Renal regenerative potential of different extra-cellular vesicle populations derived from bone marrow mesenchymal stromal cells

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Running title: extra-cellular vesicle-induced renal regeneration

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Abstract

Extra-cellular vesicles (EVs) derived from human bone marrow mesenchymal stromal cells (MSCs) promote the regeneration of kidneys in different animal models of AKI in a manner comparable to the cells of origin. However, due to the heterogeneity observed in the EVs isolated from MSC, it is unclear which population is responsible for the pro-regenerative effects.

We therefore evaluated the effect of various EV-populations separated by differential ultracentrifugation (10K population enriched with microvesicles and 100K population enriched with exosomes) on AKI recovery. Only the exosomal-enriched population induced an improvement of renal function and morphology, comparable with that of the total EV population. Interestingly, the 100K EVs exerted a pro-proliferative effect on murine tubular cells, both *in vitro* and *in vivo*. Analysis of the molecular content from the different EV populations revealed a distinct profile. The 100K population for instance was enriched in specific mRNAs (CCNB1, CDK8, CDC6) reported to influence cell cycle entry and progression; miRNAs involved in regulating proliferative/anti-apoptotic pathways and growth factors (HGF and IGF-1) that could explain the effect of renal tubular cell proliferation. On the other hand, the EV population enriched in microvesicles (10K), was unable to induce renal regeneration and had a molecular profile with lower expression of pro-proliferative molecules. In conclusion, the different molecular composition of exosome- and microvesicle-enriched populations may explain the regenerative effect of EVs observed in AKI.

Introduction

Stem cells are important in the maintenance of tissue homeostasis and restoration after injury. One of the mechanisms by which they can influence the behaviour of injured cells is through secreting soluble factors and extracellular vesicles (EVs). In particular, EVs carry a broad range of bioactive molecules including proteins, nucleic acids (mRNA, miRNA, lncRNA, DNA) and lipids, which can act on different cell types triggering a pro-proliferative and or pro-regenerative program (angiogenesis, immune tolerance, etc) (1-5). For instance, mRNA and miRNA delivered by EVs released from endothelial progenitor cells (EPC) induces endothelial cell proliferation and revascularization of injured murine tissue (6, 8). Furthermore, they can also mediate a robust pro-angiogenic program and wound healing effects in streptozotocin-induced diabetic rats through the activation of Erk1/2 signalling pathway (8). In a model of heart injury, exosomes derived from human cardiac progenitor cells inhibit apoptosis and increase cardiomyocytes and endothelial cell proliferation (9).

Among the different stem cells extensively employed in research and clinical practice, mesenchymal stem cells (MSCs) are the most widely used (10). MSCs isolated from different adult tissues (bone marrow, adipose tissue, or neonatal umbilical cord) possess immunomodulatory characteristics and migrate to injured tissues secreting factors and EVs (11, 12). For instance, human umbilical cord derived MSC-EVs have been shown to reduce fibrosis and collagen deposition in a model of drug-induced liver injury (13). Moreover, in a model of hypoxia induced pulmonary hypertension, MSC-EVs mediated the reduction of inflammation and enhanced the recovery of injured lung tissue (14, 15).

The regenerative potential of mesenchymal stem cells derived EVs (MSC-EVs) has also been investigated in different renal injury models. MSC-EVs improved survival in mice treated with cisplatin and reduced the incidence of chronic kidney disease (16). In glycerol-induced acute kidney injury (AKI), a single administration of MSC-EVs accelerated functional and histological recovery by delivery of selected miRNAs and mRNAs (17, 18). AKI and development chronic kidney

disease (CKD) were also prevented in an ischemia and reperfusion injury (IRI) model through the administration of a single dose of MSC-EVs (19). However, like other stem cells, MSCs also secrete various populations of EVs that shuttle a wide range of molecules which exhibit different effects on target cells (20). Currently, a lot of attention is being focussed on the characterization of stem cell EVs together with the cargo they carry (18, 21, 22). However, many "omic" studies are addressed only on the exosomal fraction of EVs. For instance, De Luca et al. characterized the miRNA and piRNA content of bone marrow MSC exosomes and demonstrated that the RNA content can modify the fate of umbilical cord blood derived $CD34^+$ cells (23). Furthermore, protein and miRNA carried by MSC derived exosomes have also been shown to possess tumor regulatory capacity (24).

