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AKI recovery induced by mesenchymal stromal cell- derived extracellular vesicles carrying micrnas

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1 **Acute kidney injury recovery induced by extracellular vesicles carrying miRNAs**

2 Federica Collino*^{1,4}, Stefania Bruno*², Danny Incarnato³, Daniela Dettori^{2,3}, Francesco Neri³, Paolo
3 Provero^{2,4}, Margherita Pomatto¹, Salvatore Oliviero³, Ciro Tetta⁵, Peter Quesenberry⁶ and Giovanni
4 Camussi^{1,#}.

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6 ¹ Department of Medical Sciences,² Department of Molecular Biotechnology and Healthy Sciences, ³
7 Department of Life Sciences and System Biology and HUGEF, University of Torino,⁴ Translational
8 Center of Regenerative Medicine Unito/EMEA LA Medical Board, Fresenius Medical Care, Torino,
9 Italy; ⁵ Center for Translational Genomics and Bioinformatics, San Raffaele Scientific Institute, Milan,
10 Italy; ⁶ Department of Medicine, the Warren Alpert Medical School of Brown University, Providence,
11 RI, USA.

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13 * These authors contributed equally to this work.

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20 **# Corresponding author:**

21 Dr. G. Camussi, Dipartimento di Scienze Mediche, Ospedale Maggiore S. Giovanni Battista, Corso
22 Dogliotti 14, 10126, Torino, Italy;

23 Phone +39-011-6336708, Fax +39-011-6631184.

24 E-mail: giovanni.camussi@unito.it

25

1 **Abstract**

2 Phenotypic changes induced by extracellular vesicles (EVs) have been implicated in recovery of acute
3 kidney injury (AKI) induced by mesenchymal stromal cells (MSCs). microRNAs are potential
4 candidates for cell reprogramming towards a pro-regenerative phenotype. The aim of the present study
5 was to evaluate whether microRNA de-regulation inhibits the regenerative potential of MSCs and
6 derived-EVs in a model of glycerol-induced AKI in SCID mice. For this purpose, we generated MSCs
7 depleted of Drosha, a critical enzyme of miRNA maturation, to alter miRNA expression within MSCs
8 and EVs. Drosha knock-down MSCs (MSC-Dsh) maintained the phenotype and differentiation
9 capacity. They produced EVs that did not differ from those of wild type cells in quantity, surface
10 molecule expression and internalization within renal tubular epithelial cells. However, EVs derived
11 from MSC-Dsh (EV-Dsh) showed global down-regulation of miRNAs. Whereas, wild type MSCs and
12 derived EVs were able to induce morphological and functional recovery in AKI, MSC-Dsh and EV-
13 Dsh were ineffective. RNA sequencing analysis showed that genes deregulated in kidney of AKI mice
14 were restored by treatment with MSCs and EVs but not by MSC-Dsh and EV-Dsh. Gene Ontology
15 analysis showed that down-regulated genes in AKI were associated with fatty acid metabolism. The up-
16 regulated genes in AKI were involved in inflammation, ECM-receptor interaction and cell adhesion
17 molecules. These alterations were reverted by treatment with wild type MSCs and EVs, but not by the
18 Drosha counterparts. In conclusion, miRNA depletion in MSCs and EVs significantly reduced their
19 intrinsic regenerative potential in AKI, suggesting a critical role of miRNAs.

20

1 **Introduction**

2 Several studies demonstrated that mesenchymal stromal cells (MSCs) may promote recovery from
3 acute kidney injury (AKI). The mechanism has been ascribed mainly to a paracrine action of MSC-
4 derived mediators¹. Bi et al.² demonstrated that the conditioned medium of MSCs mimics the
5 therapeutic effects of the cells. We found that extracellular vesicles (EVs) released from MSCs
6 contribute to their regenerative properties in glycerol induced AKI in SCID mice³. The regenerative
7 potential of MSC-derived EVs has been described in several models of acute and chronic kidney
8 injury^{4,5}.

9 The mechanism of action of EVs has been attributed to the transfer of information between cells⁶.
10 After internalization, EVs may deliver their proteins, lipid or nucleic acid content to recipient cells,
11 changing their fate⁷⁻⁹. EVs released by MSCs were shown to express proteins involved in MSC self-
12 renewal and differentiation capability¹⁰. Moreover, analysis of the nucleic acid composition of MSC-
13 derived EVs demonstrated the presence of MSC specific mRNA and mature miRNAs^{3, 11}. Recently,
14 extracellular RNAs have been implicated both as promising biomarkers for a variety of pathologies^{12, 13}
15 and as mediators of several biological processes¹⁴. EVs represent a mechanism for the delivery of the
16 nucleic acids in a degrading enzyme protective way¹⁵. The transfer of genetic information among cells
17 is emerging as a new paradigm of cell to cell communication¹⁶.

18 MicroRNAs (miRNAs)¹⁷ are a class of small non-coding RNAs, abundant in the cell, that function as
19 endogenous mediators of RNA silencing. miRNAs regulate protein expression by blocking both the
20 mRNA transcription or the protein translation¹⁸. Two crucial enzymes play a relevant role in the
21 regulation of miRNA maturation inside the cells, the RNase III Droscha and Dicer enzymes. The first
22 one, acts in the nucleus where it cleaves the inactive pri-miRNA into the precursor miRNA (pre-
23 miRNA), a double-stranded hairpin structure of about 70 nucleotides¹⁹. The pre-miRNA is then
24 exported from the nucleus to the cytoplasm to finally generate a functional mature miRNA by Dicer

1 cleavage²⁰. EV mediated transfer of functional miRNAs have been shown to occur in several
2 contexts²¹⁻²³.

3 Whether EV-mediated transfer of miRNAs is implicated in the healing properties of MSC-derived
4 vesicles in AKI has not yet been investigated. To address this issue, we evaluated the effect of the
5 global suppression of miRNA biogenesis²⁴ by generation of Drosha knock-down MSCs to be used as
6 EV donors. The choice of Drosha as target enzyme, was based on the knowledge that Drosha activity is
7 restricted to the generation of miRNAs, whereas Dicer is involved in the biogenesis of multiple classes
8 of small RNAs including siRNA²⁵⁻²⁷. Here, we reported that depletion of Drosha in MSCs, generated
9 EVs with a deregulation of their miRNA content and altered the intrinsic regenerative potential of
10 MSCs and their derived EVs. These data support the relevance of miRNAs shuttled by EVs in the
11 healing properties of MSCs.

12

1 **Results**

2 **Generation of Drosha knock-down MSCs**

3 miRNA reduction was obtained by MSC transduction with a lentiviral, tetracycline inducible vector,
4 containing a short hairpin RNA (shRNA) targeting Drosha (MSC-Dsh) and the red fluorescence protein
5 (RFP). Transduced MSCs maintained the classical mesenchymal markers on their surface (Fig. 1A) as
6 detected by FACS analysis and conserved their ability to differentiate in chondrocytes, adipocytes and
7 osteocytes (not shown). To quantify the knock-down of Drosha, we performed quantitative real time
8 (qRT)-PCR on Dsh-transduced MSCs, cultured with doxycycline in comparison with control
9 transduced cells (MSC-CTRL). In cell populations from three separate donors, we observed a two-fold
10 reduction ($p < 0.01$) of Drosha transcript level (Fig. 1B) in MSC-Dsh in respect to MSC-CTRL. This
11 reduction was enhanced in sorted RFP-positive MSC-Dsh (Fig. 1B, $p < 0.01$). In all experiments, sorted
12 MSC-CTRL and MSC-Dsh were used.

13 **Characterization of EVs released from MSC-Dsh**

14 EVs from MSC-Dsh (EV-Dsh) showed a size distribution comparable to the one released by MSC-
15 CTRL (EV-CTRL) with a mean diameter of 128 ± 19 and 130 ± 19 nm, respectively (detected by
16 Nanosight instrument). Cytofluorimetric analyses of EV-CTRL and EV-Dsh, showed the expression by
17 EVs of antigens characteristic of the cells of origin, such as CD29, CD44, CD105, CD73, CD90 and
18 HLA class I (Fig. 2A). EV-CTRL and EV-Dsh (3×10^9 part/ml) were equally incorporated by murine
19 tubular epithelial cells (mTEC), as observed by confocal microscopy after 24 hours of incubation (Fig.
20 2B). SYTO-RNA carried by EVs was transferred to target cells (Fig. 2B).

21 **miRNA profile in EV-Dsh**

22 miRNA screening and comparison in EV-Dsh and -CTRL was evaluated by qRT-PCR. Using a cut-off
23 < 35 Ct value in miRNA expression, we found 161 miRNAs detected in all the EV-CTRL samples

1 (n=4). Comparing the miRNA content between EV-CTRL and EV-Dsh, a global tendency to down-
2 regulation of miRNAs in EV-Dsh in respect to EV-CTRL was observed (55±15% of all the expressed
3 miRNAs). The intersection of the list of miRNAs down-regulated in different EV-Dsh in respect to
4 EV-CTRL, identified 49 miRNAs, commonly down-regulated in EV-Dsh (Table 1, $p < 0,05$).

