CD14, CD34, CD42b, L-selectin, P-selectin, CD154 (Dako, Copenhagen, Denmark), α4 integrin, (Becton Dickinson, San Jose, CA), αvß3 integrin, α6 integrin (BioLegend, San Diego, CA), HLA class I and II (Santa Cruz Biotech). RNA extraction from MVs was performed using the mirVana isolation kit (Ambion). RNA was analyzed by Agilent 2100 bioanalyzer (Agilent Tech. Inc., Santa Clara, CA). MiRNA expression levels were analyzed using the Applied Biosystems TaqMan® MicroRNA Assay Human Panel Early Access kit (Applied Biosystems, Foster City, CA) to profile 365 miRNAs by qRT-PCR (E-MEXP-2956, European Bioinformatics Institute: www.ebi.ac.uk/arrayexpress/). All reactions were performed using an Applied Biosystems 7900HT real-time PCR instrument equipped with a 384 well reaction plate (detailed protocol reported in Supplementary Information). MiRNA expression levels were analyzed by qRT-PCR in a StepOne™ Real Time System (Ambion): 200 ng of RNA was reverse-transcribed and the cDNA was used to detect and quantify specific miRNAs by qRT-PCR using the miScript SYBR Green

PCR Kit (Qiagen, Valencia, CA). In selected experiments, MVs were labeled with the red fluorescent dye PKH26 (Sigma Aldrich, St. Louis, MO) or treated with 1U/ml RNase (Ambion) and then blocked with 10 U/ml RNase inhibitor (Ambion) (18). MVs derived from cultured human fibroblasts were subjected to the same analysis and used as control (Supplementary Information).

TEnC and TEpC cultures

TEnCs and TEpCs were isolated and characterized as previously described (48, 49). TEnCs were separated by magnetic cell sorting using an anti-CD31 antibody coupled to magnetic beads (MACS system, Miltenyi Biotec, Auburn, CA) and cultured with endothelial growth factors (Lonza, Basel, Switzerland) (18).

Cell culture in hypoxic environment

TEnCs and TEpCs were cultured for 24 hr into an airtight humidified chamber flushed with a gas mixture containing 5% CO2, 94% N2 and 2% O2 at 20 atm, 37°C for 5 min.

Kidney IRI model

Experimental protocol was reported in details in Fig. 3. Male Wistar rats (250 g body weight) were anaesthetized by using an induction chamber with isoflurane and by intraperitoneal administration of ketamine (100 mg/Kg). A s.c. injection of 1-2 ml normal saline was performed to replace fluid loss during the surgical procedure. After midline abdominal incision, the right kidney was removed by a sub-capsular technique. Left renal artery and vein were then occluded by using a non-traumatic vascular clamp that was applied across the hilum of the kidney for 45 min. Animals were divided in the following groups: 1. normal (untreated); 2. sham-operated (right nephrectomy); 3. IRI (right nephrectomy + left renal pedicle clamp); 4. IRI + EPC MVs (right nephrectomy + left renal pedicle clamp + i.v. injection of 30 □g EPC MVs); 5. IRI + RNase EPC MVs (right nephrectomy + left renal pedicle clamp + i.v. injection of 30 □g EPC MVs pre-treated with 1U/ml Rnase); 6. IRI + siRNA Dicer EPC MVs (right nephrectomy + left renal pedicle clamp + i.v. injection of 30 □g MVs derived from EPCs engineered to knock-down Dicer by siRNA). 7. IRI + siRNA Control EPC MVs (right nephrectomy + left renal pedicle clamp + i.v. injection of 30 □g MVs engineered with