In the present study, we evaluated the effect of MSC-derived exosomes and microvesicles on AKI recovery. For this purpose, we purified the 10K and 100K fractions of EVs by differential ultracentrifugation and correlated their *in vivo* and *in vitro* biological effects observed with their molecular composition.

Materials and methods

Cell cultures

Bone marrow MSCs were obtained by Lonza (Basel, Switzerland) and cultured and characterized as previously described (17). MSCs derived from 5 preparations were used up to the sixth passage of culture. All of the cell preparations used were positive for the typical MSC markers (CD105, CD29, CD73, CD44 and CD90) and had the ability to differentiate into adipogenic and osteogenic lineages (not shown).

Murine tubular cells (mTECs) were obtained as previously described (17). The cells were maintained in DMEM (Euroclone) supplemented with 10% Fetal calf serum (FCS, Euroclone) and were positive for typical mTEC markers such as: cytokeratin, alkaline phosphatase and aminopeptidase A, and negative for endothelial (von Willebrand factor), hematopoietic (CD45) and glomerular (nephrin) markers (not shown).

Isolation and characterization of different fractions of MSC-EVs

EVs were obtained from supernatants of MSCs cultured overnight in RPMI deprived of FCS. The viability of MSCs at the time of EV collection was 99% as determined by trypan blue exclusion. After removal of cell debris and apoptotic bodies by centrifugation at 3,000g for 20 minutes, EVs were purified by different steps of ultracentrifugation:

- 100KTOT-EVs were purified by 2-hour ultracentrifugation at 100,000g at 4°C (Beckman Coulter Optima L-90K, Fullerton, CA, UAS);
- 10K-EVs were purified by 2-hour ultracentrifugation at 10,000g at 4°C;
- 100K-EVs were purified by 2-hour ultracentrifugation at 100,000g at 4°C of supernatant deprived of 10K-EVs.

EVs from the different fractions were used freshly or stored at -80° C after re-suspension in RPMI supplemented with 1% DMSO. Analysis of size distribution and enumeration of EVs were performed using NanoSight LM10 (NanoSight Ltd) equipped with a 405 nm laser and NTA 2.3 software. Using a laser light source the particles in the sample are illuminated and the scattered light

is captured by the camera and displayed on the connected computer running Nanoparticle Tracking Analysis (NTA). Using NTA, the particles are automatically tracked and sized based on Brownian motion and the diffusion coefficient (Dt). Three videos of 30 seconds were recorded in order to perform the analyses. EVs were characterized by cytofluorimetric analysis using FITC, PE or APC conjugated antibodies against CD73, CD44, CD105 and CD29. Conjugated mouse non-immune isotypic IgG (Miltenyi Biotec,) were used as control. Briefly, EVs ($1.5x10^8$ particles) were incubated at 4°C for 15 min with the antibodies, then diluted 1:3 and acquired immediately as described previously (25). Samples were acquired using Guava easyCyte Flow Cytometer (Millipore) and analyzed with InCyte software.

In selected experiments, the EV pellet obtained by differential ultracentrifugation was further purified using an iodixanol (Optiprep from Sigma) floating separation protocol. Briefly, the pellet was directly resuspended in 500 µl of 60% iodixanol mixed with 0.25M sucrose and transferred to an ultracentrifuge tube (Beckman Instruments). Various gradients of iodixanol: 30%, 15%, and 5% were than sequentially layered on top of the EV/60% iodixanol prep. A final volume of 10 ml was than obtained by adding saline solution on top of the 5% iodixanol fraction. The tubes were than ultra-centrifuged at 350,000g for 1 hour at 4°C without breaking in an Optima L-100K ultracentrifuge (Beckman Coulter) equipped with Type 90Ti rotor. The 15%, 30%, and 60% fractions were recovered, diluted with PBS, and ultracentrifuged again at 100,000g for 1 hour at 4°C. The pellet obtained was resuspended in PBS with 1% DMSO and frozen or used for subsequent studies. NanoSight analyses revealed that vesicles recovered by floating were mainly detected in the 15% fraction (70% of EVs) and to a lower extent in the 30% fraction (30% of EVs). Furthermore, Western blot analysis showed that the exosomal marker CD63 was mainly detected in the 15% fraction (Figure 1A).