5 **Impaired protective effect of MSC-Dsh in a model of glycerol-induced AKI**

6 At day 3 after AKI induction, 75,000 MSC-CTRL or MSC-Dsh were administered intravenously. Mice
7 were then sacrificed after 5 days from the induction of AKI. The lesions observed in AKI mice
8 included tubular hyaline casts, vacuolization, and presence of necrosis of proximal and distal tubular
9 epithelium (Fig. 3A). Morphological damage correlated with the significant rise in blood urea nitrogen
10 (BUN, Fig. 3D). Injection of MSC-CTRL resulted in decrease of tubular lesions and evidence of renal
11 tubular repair in respect to AKI mice treated with vehicle alone (Fig. 3B), in parallel with the reduction
12 of BUN levels (Fig. 3D). On the contrary, injection of MSC-Dsh did not significantly improve
13 morphological (Fig. 3C) or functional injury (Fig. 3D). The quantitative evaluation of casts (Fig. 3E)
14 and tubular necrosis (Fig. 3F) showed a significant reduction in MSC-CTRL but not in MSC-Dsh
15 treated mice.

16 We also compared the effect of EVs from MSC-Dsh (EV-Dsh) with that of EVs derived from wild type
17 MSCs (EV-CTRL). For this purpose, we injected 2.2×10^8 particles/mice of EV-CTRL and EV-Dsh at
18 day 3, after AKI induction. In mice treated with EV-CTRL but not with EV-Dsh, the tubular lesions at
19 day 5 were significantly less severe than in untreated AKI mice (Fig. 4A-C). In control healthy mice,
20 injected with EV-CTRL and EV-Dsh, the histological analysis and BUN measurement demonstrated no
21 negative effects associated with the EV-CTRL and EV-Dsh injection (Fig. 4 D-G), in the absence of
22 AKI damage. EV-Dsh injection in AKI mice didn't inhibit the rise of BUN (Fig. 4G). Also the counts
23 of hyaline casts and tubular necrosis, demonstrated the inefficiency of EV-Dsh in respect to the EV-
24 CTRL (Fig. 4 H and I).

1 **MSC-CTRL but not MSC-Dsh induced a pro-regenerative gene signature in the kidney**

2 In order to detect the molecular changes occurred in the kidneys of treated animals, we performed
3 RNA-Seq analysis (GEO number, GSE59958). We observed a strong correlation between MSC-CTRL
4 and EV-CTRL treated mice (Pearson Correlation Coefficient, PCC 0.96). Interestingly, comparison of
5 MSC-CTRL and EV-CTRL treatment with the AKI condition showed a lower degree of correlation
6 (PCC 0.72 and 0.74, respectively), indicating that both treatments partially reverted the molecular
7 changes occurring in the kidney after glycerol-induced damage. Moreover, this was confirmed by the
8 observation that healthy mice significantly correlated with MSC-CTRL and EV-CTRL samples (PCC
9 0.966 and 0.858, respectively, Fig. 5A). On the contrary, transcriptome profile of MSC-Dsh and EV-
10 Dsh treated mice showed a high correlation with the untreated AKI mice (PCC 0.997 and 0.988,
11 respectively), and low or no correlation with the healthy samples (PCC 0.486 and 0.384, respectively),
12 indicating no healing properties associated with Droscha knock-down MSC or EV-Dsh treatments (Fig.
13 5A).

14 Consistently, differential expression analysis of all the genes de-regulated in AKI versus healthy kidney
15 samples ($n=2663$, up-regulated; $n=1893$, down-regulated), showed an extremely similar pattern (Fig.
16 5B). The EV-CTRL and MSC-CTRL were clustered into two major groups supporting a relevant
17 overlap in the genes regulated by both cells and EVs during AKI repair (Fig. 5B). Interestingly, MSC-
18 Dsh and EV-Dsh kidneys showed a global gene expression pattern very similar to that of AKI (Fig.
19 5B). These results suggested that MSCs depleted of Dsh were unable to activate the pro-regenerative
20 transcriptomic program in injured kidneys. In fact, genes altered in AKI vs. healthy condition, showed
21 a significant reversion to physiological levels in EV-CTRL and MSC-CTRL treatments, compared to
22 EV-Dsh and MSC-Dsh treatments (EV-CTRL vs EV-Dsh, $p= 2.3 e^{-142}$ and $6.8 e^{-46}$ for up-regulated and
23 down-regulated genes respectively; MSC-CTRL vs MSC-Dsh, $p= 1.2 e^{-307}$ and $3.0 e^{-84}$ for up-regulated
24 and down-regulated genes respectively) (Fig. 5C).

1 **Gene ontology analysis**

2 Focusing on genes with a significant fold change among all the treatments ($FC \geq |1|$), we detected a
3 relevant group of transcripts significant reverted after MSC-CTRL ($n=702$ and 428 , respectively down-
4 and up-regulated in respect to AKI, Fig. 6A) and EV-CTRL stimulation ($n= 610$ and 706 , respectively
5 down- and up-regulated in respect to AKI, Fig. 6B). The same modulation was not observed for the –
6 Dsh treatments ($n= 33$ and 77 for MSC-Dsh and $n= 111$ and 129 for EV-Dsh, respectively up- and
7 down- in respect to AKI, Fig. 6A, B).

8 Gene Ontology analysis (GO) was performed on a subset of genes that showed comparable high
9 modulation in both MSC and EV treatments. This yield final subsets of 335 and 325 respectively up-
10 and down-regulated genes common to MSC- and EV-CTRL treatments with respect to AKI (Fig. 6C),
11 or 225 and 110 respectively up- and down-regulated genes common to MSC- and EV-CTRL treatments
12 with respect to –Dsh (Fig. 6D). The GO enrichment analysis revealed that common up-regulated
13 transcripts in EV- and MSC-CTRL in respect to AKI, were enriched for genes associated with the
14 regulation of metabolic pathways (Valine, leucine and isoleucine degradation; Tryptophan and
15 Butanoate metabolism, PPAR signaling pathway, $p= 2,36E^{-09}$, $1,74E^{-06}$ and $4,71E^{-05}$ respectively) but
16 also for genes involved in the complement and coagulation cascades (Fig. 6E, $p= 1,00E-04$). The most
17 representative GO of the down-regulated genes was associated with response to inflammation, ECM-
18 receptor interaction, cell adhesion molecules and cell cycle (Fig. 6F, $p= 1,60E^{-04}$, $1,05E^{-06}$, $1,56E^{-02}$ and
19 $4,11E^{-02}$ respectively). Cross-match of the common genes modulated after MSC- or EV-Dsh treatments
20 in respect to AKI showed no significant enrichment of these pathways (not shown). Genes co-
21 modulated in EV- and MSC-CTRL in respect to –Dsh were associated to :ECM-remodeling and focal
22 adhesion (for the down-regulated transcripts) and metabolic regulation (for the up-regulated transcripts)
23 (Supplementary Table 1).

24 **miRNA:mRNA data integration**

1 To identify potential mRNA targets for vesicle-carried miRNAs, we first clustered the miRNAs
2 expressed in EV-CTRL (n=161) into families, according to their seed sequence, and scanned the 3'-
3 UTR of AKI-expressed genes for perfect seed-match occurrences (6-8mers). Moreover, to account for
4 potential cooperative action of different miRNAs, we restricted our research to those genes targeted by
5 at least 2 expressed miRNA families. Our analysis produced a list of 16 significantly enriched miRNA
6 families ($p < 0.05$, Hypergeometric distribution), associated in 15 pairs, whose targets show significant
7 down-regulation in EV-CTRL versus AKI mice (Supplementary Table 2). For 8 of these miRNA
8 families, at least one representative miRNA was differentially expressed in EV-Dsh, supporting their
9 involvement in the EV healing process. In particular, we found a significant enrichment for miR-483-
10 5p, miR-191, miR-28-3p, miR-423-5p, miR-744, miR-129-3p, miR-24 and miR-148a families. The
11 union of the targets for the 15 pairs of miRNA families, identified 209 genes potentially modulated by
12 these miRNAs, of which 165 genes (77.8%) were not reverted in EV-Dsh treatment in respect to AKI.
13 GO enrichment analysis of these targets showed the over-representation of processes associated with
14 ECM-receptor interaction and focal adhesion ($p = 1,00E^{-04}$, $3,00E^{-03}$) and with Wnt and p53 pathways
15 (Supplementary Table 3, $p = 1,90E^{-02}$ and $3,50E^{-02}$ respectively).

16 **Effect of Drosha knock-down on the expression of markers of kidney injury**

17 Kidneys from untreated AKI mice showed elevated levels of transcripts coding for markers of tubular
18 damage such as lipocalin 2 (Lcn2)²⁸ and fibrinogen subunits (Fg)²⁹ as detected by RNA-Seq analysis.
19 In healthy mice, no expression of Lcn2 was observed. The treatment with MSC- and EV-CTRL led to
20 significant decrease of Lcn2 (Fig. 7A), while MSC- or EV-Dsh injected mice maintained elevated
21 levels of Lcn2 transcript (Fig. 7A). Lcn2 down-regulation after both MSC-CTRL and EV-CTRL
22 treatments, was confirmed by qRT-PCR (n=6 mice/group, Fig. 7B). MSC- and EV-Dsh injected
23 animals maintained elevated levels of Lcn2 (Fig. 7B).

