Stem Cell Reports

Supplemental Information

Direct Reprogramming of Human Bone Marrow Stromal Cells into Functional Renal Cells Using Cell-free Extracts

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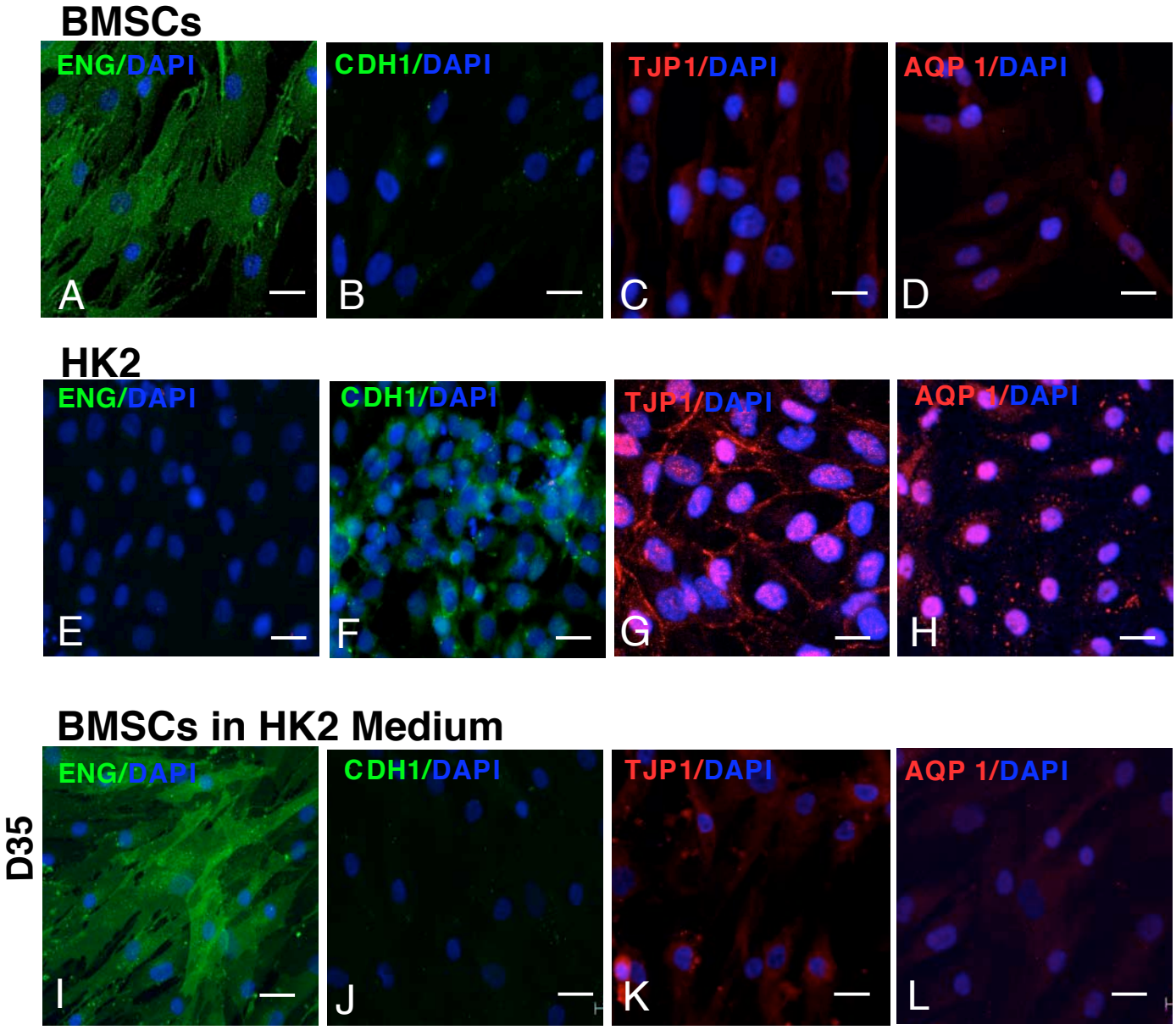

BMSCs
ENG/DAPI
CDH1/DAPI
TJP1/DAPI
AQP 1/DAPI
A
B
C
D
HK2
ENG/DAPI
CDH1/DAPI
TJP1/DAPI
AQP 1/DAPI
E
F
G
H
BMSCs in HK2 Medium
D35
ENG/DAPI
CDH1/DAPI
TJP1/DAPI
AQP 1/DAPI
I
J
K
L