an irrelevant siRNA) 8. IRI + AntagomiR-126/296 EPC MVs (right nephrectomy + left renal pedicle clamp + i.v. injection of 30 □g MVs derived from EPCs transfected with anti-miR-126 and anti-miR-296 antagomiRs); 9. IRI + fibroblast MVs (right nephrectomy + left renal pedicle clamp + i.v. injection of 30 □g MVs derived from cultured fibroblasts). For all groups, MVs were diluted in 0.9% saline and injected in the tail vein immediately after IRI. Six animals for each group were sacrificed at day 2, day 7 and day 180 (only groups from 1 to 4). Kidneys were removed for histology and immunohistochemistry. For renal histology, 5 μm-thick paraffin kidney sections were routinely stained with hematoxylin/eosin or Masson's trichrome (Merck, Darmstadt, Germany). Luminal hyaline casts and cell lose (denudation of tubular basement membrane) were assessed in non-overlapping fields (up to 28 for each section) using a x40 objective (high power filed, HPF) to evaluate the score of AKI. Number of casts and tubular profiles showing necrosis were recorded in a single-blind fashion (33). Proliferation was evaluated in rats injected with BrdU by using anti-BrdU (Dako) or anti-PCNA (Santa Cruz Biotech.) monoclonal antibodies (33). TUNEL assay (Chemicon Int., Temecula, CA) for the detection of apoptotic cells was performed according to manufacturer. Leukocyte infiltration was evaluated by staining with anti-monocyte (Chemicon Int.) or anti-granulocyte (Serotec, Oxford, UK) antibody. Immunoperoxidase staining was performed by using an anti-mouse HRP (Pierce, Rockford, IL). Confocal microscopy analysis was performed on frozen sections for localization of PKH26-labelled MVs within kidneys after staining with an anti-laminin (Sigma) or anti-RECA1 antibody (Serotec).

Blood samples for measurement of serum creatinine and BUN were collected before and 2 , 7 or 180 days after IRI. Creatinine concentrations were determined using a Beckman Creatinine Analyzer II (Beckman Instruments, Inc., Fullerton, CA). BUN was assessed in heparinized blood using a Beckman Synchrotron CX9 automated chemistry analyzer (Beckman).

In vitro internalization of MVs into renal cells

TEnCs and TEpCs were seeded on 6-well plates in normoxic or hypoxic culture conditions and incubated with PKH26-labelled MVs derived from EPCs or from fibroblasts. MV internalization

was evaluated by confocal microscopy (Zeiss LSM 5 PASCAL, Jena, Germany) and FACS in presence or absence of 1□□g/ml blocking antibodies directed to □V□□□integrin (BioLegend), □4-integrin, □5-integrin (Chemicon Int.), CD29 or L-selectin (Becton Dickinson).

In vitro assays on TEnCs and TEpCs

Angiogenesis: Formation of capillary-like structures was studied on TEnCs (5 x 104) seeded for 6 hr on Matrigel and observed under an inverted microscope (50-52). Apoptosis: TEnCs or TEpCs were subjected to TUNEL assay (Chemicon Int.). Samples were analyzed under a fluorescence microscope and green-stained apoptotic cells were counted in 10 non-consecutive microscopic fields (50). The activities of caspase-3-8-9 was assessed by ELISA (Chemicon Int.) based on the spectrophotometric detection of the cromophore p-nitroanilide (pNA) after cleavage from the labelled substrate DEVD-pNA, that is recognized by caspases. Cell lysates were diluted with an appropriate reaction buffer and DEVD-pNA was added at a final concentration of 50M. Samples were analyzed in an automatized ELISA reader at a wave length of 405 nm. Each experiment was performed in triplicate (49-50).

Gene array analysis

Human GEarray kit for the study of angiogenesis in TEnCs and apoptosis in TEpCs (SuperArray Inc., Bethesda, MD) was used to characterize the gene expression profile of cells cultured in normoxia or in hypoxia in presence or absence of MVs. Microarray data archive: (E-MEXP-2972 for TEnC angiogenesis and E-MEXP-3086 for TEpC apoptosis, European Bioinformatics Institute: www.ebi.ac.uk/arrayexpress/).