1 We also evaluated the levels of fibrinogen, as another important marker of AKI damage (29). AKI
2 induced the up-regulation of all the Fg subunits ($-\alpha$, $-\beta$ and $-\gamma$) (Fig. 7C). MSC- or EV-CTRL treated
3 mice showed the down-regulation of the Fg- β , but less relevant reduction of α and γ subunits. MSC- or
4 EV-Dsh injected mice maintained elevated levels of all the Fg subunits (Fig. 7C-D). In healthy mice no
5 or minimal expression of all the Fg-subunits was observed. qRT-PCR of whole kidney confirmed
6 minimal expression of Fg- β in control kidneys (Fig. 7E). A robust increase of Fg- β was observed after
7 5 days in AKI-untreated as well as in mice treated with MSC-Dsh and EV-Dsh. MSC-CTRL and EV-
8 CTRL reverted the Fg- β expression showing transcript expression comparable to that of healthy mice
9 (Fig. 7E). Healthy mice injected with EV-CTRL and EV-Dsh showed levels of expression of Lcn2 and
10 Fg- β , comparable to that of healthy untreated animals (not shown). These data collectively indicated
11 that injured renal tubules can initiate local fibrinogen synthesis ²⁹. Immunohistochemistry analysis of
12 Fg- β in untreated AKI mice, showed a strong peritubular staining for fibrinogen and some positivity in
13 the tubular cell cytoplasm (Fig. 7F). Tubular staining was not observed in kidneys from healthy mice
14 and was markedly reduced in mice treated with MSC- and EV-CTRL but not -Dsh (Fig. 7F).

15

1 **Discussion**

2 The results of the present study demonstrated that the down-regulation of miRNAs in human MSCs,
3 reduced the intrinsic kidney pro-regenerative properties of these cells and of their derived-EVs. This
4 effect was associated with changes in miRNA composition of cells and EVs. Previous studies
5 demonstrated that EVs derived from human MSCs were able to favor recovery of AKI by activation of
6 anti-apoptotic and pro-regenerative programs in tubular epithelial cells^{3, 30, 31, 32}. Transfer of mRNAs
7 from MSCs to tubular epithelial cells was observed both *in vitro* and *in vivo*³. The mRNA transfer,
8 observed also in other models^{33,34}, was shown to be followed by their transient transduction into
9 proteins. We recently demonstrated an EV-mediated transfer of miRNAs from MSCs to tubular
10 epithelial cells in an *in vitro* model of renal tubular cell injury induced by ATP depletion³⁵. miRNA
11 modulation in renal tubular cells, was associated with functional recovery and phenotypic changes³⁶. In
12 the present study, we evaluated whether EV-shuttled miRNAs play a critical role in the healing
13 properties of MSCs in an *in vivo* model of AKI induced by glycerol administration. We observed that
14 knock-down of Drosha in MSCs, globally reduced miRNA content in the EVs. Moreover, MSC and
15 EV-Dsh exhibited a significantly reduced pro-regenerative effect in AKI mice in respect to the wild
16 type MSCs and EVs. The lack of EV-Dsh efficacy was not dependent on reduced internalization or
17 expression of surface molecules but rather to the molecular content of EVs. The efficacy of wild type
18 MSCs and EVs in the recovery from AKI was observed not only at functional and morphological but
19 also at molecular level. Transcriptome analysis of the kidney from AKI mice showed the alteration of
20 about 4.556 genes in respect to healthy mice. The genes down-regulated by AKI were associated with
21 metabolism such as Valine, leucine and isoleucine degradation; Tryptophan and Butanoate and fatty
22 acid metabolism. Different reports documented the importance of mitochondrial fatty acid oxidation
23 (FAO) in the cytoprotection of proximal tubule cells during the development of AKI³⁶. Among the
24 genes positively modulated by wild type MSCs and EV treatment, the enrichment of genes acting in

1 metabolic pathways associated with fatty acid oxidation, glycolysis, gluconeogenesis and ketone bodies
2 generation has been detected. These processes has been involved in the regulation of energy
3 metabolism of damage tubular epithelial cells by MSC treatment³⁷.

4 Moreover, AKI damage was associated with the up-regulation of genes associated with inflammation,
5 ECM-receptor interaction, cell adhesion molecules and cell cycle in respect to healthy controls.
6 Treatment with wild type MSC and EVs restored a normal transcriptome pattern. In contrast, Dsh
7 knock-down cells and EVs were unable to inhibit the molecular alteration observed in AKI and the
8 RNA-Seq analysis showed a superimposable pattern between AKI and -Dsh treated mice. Recent
9 studies correlated Lcn2 and Fg- β expression with AKI progress in mice^{28, 29}. Over-expression of these
10 markers of renal injury was reduced by wild type MSCs and EVs but not by MSC- and EV-Dsh.
11 Inefficiency of Drosha knock-down cells and EVs in inducing renal repair was observed not only at
12 morphological level but also at functional levels as indicated by the persistence of elevated BUN level.
13 In the attempt to identify the possible healing miRNAs shuttled by EVs, we showed statistically
14 significant correlation of 16 miRNA family (associated in 15 pairs) with genes, whose targets show
15 significant down-regulation in EV-CTRL treated animals in respect to AKI. Of them, 8 miRNA
16 families resulted significant reduced in EV-Dsh (miR-483-5p, miR-191, miR-28-3p, miR-423-5p, miR-
17 744, miR-129-3p, miR-24 and miR-148a families). Union of genes of the 15 pairs of miRNAs
18 identified 209 genes, potentially modulated by these miRNAs. GO enrichment analysis of these targets
19 showed the over-representation of processes associated with the first stages of kidney damage and
20 repair by stem cell administration such as the cell adhesion molecules (CAM) and MAPK signaling³⁸.
21 Interestingly, Wnt signaling pathway³⁹, ECM-remodeling⁴⁰ and p53 pathway⁴¹ were also present
22 among the significant biological process identified (not shown). These processes are usually associated
23 to relevant stages in the transition from acute to chronic injury such as vascular failure, interstitial

1 fibrosis, and glomerulosclerosis development³⁸⁻⁴¹. By selecting in this set, only genes not reverted in
2 EV-Dsh treatment in respect to AKI, 165 genes resulted the possible miRNA targets. GO enrichment
3 analysis of these genes showed that EV-Dsh treated mice overexpressed genes associated with
4 processes related with the progression of kidney damage such as Wnt pathway, p53 signaling, ECM-
5 remodeling and focal adhesion processes as the untreated AKI.

6 In conclusion, the results of the present study indicate that miRNAs play a critical role in the
7 regenerative potential of MSCs. The global down-regulation of miRNAs obtained by Droscha knock-
8 down in MSCs abrogated the ability to promote AKI recovery both by cells and EVs.

1 **Concise methods**

2 **Culture of bone marrow MSC**

3 Bone marrow cells were obtained by Lonza (Basel, Switzerland) and cultured as previously described
4 (3). Cells were seeded at a density of 10,000 cells/cm² and used within the seven passage.
5 Cytofluorimetric analysis was performed as described ³ and the following monoclonal antibodies
6 conjugated with Allophycocyanin (APC) or Fluorescein (FITC), were used: anti-CD146, -CD44, -
7 CD105, -CD29 and -CD90. Mouse IgG isotypic controls were from Miltenyi (Bergisch Gladbach,
8 Germany). All of the cell preparations at different passages of culture expressed the typical MSC
9 markers: CD105, CD73, CD44, CD90, CD166, and CD146. They also expressed HLA class I. The
10 adipogenic and osteogenic differentiation ability of MSC was determined as previously described ³.

11 **Transduction of MSC with lentiviral particles**

12 Lentiviral particles were produced using the 3th generation core packing plasmids kindly provided by
13 Dr. Calautti (Department of Molecular Biotechnology and Life Sciences, Torino). Virus particles were
14 released by 293T cells after overnight collection, filtered through a 0.45 mm filter and concentrated by
15 ultracentrifugation as previous described ⁴². For the construct targeting Drosha, approximately 10⁵
16 passage 0 hMSCs were transduced with particles containing the pTRIPZ lentiviral inducible vector
17 shRNAmiR targeting Drosha (Open Biosystem, clone V2THS_71783) with a single infection.
18 Transduction efficiency was determined by measuring the % of RFP positive cells using different doses
19 of lentivirus. Wild type cells were transduced with a pTRIPZ lentiviral inducible vector scramble-
20 shRNAmiR. Following transduction, cells were grown out to 70% confluence in complete medium
21 containing doxycycline (1µg/ml). RFP-positive cells were selected using a MoFlo™ Cell Sorter
22 (Dakocytomation, Copenhagen, Denmark). Sorted RFP⁺ cells were characterized by incubation with
23 FITC- or APC-conjugated antibodies. Ten thousand cells were analyzed at each experimental point.

1 Doxycycline was added to cultured cells every time they reached the semi-confluent stage and the
2 medium was changed after 72 hours during the duration of the experiment to avoid fluctuation in the
3 Drosha transcript level. Drosha expression was evaluated by quantitative real time PCR using the
4 following primers: F1- 5-CATGCACCAGATTCTCCTGTA-3 and R1 5-
5 GTCTCCTGCATAACTCAACTG-3 as previous described ⁴³.