Figure S1

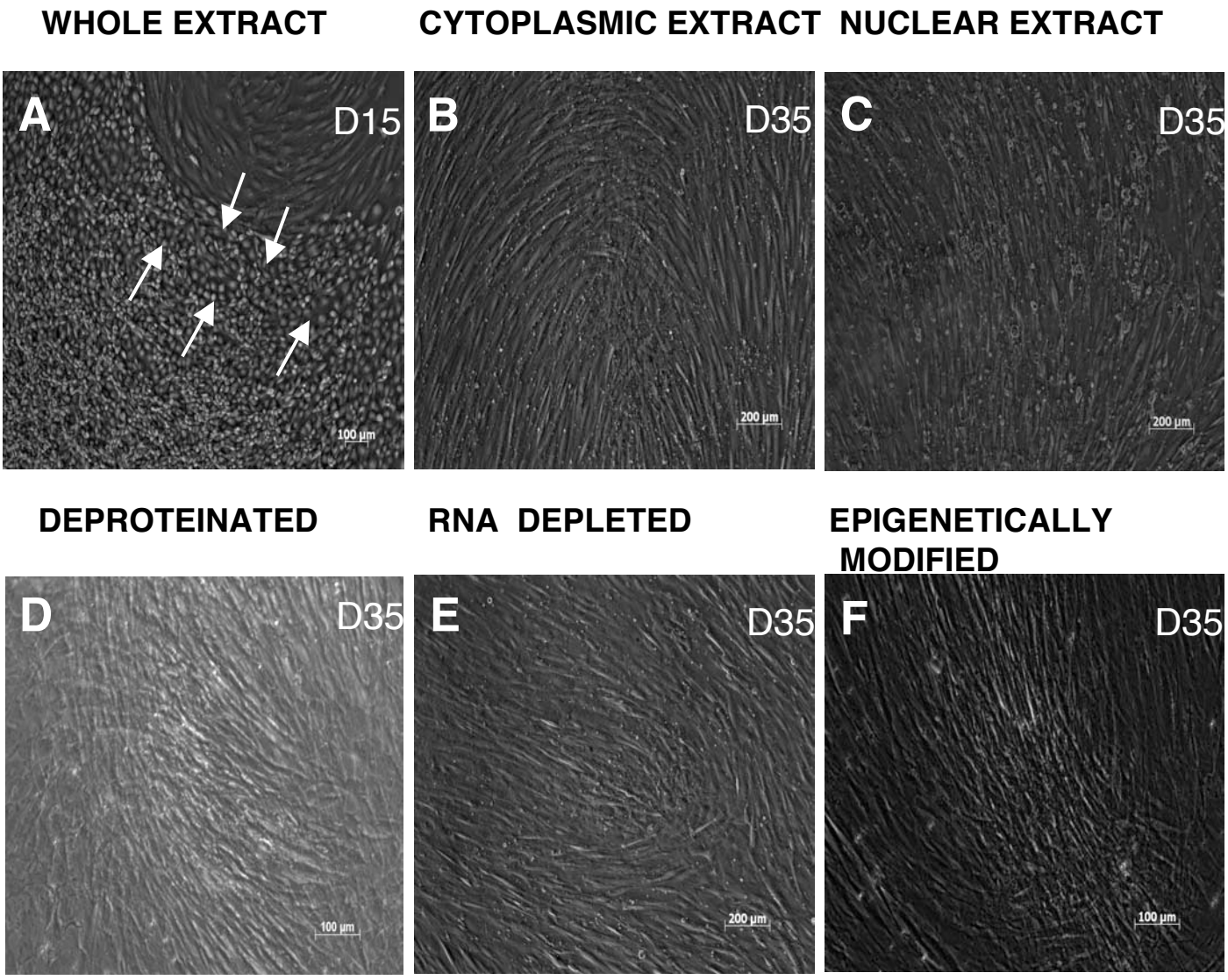

WHOLE EXTRACT
CYTOPLASMIC EXTRACT
NUCLEAR EXTRACT
A
D15
100 µm
B
D35
200 µm
C
D35
200 µm
DEPROTEINATED
RNA DEPLETED
EPIGENETICALLY MODIFIED
D
D35
100 µm
E
D35
200 µm
F
D35
100 µm

