Mesenchymal Stem Cell-Derived Microvesicles Protect Against Acute Tubular Injury

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ABSTRACT

Administration of mesenchymal stem cells (MSCs) improves the recovery from acute kidney injury (AKI). The mechanism may involve paracrine factors promoting proliferation of surviving intrinsic epithelial cells, but these factors remain unknown. In the current study, we found that microvesicles derived from human bone marrow MSCs stimulated proliferation in vitro and conferred resistance of tubular epithelial cells to apoptosis. The biologic action of microvesicles required their CD44- and /H92521-integrin-dependent incorporation into tubular cells. In vivo, microvesicles accelerated the morphologic and functional recovery of glycerol-induced AKI in SCID mice by inducing proliferation of tubular cells. The effect of microvesicles on the recovery of AKI was similar to the effect of human MSCs. RNase abolished the aforementioned effects of microvesicles in vitro and in vivo, suggesting RNA-dependent biologic effects. Microarray analysis and quantitative real time PCR of microvesicle-RNA extracts indicate that microvesicles shuttle a specific subset of cellular mRNA, such as mRNAs associated with the mesenchymal phenotype and with control of transcription, proliferation, and immunoregulation. These results suggest that microvesicles derived from MSCs may activate a proliferative program in surviving tubular cells after injury via a horizontal transfer of mRNA.


Several studies demonstrate that the administration of exogenous mesenchymal stem cells (MSCs) contributes to the recovery of tissue injury in several organs such as heart, liver, brain, and pancreas.1 Recently, the administration of in vitro expanded MSCs was shown to improve acute kidney injury (AKI).2–8 Indeed, the infusion of MSCs protected and accelerated the recovery from AKI induced by cis-platinum,3,4,9 glyceral,5,6 and ischemia-reperfusion injury.7,8 The mechanisms involved remain controversial. Some experiments based on bone marrow transplantation indicated that bone marrow-derived stem cells may contribute to repopulate the injured nephrons.10,11 MSCs were shown to localize within the injured kidneys when injected in mice with AKI.3,5,6,12 However, several reports indicate only a transient recruitment of MSCs in the renal vasculature without a direct incorporation within the regenerating tubules.7,8,13 It was suggested that the transient presence of MSCs within the injured kidney may provide a paracrine support to the repair, which is mainly sustained by intrinsic epithelial cells surviving injury.2,14 Recently Bi et al.9

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showed that MSCs may protect the kidney from toxic injury by producing factors that limit apoptosis and enhance proliferation of the endogenous tubular cells, suggesting that the tubular engraftment of MSCs is not necessary for their beneficial effect.

Besides soluble factors, cell-derived microvesicles (MVs) were described as a new mechanism of cell-to-cell communication.15 MVs are released by various cell types including stem cells and progenitors.15,16 MVs may interact with target cells by surface-expressed ligands, transfer surface receptors, deliver proteins, mRNA, and bioactive lipids.15 Ratajczak et al.17 showed that MVs derived from embryonic stem cells may reprogram hematopoietic progenitors by an mRNA-dependent mechanism. Recently, we demonstrated that MVs derived from human endothelial progenitor cells (EPCs) are able to trigger angiogenesis in human vascular endothelial cells by a horizontal transfer of mRNA.18

In the present study, MVs obtained from human MSCs were characterized for the expression of surface molecules and for their mRNA content. Moreover, we evaluated whether administration of MSC-derived MVs in SCID mice with glycerol-induced AKI may favor functional and morphologic recovery. The effect of MVs was compared with that of MSC administration.

RESULTS

Characterization of MSC-Derived MVs

By cytofluorimetric analyses, MVs were detected mainly below the forward scatter signal corresponding to 1-μm beads (Figure 1A). When determined by Zetasizer, the size of MVs ranged from 80 nm to 1 μm, with a mean value of 135 nm. Transmission and scanning electron microscopy performed on purified MVs showed their spheroid morphology and confirmed their size (Figure 2 A and B). When electron microscopy was performed on MSCs cultured overnight in serum-free condition, structures resembling MVs were found within larger vesicles in the cytoplasm or dismissed from the cell surface (Figure 2 C, D, and E). Cytofluorimetric analyses showed the presence of several adhesion molecules known to be expressed on MSC plasma membrane such as CD44, CD29, α4- and α5 integrins, and CD73, but not α6-integrin (Figure 1A). In addition, MVs did not express HLA-class I at variance with the cells of origin.

Gene Array Analyses of MSC-Derived MVs

MVs contained mRNA that was submitted to microarray analysis, not to define the amount of mRNA, but only to define which transcripts were present.18,19 A total of 239 transcripts were found with this procedure; 132 were associated to Entrez Gene identifiers by IPA 6.0 analysis (additional information: Table 1). This observation indicated that MVs shuttled a specific subset rather than a random sample of cellular mRNA. Several mRNA characteristic of mesenchymal cell lineages, such as neural, osteogenic, epithelial, and hematopoietic, were present (Table 1). Moreover, MVs contained mRNA related to several cell functions involved in the control of transcription, cell proliferation, and immune regulation (Table 2). Quantita-
Incorporation of MSC-Derived MVs in Tubular Cells

MVss labeled with PKH26 dye were incorporated by cultured tubular epithelial cells (TECs) as shown by confocal microscopy and FACS analysis (Figure 3 A,B). MV treatment with soluble hyaluronic acid and anti-CD44 and -CD29 blocking antibodies inhibited MV incorporation in TECs, whereas anti-α4-integrin (Figure 3) and α5-integrin (not shown) did not prevent MV internalization, suggesting that expression of CD44 and CD29 is critical for their internalization. Moreover, removal of surface molecules by trypsin treatment of MVs inhibited their incorporation in TECs, confirming the relevance of surface molecules in MV internalization (Figure 3 A,B).