Statistical analysis

All data of different experimental procedures are expressed as average ± SD. Statistical analysis was performed by Kruskal-Wallis statistical test for in vivo studies and by Student’s t-test or ANOVA with Newmann-Keuls or Dunnet’s multicomparison test where appropriated for in vitro experiments. For FACS data, Kolmogorov Smirnov nonparametric statistical test was performed.

DISCLOSURES

SG, MCD and GC received funding for research from Fresenius Medical Care. SB (SisTer SpA) and CT (Fresenius Medical Care) are employed by a commercial company and contributed to the study as researchers. VC, MCD, SB, CT and GC are named as inventors in related patents.

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FIGURE LEGENDS

Figure 1: Characterization of EPC-derived MVs. (**a, b**) transmission electron microscopy performed on cultured EPCs showing MV shedding by a membrane sorting process. (**c**) Schematic representation of shedding MV formation by budding of plasmamembrane**.** (**d**) transmission electron microscopy analysis of purified MVs showing a spheroid shape. In a,b and d bars indicate 100 nm. (e) Nanosight analysis of purified MVs showing MV size distribution (f) FACS analysis of MV protein surface expression. (g) Bioanalyzer RNA profile of EPCs and EPC-derived MVs. (h) Analysis of miRNAs (miRNA array) present in EPCs and EPC-derived MVs (white circle: EPCs; gray circle: EPC-derived MVs).

Figure 2: Representative qRT-PCR for miR-126 and miR-296 in EPCs and EPC-derived Mvs (a, b) (a) qRT-PCR analysis of miR-126 and miR-296 content in EPCs cultured with vehicle alone (wild-type), subjected to siRNA for Dicer (siRNA Dicer) or transfected with anti-miR-126 and anti-miR-296 antagomiRs (AmiR 126/296). (b) qRT-PCR analysis of miR-126 and miR-296 content in MVs derived from EPCs cultured with vehicle alone (wild-type), treated with 1U/ml Rnase (RNase), subjected to siRNA for Dicer (siRNA Dicer) or transfected with anti-miR-126 and anti-miR-296 antagomiRs (AmiR 126/296).

Figure 3: Representative scheme of the experimental plan of acute renal IRI in male Wistar rats. Schematic representation of IRI model, experimental groups, number of animals treated, modality and dose of MV injection, timing of sacrifices and functional/histological analysis performed.

Figure 4: Protective effect of EPC-derived MVs on acute kidney IRI. (a, b) evaluation of serum creatinine (a) and BUN (b) in different experimental groups. IRI induced a significant increase of serum creatinine and BUN (*$p<0.05$ IRI vs. Sham or Normal). EPC-derived MVs significantly decreased serum creatinine and BUN (#$p<0.05$ IRI + MV EPC vs. IRI). The pre-treatment of EPC MVs with 1 U/ml RNase or the use of MVs released from EPCs transfected with siRNA Dicer or with antagomiRs-126/296 (AmiR126/296) did not reduce serum creatinine and BUN (†$p<0.05$ IRI

+ MV EPC RNase, IRI + MV EPC siRNA DICER or IRI + MV EPC AmiR126/296 vs. IRI + MV EPC). MVs released from EPCs transfected with an irrelevant control siRNA (siRNA Control) significantly decreased serum creatinine and BUN (#p<0.05 IRI + MV EPC siRNA Control vs. IRI). The specificity of EPC-derived MVs was confirmed by the lack of renoprotective effect of MVs derived from control human fibroblasts (*p<0.05 IRI + MV fibroblasts vs. Sham or Normal). (c) Hematoxylin/eosin staining of representative kidney sections from different experimental groups (magnification x100).