Figure S2

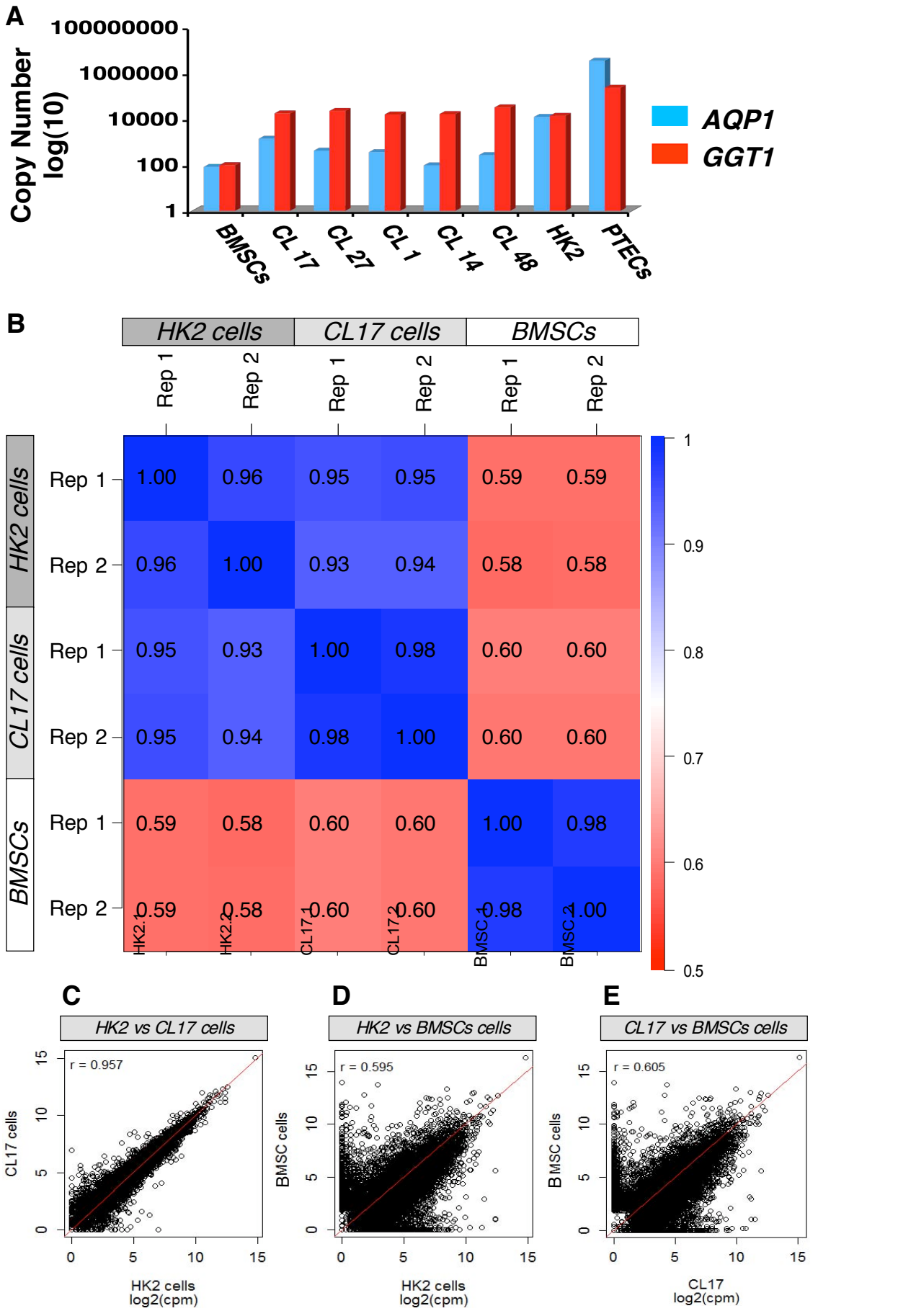

A
Copy Number log(10)
100000000
1000000
10000
100
1
BMSCs
CL 17
CL 27
CL 1
CL 14
CL 48
HK2
PTECs
AQP1
GGT1
B
HK2 cells
CL17 cells
BMSCs
Rep 1
Rep 2
1.00 0.96 0.95 0.95 0.59 0.59
0.96 1.00 0.93 0.94 0.58 0.58
0.95 0.93 1.00 0.98 0.60 0.60
0.95 0.94 0.98 1.00 0.60 0.60
0.59 0.58 0.60 0.60 1.00 0.98
0.59 0.58 0.60 0.60 0.98 1.00