In Vitro Proliferative and Antiapoptotic Effects of MSC-Derived MVs

Incubation of TECs with different doses of MVs promoted cell proliferation compared with control cells incubated with vehicle alone (Figure 4A) and induced synthesis of hepatocyte growth factor (HGF) and macrophage-stimulating protein (MSP) (Figure 4B). In addition, incubation of TECs with MVs significantly inhibited apoptosis induced by serum deprivation (Figure 4C), vincristine, and cis-platinum (Figure 4D). MV treatment with soluble hyaluronic acid or trypsin, which inhibited MV incorporation, also inhibited the proliferative and antiapoptotic effects on TECs (Figure 4 A and B), suggesting that MV incorporation was required for their activity. However, when MVs were incubated with RNase that induced a complete degradation of the RNA shuttled by MVs,18 the proliferation and the anti-apoptotic effects elicited by MVs were significantly reduced (Figure 4 A, C, and D). Figure 1B shows by FACS analyses that size and expression of surface adhesion molecules did not change in RNase-treated MVs. Moreover, internalization of RNase-treated MVs in TECs did not differ from that of untreated MVs (Figure 3B). DNase treatment was ineffective (not shown). RNase treatment of MVs did not interfere per se with TEC proliferation induced by the EGF (Figure 4A). These results suggest that the MV biologic effects were mediated by the transfer of mRNA following MV internalization as described previously for EPC-derived MVs.18 MVs derived from human fibroblasts did not stimulate TEC proliferation nor inhibited apoptosis (data not shown).

In Vitro Evidence of De Novo Human Protein Expression in Murine TECs by MV-Mediated Horizontal Transfer of mRNA

We used as reporter genes POLR2E and SUMO-1, which were present in MVs derived from human MSCs. Human POLR2E and SUMO-1 were detected by real time PCR (RT-PCR) after 1 and 3 h of MV incubation with TECs (Figure 5A). The primers used did not recognize murine mRNA, as seen by negative RT-PCR in RNA extracted from control murine TECs. De novo cytoplasmic expression of human POLR2E protein and cytoplasmic and nuclear expression of SUMO-1 protein were detected in murine TECs after 24 h incubation with MVs (Figure 5 B). Nuclear localization of both proteins was observed after 48 h (Figure 5B).

MSC-Derived MVs Protect Against Glycerol-Induced AKI

We compared the effect of human MSCs and MSC-derived MVs injected intravenously in glycerol-induced AKI in SCID mice (Figure 6). Three days after glycerol injection, we observed a significant rise in blood urea nitrogen (BUN) and creatinine (Figure 7A) associated with a marked tubular epithelial injury, whereas control mice injected with saline alone displayed no histologic alterations (not shown). At day 3, MSCs or MSC-derived MVs were injected intravenously at doses of 75,000 cells (an amount of cells releasing approximately 15 μg MVs overnight) or 15 μg MV proteins, respectively. Mice were sacrificed at 4, 5, 8, and 15 d after induction of AKI (Figure 6). The lesions observed in mice with AKI at days 4, 5, and 8 included tubular hyaline casts, vacuolization, and widespread necrosis of proximal and distal tubular epithelium (Figure 7B). Proximal tubules showed cytoplasmic vacuolization, swelling and disorganization of mitochondria, loss of brush border, and denudation of basal membrane (Figure 8 B and C). When mice were treated with MSCs or MVs, the tubular lesions were less severe at day 5 and almost absent at day 8 compared to those of mice treated with vehicle alone.
(Figure 7B). The quantitative evaluation of casts and tubular necrosis at day 5 showed a significant reduction in MV- and MSC-treated mice in parallel with the reduction of BUN (Table 3). The recovery was complete at day 15 (not shown). By electron microscopy, tubular cells in MV-treated AKI mice showed a marked increase of mitochondria at day 5 that was decreased at day 8. In addition, the brush border was already restored at day 5, and the ultrastructure of tubules was almost

Table 1. MV-shuttled mRNA involved in cell differentiation

<table>
<thead>
<tr>
<th>Differentiation</th>
<th>Gene Name</th>
<th>Description</th>
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<tr>
<td>Neural</td>
<td>RAX2</td>
<td>Retina and anterior neural fold homeobox 2</td>
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<tr>
<td></td>
<td>OR11H12</td>
<td>Olfactory receptor, family 11, subfamily H, member 12</td>
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<td></td>
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<td>Olfactory receptor, family 2, subfamily M, member 3</td>
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<td>DDN</td>
<td>Dendrin</td>
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<td></td>
<td>GRIN3A</td>
<td>Glutamate receptor, 3A</td>
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<tr>
<td>Bone</td>
<td>NIN</td>
<td>Ninein (GSK3B interacting protein)</td>
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<td></td>
<td>BMP15</td>
<td>Bone morphogenetic protein 15</td>
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<td>IBSP</td>
<td>Bone sialoprotein II</td>
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<td>Endo/epithelial</td>
<td>MAGED2</td>
<td>Melanoma antigen family D, 2</td>
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<td></td>
<td>CEACAM5</td>
<td>Carcinobryonic antigen-related cell adhesion molecule</td>
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<td>COL4A2</td>
<td>Collagen, type IV, alpha 2</td>
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<td>SCNN1G</td>
<td>Sodium channel, nonvoltage-gated1, gamma</td>
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<td>Hexokinase 3 (white cell)</td>
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<td></td>
<td>EPX</td>
<td>Eosinophil peroxidase</td>
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Table 2. MV-shuttled mRNA involved in transcription, cell proliferation, and immune regulation