6 **Isolation of EVs**

7 EVs were obtained from supernatants of MSCs cultured in RPMI deprived of FCS, supplemented with
8 0.5% of BSA (Sigma) and in the presence of doxycycline. The viability of cells incubated overnight
9 without serum was > 98% as detected by trypan blue exclusion. No apoptotic cells were detected by
10 TUNEL assay. Supernatants of MSCs were first centrifuged at 300g and 10.000 g to remove debris,
11 apoptotic bodies, large vesicles and then submitted to ultracentrifugation for 1 hours at 4°C at 100.000g
12 (Beckman Coulter Optima L-90K ultracentrifuge; Beckman Coulter, Fullerton, CA) as previously
13 described ⁴⁴.

14 EV dimension and profile were analyzed by Nanosight LM10 (NanoSight Ltd, Minton Park, UK) and
15 the protein content was quantified by Bradford (BioRad, Hercules, CA). Endotoxin contamination of
16 EVs was excluded by Limulus testing according to the manufacturer's instruction (Charles River
17 Laboratories, Inc., Wilmington, MA), and EVs were stored at -80 °C. To trace EVs by fluorescence
18 microscopy, MSCs were labeled with the SYTO-RNA Select green fluorescent cell stain (Molecular
19 Probes, Life Tecnology) that specifically stain RNA, and with a lipophilic membrane stain that
20 diffuses laterally to stain the entire cell (Life Tech) ⁴⁵. EVs obtained from labeled cells were washed by
21 ultra-centrifugation as described below.

22 **EV characterization**

23 EVs were characterized by cytofluorimetric analysis using FITC or APC conjugated antibodies against
24 CD73, CD44, CD105, CD90, CD29 and HLA-class I. FITC or APC mouse non-immune isotypic IgG

1 (Miltenyi Biotec,) were used as control. Briefly, EVs (1.5×10^8 particles) were incubated for 15 min at
2 4°C with antibodies then diluted in 1 to 3 and immediately acquired as described by as previously
3 described ⁴⁶. FACS analysis was acquired using Guava easyCyte Flow Cytometer (Millipore, Billerica,
4 MA, USA) and analyzed with InCyte software ⁴⁶.

5 **Isolation and culture of mTEC**

6 Kidneys were obtained from healthy female C57 mice. mTEC were isolated, cultured and characterized
7 for expression of tubular and negative for endothelial and glomerular markers as previously described
8 by ³. To determine the incorporation efficacy of EV-Dsh in respect to EV-CTRL by mTEC, we
9 incubated EVs (3×10^9 part/ml) double-labeled with SYTO-RNaselect and Vybrant® Dil (both
10 Molecular Probes) ⁴⁵ with mTEC for 24 hours. The up-take of EVs was analyzed confocal microscopy
11 (Zeiss LSM 5 Pascal, Carl Zeiss, Oberkochen, Germany).

12 **RNA isolation and screening**

13 Total RNA was isolated from cells and EVs using the mirVana RNA isolation kit (Applied Biosystem)
14 according to the manufacturer's protocol. For the extraction of the RNA from kidneys Trizol reagent
15 was used. RNA was then quantified spectrophotometrically (Nanodrop ND-1000, Wilmington DE) and
16 the RNA quality was assessed by capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent
17 Technologies, Inc, Santa Clara, CA) using the Total Eukaryotic Nano and Small RNA kits.

18 **RNA-Seq**

19 For RNA-Seq library preparation, approximately 2 μg of total RNA from mouse kidney was subjected
20 to poly(A) selection, and libraries were prepared using the TruSeq RNA Sample Prep Kit (Illumina)
21 following the manufacturer's instructions. Sequencing was performed on the Illumina HiScanSQ
22 platform. Reads were mapped to the *Mus musculus* mm9 reference assembly using TopHat v2.0.10 ⁴⁷,
23 and RPKM for transcripts were calculated with Cufflinks v2.1.1 ⁴⁸. Transcripts with $\text{RPKM} \geq 1$ were

1 further considered for differential expression analysis. Genes with $\log_2(\text{FC}) \geq 1$ and $\log_2(\text{FC}) \leq -1$ were
2 respectively considered as up- or down-regulated. Gene Ontology analysis was performed using
3 DAVID Bioinformatics Resource ⁴⁹.

4 RNA-Seq data have been deposited in the Gene Expression Omnibus (GEO number, GSE59958,
5 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59958>).

6 **RNA and miRNA expression analysis.**

7 RNA expression confirmation was conducted by real-time PCR (qRT-PCR). First-strand cDNA
8 preparation and qRT-PCR experiments were performed as previous described ³⁵. qRT-PCR was
9 performed using a 96-well StepOne™ Real Time System (Applied Biosystems) Fold change in mRNA
10 expression was calculated as $2^{-\Delta\Delta\text{Ct}}$, using mGAPDH gene as normalizer. All the sequence-specific
11 oligonucleotide primers were purchased from MWG-Biotech AG, Ebersberg, Germany ([www.mwg-](http://www.mwg-biotech.com)
12 [biotech.com](http://www.mwg-biotech.com)).

13 To analyze the miRNA content in MSC and EV-CTRL and –Dsh, the Applied Biosystems TaqManH
14 MicroRNA Assay Human Panel Early Access kit (Life Technologies) was used to profile 754 mature
15 miRNAs by sequential steps of reverse transcription (Megaplex RT Pools; Life Technologies) using an
16 Applied Biosystems 7900H qRT-PCR instrument. Raw Ct values, automatic baseline and threshold
17 were calculate using the SDS software version 2.3. Comparison of miRNA expression was conducted
18 using the Expression Suite software (Life Technologies). Fold change (Rq) in miRNA expression in
19 EV-Dsh in respect to EV-CTRL was calculated as $2^{-\Delta\Delta\text{Ct}}$, using the RNU-48 as normalizer. Rq was
20 measured for each sample and obtained comparing eight EV samples ($n=4/\text{each group}$). Statistical
21 significance was set at $p < 0.05$ and measured by *pair Student's t-test*. Only statistically reduced
22 miRNAs in all samples tested would be considered for further studies.

1 **miRNA target sites enrichment analysis**

2 For miRNA target sites analysis, miRNAs expressed in EV-CTRL were clustered in families
3 according to their seed sequence (nt 2-7). For each down-regulated gene with a fold-change of at least
4 -1 in EV-CTRL vs. AKI mice , we extracted the 3'-UTR sequence, and performed a screening for
5 perfect targets (6-8mers) for each miRNA family. For genes with multiple isoforms, only the isoform
6 with the longest 3'-UTR was used. To account for potential miRNA cooperatively, we searched for
7 targets enriched for seed-match sequences of at least 2 expressed miRNA families. The expected
8 number of targets (used as background) for each pair of miRNA families was calculated on the whole
9 Refseq annotation (21,164 genes). Statistical significance was assessed using an Hypergeometric test.

10 **SCID Mice Model of AKI**

11 Studies were approved by the Ethic Committee of Turin University and conducted in accordance with
12 the National Institute of Health Guide for the Care and Use of Laboratory Animals. A model of
13 rhabdomyolysis-induced acute kidney injury (AKI) was performed in male SCID mice as previous
14 described³. On day 3 after glycerol administration, mice received an intravenous injection into the tail
15 vein of 75.000 MSC-CTRL or MSC-Dsh cells; and EV-CTRL or EV-Dsh in 150 µl saline, or saline
16 alone. The number of particles injected (2.2×10^8 particles/mice) corresponds to an average of the
17 amount of EVs released overnight by 75.000 MSCs (average of 2.900 EVs/cell), which showed a
18 therapeutic effect on this AKI model³. Mice were killed at day 5 (n =8 per group) after glycerol
19 administration and blood samples for BUN determination and kidney specimens were collected. For
20 gene expression, small pieces of the kidney cortical regions were stored in RNA later solution (Applied
21 Biosystem) at -20 °C until required.

22 **Statistical analysis**

1 Data were analyzed using the GraphPad Prism 6.0 Demo program. Statistical analyses were conducted
2 using One-way ANOVA with Newman-Keuls or Dunnett post-tests and *Student's t-test* where
3 appropriated. Statistical significance was established at $p < 0.05$.

4

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8

9 **Statement of competing financial interests**

10 C.T. and F.C. (Fresenius Medical Care) are employed by a commercial company and contributed to the
11 study as researchers. C.T. and G.C. are named inventors in related patents.

12

13

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1 **Figure legends**

2 **Figure 1.** Characterization of MSC-Dsh cells. (A) FACS analysis of transduced wild type MSCs
3 (MSC-CTRL, upper panel) and Drosha knock-down MSCs (MSC-Dsh, lower panel). Efficiency of
4 transduction was measured as % of red fluorescence protein (RFP) positive cells. (B) Comparison of
5 Drosha transcript expression in unsorted or RFP-sorted MSC-CTRL and MSC-Dsh measured by qRT-
6 PCR. GAPDH transcript was used to normalize RNA input. (* $p < 0.05$). Three different lines were
7 tested. Data are mean \pm SD of three different experiments.