RNA extraction and mRNA analysis

RNA was extracted from different EV populations using the All in one kit (Norgen) according to the manufacturer's protocol. Human Cell Cycle RT² *Profiler*™ PCR Array (SABioscience-Qiagen) was performed to profile the expression of 84 genes related to cell cycle regulation.

To confirm the mRNA expression of selected genes, cDNA was obtained using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 5 ng of cDNA and 200 nM of each primer (Table 1) were added to 1X SYBR GREEN PCR Master Mix (Applied Biosystems) and run on a 96-well StepOne quantitative Real Time PCR (qRT-PCR) System (Applied Biosystems). GAPDH was used as a housekeeping gene.

Small RNA seq

Small RNA libraries for micro-RNA sequencing were prepared using the TruSeq Small RNA Library Preparation Kit according to the manufacturer's protocol. The size of the library was checked by using a Fragment Analyzer instrument. Samples were sequenced on an Illumina HiScanSQ platform (Illumina). Sequencing reads were trimmed out of the low-quality bases and adapter clipped with Fastx Toolkit. Filtered sequences were mapped and quantified by using miRDeep2 software (26) on the most updated version of the human miRBase database (27). For downstream analysis, miRNAs with RPM < 100 in all the samples were filtered out. miRNAs with a Fold change of log2 (FC)≤ 1 and log2(FC) ≥ 1 were considered as up-regulated or down-regulated in the different populations, respectively.

miRNA prediction and network analysis.

Target prediction and biological process enrichment analysis was performed using the Funrich analysis tool (28). Only biological processes of selected genes showing a *p* value *<0.05* were considered as significantly enriched. Network analysis to define the associated pathways of the 100K and 10K miRNA-predictive genes, was conducted using the Funrich interaction plug-in (*p* value *<0.01* in the enriched pathways). A filter of a minimum of 6 interactions among different nodes was applied.

Protein expression analysis

Proteins were extracted from different EV populations by RIPA buffer (20 nM Tris-HCl, 150 nM NaCl, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.8) supplemented with a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich). The protein content was quantified by BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific). 30 μg of proteins were separated by electrophoresis using a 4% to 15% gradient sodium dodecyl sulfate–polyacrylamide gel. The proteins were transferred to a PVDF membrane by the iBlot™ Dry Blotting System (Life Technology) and then immunoblotted with the following antibodies: CD63 and CD9 (Santa Cruz Biotechnology) and hepatocyte growth factor (HGF) (Thermo Fisher Scientific). The protein bands were visualized using a ChemiDoc™ XRS + (BioRad) with an enhanced chemiluminescence detection kit (ECL) (GE healthcare, Amersham).

In order to further analyse the protein content of the different EV populations, 100 μg of EV protein was hybridized on glass slides from the Human Cytokine Array kit (Ray Biotech) according to the manufacturer's protocol. The kit allows detecting the expression of 80 different cytokines and growth factors.

Electron microscopy.

Transmission electron microscopy was performed by loading EVs onto 200 mesh nickel formvar carbon coated grids (Electron Microscopy Science) for 20 minutes. EVs were then fixed with a solution containing 2.5 % glutaraldehyde and 2% sucrose followed by repeated washings in distilled water. The samples were than negatively stained with NanoVan (Nanoprobes, Yaphank) and examined by Jeol JEM 1010 electron microscope.