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<td></td>
<td>LHX6</td>
<td>LIM homeobox 6</td>
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<td>TCFP2</td>
<td>Transcription factor CP2</td>
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<td>BCL6B</td>
<td>B-cell CLL/lymphoma 6, member B</td>
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<td>High mobility group nucleosomal binding domain 4</td>
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<td>TOPO3</td>
<td>Topoisomerase I binding, arginine/serine rich</td>
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<td>ESF1</td>
<td>Nuclear pre-rRNA processing protein</td>
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<td>POLR2E</td>
<td>Polymerase (RNA) II (DNA-directed) polypeptide E, 25 kDa</td>
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<td>Fucosyltransferase 3</td>
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<td>Adrenomedullin 2</td>
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<td>LT4AH</td>
<td>Leukotriene A4 hydrolase</td>
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<tr>
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<td>BDH2</td>
<td>3-Hydroxybutyrate dehydrogenase, type 2</td>
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<td>CRLF1</td>
<td>Cytokine receptor-like factor 1</td>
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<td></td>
<td>IL1RN</td>
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<td>MT1X</td>
<td>Metallothionein 1X</td>
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<td>Cytoskeleton</td>
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<tr>
<td></td>
<td>MSN</td>
<td>Moesin</td>
</tr>
<tr>
<td></td>
<td>CTNNA1</td>
<td>Catenin (cadherin-associated protein)</td>
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<td>Extracellular matrix</td>
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<td>Collagen type IV, alpha 2</td>
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<tr>
<td></td>
<td>IBSP</td>
<td>Bone sialoprotein II</td>
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indistinguishable from that of control mice without AKI (Figure 8). In addition, MSC- and MV- treated mice showed a significant reduction of both BUN and creatinine (Figure 7A). There was no significant difference between the treatment with vehicle alone, as detected by PCNA- and 5-bromo-2’-deoxy-uridine (BrdU)-positive cells. The enhanced proliferation was detected 24 h (day 4 of AKI) and 48 h (day 5 of AKI) after MV administration and found to decrease thereafter. MSC treatment induced a comparable enhancement of cell proliferation (Figure 9). The majority of PCNA- and BrdU-positive cells, seen in MV- and in MSC- injected animals, were located in distal and proximal tubules. On the contrary, in untreated mice with AKI, proliferation was delayed as the number of PCNA- and BrdU positive cells increased only at day 8 when no further proliferation was detected in mice with AKI treated with MVs or MSCs. sHA-treated MVs as well as fibroblast-derived MVs did not significantly enhance proliferation (Figure 9A and B).

Evaluation of MV Localization After In Vivo Injection

When labeled MVs were injected, a significant accumulation in the kidney was observed at 6 h only in AKI mice (Figure 10A). After 1 h, MVs were detectable within the endothelial cells of large vessels and within the lumen of some injured tubules (Figure 10B). After 3 h, several tubular cells contained labeled MVs (Figure 10 C and D). The amount of tubular cells containing MVs was markedly enhanced at 6 h (Figure 10 E and F). When injected in normal control mice, the accumulation was significantly lower than in AKI, and MVs were not detected in tubular cells (Figure 10 A and H). In plasma, the concentration of MVs significantly decreased in AKI but not in normal controls (Figure 10A). Trypsin-treated MVs were not detected in the kidney of AKI mice and remained constant in plasma at any time (Figure 10 A,G). MVs were minimally detected in the lung (Figure 10A) and as shown by confocal microscopy were mainly located in the endothelial cells of large vessels and not in the alveolar capillaries (Figure 10 J and K). In contrast, liver accumulation of MVs was detected both in normal controls and in AKI mice (Figure 10 A and L). Trypsin-treated MVs did not localize in any of the examined organs (Figure 10A).

RNA Shuttled by MV Mediates MV-Induced Recovery of AKI

As shown in Figure 7 and in Table 3, RNase treatment significantly reduced the recovery of BUN, creatinine, and tubular lesions that did not differ from those of untreated mice with AKI. Moreover at (Table 3). The specificity of MSC-derived MVs was also indicated by the absence of protective effects of MVs derived from human fibroblasts (Table 3).

As shown in Figure 9, MV treatment of mice with AKI significantly enhanced tubular cell proliferation compared to treatment with vehicle alone, as detected by PCNA- and 5-bromo-2’-deoxy-uridine (BrdU)-positive cells. The enhanced proliferation was detected 24 h (day 4 of AKI) and 48 h (day 5 of AKI) after MV administration and found to decrease thereafter. MSC treatment induced a comparable enhancement of cell proliferation (Figure 9). The majority of PCNA- and BrdU-positive cells, seen in MV- and in MSC- injected animals, were located in distal and proximal tubules. On the contrary, in untreated mice with AKI, proliferation was delayed as the number of PCNA- and BrdU positive cells increased only at day 8 when no further proliferation was detected in mice with AKI treated with MVs or MSCs. sHA-treated MVs as well as fibroblast-derived MVs did not significantly enhance proliferation (Figure 9 A and B).