8 **Figure 2.** Characterization of EV-Dsh surface markers and up-take by murine tubular epithelial cells
9 (mTEC). (A) Representative FACS analyses of the expression of classical MSC markers (CD29, CD44,
10 CD105, CD73, CD90 and HLA-class I) by EV-CTRL and EV-Dsh. Dot lines indicate the isotype
11 controls. Three different EV preparations were tested with similar results. (B) Representative
12 micrograph of incorporation of EV-CTRL and EV-Dsh by mTEC after 24 h of incubation. EVs were
13 collected from MSC double-stained with Vybrant Dil (red) and SYTO-RNA (that labels the RNA,
14 green). Original magnification X 200 and enlarged image X 630. Three experiments were performed
15 with similar results.

16 **Figure 3.** Effect of intravenous injection of MSC-CTRL and MSC-Dsh in AKI mice. (A-C)
17 Representative micrographs of renal histology of untreated AKI mice injected with PBS (A), and
18 treated AKI injected with MSC-CTRL (B) or MSC-Dsh (C) cells (75.000 cells) and sacrificed at day 5
19 after glycerol administration. Original magnification, X 400 (D). Blood urea nitrogen (BUN, mg/dl)
20 levels measured in healthy, untreated AKI and AKI mice treated with MSC-CTRL or MSC-Dsh, 5 days
21 after glycerol administration. ANOVA with Newman–Keuls multicomparison test: * $p < 0.05$ AKI and
22 MSC-Dsh vs CTRL; ** $p < 0.05$ MSC-CTRL vs MSC-Dsh. (E-F) Morphometric evaluation of hyalin
23 casts (E) and tubular necrosis (F) in untreated AKI and AKI mice treated with MSC-CTRL or MSC-
24 Dsh, 5 days after glycerol administration. Results are expressed as mean \pm SEM (8 mice/group).

1 ANOVA with Newman–Keuls multicomparison test: *, $p < 0.05$ MSC-CTRL vs AKI and **, $p < 0.05$
2 MSC-Dsh vs MSC-CTRL.

3 **Figure 4.** Effect of intravenous injection of EV-CTRL and EV-Dsh in AKI mice. (A-C) Representative
4 micrographs of renal histology of untreated AKI (A) and AKI mice treated with EV-CTRL (B) or EV-
5 Dsh (C) and sacrificed at day 5 after glycerol administration. Original magnification, X 400. (D-F)
6 Representative micrographs of renal histology of untreated healthy (D) and healthy mice injected with
7 EV-CTRL (E) or EV-Dsh (F) and sacrificed at day 2 after EV administration. (G) Blood urea nitrogen
8 (BUN, mg/dl) levels measured in healthy, untreated AKI and AKI mice treated with EV-CTRL or EV-
9 Dsh (5 healthy mice/group and 16 AKI mice/group). ANOVA with Newman–Keuls multicomparison
10 test: * $p < 0.05$ versus AKI; ** $p < 0.05$ AKI EV-Dsh vs AKI EV-CTRL. (H-I) Morphometric evaluation
11 of hyalin casts (H) and tubular necrosis (I) in untreated AKI and AKI-treated mice injected EV-CTRL
12 or EV-Dsh 5 days after glycerol administration. Results are expressed as mean \pm SEM; ANOVA with
13 Newman–Keuls multicomparison test: *, $p < 0.05$ EV-CTRL vs AKI and ** $p < 0.05$, EV-Dsh vs EV-
14 CTRL.

15 **Figure 5.** MSC-Dsh treatment induced in the kidney a different gene expression signature in respect to
16 MSC-CTRL. (A) Pearson correlation of the global transcriptome profile of kidneys of healthy,
17 untreated AKI and AKI treated with MSC-CTRL, MSC-Dsh, EV-CTRL and EV-Dsh. EV-CTRL and
18 MSC-CTRL clustered into two major groups supporting an overlap in the genes regulated by both cells
19 and EVs in AKI. Moreover, healthy mice significantly correlated with MSC-CTRL and EV-CTRL
20 samples. On the contrary, transcriptomic profile of MSC-Dsh and EV-Dsh treated mice showed a high
21 correlation with the untreated AKI mice. (B) Heatmap of the distribution of AKI-deregulated genes in
22 –CTRL and –Dsh samples ($n=2663$, up AKI vs healthy; $n=1893$, down AKI vs healthy). (C) Median
23 distribution of the fold changes in MSC- and EV-CTRL, MSC- and EV-Dsh in respect to AKI samples
24 showing statistically different global distribution of all genes in the two groups.

1 **Figure 6.** Analysis of differentially expressed genes in MSC- or EV-CTRL treated mice versus AKI.
2 (A, B) Venn diagram showing the number of genes up- or down-regulated ($FC \geq |1|$) in the kidneys
3 from AKI mice treated with MSC-CTRL and MSC-Dsh (A) or EV-CTRL and EV-Dsh (B) in respect
4 to untreated AKI. (C, D) Venn diagram showing the number of genes commonly up- or down-regulated
5 (C) in the kidneys from AKI mice treated with MSC- or EV-CTRL in respect to untreated AKI and (D)
6 in the kidneys from AKI mice treated with MSC- or EV-CTRL in respect to AKI treated with MSC- or
7 EV-Dsh. (E, F) Gene ontology analysis reveals that common modulated genes in AKI mice treated
8 with MSC- or EV-CTRL, in respect to untreated AKI, are enriched (E) for genes associated with
9 metabolic pathways, complement and coagulation cascades response (for the up-regulated) and (F) for
10 genes associated with response to inflammation, ECM-receptor interaction, cell adhesion molecules
11 and cell cycle (for the down-regulated).

12 **Figure 7.** Effect of wild type and Drosha-depleted MSC and EVs on markers of AKI. (A)
13 Representative genomic occupancy profile of the *Lcn2* gene among healthy, untreated AKI, MSC-
14 treated (left panel) or EV-treated AKI (right panel). (B) qRT-PCR confirmation of *Lcn2* modulation in
15 mice after MSC- and EV- treatments in respect to untreated AKI mice (n=6 mice/group). Healthy mice
16 are used as negative control. (C-D) Expression level of the three Fg subunits, detected by RNA-Seq
17 analysis, among healthy, untreated AKI, MSC-treated (C) or EV-treated (D) AKI mice. (E) qRT-PCR
18 confirmation of Fg- β expression in mice after MSC- and EV- treatments in respect to untreated AKI
19 mice (n=6 mice/group). In healthy mice no or minimal expression of all the Fg-subunits was observed.
20 (F) Immunohistochemistry analysis of Fg- β among healthy, untreated AKI, MSC- and EV- treated
21 animals. Tubular staining was not observed in kidneys from healthy mice and was markedly reduced in
22 mice treated with MSC- and EV-CTRL but not in MSC and EV-Dsh injected mice. AKI untreated,
23 MSC- and EV-Dsh treated animals showed a strong peritubular and some cytoplasmic tubular staining.

1 For qRT-PCR analysis, data are expressed as mean \pm SD; ANOVA with Newman–Keuls
2 multicomparison test: * $p < 0.05$ vs AKI and ** $p < 0.05$ vs –CTRL.

3

1 **Table 1.** miRNAs down-regulated in EV-Dsh in respect to EV-CTRL. The relative expression of
2 miRNAs in EV-Dsh was defined as fold change evaluated as $2^{-\Delta\Delta Ct}$ in respect to the reference sample
3 (EV-CTRL) as described in Materials and Methods (n=4/each group, $p < 0,05$).

Target Name	Rq mean	SD	T test	Target Name	Rq mean	SD	T test
hsa-miR-215	0,04	0,04	2,27E-05	hsa-miR-483-5p	0,28	0,28	0,014658
hsa-miR-28-3p	0,28	0,08	0,000415	hsa-miR-106a	0,50	0,20	0,015468
hsa-miR-376a	0,43	0,09	0,001068	hsa-miR-590-5p	0,30	0,29	0,017129
hsa-miR-24	0,67	0,06	0,001268	hsa-miR-376c	0,57	0,18	0,017469
hsa-miR-1227	0,23	0,13	0,001392	hsa-miR-133a	0,47	0,22	0,018088
hsa-miR-30e-3p	0,53	0,09	0,001867	hsa-miR-423-5p	0,22	0,19	0,018506
hsa-miR-411	0,45	0,11	0,002006	hsa-miR-425-5p	0,46	0,24	0,020084
mmu-miR-129-3p	0,13	0,18	0,002474	hsa-miR-29a	0,67	0,15	0,022468
hsa-miR-486	0,22	0,17	0,00274	hsa-miR-574-3p	0,57	0,20	0,024011
hsa-miR-218	0,33	0,15	0,002831	hsa-let-7d	0,59	0,19	0,024324
hsa-miR-889	0,22	0,17	0,002908	hsa-miR-150	0,64	0,18	0,026056
hsa-miR-1243	0,19	0,19	0,003278	hsa-miR-19b	0,62	0,19	0,029332
hsa-miR-345	0,38	0,15	0,003789	hsa-miR-33a#	0,24	0,40	0,031748
mmu-miR-140	0,50	0,13	0,004866	hsa-miR-1208	0,54	0,24	0,032548
hsa-miR-186	0,34	0,19	0,005884	hsa-miR-191	0,63	0,20	0,033122
hsa-miR-382	0,22	0,22	0,00613	hsa-miR-203	0,58	0,23	0,035758
hsa-miR-146b	0,55	0,13	0,006271	hsa-miR-200c	0,40	0,34	0,03695
hsa-miR-202	0,18	0,24	0,006639	hsa-miR-181a	0,55	0,25	0,038543