***In vitro* experiments on mTECs**

For proliferation experiments, mTECs were seeded in 96-well plates at 1,500 cells/well in either: 100 μl/well of DMEM with 10% FCS (positive control), or 0% FCS (negative control), or 0% FCS in the presence of different concentrations of EVs ($0.5x10^7$ and $1x10^7$ EVs/ml). DNA synthesis was detected through the incorporation of 5-bromo-2'-deoxy-uridine (BrdU) into the cellular DNA after 48 hours of culture with EVs as per manufacturer's protocol (Roche Applied Science).

SCID mouse model of AKI

Animal studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Ethics Committee of the University of Turin and the Italian Health Ministry (authorization number: 211/2016-PR).

AKI was induced by an intramuscular (*i.m*) injection of glycerol (Sigma) in SCID mice as described previously (17). Glycerol induces myolysis and hemolysis thereby exposing the kidneys to large toxic amounts of myoglobin and hemoglobin (29). Briefly, mice were anesthetized with an *i.m.* injection of zolazepam (80 mg/Kg) and xilazina (16 mg/Kg) and then injected with 8 ml/kg of 50% glycerol in water. Half the dose was injected into each muscle of the inferior hind limbs. Three days after glycerol injection, the mice were treated intravenously with either 120 μl of the vehicle alone (n=10) or containing $165x10^6$ particle of the different populations of EVs (n =10 for each populations). The mice were sacrificed 5 days after glycerol injection (2 days after EV treatment).

Blood samples were collected 5 days after glycerol-induced AKI for the measurement of blood urea nitrogen (BUN) and creatinine. BUN was measured by direct quantification of serum urea with a colorimetric assay kit according to the manufacturer's protocol (Arbor Assays). Serum creatinine was measured using a colorimetric microplate assay based on the kinetic Jaffe reaction as per manufacturer's protocol (Quantichrom Creatinine Assay, BioAssay Systems).

Renal morphology was evaluated through formalin fixed parafilm embedded tissue staining. Briefly, 5 μm-thick paraffin kidney sections were routinely stained with hematoxylin and eosin (H&E, Merck) for microscopic evaluation. Luminal hyaline casts and cell necrosis (denudation of tubular basement membrane) were assessed in non-overlapping fields (10 for each section) using a 40x objective (high power filed, HPF). The number of casts and tubular profiles showing necrosis were recorded in a single-blind fashion, as described previously (16, 17).

Immunohistochemistry for the detection of proliferating tubular cells was performed as described previously (16, 17). Briefly, paraffin kidney sections were subjected to antigen retrieval, followed by blocking and labelling with 1:400 of anti-PCNA (Santa Cruz Biotechnology) and 1:200 of anti-

BrdU (Dakocytomation). Immunoperoxidase staining was then performed using a 1:300 dilution of secondary HRP antibody (Pierce). Scoring for PCNA and BrdU-positive cells was carried out by counting the number of positive nuclei per HPF (40x) in 10 randomly chosen sections of the kidney cortex.

Statistical analyses

Data were analyzed using the GraphPad Prism 6.0 program. Statistical analysis was performed by employing either: student's t-tests, analysis of variance (ANOVA) with Newmann‑Keuls' or ANOVA with Dunnet's multi-comparison tests as deemed appropriate. A *p*-value of <0.05 was considered significant.

Results

Characterization of different populations of MSC-EVs

Different EV populations were analyzed by NTA to determine their size. The 100K-EVs appeared to be homogenous with a mean size of 160±72 nm (Figure 1B). On the other hand, the 10K-EVs were more heterogeneous in size containing EVs which on average were much larger (215±110 nm) (Figure 1B). Variability in size of the EVs that were fixed as seen by transmission electron microscopy was observed, whereby they were smaller than that observed with the NanoSight. This could be due to the influence of both temperature and Brownian motion incorporated in the nanosight method of EV characterization. In addition, transmission electron microscopy showed the presence of bigger EVs in the 10K fraction compared to the 100K fraction (Figure 1C).