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As shown in Figure 7 and in Table 3, RNase treatment significantly reduced the recovery of BUN, creatinine, and tubular lesions that did not differ from those of untreated mice with AKI. Moreover at
day 8, in mice injected with MVs treated with RNase, the lesions persisted, with an increase in tubular casts and aspect of epithelial atrophy (Figure 7B). Tubular cell proliferation detected as BrdU- or PCNA-positive cells in mice injected with RNase-treated MVs did not differ from that of untreated mice with AKI (Figure 9).

**DISCUSSION**

In the present study, we demonstrated that MVs derived from human MSCs are able to stimulate in vitro proliferation and apoptosis resistance of TECs and to accelerate in vivo the recovery of glycerol-induced AKI in SCID mice. The effect of
administration of MVs was comparable to that of human bone marrow-derived MSCs. The RNase treatment of MVs abrogated both the in vitro and in vivo effects of MVs, suggesting that the mRNA shuttled by MVs is the final effector of their biologic effects.

Several studies demonstrated that the administration of bone marrow-derived MSCs may protect and reverse AKI in different experimental models.2–9 The low number of MSCs detectable within tubules after injury together with the increase in proliferating tubular cells throughout the kidney implies a trophic effect of MSCs on resident tubular cells that have survived injury, rather than a direct repopulation.7,8,14 It was suggested that MSCs may exert their effects by a paracrine action on resident cells.7,13,21,22 Recently, soluble factors were implicated in the MSC protective effect.23 Bi et al.9 showed that MSCs may protect the kidney from toxic injury by producing factors that limit apoptosis and enhance proliferation of the endogenous tubular cells, indicating that tubular engraftment of the MSCs is not necessary for their beneficial effect.

We demonstrated that intravenous administration of MVs derived from human MSCs has the same efficacy of MSCs on the functional and morphologic recovery of glycerol induced AKI in SCID mice. MVs are small vesicles released by cells that express the characteristic antigens of the cell from which they originate and carry membrane and cytoplasmic constituents.15,16 MVs may interact with target cells through specific receptor–ligand interactions and transfer receptors, proteins, and bioactive lipids.16 Moreover, several studies showed that MVs may shuttle selected patterns of mRNA and proposed this as a new mechanism of genetic exchange between cells.17–19,24

Figure 5. mRNA horizontal transfer and human protein expression in tubular epithelial cells (TECs) treated with mesenchymal stem cell (MSC)-derived microvesicles (MVs). (A) 1 × 10⁵ TECs cultured in the absence (TEC) or in the presence (TEC + MV) of 30 μg MVs for 1 and 3 h were analyzed by RT-PCR for specific human mRNA. Bands of PCR products specific for human POLR2E and SUMO-1 of the expected size (90 bp) were detected in a 4% agarose gel electrophoresis. As positive control the extract of human bone marrow-derived MSCs (BM-MSC) was used. The asterisk indicates the control without cDNA. B: Representative micrographs showing the expression of human POLR2E and SUMO-1 proteins by TECs, cultured in the absence or in the presence of 30 μg MVs for 24 and 48 h. After 24 h, POLR2E protein was detected in the cytoplasm and SUMO-1 in the cytoplasm and nuclei of TECs. After 48 h, both proteins were translocated to the nucleus. Nuclei were counterstained with Hoechst dye. Independent experiments using four different MV preparations were performed with similar results. Original magnification: ×630.

Figure 6. Schematic representation of the protocol of glycerol induced acute kidney injury (AKI) and treatment with mesenchymal stem cells (MSCs) or MSC-derived microvesicles (MVs). Glycerol was injected intramuscularly at time 0; the arrow at day 3 indicate the administration of 75,000 MSCs; or 15 μg of MSC-derived MVs; or MSC-derived MVs treated with RNase, trypsin, or sHA; or fibroblast-derived MVs; or vehicle alone; the subsequent arrows indicate the time of sacrifice.
effects were specific for MSC-derived MVs as MVs obtained form fibroblasts were ineffective, and involved MV adhesion molecules as trypsin treatment abrogated MV accumulation in the kidney. MVs accumulated within the lumen of injured tubules, thus allowing an uptake from the apical part of tubular cells that survived injury. In addition, an uptake by endothelial cells was observed, suggesting that MVs may also reach the basolateral side of tubular cells via peritubular capillaries. Apart from inducing TEC proliferation, MVs may act by a mechanism of renal protection that limits the extent of injury. However, in the present experimental setting, MVs were administered at the peak of functional and morphologic alterations.5,6