hsa-miR-10a	<i>0,28</i>	<i>0,22</i>	<i>0,007002</i>	hsa-miR-487b	<i>0,43</i>	<i>0,32</i>	<i>0,03889</i>
hsa-miR-323-3p	<i>0,27</i>	<i>0,22</i>	<i>0,007399</i>	hsa-miR-17	<i>0,63</i>	<i>0,21</i>	<i>0,04191</i>
hsa-miR-214	<i>0,49</i>	<i>0,16</i>	<i>0,007832</i>	hsa-miR-532	<i>0,54</i>	<i>0,27</i>	<i>0,04398</i>
hsa-miR-744	<i>0,45</i>	<i>0,18</i>	<i>0,008342</i>	hsa-miR-148a	<i>0,34</i>	<i>0,25</i>	<i>0,044801</i>
mmu-miR-134	<i>0,39</i>	<i>0,20</i>	<i>0,008937</i>	hsa-miR-328	<i>0,48</i>	<i>0,31</i>	<i>0,044856</i>
hsa-miR-199a	<i>0,27</i>	<i>0,25</i>	<i>0,00985</i>	hsa-miR-29c	<i>0,56</i>	<i>0,28</i>	<i>0,049784</i>
hsa-let-7b	<i>0,60</i>	<i>0,15</i>	<i>0,01334</i>				

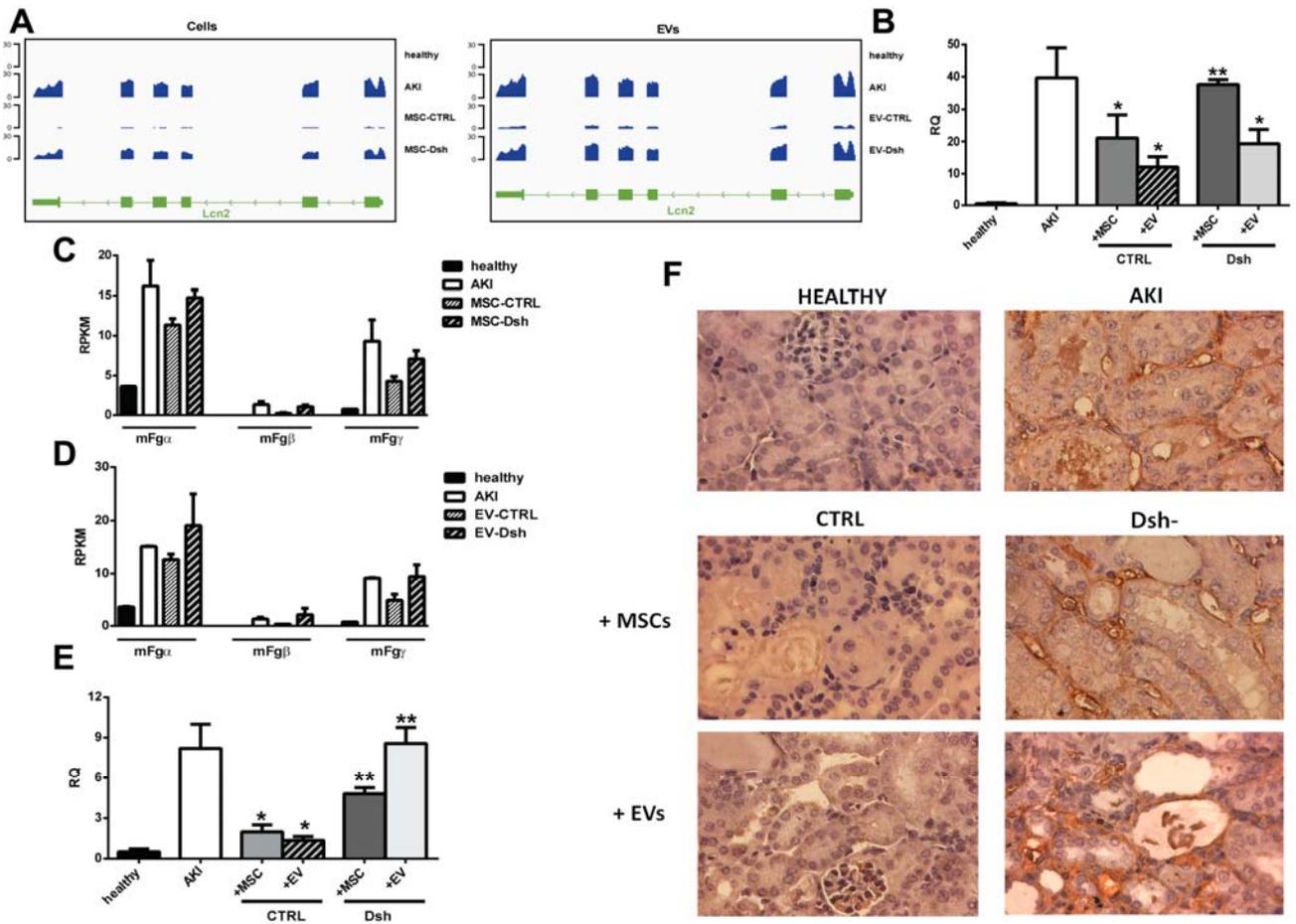
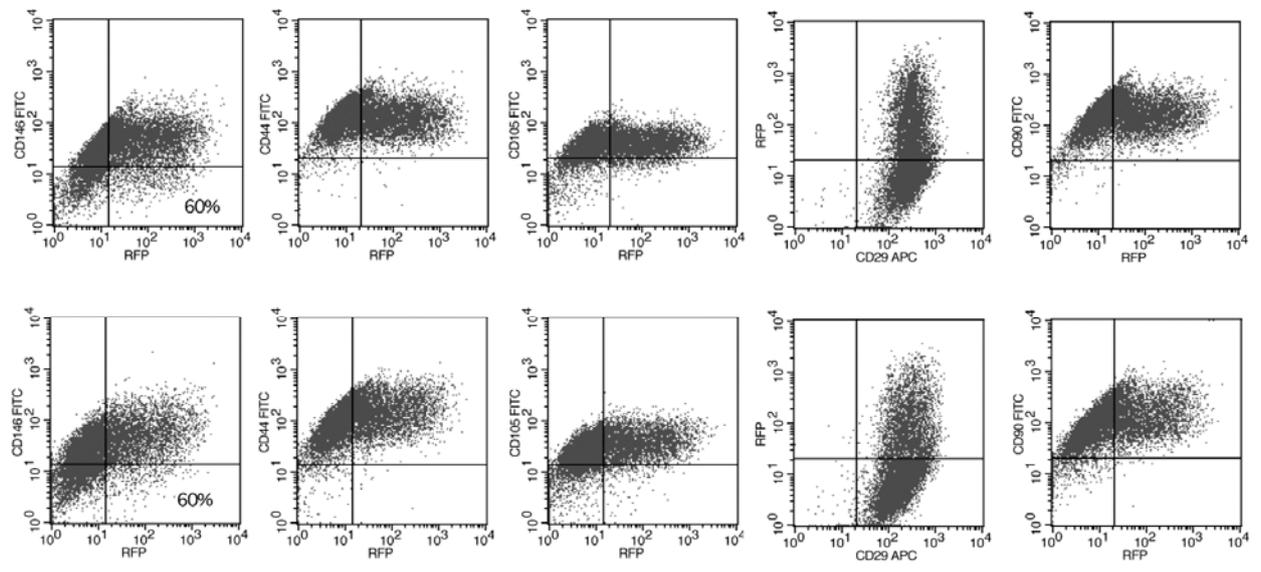