Figure 5: Enhancement of cell proliferation induced by EPC-derived MVs in ischemic kidneys. Count (a, b) and representative micrographs (c, d) of BrdU-positive (a, c) or PCNA-positive (b, d) cells in different experimental conditions. MVs induced a significant increase of BrdU- and PCNA-positive cells (*p<0.05 IRI + MV vs. IRI). The pre-treatment of MVs with 1 U/ml RNase or the use of MVs released from EPCs transfected with siRNA Dicer or with antagomiRs-126/296 (AmiR126/296) significantly reduced the number of proliferating cells (#p<0.05 IRI + MV RNase, IRI + MV siRNA DICER or IRI + MV AmiR126/296 vs. IRI + MV). MVs released from EPCs transfected with an irrelevant control siRNA (siRNA Control) significantly enhanced the number of BrdU- and PCNA-positive cells (#p<0.05 IRI + MV siRNA Control vs. IRI).All sections were counterstained with hematoxylin; original magnification x100.

Figure 6: Decrease of tubular cell apoptosis and leukocyte infiltration induced by EPC-derived MVs in ischemic kidneys. (a, b) Count (a) and representative micrographs (b) of TUNEL-positive cells in different experimental conditions. A significant increase of TUNEL-positive cells was observed in IRI in comparison to sham-treated animals (*p<0.05 IRI vs. Sham). MVs induced a significant decrease of apoptotic cells (#p<0.05 IRI + MV vs. IRI). The pre-treatment of MVs with 1 U/ml RNase or the use of MVs released from EPCs transfected with siRNA Dicer or with antagomiRs-126/296 (AmiR126/296) significantly reduced their anti-apoptotic effect (†p<0.05 IRI + MV RNase, IRI + MV siRNA DICER or IRI + MV AmiR126/296 vs. IRI + MV). MVs released from EPCs transfected with an irrelevant control siRNA (siRNA Control) significantly reduced the

number of TUNEL-positive cells (#p<0.05 IRI + MV siRNA Control vs. IRI. All sections were counterstained with hematoxylin; original magnification x100. (c, d) Counts of infiltrating granulocytes (c) and monocytes (d) in different experimental conditions. IRI induced an enhancement of granulocyte and monocyte infiltration in the kidney (*p<0.05 IRI vs. Sham) that was not observed in MV-treated animals (#p<0.05 IRI + MV vs. IRI). By contrast, RNase pre-treatment inhibited the decrease of granulocyte and monocyte infiltration induced by MVs (†p<0.05 IRI + MV RNase vs. IRI MV).

Figure 7: Long-term preservation of renal function and inhibition of glomerulosclerosis, tubulo-interstitial fibrosis and capillary rarefaction induced by EPC-derived MVs. (a) Evaluation of serum creatinine 180 days after IRI in different experimental groups. Rats treated with EPC-derived MVs showed lower serum creatinine levels than those observed in IRI animals (*p<0.05 IRI vs. Sham; #p<0.05 IRI + MV vs. IRI). (b) Representative Masson's trichrome (upper panels) and hematoxylin/eosin (lower panels) staining of kidney sections of rats sacrificed at 180 days after IRI in different experimental groups. Original magnification x100 in the upper panel, x200 in the lower panel. (c-f) Mean fluorescence intensity (c, e) and representative confocal microscopy micrographs showing the staining for RECA-1 antigen (d, f) in tubulo-interstitial structures (c, d) and within the glomeruli (e, f) in kidney sections of rats sacrificed at 180 days after IRI in different experimental groups. Original magnification x200 in d and f: nuclei were counterstained with 2.5 □g/ml Hoechst (*p<0.05 IRI vs. Sham; #p<0.05 IRI + MV vs. IRI).

