SUPPLEMENTARY MATERIALS

Quantitative evaluation of human bone mesenchymal stem cells rescuing fulminant hepatic failure in pigs

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SUPPLEMENTARY MATERIALS AND METHODS

Isolation, culture and phenotypic identification of hBMSCs

hBMSCs were isolated by bone marrow aspiration from the iliac crest of 50 healthy male volunteers. Isolation and culture of the BMSCs derived from each volunteer were performed as described previously[1]. Phenotypic analyses of the cultured hBMSCs using standard flow cytometry methods were performed prior to transplantation. In particular, the 7th passage of hBMSCs (1×10^6 cells) was incubated with directly PE- or FITC-conjugated mouse monoclonal antibodies against human CD34, CD45, CD29 and CD90 for 60 minutes in the dark at 4℃, followed by washing and resuspension in phosphate buffer solution. Immunoglobulin isotype incubation was performed as a negative control. Flow cytometry was then performed with a FACSCalibur system (FC500, Beckman Coulter, Fullerton, CA, USA).

Multilineage differentiation assays

Osteogenic, adipogenic and hepatogenic differentiation was performed to characterize the multipotent differentiation of the hBMSCs, as described previously[1]. To induce osteogenic and adipogenic differentiation, hBMSCs were cultured in commercially available osteogenic and adipogenic differentiation media. The alkaline phosphatase activity and Oil Red O staining of the cultured cells were assessed at day 20. Hepatogenic differentiation was performed as described previously[1]. On day 20, the cultured cells were characterized by RT-PCR using hepatic-specific gene primers (specific to ALB, cytokeratin 8, glucose-6-phosphate dehydrogenase and hepatocyte nuclear factor-1α). Glyceraldehyde phosphate dehydrogenase (*GAPDH*) was used as an internal control.

Biochemical function evaluation

Biochemical tests of liver function, kidney function, metabolism and coagulation were evaluated by analysing 35 markers prior to hBMSC transplantation (day 0) and then at 6 and 12 hours and at 1, 2, 3, 5, 7, 10 and 14 days after transplantation using an automatic clinical chemistry analyser (AU5800, Beckman, Jersey City, NJ, USA).

qRT-PCR

qRT-PCR was performed using a two-step protocol with specific primers, SYBR Green dye, and the ABI 7500HT instrument (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), as described previously[1]. The amount of cDNA was optimized so that amplification of both control genes and the cDNAs of interest were in the exponential phase. Transcripts were quantified using the comparative Ct method and normalized to the levels of the endogenous control (*GAPDH*)[2].

IHC and H&E staining

For the observation of hBMSC transdifferentiation, IHC was performed to detect the expression of hBMSC-specific markers (CD90 and CD29) and human hepatic-specific markers (ALB and HSA) in liver tissue from pigs in the transplantation group at days 3 and 7 after hBMSC transplantation, as described previously[1]. Briefly, liver tissue sections (4 μm) were applied to poly-L-lysine-coated slides. After the sections were dewaxed, rehydrated and washed, endogenous peroxidases were inactivated with 3% H2O2 for 10 minutes at room temperature. The sections were then incubated overnight with primary anti-human antibodies with no cross-reactivity to pig tissues. The sections were subsequently washed with PBS three times and incubated with the appropriate secondary antibodies at 37℃ for 1 hour.

After reaction with the DAB chromogen, the sections were rinsed with distilled water, counterstained with haematoxylin and mounted using Histomount. The labelled cells were visualized using an inverted microscope (Eclipse E200, Nikon, Tokyo, Japan), and digital images were captured using NIS-Elements F 3.0 software. Omission of the primary antibody or substitution with an unrelated IgG served as a negative control. Brown staining indicated positive expression. Counting of cells stained for ALB and HSA at days 7 and 14 was performed in 20 random fields from four pigs using NDP.scan 2.5 software (NanoZoomer 2.0-RS, Hamamatsu Photonics, Hamamatsu, Japan). For H&E staining, each liver tissue section was heat fixed at 60℃ for 1 hour and then stained with H&E, as described previously[1]. Liver tissue samples were harvested from five pigs in the normal, transplantation, and control groups, two surviving pigs and five surviving rats in the DLL4 intervention groups; and five pigs and five rats in the normal and control groups.

UPLC-MS quantification of the relative abundance of human-derived proteins

To quantify the relative abundance of total human proteins in pigs, the proteins in serum or liver tissue samples were digested with sequencing-grade trypsin. The resulting peptide mixtures were subjected to nanoflow liquid chromatography (ACQUITY UPLC system, Waters Co., Milford, MA, USA) coupled with mass spectrometry (Q Exactive mass spectrometer, Thermo Fisher Scientific, Bremen, Germany)[3]. The spectra were then searched against *Homo sapiens* and *Sus scrofa* proteins in the UniProt database (as of 07-09-2015) using the MaxQuant search engine. The relative abundance of total human proteins was calculated as the fraction of unequivocal human peptides among all peptides with unequivocal origins.