HK2.1
HK2.2
CL17.1
CL17.2
BMSC.1
BMSC.2
1
0.9
0.8
0.7
0.6
0.5
C
HK2 vs CL17 cells
r = 0.957
CL17 cells
HK2 cells log2(cpm)
D
HK2 vs BMSCs cells
r = 0.595
BMSC cells
HK2 cells log2(cpm)
E
CL17 vs BMSCs cells
r = 0.605
BMSC cells
CL17 log2(cpm)

Figure S3

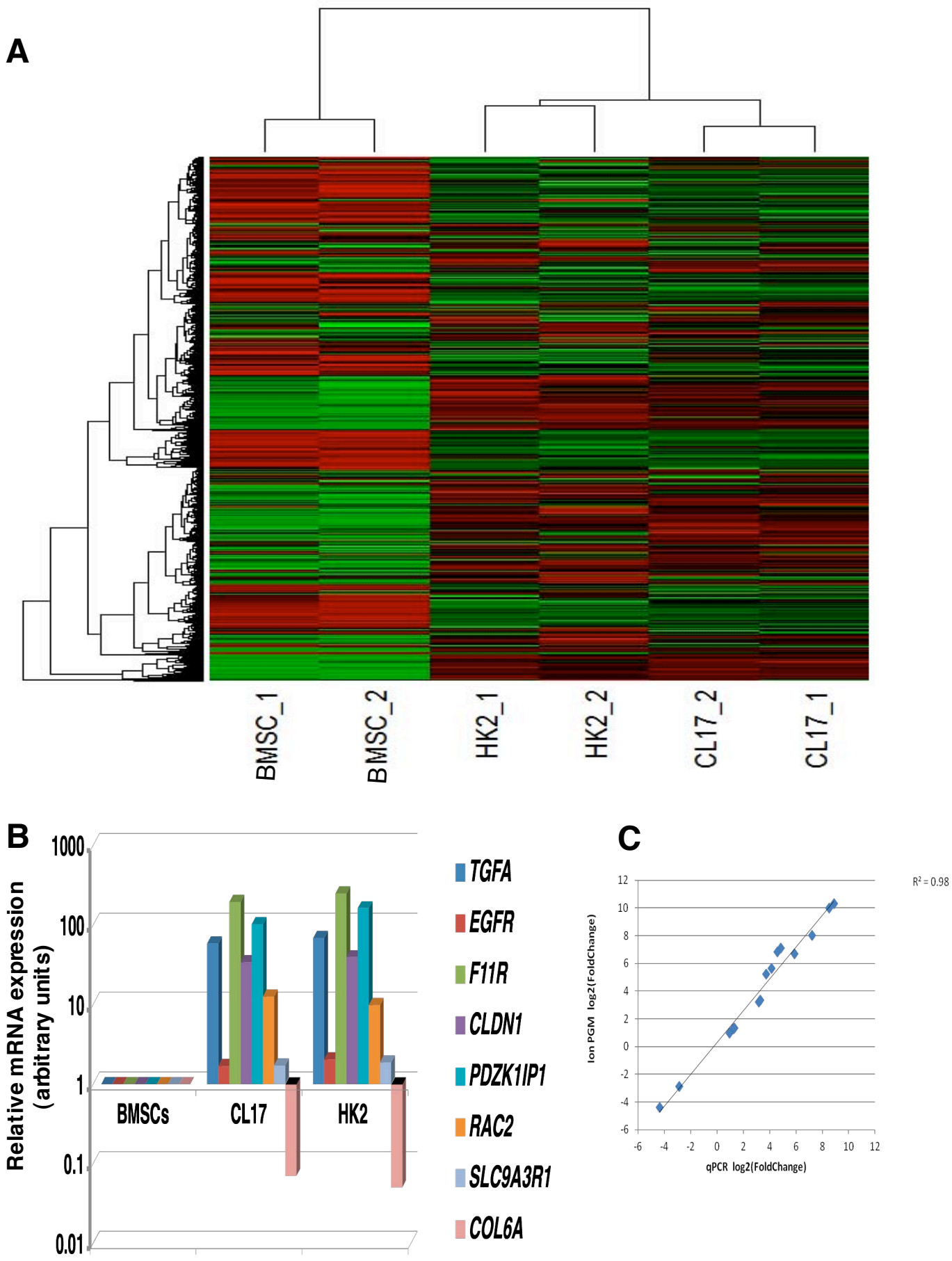
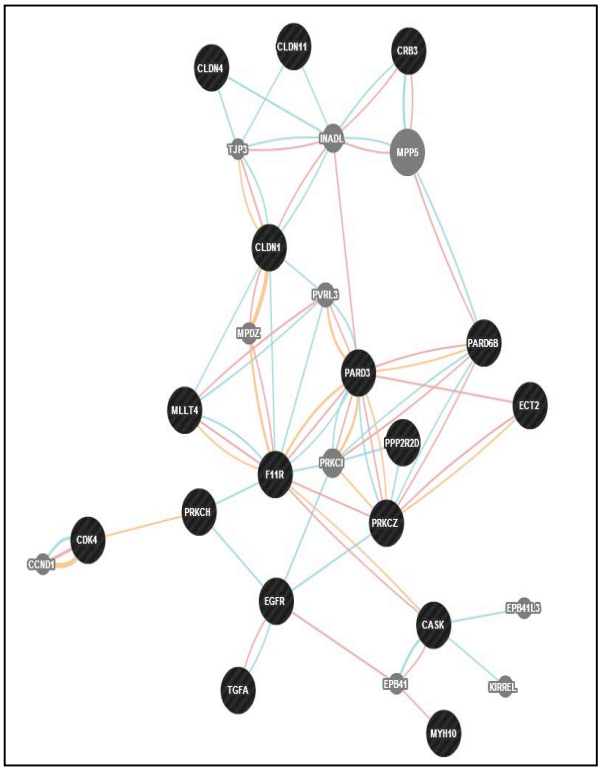
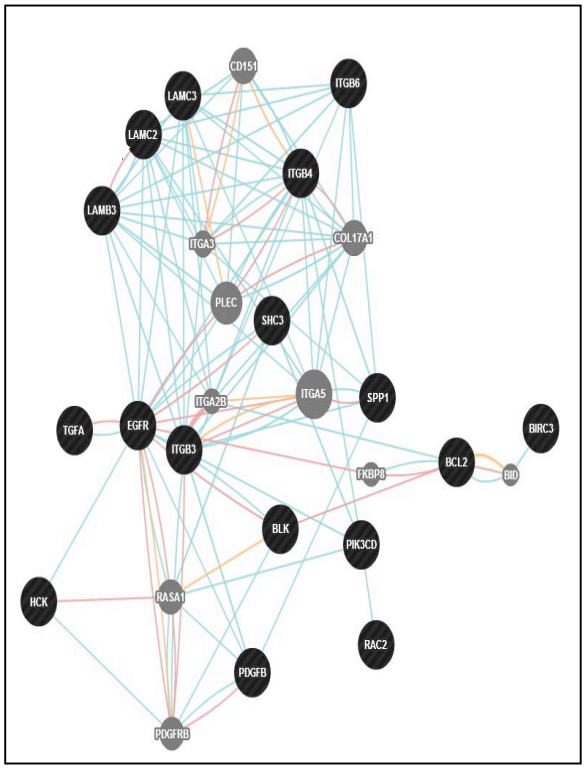