The expression of typical exosomal markers was evaluated in the different EV-populations by Western blot analyses. CD63, a fairly common marker for EVs was expressed at low levels in the 10K-EV population compared to the 100K-EVs (Figure 1D). Furthermore, a similar pattern was also observed for the CD9 marker (Figure 1D). FACS analyses showed the presence of several markers characteristic of MSCs, such as CD29, CD44, CD73 and CD105, in all the EV populations tested (not shown), as described previously for 100KTOT-EVs (17, 25).

In vivo effects of different populations of MSC-EVs in experimental AKI

Analyses of renal function indicated that the 100KTOT-EVs significantly improved renal function by reducing BUN and creatinine levels compared to AKI mice treated with the vehicle alone (Table 2). Furthermore, mice treated with the 100K-EVs also improved renal function in a similar fashion to the 100KTOT-EV fraction. However, no significant improvement in renal function was observed in mice treated with the 10K-EV fraction (Table 2). This was further confirmed by histological analyses, whereby, the 10K-EV treatment did not induce a significant recovery in renal histology as the formation of hyaline cast and necrotic renal tubules was comparable to AKI mice without EV treatment (Figure 2). On the other hand, the 100K-EVs significantly reduced morphological injuries in a similar manner to the 100KTOT-EVs (Figure 2). Moreover, both 100K-EVs and 100KTOT-

EVs significantly enhanced tubular cell proliferation, compared to the treatment with the vehicle alone or with 10K-EVs (Figure 3A). Furthermore, this proliferative effect of 100K-EVs and 100KTOT-EVs was also observed *in vitro* on mTECs (Figure 3B). In contrast, the 10K-EVs were unable to trigger proliferation of mTECs *in vitro* (Figure 3B).

Analyses of EV content: RNAs

The differential expression of cell cycle specific mRNAs by different EVs was evaluated (Supplementary Table 1). The 100K-EVs fraction significantly expressed mRNAs involved in cell cycle progression such as CCNB1, CDK8 and CDC6. Conversely, the 10K-EVs, significantly expressed only TP53 which is a negative regulator of the cell cycle. Gene expression was further confirmed by qRT- PCR (Figure 4).

Bioanalyzer profiling revealed an enrichment of small RNAs in both the 10K and 100K-EVs (Figure 5A). This was followed by comparative miRNA sequencing analysis which was performed on different EV populations as follows: (i) 10K vs 100KTOT-EVs, (ii) 100K vs 100KTOT-EVs and (iii) 10K vs 100K-EVs. By using a cut-off of RPM $\geq$ 100 to define the expressed vs not expressed miRNAs, we found 160 miRNAs to be present in the 10K-EV population and 164 in the 100K-EVs (Supplementary Table 2). On comparing with the 100KTOT-EVs, the two EV populations shared 124 common miRNAs (Figure 5B). Moreover, we identified 32 miRNAs that were up-regulated (FC$\geq$ 1) in the 10K-EV fraction with respect to the 100K-EVs (Table 3). On the other hand, 37 miRNAs were up-regulated in the 100K-EVs (FC$\leq$ -1) (Table 4).

Predictive target analysis using Funrich analysis tool conducted on the up-regulated miRNAs in one fraction with respect to the other revealed a group of 2,228 selective target genes for the 100K-EVs and 1,744 for the 10K-EVs. The biological processes over-represented by the 100K-EV predictive genes were mainly associated with protein transport, vitamin metabolism, nucleic acid metabolism, cytoskeleton organization, DNA repair, cell differentiation and cell growth (Figure 5C). Selective processes represented by the 10K-EV predictive genes were: transport, cell-to-cell communication,

apoptosis, fatty acid metabolism, cell growth and cell cycle regulation and matrix adhesion (not shown).

A network analysis of the 100K-EV predictive genes was performed to define their associated pathways. What was observed was a high representation of proliferative/anti-apoptotic pathways, such as PI3K and mTOR associated signaling events. A robust representation of protein nodes clustered around growth factor pathways such as: proteoglycan-syndecan signaling events, hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1) mediated pathway and signaling were over-represented in the interactome network created ($p<0.001$) (Figure 5D). Furthermore, signaling associated with cytoskeleton remodeling such as Arf6, integrins and focal adhesion kinase were also highly represented, together with angiogenic processes such as of PDGFRβ and EGFR signaling events. The same analysis conducted on the 10K predictive genes showed a high representation of cell cycle associated pathways such as G1/S transition, S phase and cell cycle checkpoint (not shown).