Figure 7

A



B

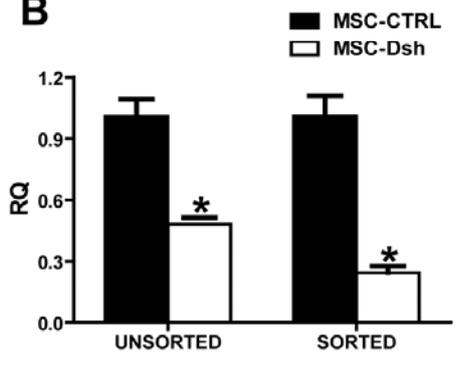


Figure 1

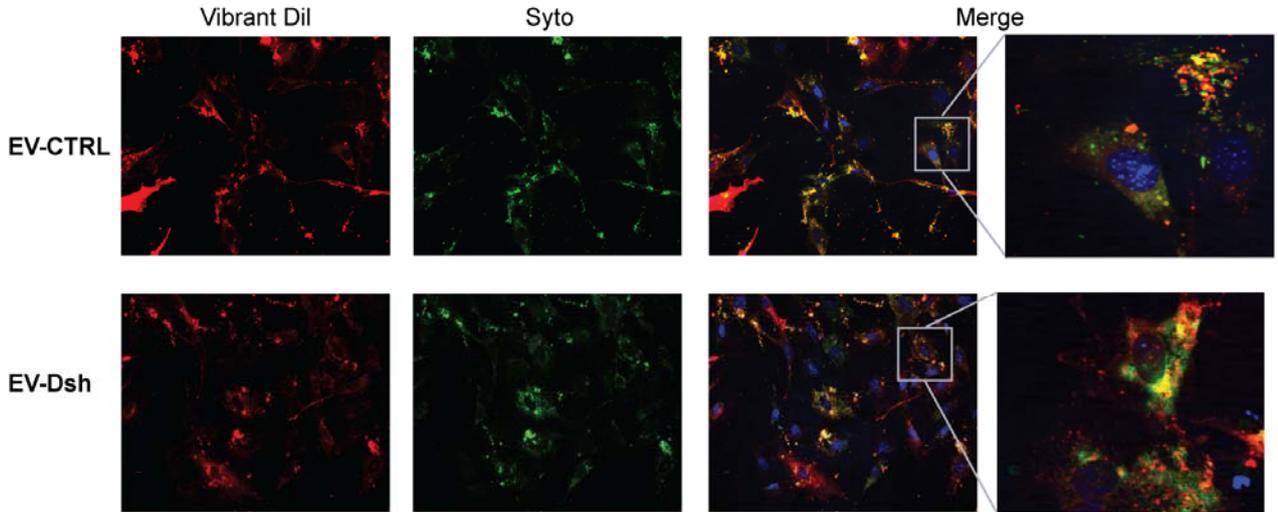
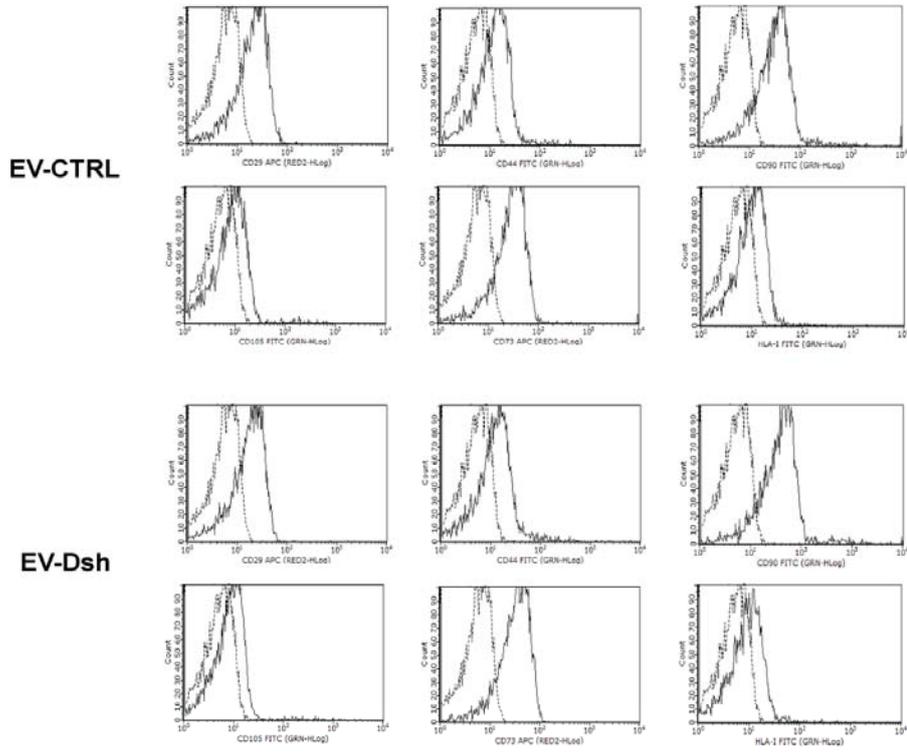
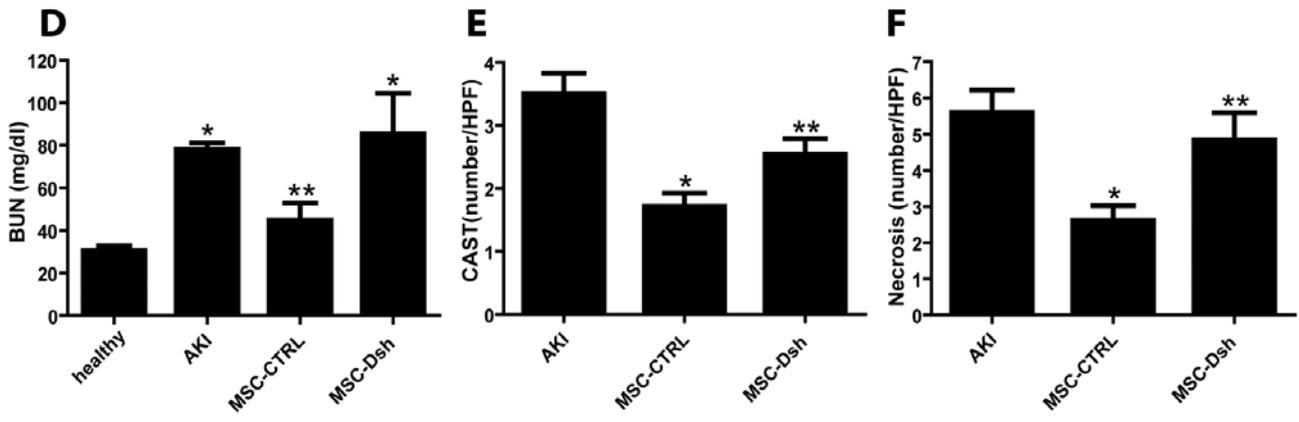
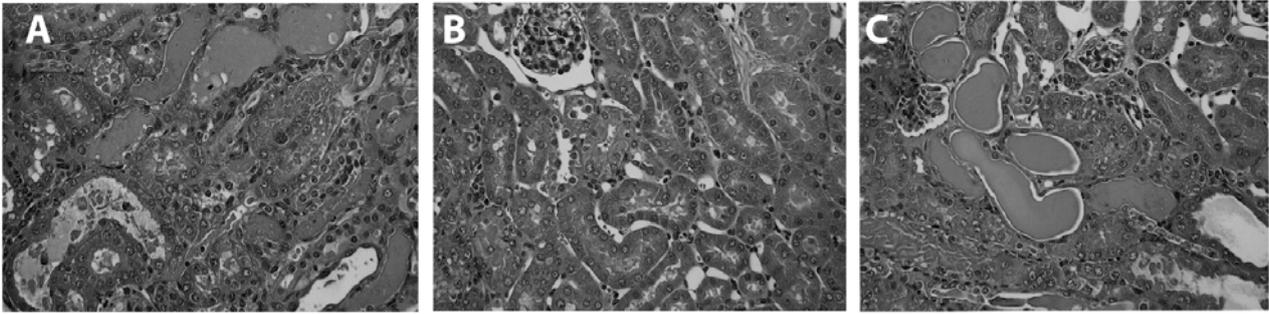
A