A
BMSC_1
BMSC_2
HK2_1
HK2_2
CL17_2
CL17_1
B
Relative mRNA expression (arbitrary units)
1000
100
10
1
0.1
0.01
BMSCs
CL17
HK2
TGFA
EGFR
F11R
CLDN1
PDZK1IP1
RAC2
SLC9A3R1
COL6A
C
R² = 0.98
Ion PGM log2(FoldChange)
qPCR log2(FoldChange)
12
10
8
6
4
2
0
-2
-4
-6
-6
-4
-2
0
2
4
6
8
10
12

Figure S4

EGFR – Tight Junction



EGFR –Focal adhesion



EGFR – Brush border

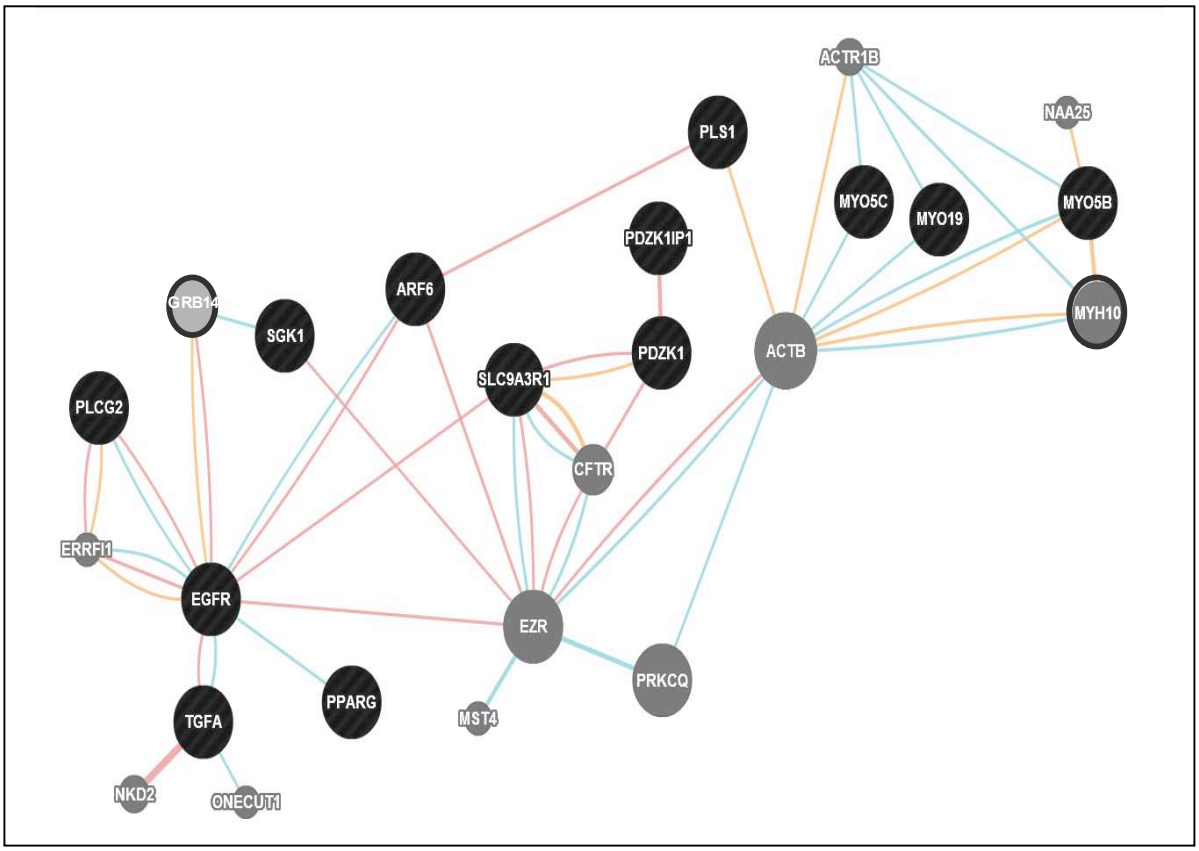


Figure S5

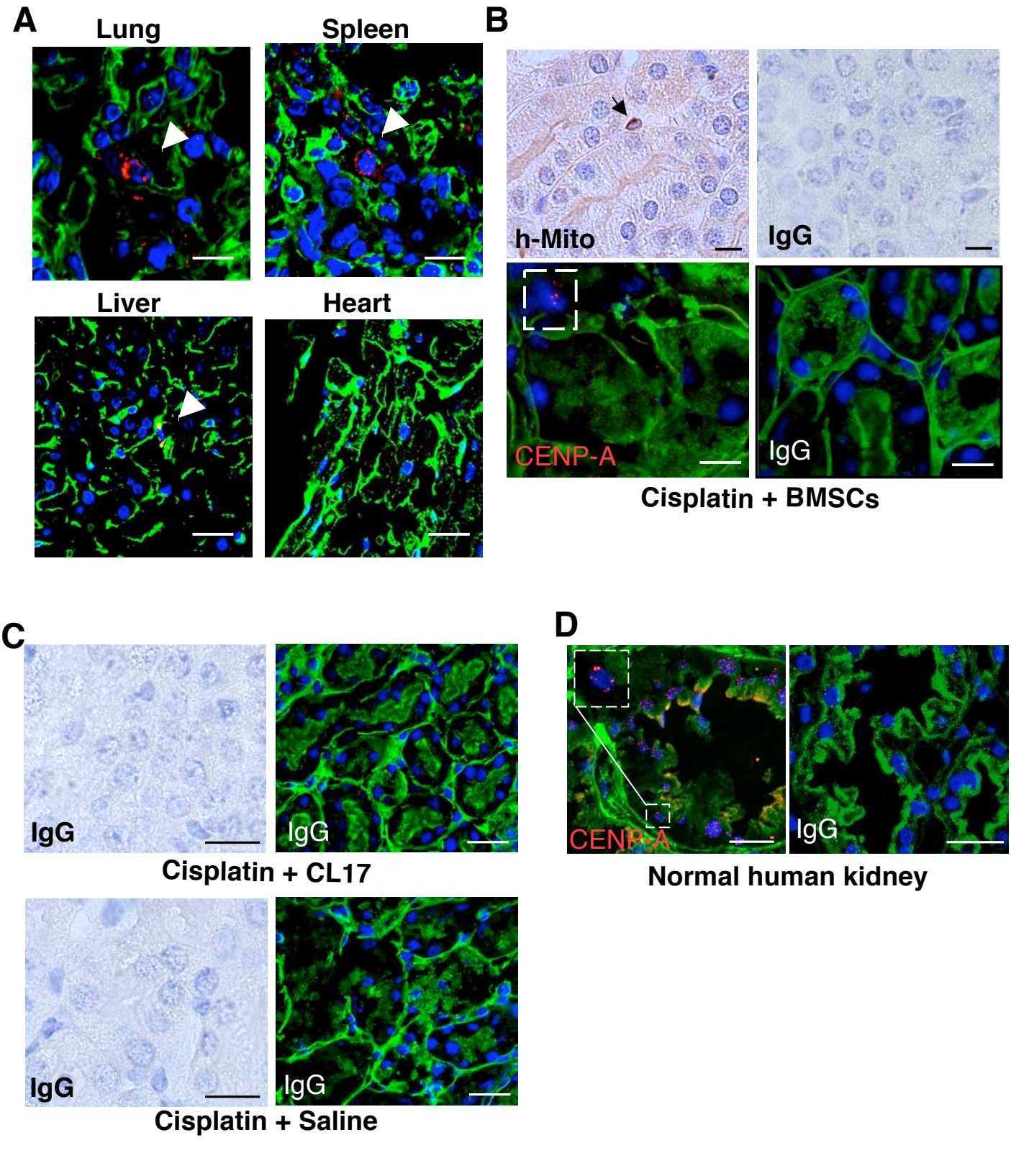

A
Lung
Spleen
Liver
Heart
B
h-Mito
IgG
CENP-A
IgG
Cisplatin + BMSCs
C
IgG
IgG
Cisplatin + CL17
IgG
IgG
Cisplatin + Saline
D
CENP-A
IgG
Normal human kidney

Figure S6

SUPPLEMENTAL FIGURE LEGENDS

Figure S1, Related to Figure 2: Antigenic profile of BMSCs, HK2 cells and BMSCs in HK2 medium.

Expression of ENG, CDH1, TJP1 and AQP1 in BMSCs **(A-D)** HK2 cells **(E-H)** and in BMSCs grown in HK2 specific media for 35 days **(I-L)**. Scale bar: 50μm. Images are representative of two independent experiments.

Figure S2: Reprogramming of BMSCs with modified extracts.

BMSC treated with whole extract changed their spindle like shape into cobble stone colonies at day 15 (**A,** arrows). When BMSCs were treated with cytoplasmic or nuclear extract alone no cell change occurred after 35 days **(B-C)**. Deproteinated or RNA depleted extract did not produce any change at 35 days **(D-E)**. Treatment of HK2 extracts with the epigenetic modifiers Trichostatin A and 5-aza-cytidine did not lead to any cell type change in BMSC at 35 days post-treatment **(F)**. Images are representative of two independent experiments for each condition.