As seen in vitro, pretreatment of MVs with RNase abrogated the protective effect of MVs. These results suggest that the transfer of small amounts of exogenous mRNA may stimulate tubular cell regeneration. Indeed, MVs derived from MSCs contained mRNA associated with the mesenchymal differentiative phenotype and with several cell functions involved in the control of transcription, proliferation, and cell immune regulation. Interestingly, as in EPC-derived MVs, MVs from MSCs carried a gene encoding the polymerase responsible for synthesizing mRNA in eukaryotes.28 In vitro evidence for an effective horizontal transfer of mRNA was obtained by the presence of the human-specific mRNA for POLR2E and SUMO-1 and by their de novo protein expression in MV-treated TECs. In vivo expression of human POLR2E and SUMO-1 proteins in tubular cells was also detected in mice with AKI treated with MVs. MV-mediated transfer of mRNA/proteins derived from stem cells may therefore induce de-differentiation of mature cells, triggering a proliferative program that may contribute to the repair of tissue injury. Moreover, stimulation of TECs with MVs induced synthesis of HGF and MSP, although their mRNA was not included in MVs, suggesting that the activation of cellular pathways that generate a cascade of multiple mediators that may be concurrent with recovery from acute tubular injury.29–31

Figure 7. Effects of intravenous injection of microvesicles (MVs) or mesenchymal stem cells (MSCs) into acute kidney injury (AKI) mice. Mice were given intramuscular injection of 8 ml/kg of 50% glycerol on day 0, followed by intravenous injection of MVs or RNase-treated MVs or MSCs or vehicle as control on day 3. (A and B) Creatinine and blood urea nitrogen values at the beginning of the experiments and on day 3, 5, 8, and 15 after glycerol administration. ANOVA with Dunnet’s multicomparison test: *P < 0.05 MV- or MSC-treated AKI mice versus control AKI mice. (C) Representative micrographs of renal histology at day 5 and 8 after glycerol administration in control AKI mice, in AKI mice injected with 15 μg of MVs or RNase-MV or with 75,000 MSCs. Magnification: ×400.
Injection of 75,000 MSCs, of 15 µg of MSC-derived MVs with or without treatment with sHA or trypsin and 15 mg of fibroblast-derived MVs. Results are expressed as mean ± SD; ANOVA with Dunnet’s multicomparison test. *P < 0.05 treatments vs untreated AKI. AKI; AKI untreated with MVs; AKI + MV; AKI treated with MSC-derived MVs; AKI + RNase MV; AKI treated with RNase inactivated MSC-derived MVs; AKI + MSC; AKI treated with MSCs; AKI + MV sHA; AKI treated with MSC-derived MVs preincubated with sHA; AKI + F-MV; AKI treated with fibroblast-derived MVs.

Table 3. Effect of MSCs, MSC-derived MVs, and fibroblast-derived MVs on renal morphology and function at day 5 after AKI induction

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<tr>
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<th>Control</th>
<th>AKI</th>
<th>AKI + MV</th>
<th>AKI + MV RN asi</th>
<th>AKI + MSC</th>
<th>AKI + F-MV</th>
<th>AKI + MV sHA</th>
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<tr>
<td>Cast (n/HPF)</td>
<td>0</td>
<td>3.93 ± 1.02</td>
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<td>0.35 ± 0.31*</td>
<td>3.03 ± 0.32</td>
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<tr>
<td>Tubular necrosis (n/HPF)</td>
<td>0</td>
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<td>BUN (mg/dl)</td>
<td>30 ± 10</td>
<td>145 ± 20</td>
<td>60 ± 12*</td>
<td>135 ± 17</td>
<td>52.8 ± 14*</td>
<td>139 ± 18</td>
<td>133 ± 14</td>
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</tbody>
</table>

Injection of 75,000 MSCs, of 15 µg of MSC-derived MVs with or without treatment with sHA or trypsin and 15 mg of fibroblast-derived MVs. Results are expressed as mean ± SD; ANOVA with Dunnet’s multicomparison test. *P < 0.05 treatments vs untreated AKI. AKI; AKI untreated with MVs; AKI + MV; AKI treated MSC-derived MVs; AKI + RNase MV; AKI treated with RNase inactivated MSC-derived MVs; AKI + MSC; AKI treated with MSCs; AKI + MV sHA; AKI treated with MSC-derived MVs preincubated with sHA; AKI + F-MV; AKI treated with fibroblast-derived MVs.
Isolation of MVs

MVs were obtained from supernatants of MSCs and of fibroblasts cultured in RPMI deprived of FCS and supplemented with 0.5% of BSA (Sigma). The viability of cells incubated overnight without serum was > 99% for MSCs and 85% ± 4.3% for fibroblast as detected by trypan blue exclusion. No apoptotic cells were detected by TUNEL assay in MSCs, and 3.2% ± 1.3% apoptotic cell were detected for fibroblast. After centrifugation at 2000 g for 20 min to remove debris, cell-free supernatants were centrifuged at 100,000 g (Beckman Coulter Optima L-90K ultracentrifuge) for 1 h at 4 °C, MV pellets were washed in medium 199, and the protein content was quantified by the Bradford method (BioRad, Hercules, CA). Endotoxin contamination of MVs was excluded by Limulus testing according to the manufacturer’s instruction (Charles River Laboratories, Inc., Wilmington, MA), and MV were stored at −80 °C. The morphologic analyses performed on MV suspension after staining with propidium iodide did not show the presence of apoptotic bodies.