Figure 8: In vivo localization and in vitro internalization of EPC-derived MVs in isolated human TEnCs and TEpCs. (a, b) Confocal microscopy analysis of PKH26-labelled MV localization in endothelial cells (green staining for RECA; arrows) of large vessels and peritubular capillaries 2 hr after injection. (c) Confocal microscopy analysis of PKH26-labelled MV localization in tubular epithelial cells (green staining for laminin). (d, e) Representative micrograph of PKH26-labelled MVs in kidney glomeruli (d) and liver (e) of sham-operated animals. In merge images, nuclei were counterstained with 2.5 □g/ml Hoechst. Original magnification x100 in a, b, c, d and x200 in e. (f,

g) Confocal microscopy analysis of PKH26-labelled MVs in TEnCs (f) and TEpCs (g). Nuclei were counterstained with 2.5 □g/ml Hoechst. Original magnification x400. (h, i) Representative FACS analysis of PKH26-labelled MV internalization in TEnCs (h) and TEpCs (i) cultured in normoxia or hypoxia in presence or absence of different blocking monoclonal antibodies. Hypoxia enhanced MV internalization in TEnCs as well as in TEpCs ($p<0.05$ Normoxia vs. Hypoxia). Anti-L-selectin mAb significantly decreased MV internalization in both cell types ($p<0.05$ Hypoxia + L-selectin mAb vs. Hypoxia). Three different experiments were performed with similar results. Kolmogorov-Smirnov statistical analysis was performed on FACS data.

Figure 9: Effect of EPC-derived MVs on apoptosis, angiogenesis and mRNA expression profile of TEnCs cultured in hypoxic conditions. (a) TUNEL assay of TEnCs cultured in different experimental conditions. In respect to normal culture (Control), hypoxia induced a significant increase of TEnC apoptosis (*$p<0.05$ Hypoxia vs. Control). MVs significantly decreased hypoxia-induced TEnC apoptosis (#$p<0.05$ Hypoxia + MV vs. Hypoxia). By contrast, pre-incubation of MVs with 1U/ml RNase or the use of MVs released from EPCs transfected with siRNA Dicer or with antagomiRs-126/296 (AmiR126/296) significantly inhibited the anti-apoptotic effect of MVs (†$p<0.05$ Hypoxia + MV RNase, Hypoxia + MV siRNA Dicer or Hypoxia + MV AmiR126/296 vs. Hypoxia + MV). MVs released from EPCs transfected with an irrelevant control siRNA (siRNA Control) significantly reduced the number of apoptotic cells (#$p<0.05$ Hypoxia + MV siRNA Control vs. Hypoxia). Results are given as mean±SD of green stained apoptotic cells in 10 microscopic fields (magnification x100) of five independent experiments. (b) In vitro angiogenesis assay of TEnCs cultured on Matrigel-coated plates in different experimental conditions. In respect to normal culture (Control), hypoxia induced a significant decrease of TEnC angiogenesis (*$p<0.05$ Hypoxia vs. Control). MVs enhanced angiogenesis of hypoxic TEnCs (#$p<0.05$ Hypoxia + MV vs. Hypoxia). By contrast, pre-incubation of MVs with 1U/ml RNase or the use of MVs released from EPCs transfected with siRNA Dicer or with antagomiRs-126/296 (AmiR126/296)significantly inhibited the pro-angiogenic effect of MVs (†$p<0.05$ Hypoxia + MV RNase, Hypoxia + MV siRNA

Dicer or Hypoxia + MV AmiR126/296 vs. Hypoxia + MV). MVs released from EPCs transfected with an irrelevant control siRNA (siRNA Control) significantly increased TenC angiogenesis (#p<0.05 Hypoxia + MV siRNA Control vs. Hypoxia). Results are given as mean±SD of 20 different microscopic fields (magnification x100). Three independent experiments were performed with similar results. (c) Gene array profiling of TEnCs cultured in different experimental conditions (angiogenesis-related genes). The graph shows the fold-variation of angiogenesis-related genes between TEnCs cultured in hypoxia in absence (white columns) or presence (black columns) of MVs in comparison to TEnCs cultured in normoxic conditions. Samples were normalized for the signals found in house-keeping genes (actin, GAPDH). Three independent experiments were performed with similar results. Gene table: CCL2: Chemokine (C-C motif) ligand 2; CXCL5: C-X-C motif chemokine 5; TYMP: thymidine phosphorylase; IL8: Interleukin-8; MMP2: Matrix metalloproteinase-2; NRP1: Neuropilin-1; PECAM1: Platelet Endothelial Cell Adhesion Molecole (CD31); PLAU: Urokinase-type plasminogen activator; SPHK1: Sphingosine kinase 1; VEGFa: vascular endothelial growth factor A; FGFR3: *Fibroblast growth factor receptor 3*