Figure 2



1

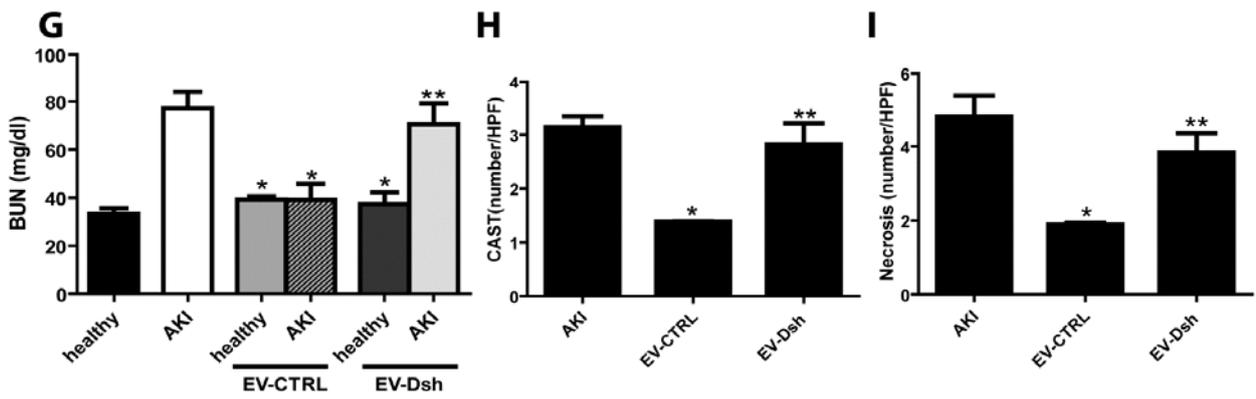
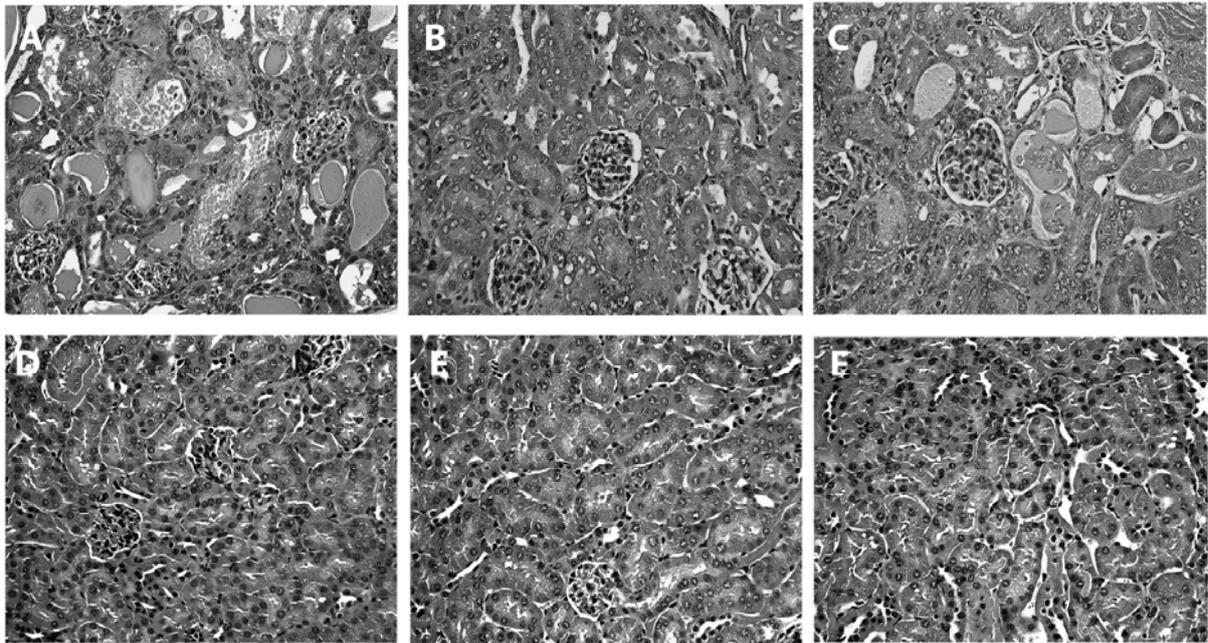


Figure 4

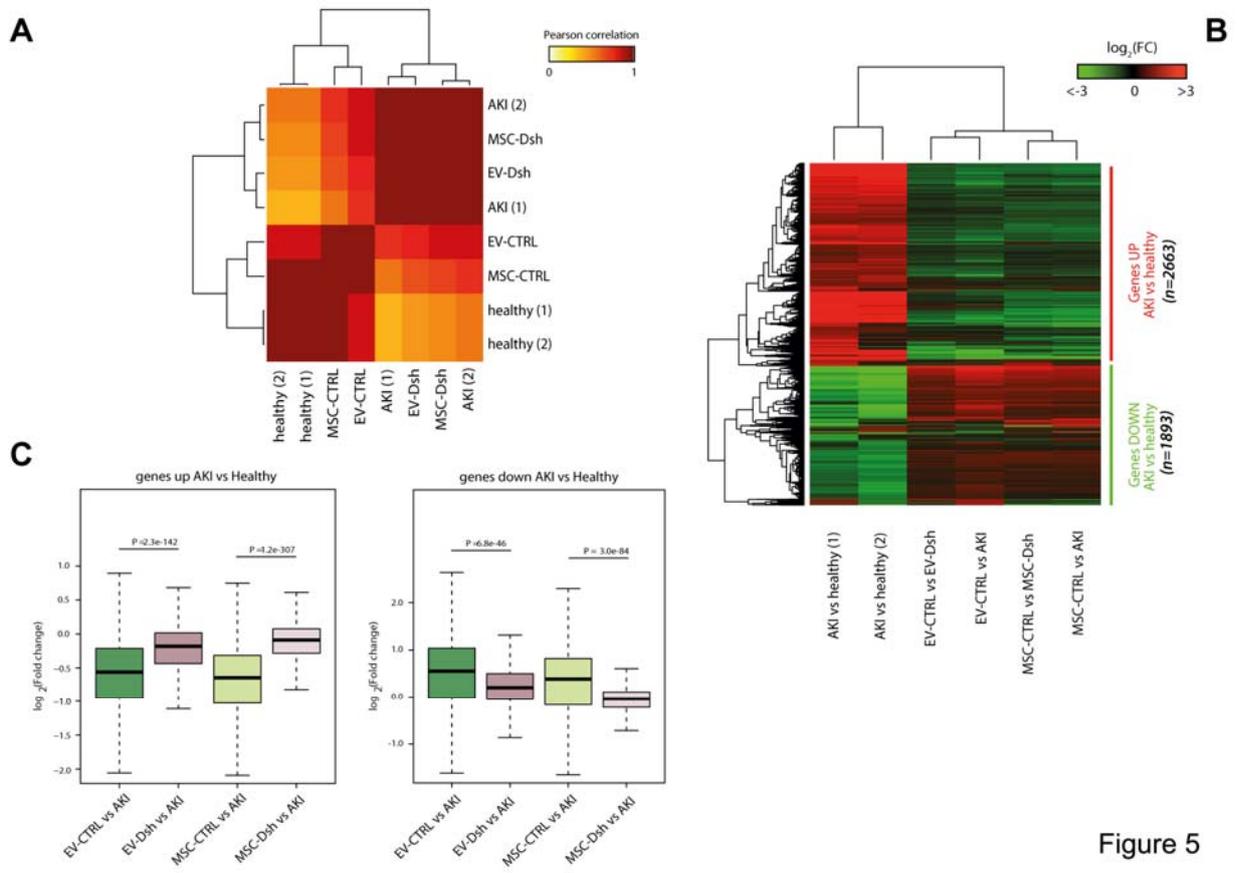


Figure 5

1

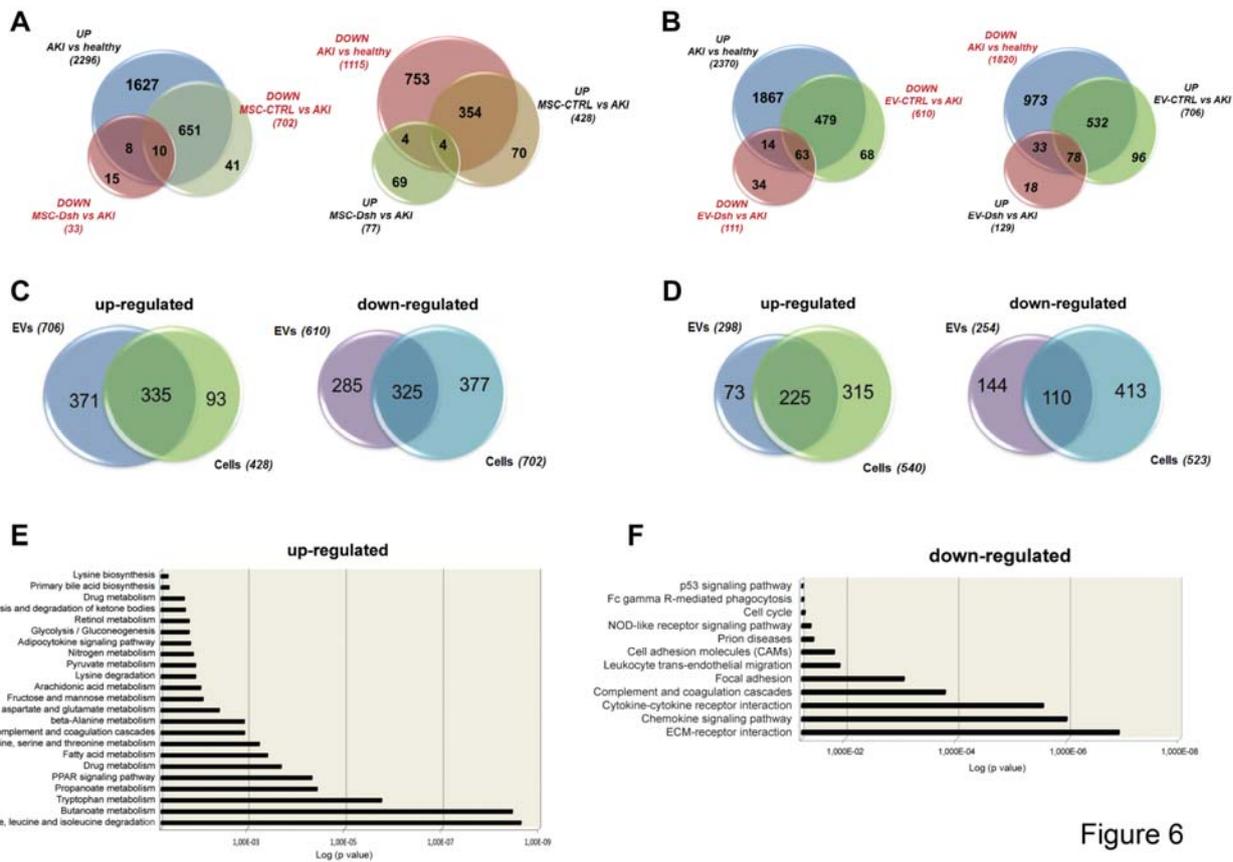


Figure 6