Analyses of EV content: Protein

Pro-proliferative effects of EVs could also depend on their protein content such as specific growth factors. For this reason, we analyzed the protein content of the different EV populations by an antibody array that can evaluate the presence of 80 different cytokines and growth factors semi-quantitatively. This analysis revealed the presence of 13 cytokines in both the EV-populations (Table 5). Growth factors involved in the regulation of biological processes such as hematopoiesis, osteocyte differentiation and neuronal development, were differentially expressed in the 10K and 100K-EVs. The 10K-EVs were positive for several cytokines and chemokines involved in the control and regulation of inflammation and immuno-modulation such as IFN-γ and TGF-β. The 100K-EVs were enriched with HGF (Figure 4C) and IGF-1, two growth factors known to be involved in renal regeneration mediated by MSCs. Furthermore, qRT-PCR also confirmed an enrichment of HGF mRNA in the 100K-EVs with respect to the 10K-EVs (Figure 4B). No

significant difference however, was observed in the mRNA levels of IGF-1 between the 10K and 100K-EVs (Figure 4B).

Discussion

Human bone marrow MSC-EVs are able to promote regeneration in different animal models of AKI and mimic the efficacy of MSCs (16-19, 30, 31). EVs isolated from cultured bone marrow MSCs contain vesicles of different size and density. Thus, it was unclear which population of the EVs was responsible for the pro-regenerative effect of MSC-EVs observed in AKI mice models. Through this study, we have demonstrated that different EV populations can exhibit diverse regenerative capabilities on AKI. The 100K population, positive for typical exosomal markers (CD63 and CD9), markedly improved both renal function and morphology in the presented AKI model. In contrast, the 10K-EVs which were enriched in microvesicles expressing only the MSC markers, did not have any significant effect on renal function and morphology. These results therefore suggest that the pro-regenerative effect of MSC-EVs observed is mainly ascribed to exosomes. However, as the total EV population has the same effect as the exosomal fraction, it can be speculated that microvesicles do not interfere in a negative manner.

Several studies have reported different biological properties of exosomes and microvesicles. For instance, exosomes obtained from murine MSCs were able to not only prevent but also reverse monocrotaline-induced pulmonary hypertension, whereas the MVs were ineffective (15). Another study showed that exosomes and microvesicles derived from endothelial colony forming cells had different regenerative properties when injected in an experimental murine model of AKI induced by ischemia reperfusion injury (IRI). Exosomes showed pro-regenerative effects in IRI-mice, whereas microvesicles were ineffective (32). In addition, exosomes and microvesicles derived from human colon cancer cells exhibited distinct biological activities; in particular, exosomes which promoted greater invasion compared to microvesicles (33). EVs derived from murine and/or human BM-MSCs reversed the radiation damage to marrow hematopoietic cells both *in vitro* and *in vivo*. In this case, preparations containing both exosomes and microvesicles were more effective as oppose to either microvesicles or exosomes alone (34).

In this study, we observed that the exosome-enriched 100K-EVs, but not the 10K-EVs, exerted a pro-proliferative effect on murine tubular cells, both *in vitro* and *in vivo*. The differential biological activity of different vesicle populations has been ascribed to their distinct molecular content. In the 100K-EVs, we found the presence of CCNB1, CDK8 and CDC6 transcripts. CCNB1 mRNA, encodes for the protein named G2/mitotic-specific cyclin-B1 (35). Cyclin B1 is involved in the early events of mitosis, such as chromosome condensation, nuclear envelope breakdown, and spindle pole assembly. Once activated, cyclin B1 promotes several events involved in the early stages of mitosis. CDK8 encodes for a protein member of the cyclin-dependent protein kinase (CDK) family that influences binding of RNA polymerase II to the mediator complex (36). CDC6, or cell division cycle 6, encodes for a protein that is an essential regulator of DNA replication and also plays an important role in the activation and maintenance of checkpoint mechanisms in the cell cycle that co-ordinates S phase and mitosis (37).