Figure 10: Effect of EPC-derived MVs on apoptosis and mRNA expression profile of TEpCs cultured in hypoxic conditions. (a, b) TUNEL assay (a) and ELISA for caspase-3, -8 and -9 activities (b) of TEpCs cultured in different experimental conditions. Hypoxia induced a significant increase of TEpC apoptosis (*p<0.05 Hypoxia vs. Control). MVs significantly decreased hypoxia-induced TEpC apoptosis (#p<0.05 Hypoxia + MV vs. Hypoxia). By contrast, pre-incubation of MVs with 1U/ml RNase or the use of MVs released from EPCs transfected with siRNA Dicer or with antagomiRs-126/296 (AmiR126/296) significantly inhibited the anti-apoptotic effect of MVs (†p<0.05 Hypoxia + MV RNase, Hypoxia + MV siRNA Dicer or Hypoxia + MV AmiR126/296 vs. Hypoxia + MV). MVs released from EPCs transfected with an irrelevant control siRNA (siRNA Control) significantly reduced the number of apoptotic cells (#p<0.05 Hypoxia + MV siRNA Control vs. Hypoxia). Results are given as mean±SD of green stained apoptotic cells in 10 microscopic fields (magnification x100) of five independent experiments. Similar results were

observed for caspase activities (*p<0.05 Hypoxia + MV or Hypoxia + MV siRNA Control vs. Hypoxia; #p<0.05 Hypoxia + MV RNase or Hypoxia + MV siRNA Dicer vs. Hypoxia + MV). Results are given as mean±SD of 5 independent experiments and expressed as % increase of caspase activity in respect to normal culture conditions. (c, d) Gene array profiling of TEpCs cultured in different experimental conditions (apoptosis-related genes). The graph shows the fold-variation of apoptosis-related genes between TEpCs cultured in hypoxia in absence (black columns) or presence (white columns) of MVs in comparison to TEpCs cultured in normoxic conditions. Samples were normalized for the signals found in house-keeping genes (actin, GAPDH). Three independent experiments were performed with similar results. Gene table in c: *CASP1: Caspase-1; CASP10: Caspase-10; CASP14: Caspase-14; CASP3: Caspase-3; CASP4: Caspase-4; CASP5: Caspase-5; CASP6: Caspase-6; CASP7: Caspase-7; CASP8: Caspase-8; CASP9: Caspase-9.*

Gene table in d: APAF1: Apoptotic protease activating factor 1; BAK1: BCL2-antagonist/killer 1; BAX: BCL2-associated X protein; BIK: Bcl-2-interacting killer; NOD1: Nucleotide-binding oligomerization domain-containing protein 1; CD40: CD40; CD40LG: CD40 ligand (CD154); CRADD: death domain (CARD/DD)-containing protein; FADD: Fas-Associated protein with Death Domain; FASLG: Fas ligand (TNF superfamily, member 6); LTA: lymphotoxin alpha; LTBR: lymphotoxin beta receptor (TNFR superfamily, member 3); PYCARD: Apoptosis-associated speck-like protein containing a CARD or ASC; RIPK2 : Receptor-interacting serine/threonine-protein kinase 2; TNF: tumor necrosis factor; CD27: CD 27; TNFSF10: TNF-related apoptosis-inducing ligand (TRAIL); TRADD: Tumor necrosis factor receptor type 1-associated DEATH domain protein; TRAF2: TNF receptor-associated factor 2; TRAF3: TNF receptor-associated factor 3; TRAF4: TNF receptor-associated factor 4.