Figure S3, Related to Figure 3A and 3E

(A) Absolute quantification of *AQP1* and *GGT1* mRNA by qRT-PCR in BMSCs, in 5 selected clones, in HK2 cells and in primary PTECs.

(B) Pearson correlation coefficients among all analyzed samples. The higher and the lower coefficients are coloured in a blue to red gradient. **(C-E)** Scatter plots representing the expression of each gene in the indicated pairs of cell types and Pearson correlation coefficients among samples.

Figure S4, Related to Figure 3G

(A) Heatmap of all the genes with ≥5 reads in at least one replicate of each cell lineage. Columns represent biological replicates, and rows represent genes. Green and red colours indicate high and low expression, respectively. The dendrogram of the unsupervised hierarchical clustering of the analyzed samples is shown in the upper part of the heatmap. **(B)** Relative expression of mRNA of human *TGFA, EGFR, F11R, CLDN1, PDZK1 IP1, RAC2, SLC9A3R1, COL6A* by qRT-PCR in CL17 and HK2 in comparison to BMSCs. BMSC mRNA expression was used as the reference sample. The qRT-PCR of these markers, involved in pathways of the BMSC reprogramming, confirmed the fold inductions observed by RNA-seq validating the approach. (C) Comparison between qPCR and Ion PGM data revealed a very high concordance with an R-squared correlation coefficient of 0.98.

Figure S5, Related to Figure 3G

EGFR pathway upregulation and its correlation with genes involved in tight junction, focal adhesion and brush border networks in the reprogrammed BMSCs. Black filled circles represent up-regulated genes of the specific pathway emerged from the functional network analysis with ToppFun and GraphiteWeb. In each pathway 10 genes have been added by GeneMania software to complete the network (filled grey circles). Some of the genes added by GeneMania are up-regulated in CL17 cells (filled grey bold circles). Pink lines represent known physical interactions, blue lines indicate that they belong to same pathway, and yellow lines that are predicted to belong to the same pathway.

Figure S6, Related to Figure 6

(A) Detection of $PKH26^{+ve}$ CL17 cells in different organs than the kidney of AKI mice at day 4. Representative images of $PKH26^{+ve}$ CL17 cells in the lung, spleen, liver (arrowheads), and heart. Scale bars: upper panels 10μm; lower panels: 20 μm. (B) Representative pictures

for h-Mito (upper left panel) and CENP-A staining (bottom left panel) and the respective negative controls (right panels) in renal tissue of mice with cisplatin-induced AKI receiving control BMSCs. Scale bars: 10 μm. Nuclei were stained with DAPI (blue) and renal structures were labelled with wheat germ agglutinin (WGA, green). (C) Representative pictures showing negative controls for h-Mito (left panels) and CENP-A staining (right panels) in AKI animals receiving CL17 (upper panels) or saline (bottom panels). Scale bars: 10 μm for h-Mito and 20 μm for CENP-A staining. (D) Representative pictures showing CENP-A staining in normal human kidney used as positive sample (left panel) and the respective negative control (right panel). Scale bars: 20 μm.

SUPPLEMENTAL TABLES

Table S1. Top 20 down-regulated genes (min fold change) in CL17 vs BMSCs.

Gene	logFC	Fold change	p-adjusted	Down-regulated in HK2 vs BMSCs
COL1A2	-18.5	376095	3.5E-43	Yes
MMP2	-16.4	87057	1.6E-181	Yes
COL6A3	-16.3	78144	8.9E-108	Yes
SULF1	-16.2	74199	1.4E-178	Yes
COL3A1	-15.6	51079	5.2E-24	Yes
TIMP3	-15.5	45853	2.1E-158	Yes
ACAN	-15.3	39800	3.5E-19	Yes
POSTN	-15.2	37533	3.4E-27	Yes
ITGBL1	-14.5	22743	2.3E-132	Yes
CXCL12	-14.4	21579	1.9E-109	Yes

ADAMTS2	-14.2	19048	1.8E-125	Yes
CDH11	-14.2	18687	1.6E-124	Yes
PENK	-14.1	17999	1.7E-09	Yes
CD248	-13.8	14240	1.4E-113	Yes
ITGA11	-13.7	13259	4.0E-14	Yes
HSPB7	-13.5	11573	4.0E-104	Yes
COL5A1	-13.3	10432	6.7E-36	Yes
BGN	-13.2	9284	8.7E-21	Yes
SCUBE3	-13.2	9169	1.3E-50	Yes
LAMA4	-13.0	8024	1.8E-48	Yes

Table S2. Top 20 up-regulated genes (max fold change) in CL17 vs BMSCs.

Gene	**logFC**	**Fold change**	**p-adjusted**	**Up-regulated in HK2 vs BMSCs**
CXCL5	13.4	10840	2.8E-58	Yes
SERPINA1	13.2	9600	3.8E-36	Yes
LCN2	12.4	5340	1.2E-05	Yes
GNG4	12.3	4964	6.0E-60	Yes
CLEC4E	12.2	4796	1.6E-07	Yes
IGFN1	12.1	4368	2.4E-43	Yes
ELF3	11.9	3821	1.9E-31	Yes
HKDC1	11.9	3798	4.4E-37	Yes
EEF1A2	11.9	3713	3.0E-110	Yes
GALNT14	11.7	3427	2.2E-32	Yes

TM4SF18	11.7	3420	8.4E-39	Yes
ARHGAP40	11.7	3293	1.1E-48	Yes
HNF1B	11.6	3089	1.5E-48	Yes
CSF2	11.5	2894	8.8E-27	Yes
SLCO4A1	11.5	2833	1.9E-48	Yes
DCDC2	11.3	2459	6.3E-42	Yes
F11R	11.3	2445	1.5E-41	Yes
COCH	11.2	2407	1.6E-41	Yes
SLCO2A1	11.2	2346	7.2E-09	Yes
LRRN4	11.1	2270	2.2E-41	Yes

Table S3. Functional characterization DEG for selected pathways.xls

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Transmission Electron microscopy

Human BMSCs, clone 17 (CL17), and HK2 cells grown on thermanox slides (Nunc), were fixed for 4 hours in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and washed repeatedly in the same buffer. After postfixation in 1% OsO4, specimens were dehydrated through ascending grades of alcohol and embedded in Epon resin (Fluka, Sigma-Aldrich). Ultra thin sections were stained with uranyl acetate and lead citrate and examined by using transmission electron microscope (Morgagni 268D, Brno, Czech Republic).