In the 10K-EVs, we detected only the presence of TP53 that is a well-known negative regulator of the cell cycle (38), thus explaining the absence of a pro-proliferative effect. It has been previously shown that miRNAs shuttled by EVs, derived from different stem cell types, influence EV-pro-regenerative effect in different AKI-models (18, 39, 40). In addition, the miRNA content in the exosome and microvesicle enriched fractions in this study was distinct. Bio-informatic analyses indicated that miRNA present in the 100K-EVs might account for regulation of proliferative/anti-apoptotic pathways. Signaling associated with cytoskeleton remodeling and angiogenic processes were also highly represented in this fraction. A robust representation of protein nodes clustered around growth factor pathways, in particular signaling mediated by IGF-1 and HGF were highly represented in the 100K-EVs interactome network. Moreover, some miRNAs particularly enriched in 100K-EVs are known to be involved in renal regeneration. For instance, miR-486-5p has been implicated in reparative process induced by exosomes, derived from endothelial colony forming cells, in IRI (40). Likewise, miR-126 has also been associated with kidney regeneration induced by

EPC-derived EVs in an IRI model of kidney injury (39). Moreover, miR-34a induced via p53 in cisplatin nephrotoxic AKI, has been shown to protect tubular cells from injury and death (41).

The pro-proliferative effects of 100K-EVs could also be partly attributed to their content in specific growth factors. We found that growth factors involved in different biological processes such as the control of hematopoiesis, neuronal development and osteocyte differentiation were differentially distributed in the 10K and 100K-EVs. The 10K-EVs shuttled cytokines involved in the control and regulation of inflammation as well as immuno-modulation, and chemokines/chemokines receptors known to be expressed by MSCs and to be involved in the MSC recruitment (42). Nonetheless, they contained less pro-proliferative growth factors compared to the 100K-EVs. The latter on the other hand was particularly enriched in growth factors known to be involved in renal regeneration such as IGF-1 and HGF. For instance, Imberti et al has reported that MSCs exert beneficial effects on tubular cell repair by producing the mitogenic and pro-survival factor IGF-1 in an AKI model induced by cisplatin (43). The horizontal transfer of IGF-1 receptor mRNA to tubular cells through MSC-derived exosomes potentiates tubular cell sensitivity to locally produced IGF-1 providing a support for renal repair post AKI (44). We also found that 100K EVs were enriched in both HGF protein and mRNA. Further to this, it has been shown previously that HGF mRNA present in EVs derived from both human Wharton jelly-derived MSCs and rat BM-MSCs, was delivered to tubular cells and then translated into protein (45, 46). Transfer of HGF to resident cells has been shown to promote renal repair preventing fibrogenesis in models of IRI (45, 46).

In conclusion, this study demonstrated that despite their heterogeneity EVs derived from MSCs are able to promote AKI recovery. This biological effect has been ascribed to the ability of EVs to induce tubular cell proliferation leading to the repopulation of injured tubules. The EV fraction enriched in exosomes contained mRNAs, miRNAs and growth factors that may act synergistically to induce the pro-proliferative effects observed and may explain the ability of this fraction to promote AKI recovery. In contrast, the 10K population, which was not effective, displayed a molecular composition that was low in the expression of pro-proliferative factors. Since 100KTOT-

EVs and 100K-EVs did not significantly differ in terms of functional, morphological and proliferative effects, we can speculate that the 10K EV population did not interfere with the regenerative capacity of the exosomal population in AKI. This observation may be relevant for a future clinical application of EVs, which would require the set up in GMP conditions with minimal manipulation and loss/damage of EVs. Our data indicated that it is not necessary to remove microvesicles from MSC-EV preparation to obtain a regenerative effect.

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Author disclosure statement

The authors have declared that no competing interests exist.

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