In selected experiments, MVs from MSCs were treated with 1 U/ml RNase (Ambion Inc., Austin, TX) for 1 h at 37 °C, the reaction was stopped by addition of 10 U/ml RNase inhibitor (Ambion Inc.) and MVs were washed by ultracentrifugation.17,18 The effectiveness of RNase treatment was evaluated after RNA extraction using TRIZOL reagent (Invitrogen, Carlsbad, CA) by spectrophotometer analysis of total extracted RNA (untreated: 1.3 ± 0.2 µg RNA/mg protein MV; RNase treated: <0.2 µg RNA/mg protein MV). In addition, RNA extracted from RNase-treated and untreated MVs was labeled by oligo dT driven retrotranscription and analyzed on 0.6% agarose gel to show the complete degradation of RNA by RNase treatment, as described previously.18 As control, MVs were treated with 1 U/ml DNase (Ambion Inc.) for 1 h at 37 °C.

FACS Analysis of MVs

The size of MVs was determined by cytofluorimetric analyses. The instrument was rinsed with particle-free rinse solution for 15 min to eliminate the background. The beads of different sizes (1, 2, 4, and 6 µm, Molecular Probes, Invitrogen) were used as the size markers, and analysis was performed using a log scale for forward scatter and side scatter parameters. Moreover, the size of MV was evaluated by the Zetasizer Nano (Malvern Instruments, Malvern Worcestershire, UK) instrument, which permits discrimination of microparticles less than 1 µm in diameter. Cytofluorimetric analysis was performed as described previously,18,37 using the following FITC- or PE-conjugated antibodies: CD146, CD44 (Dako Cytomation), CD133 (Miltenyi Biotec), CD73, ICAM-1, αβ-integrin, (Becton Dickinson, αβ3-inte-
In vitro Isolation and Expansion of TECs
Kidneys were obtained from healthy female C57 mice. Kidneys were finely minced with scissors and then were forced through a 40-μm pore filter (Becton Dickinson, San Jose, CA); the glomerular part and aggregated remained on the surface of filter while tubular cells were collected. After 2 washings in PBS (Lonza), the cell suspension was plated in a T25 flask (Becton Dickinson) in the presence of DMEM (Sigma) and 10% FCS. We changed media after 5 days to eliminate non-living cells. TECs were characterized for their positive staining of cytokeratin, actin, alkaline phosphatase, aminopeptidase A, and megalin, and for negative staining for von Willebrand factor, CD45, nephrin, and desmin.

Immunofluorescence for Human Protein Expression in TECs
Indirect immunofluorescence was performed on TECs cultured on chamber slides (Nalgen Nunc International, Rochester, NY) and stimulated for 1 and 2 days in the presence of 30 μg/ml of different preparations of MVs. The cells were fixed in 4% paraformaldehyde containing 2% sucrose and permeabilized with HEPES-Triton X100 buffer (Sigma). The following antibodies were used: rabbit anti-human POLR2E (Abcam, Cambridge Science Park, UK) and rabbit anti-human Sumo-1 (AbCam). Omission of the primary antibodies and substitution with nonimmune rabbit IgG were used as controls. Alexa
cor, Gaithersburg, MD) as described previously. As apoptotic stim-

Figure 11. Detection of human mRNA and human protein expression in kidneys of mice treated with human mesenchymal stem cell (MSC)-derived microvesicles (MVs). (A) Representative RT-PCR of acute kidney injury (AKI) mice untreated (AKI) or treated with 15 μg of MSC-derived MVs (AKI+MV) and sacrificed 1 and 3 h after MV injection. Bands of PCR products specific for human POLR2E of the expected size (90 bp) were detected in a 4% agarose gel electrophoresis in AKI treated with MVs but not in untreated AKI or in normal murine kidney (Ctrl). As positive control the extract of human bone marrow-derived MSCs (BM-MSC) was used. The asterisk indicates the control without cDNA. (B) Representative confocal micrographs showing the nuclear expression of human POLR2E and SUMO-1 proteins in kidney sections of AKI mice treated or not with MVs and sacrificed 48 h later. Nuclei were counterstained with Hoechst dye. Original magnification: ×400. Arrows indicate positive nuclei. The right panels of each show merge for the SUMO-1 or POLR2E staining and the nuclear staining with Hoechst. Eight animals per groups were examined with similar results.

Fluor 488 anti-rabbit (Molecular Probes) was used as secondary antibody. Confocal microscopy analysis was performed using a Zeiss LSM 5 Pascal Model Confocal Microscope (Carl Zeiss International, Germany). Hoechst 33258 dye (Sigma) was added for nuclear staining.

Cell Proliferation, Apoptosis Assays, and Release of HGF and MSP

TECs were seeded at 4000 cells/well into 96-well plates in DMEM (Sigma) deprived of FCS. DNA synthesis was detected as incorporation of BrdU into the cellular DNA after 48 h of culture. Cells were then fixed with 0.5 M ethanol/HCl and incubated with nuclease to digest the DNA. BrdU incorporated into the DNA was detected using an anti-BrdU peroxidase-conjugated antibody and visualized with a soluble chromogenic substrate (Roche Applied Science, Mannheim, Germany). Optical density was measured with an ELISA reader at 405 nm. Apoptosis was evaluated using the TUNEL assay (ApopTag Oncor, Gaithersburg, MD) as described previously. As apoptotic stimuli, we used serum deprivation or stimulation with 100 ng/ml of vincristine (Sigma) and 5 μg/ml of cis-platinum (Sigma) in DMEM plus 3% FCS.