Scanning electron microscopy

For scanning electron microscopy (SEM) analysis, BMSCs, HK2 and CL17 cells, seeded on thermanox slides, were fixed in 2.5% glutaraldehyde (buffered with 0.1 M sodium cacodylate buffer, pH 7.4) for 1 hour, postfixed in 1% osmium tetroxide and dehydrated

through an increasing ethanol series. Cell monolayers were then dried with pure hexamethyldisilazane (HMDS, Fluka Chemie AG, Buchs, Switzerland) (twice for 30 minutes), sputter-coated with gold and observed at SEM (Supra 55, Zeiss, Oberkochen, Germany).

Immunocytochemistry

Immunocytochemistry was performed following standard procedures. Primary antibodies to CD105 (1:100, Dako, catalogue n (cat. n): M3527), CDH1 (1:50, Beckton Dickinson, cat. n: 610182), TJP1 (1:50, cat. n: sc-10804), AQP1 (1:50, cat. n: sc-20810) and GGT1 (1:25, cat. n: sc-20638) from Santa Cruz, were incubated overnight at 4^{o}C. Secondary antibodies (1:50, anti-IgG FITC and 1:50 anti-IgG Cy3 Jackson Laboratories) were incubated for 1 hour at room temperature. 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (1:10000, Molecular Probes) was used to visualise the nuclei. Cells were mounted (Dako mounting solution) and fluorescent images were acquired using an Apotome microscope, Axion Vision, Imager 2Z, Zeiss

Flow cytometry

Flow cytometry analysis was performed following standard procedures using a FACSAria (BD) machine. The following conjugated antibodies were used: ENG-PE (Caltag, Invitrogen, cat. n: MHCD10504), CDH1-Alexa-488 (Cell Signaling, Invitrogen, cat. n: 31995), Aquaporin 1-Fluorescein Isothyocyanate (AQP1-FITC) (Santa Cruz, cat. n: sc-32737 FITC) or isotype-matched control antibodies.

Generation of Clones using limiting dilution

Human BMSC treated with HK2 extracts were used for limiting dilution at first passage to

generate clones. Cells were dissociated with trypsin-EDTA and plated into single cell suspension into four 96-well plates (BD) in HK2 medium. Wells containing more than one cell were excluded from the study. When the 96 wells were confluent the clones were subcultured in higher dimension plates/flasks sequentially. Fifty clones (out of total 240 individual cells inoculated) were isolated and expanded. One half of each of fifty clones was frozen at liquid N2 and the other half was collected into Trizol (Ambion, Invitrogen) and kept at -20^{o}C until further analysis. Clones were generated from one reprogramming experiment using one human BMSC donor.

Duplication time measurement

Duplication time was evaluated as previously described (Korzynska and Zychowicz, 2008). Human BMSCs, CL17 and HK2 cells were plated at the density of 30,000 cells/well (n=5), and the number of cells was counted at different time intervals. Growth curves were produced for each cell line and the duplication time was calculated at the linear part of the curve between time intervals of 24-48 hours for HK2 and CL17 cells and 7-14 days for BMSCs. The equation $T2=\Delta t/\log_2(\Delta N/N_0+1)$ was used for the calculation of the duplication time. In this equation N_0 is the number of cells at the beginning of observation (seeding density) and ΔN is the increase in the number of cells during the period of time of the length Δt (Korzynska and Zychowicz, 2008).

RNA extraction, qRT-PCR

Total RNA was prepared from BMSCs, HK2 cells and five of the clones obtained by limiting dilution, using TRIzol reagent according to the manufacturer's instructions. Contaminating genomic DNA was removed by RNase-free DNase (Promega) for 1h at 37°C. Two μg of purified RNA was reverse transcribed using a mixture of oligodT and random

examers oligonucleotides and 200U of SuperScript II RT (Invitrogen) for 1h at 42°C. No enzyme was added for reverse transcriptase-negative controls.

To amplify human cDNAs, we used SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instruction. qRT-PCR was performed on a 7300 Real Time PCR System (Applied Biosystems). The amplification profile consisted of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. We used the ΔΔCt technique to calculate cDNA content in each sample using the cDNA expression in BMSCs or HK2 cells as calibrator.

For absolute quantification of the copy number of human *AQP1* and human *GGT1* transcripts, commercially synthesized oligonucleotides (450 bp) were obtained (Sigma-Aldrich). The DNA was serially diluted (40, $4x10^2$, $4x10^3$, $4x10^4$, $4x10^5$ and $4x10^6$ copies) to make a standard curve (Bustin, 2000).

From the standard series included in the PCR, a linear relationship between Ct and log10 (DNA copy number) was plotted for a target transcript. Based on the relationship, the *C*t value for a given sample was used to infer its corresponding amount of template. Because the target gene sequence was known, it was possible to calculate the copy numbers by the molecular weight of the sequence, which estimated the copy number in an unknown sample (Wang et al., 2014).