TABLES

Table 1. miRNA array analysis in EPCs (A: pro-angiogenic, B: proliferative; C: anti-apoptotic; D: stem cells)

let-7a	199a [D]	34c

let-7b [A]
let-7c
let-7d
let-7e
let-7f [A]
let-7g
100
103
106 b [C, D]
10 a
10b
125a [B]
125b [B]
126 [A]
127
130a [A]
130b [D]
132
134
135b
137
140
143
145
146a
146b
148a
148b
149
151
152
155
15a
15b
16
17-5p [B, C, D]
181b
181d
186
18a [B]
191
193a
193b
195
196b
197
199b
19a [B, D]
19b [B, D]
20a [B]
21 [B, C]
213
214
218
22
221
222
224
23a
23b
24 [D]
25 [D]
26a
26b
27a [B]
27b
28
296 [A]
299-5p
29a
29c
301 [D]
30a-3p
30a-5p
30b
30c [D]
30d
30e-3p
31
32
320
323
324-3p
324-5p
328
330
331
335
340
342
345
34a [C, D]
361
362
365
369-5p [D]
374
376a
376b
378 [A]
379
380-5p
382
410
411
423
424
425
425-5p
432
433
452
452
484
485-3p
485-5p
487b
491
493
494
500
503
532
550
565
594
615
646
650
656
660
7
92 [B]
93
98 [C]
99a
99b

Table 2: miRNA array analysis in EPC MVs (A. pro-angiogenic, B:proliferative; C;anti-apoptotic; D: stem cells)

let-7a
let-7b [A]
let-7c
196b
197
199a [D]
335
339
342

let-7d
let-7f [A]
let-7g
100
101
103
106 b [D]
10 a
10b
125a [B]
125b [B]
126 [A]
126 *
127
130a [A]
130b [D]
134 [*D]
137
140
142-3p
142-5p
143
145
146a [B]
146b
148a
148b
149
151
152
155
15a
15b
16
17-3p
17-5p [B, C, D]
181b
181d
186
18a [B]
191
192
193a
193b
194
195
199b
19a [B, D]
19b [B, D]
20a [B]
204
200c [D]
21 [B, C]
210 [A]
213
214
218
22
221
222
223
224
23a
23b
24 [D]
25 [D]
26a
26b
27a
27b [C]
28
296 [A]
299-5p
29a
29c
301 [D]
30a-3p
30a-5p
30b
30c [D]
30d
30e-3p
30e-5p
31
320
323
324-3p
324-5p
328
330
331
345
34a [D]
361
362
365
369-5p [D]
374
376a
378 [A]
379
382
409-5p
410
411
423
424
425
425-5p
432
433
451
452
484
486
485-3p
485-5p
487b
491
493
494
500
503
518d
532
550
564
565
575
650
660
9
92 [B]
93
99a
99b

Table 3: Morphologic evaluation. Renal morphology score in different experimental groups:

n/HPF: number/high power field. *p<0.05 IRI + EPC MV, IRI + EPC MV siRNA Control or IRI + EPC MV AmiR126/296 vs. IRI; [#]p<0.05 IRI + EPC MV AmiR126/296 vs. IRI + EPC MV

	Casts (n/HPF)	Tubular necrosis (n/HPF)
Normal	0	0
Sham	0	0
IRI	2.6 ± 1.2	2.9 ± 0.42
IRI + MV EPC	*0.48 ± 0.21	*0.38 ± 0.16
IRI + MV EPC RNase	2.93 ± 0.84	2.82 ± 0.89
IRI + MV EPC siRNA Dicer	2.26 ± 1.28	1.83 ± 1.19
IRI + MV EPC siRNA Control	*0.38 ± 0.23	*0.42 ± 0.11
IRI + MV EPC AmiR126/296	* #1.36 ± 0,56	* #1.76 ± 0.79
IRI + MV Fibroblasts	2.2 ± 0.94	2.3 ± 0.82