To evaluate the production of HGF and MSP, 1 × 10^5 TECs were cultured with or without MV (30 μg/ml) and, after 24 and 48 h of incubation, the supernatants were recovered and MSP-1and HGF production measured by ELISA (Raybiotech, Norcross GA) in accordance with manufacturer’s instruction.

Gene Array Analysis

RNA extraction, Samples Labeling and Hybridization on BeadChips

RNA was extracted from MVs using TRIZOL reagent (Invitrogen) following the procedure suggested by the manufacturer. Total RNA was quantified spectrophotometrically (Nanodrop ND-1000, Wilmington DE). cRNA was synthesized using three different quantities of total RNA (0.25 μg, 0.5 μg, and 1 μg). cRNA synthesis and labeling was done using Illumina RNA Amplification Kit (Ambion) following the procedure suggested by manufacturer. Sentrix Human-6 Expression BeadChip hybridization, washing, and staining were also done as suggested by the manufacturer. Arrays were scanned on Illumina BeadStation 500 (Illumina, San Diego, CA). Transcripts present in the MVs were defined as those characterized by a positive linear relation between the transcript signal detected by the microarray analysis and the amount of total RNA hybridized.

Microarray Data Analysis

The analysis was done hybridizing arrays with labeled-cRNA produced using three different concentrations of total RNA extracted from two independent preparations of MVs. BeadChip array data quality control was performed using Illumina BeadStudio software, version 1.3.1.5. Transcript average intensity signals were calculated with BeadStudio without background correction. Raw data were analyzed using Bioconductor. Average transcript intensities were log2 transformed and normalized by the Loess method.

A simple statistical linear model was used to identify transcript signals linearly correlated to the increment of total RNA concentration used to prepare cRNA. In equation (1), y_{ij} is the observed expression level for transcript i in sample j (j = 1, ..., 6), μ is the average expression level of transcript I, and βRNA represents the effect of total RNA concentration on the expression level of transcript i. ε represents random error for transcript i and sample j, and it is assumed to be independent for each transcript and sample, and normally distributed with mean 0 and variance σ^2.

\[ y_{ij} = μ + β_{RNA} + ε_{ij} \]  

Transcripts characterized by a model with \( P \leq 0.05 \), \( r^2 \geq 0.8 \), and a positive slope were selected (239).

Transcripts annotation and data mining were performed using IPA 4.0 software (www.ingenuity.com). Microarray data were deposited on GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/) as geo accession GSE12243.
Quantitative Real Time PCR
Quantitative real-time PCR was performed as described previously. Quantitative real-time PCR was performed on total RNA extracted from cells used to produce MVs and from an MV preparation different from those used for microarray analysis. Cell cDNA was used to evaluate primers efficiency. The primers used for real-time PCR are shown in Table 4. First-strand cDNA was produced from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Briefly, 200 to 400 ng mRNA, 2 μl RT buffer, 0.8 μl dNTP mixture, 2 μl RT random primers, 1 μl MultiScribe reverse transcriptase, and 4.2 μl nuclease-free water were used for each cDNA synthesis. After the reverse transcription, cDNA was stored at −20 °C. Twenty microliters of RT-PCR mix, containing 1X SYBR GREEN PCR Master Mix (Applied Biosystems), 100 nM of each primer, and 0 μl, 1 μl, and 2 μl of MV cDNA, were assembled using a 48-well StepOne Real Time System (Applied Biosystems). Negative cDNA controls (no cDNA) were cycled in parallel with each run.

Reverse Transcriptase PCR (RT-PCR)
Total RNA extracted from TECs or from kidneys of SCID mice was submitted to RT-PCR using the primer for human POLR2E and SUMO-1 reported in Table 4. Bands of the expected size (90 bp) were detected in a 4% agarose gel after electrophoresis. cDNA from a preparation of human bone marrow MSC was used as positive control.

SCID Mice Model of AKI
Studies were approved and conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. As described previously, a model of rhabdomyolysis-induced AKI was performed in male SCID mice (7 to 8 wk old) (Charles River Laboratories), by intramuscular injection with hypertonic glycerol (8 ml/kg body weight of 50% glycerol solution) into the hind limbs (Figure 6). Intramuscular injection of glycerol induces myolysis and hemolysis causing toxic and ischemic tubular injury. On day 3 after glycerol administration, mice received an intravenous injection into the tail vein of 15 μg of MVs from MSCs treated with sHA, or trypsin or RNase-MVs, or 15 μg of MVs from human fibroblasts, or 75,000 BM-MSCs in 150 μl saline, or saline alone. The following groups were studied: group 1, AKI group; group 2, AKI plus 15 μg MVs injected 3 d after injection of renal injury; group 3, AKI plus 15 μg RNase-treated MVs; group 4, AKI plus 75,000 human MSCs; group 5, AKI plus 15 μg of MVs from fibroblasts; group 6, AKI plus 15 μg MVs from MSCs treated with sHA; group 7, AKI plus 15 μg MVs from MSCs treated with trypsin. The amount of 15 μg MVs was chosen because it corresponds to the amount released overnight by 75,000 MSCs. For in vivo detection of proliferation, mice were administrated an injection of BrdU (100 mg/kg) intraperitoneally for 2 successive days before being killed. In each group, mice were killed at different time intervals (day 3 [n = 10], day 4 [n = 8 per group], day 5 [n = 8 per group], day 8 [n = 8 per group] and day 15 [n = 6 per group] after glycerol administration) and kidneys and samples for BUN and creatinine determination were collected.