The primers that were used are listed following:

Gene	***Sequences***
RPL29	For 5'-AGCCCCTTTCTCTTCCGGTT-'3
(NM_000992.2)	Rev 5'-GTGCCATTTTCGGGACTGGT-'3
AQP1	For 5'-CCCTCATGTACATCATCGCCC-'3
(NM_198098.2)	Rev 5'-ACACCATCAGCCAGGTCATT-'3
GGT1	For 5'-AGCCTGTCTTGTGTGAGGTG-'3
(NM_001032364.2)	Rev 5'-TGAGGCTGCCGTTGTAGAAG-'3
ENG	For 5'-AGCCCTGACCTGTCTGGTTG-'3
(NM_001114753.1)	Rev 5'-GAACGCGTGTGCGAGTAGA-'3

TGFa	For 5'-TCCCCGCTGAGTGACCC-'3
(NM_001099691.2)	Rev 5'-CTCCTGCACCAAAAACCTGC-'3
EGF-R	For 5'-GAGCAGCGATGCGACCCT-'3
(NM_005228.3)	Rev 5'-CTTGGCAAACTTTCTTTTCCTCCA-'3
F11R	For 5'-GGCTTTTCTTCTCCCCGTGT-'3
(NM_016946.4)	Rev 5'-CCGGTCCTCATAGGAAGCTGT-'3
CLDN1	For 5'-CAGTCAATGCCAGGTACGAA-'3
(NM_021101.4)	Rev 5'-ACAGCAAAGTAGGGCACCTC-'3
PDZK1IP1	For 5'-CGTCGGAAACAAGGCAGATG-'3
(NM_005764.3)	Rev 5'-CATGCTCACTGGACCTGAAAC-'3
RAC2	For 5'-TCACCACCGACACTCTCCAG-'3
(NM_002872.4)	Rev 5'-CCACGGCCCCATCTCCC-'3
SLC9A3-R1	For 5'-CGAGGAGCTGAATTCCCAAGA-'3
(NM_004252.4)	Rev 5'-AGTCTAGGATGGGGTCGGAG-'3
COL6A2	For 5'-ATGACGCTGTTCTCCGACCT-'3
(NM_001849.3)	Rev 5'-ACGGACAGCTCTGTTTGGCA-'3

Albumin binding and uptake assay

Albumin binding and uptake assays were performed as previously described (Gekle et al 1998, Morigi et al., 2005) with small modifications. Human BMSCs, CL17 and HK2 cells were incubated with serum free medium over night. Cells were washed 3x with Ringer's buffer pH 6.0 and then were exposed to 50μg/ml BSA-FITC (Bovine Serum Albumin, Sigma-Aldrich) alone or in the precence of an excess of unlabeled BSA (5mg/ml) for 15 min at 4^{o}C for binding studies and for 90 min at 37^{o}C for uptake experiments. The cells were then washed 3x with Ringer's buffer pH 7.4, fixed in 2% PFA and 4% sucrose for 10 min at RT. Cells were mounted with mounting solution and images were acquired using an Apotome microscope, Axion Vision, Imager 2Z, Zeiss.

Renal organoids

Briefly, E11.5 CD1 mouse embryonic kidneys were dissected in MEM (Sigma-Aldrich) placed in 1x trypsin/EDTA (Biochrom AG) for 3 min at 37°C and then dissociated by trituration into single-cell suspensions that were filtered through a 40-μm cell strainer

(Falcon, BD). Human cells were labeled with Cell Tracker (Molecular Probes) following the manufacturer's instructions and labelling efficacy and viability were evaluated by Trypan blue (Sigma-Aldrich) exclusion. A total of $1x10^5$ freshly dissociated renal cells were centrifuged at 900 g for 4 min in the presence of $1x10^4$ CL17, HK2 or BMSCS then the pellet was placed on a top of a 5 μm filter (Millipore) supported by a metal grid, at the air–medium interface in a humidified atmosphere with 5% CO_2 and at 37°C and maintained in Advanced DMEM supplemented with 2% Embryonic Stem cells Fetal Bovine Serum, 1% L-glutamine and 1% penicillin/streptomycin (all from Gibco, Invitrogen). During the first 24 h, aggregates were cultured in the presence of 1.25 μmol/l Glycyl-H1152 dihydrochloride (Tocris), a Rho kinase inhibitor. Renal chimeras were cultured *in vitro* for 1 or 5 days, then fixed and finally processed for immunofluorescence analysis, as previously described (Xinaris et al., 2012).

Renal histology and Immunohistochemistry Analysis

Duboscq-Brazil fixed, paraffin embedded kidney samples were stained with periodic acid–Schiff's reagent (PAS) and observed by light microscopy (Primo Star, Zeiss, Jena, Germany). Luminal hyaline casts and tubular necrosis (denudation of tubular basement membrane) were assessed in up to 25 non overlapping fields/section for each animal (40x, high power field, HPF).

For immunofluorescence staining, acetone-fixed cryosections were subjected to antigen retrieval using microwave (twice for 5 min in citrate buffer 10mM pH 6.0 at operating frequency of 2450 MHz and 600W power output). After blocking with 1% BSA, sections were incubated with rabbit anti-human CENP-A (Cell Signalling Technology, Danvers, MA, USA, dilution 1:100; cat. n: 2186) followed by goat anti-rabbit Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Cell nuclei were

labelled with DAPI and renal structures with fluorescein wheat germ agglutinin (WGA, VECTOR Labotatories, cat. n: FL-1021). Human tissue was used as positive control (Figure S 6D). Fluorescence was examined using an inverted confocal laser-scanning microscope (LSM 510 Meta).

For immunoperoxidase experiments, Duboscq-Brazil fixed, paraffin embedded renal sections (3μm) were deparaffinised, hydrated and incubated for 5 min with Peroxidased1 solution (Biocare, Concord, CA) to quench endogenous peroxidases. Antigen retrieval was performed by boiling sections using microwave in Rodent Decloacker solution. After blocking with Rodent Block M (Biocare), sections were incubated with anti-human mitochondria (Millipore, Temecula, CA, USA, dilution 1:50; cat. n: mab1273) followed by specific mouse-on-mouse polymer kit (Biocare) and diaminobenzidine (Merck, Darmstadt, Germany) substrate solution. Slides were finally counterstained with hematoxylin, dehydrated in graded alcohols, mounted with coverslips, and observed by light microscopy (Apotome Axio Imager Z2, Zeiss, Jena, Germany). Negative controls for immunofluorescence and immunoperoxidase experiments were obtained by incubating the adjacent section of examined renal tissue with non-immune immunoglobulin of the same isotype of the primary antibody.

SUPPLEMENTAL REFERENCES

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