Kidney tissues were processed for histology, immunohistochemistry, immunofluorescence and transmission electron microscopy.

Morphologic Studies
For renal histology 5-μm-thin paraffin kidney sections were routinely stained with hematoxylin and eosin (Merck, Darmstadt, Germany). Luminal hyaline casts and cell lose (denudation of tubular basement membrane) were assessed in nonoverlapping fields (up to 28 for each section) using a 40x objective (high power filed, HPF). Number of casts and tubular profiles showing necrosis were recorded in a single-blind fashion.

Transmission electron microscopy was performed on Karnovsky’s-fixed, osmium tetraoxide-postfixed tissues and embedded in epoxy resin according to standard procedures. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with a Jeol JEM 1010 electron microscope. Transmission electron microscopy was also performed on MVs or on cultured MSCs releasing MVs processed as described above. For scanning electron microscopy MVs were fixed in Karnovsky fixative, dehydrated in alcohol, dried on glass surface and coated with gold by sputter coating. The specimens were examined in a scanning Jeol T300 electron microscope. Images were obtained via secondary electron at a working distance of 15 to 25 mm and an accelerating voltage of 20 to 25 kV.

Immunohistochemistry for detection of proliferation of tubular cells was performed as described previously. Kidney sections were subjected to antigen retrieval, and slides were blocked and labeled with 1:25 dilution of monoclonal anti BrdU antibody (Dako Cytomation) or 1:400 of monoclonal anti-PCNA (Santa Cruz Biotechnology, Santa Cruz CA). Immunoperoxidase staining was performed using 1:300 dilution of anti-mouse HRP (Pierce, Rockford IL). Scoring for BrdU- and PCNA-positive cells was carried out by counting the number of positive nuclei per field in 10 randomly chosen sections of kidney cortex using 40x magnification. Confocal microscopy analysis was performed on frozen sections for localization of PKH26-labeled MVs in different murine organs and for detection of specific human proteins POLR2E and SUMO-1. Section were blocked and labeled with rabbit anti-human POLR2E (Abcam) (1:300 dilution) or rabbit anti-human Sumo-1 (Abcam) (1:300 dilution) or rabbit anti Laminin (Sigma) (1:100 dilution) or rabbit anti vWillebrand factor (Dako Cytomation) (1:100 dilution). Omission of the primary antibodies or substitution with nonimmune rabbit IgG were used as controls. Alexa Fluor 488 anti-rabbit (Molecular Probes) was used as secondary antibody.

Table 4. Human primers for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>POLR2E</td>
<td>5'-GCTCTGAAAAATCCGCAAGA-3'</td>
</tr>
<tr>
<td>SENP2/SUMO1</td>
<td>5'-AAATAAGATCGACCAATGCGAGT-3'</td>
</tr>
<tr>
<td>RBL1</td>
<td>5'-GGAGAAGTGGCTTCTACCATTTT-3'</td>
</tr>
<tr>
<td>CXXX7</td>
<td>5'-TGCTTTACAAAGCTGGCCCATCTA-3'</td>
</tr>
<tr>
<td>LT4A4H</td>
<td>5'-CCATATGGCGTGGATGAGCAAAATCA-3'</td>
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Plasma BUN and Creatinine
Blood samples for measurement of BUN and plasma creatinine were collected before and 3, 4, 5, 8 and 15 d after glycerol-induced AKI. Creatinine concentrations were determined using a Beckman Creatinine Analyzer II (Beckman Instruments, Inc., Fullerton, CA). Creatinine levels that exceeded 0.3 mg/dl were considered abnormal (normal range in our laboratory: 0.1 to 0.3 mg/dl). BUN was assessed in heparinized blood using a Beckman Synchrotron CX9 automated chemistry analyzer (Beckman).

Spectrofluorimetric Detection of MVs in Different Tissues after In Vivo Injection
To evaluate the amount of MVs in different murine organs, we injected intravenously AKI or healthy mice with 100 μg of PKH-26 labeled MVs treated or not with trypsin. Mice were sacrificed after 15 min, 1, 3, and 6 h and liver, lungs, kidneys, and blood were recovered. The content of PKH-26 in different samples was measured after lipid extraction with chloroform-isopropyl alcohol (1:1 vol/vol) and 0.125% SDS (Sigma) of homogenized tissues (Tissue Ruptor, Quiagen) or plasma. The fluorescence intensity of PKH-26-containing lipid extracts were measured with Fluoromax-4 spectrofluorimeter (Horiba Jobin Yvon, Edison NJ). Excitation wavelength was positioned at 550 nm; emission wavelength was set at 567 nm. Calibration values for maximum and minimum were obtained using 20 μg/ml and decreasing concentration until 1 μg/ml of lipid extracts of PKH26-labeled MV. Each lipid sample (from different organs and plasma) was analyzed in PBS (Sigma) containing 0.1% triton X100 (Sigma). As negative control, we measured fluorescence intensity in lipids extracted from untreated mice. Data were expressed as micrograms per gram of dry tissue or micrograms per microliter plasma.

Statistical Analysis
Statistical analysis was performed by using the t test, ANOVA with Newmann-Keuls, or ANOVA with Dunnet’s multicomparison tests, as appropriate. A p value of <0.05 was considered significant.

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DISCLOSURES
M. C. D. and G. C. are named inventors on related patent applications.

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