To quantify the relative abundance of total human ALB, proteins from liver tissue were fractionated by high-pH reversed-phase liquid chromatography (RPLC). Each fraction was then subjected to deep analysis, as described previously[3]. The percentage of human ALB was estimated using three pairs of peptides that distinguish human ALB from pig ALB.

UPLC-MS profiling of serum metabolites

To profile the hepatic metabolic functions of two groups' animals, serum samples were precipitated with acetonitrile to remove proteins, and the supernatants were analysed by UPLC-MS, as described above. The spectral reads from UPLC-MS were processed with MassLynx 4.1 to obtain peak intensities. Temporal changes in metabolite profiles (in the form of peak intensities) were analysed using principal component analysis (PCA), as described previously[4].

Cytokine arrays for measuring serum cytokine levels

To identify significantly expressed cytokines in pigs with FHF in the transplantation and control groups, serial serum samples harvested from randomly chosen pigs (n=3/group) on days 0 and 3 after the sham procedure in the control group and on days 0, 3, 7 and 14 after transplantation in the transplantation group were analysed using human cytokine arrays (RayBiotech, Inc., Norcross, GA, USA) containing antibodies against 215 cytokines according to the manufacturer's instructions and as described previously[5]. All cytokine array assays contained positive, negative, blank and internal controls. The levels of each protein were measured in duplicate.

Annotation enrichment analysis

Annotation enrichment analysis was performed using Panther (http://pantherdb.org) against all GO biological process terms for humans. The significance cut-off was set to $P<0.05$. When analysing altered cytokines, the collection of 215 cytokines in the antibody arrays was used as background. When analysing differentially expressed genes, the collection of all human genes was used as background (default option).

ELISA measurements

To confirm the results of the cytokine array analysis, six significantly regulated proteins were validated by ELISA using the same samples as had been used in the antibody array assays. The expression level of DLL4 in the pigs in the DLL4 intervention group was also validated by ELISA, as described previously for other proteins[6].

Total RNA extraction and mRNA-seq

For mRNA-seq, PBMCs were collected from pigs using Ficoll-Histopaque medium on days 0 and 3 in the C group and on days 0, 7 and 14 in the T group. As many unforeseen health risks exist in animals with FHF that undergo several partial hepatectomies and to ensure an adequate number of surviving animals for follow-up, liver tissues (one section each from the left, middle and right lobes; each 10-20 g) and serial PBMC samples were harvested from two randomly chosen pigs in each group at each time point. Total RNA extraction from the liver tissues and PBMCs was performed using TRIzol reagent following the manufacturer's instructions. A sequencing library was then prepared according to the manufacturer's instructions (TruSeq™ Small RNA Sample Preparation Kit, Illumina, San Diego, CA, USA), including adapter ligation, reverse transcription, PCR amplification and pooled gel

purification steps. The pooled library consisted of sequences with lengths of approximately 250 nucleotides. The library was sequenced using the HiSeq 2000 sequencing system (Illumina, San Diego, CA, USA). The average number of sequencing reads was approximately 22.5 million per RNA sample.

Sequencing reads were mapped to the pig reference genome (Sscrofa10.2) or to the human reference genome (GRCh37.71) using TopHat v2.0.10[7] with default parameters. HTSeq v0.6.1[8] was used to compute the raw read counts for each gene. DESeq2 v1.8.1[9] was used to call differentially expressed genes. Significance was defined as $P<0.05$ for testing individual expression changes or as FDR<0.05 for the discovery of significant expression changes based on a profile. The Benjamini–Hochberg–Yekutieli procedure (as implemented in the R function p.adjust) was used for multiple-test correction.

Quantification of pig- and human-derived gene expression

Pig- and human-originated sequencing reads were separated in the above mRNA-seq results for the two pigs in the transplantation group. To maximize separation of pig- and human-derived expression, we reconstructed the individual genomes of the pigs. Strict alignment was used to identify sequencing reads from these pigs. To construct individual pig genomes, the mRNA-seq results from PBMCs at day 0 were mapped to the pig reference genome (Sscrofa10.2) using TopHat v2.0.10 with default parameters. Single nucleotide polymorphisms (SNPs) were called using Samtools v0.1.18[10] with default parameters. For each pig, the detected SNPs were merged with the reference genome.

The hBMSCs used in this study were pooled from different donors. Alignment with the human reference genome (GRCh37.71) with default tolerance of mismatch was used to identify sequencing reads that potentially represented human expression. Reads mapped to only one species were considered species-specific reads. Reads mapped to both species were considered ambiguous reads and were used to compute the lower and upper bounds of species-specific expression.

Gene expression analysis and functional interpretation of expression variations

Genes differentially expressed between two samples were detected using the methods described above. The R package coXpress v1.4 was used to detect the gene clusters that were differentially co-expressed between the control and the transplantation groups. The expression profiles of two control group pigs at days 0 and 3 were used to assess co-expression in the control group, and the expression profiles of two transplantation group pigs at days 0 and 7 were used to assess co-expression in the transplantation group. Gene expression variations were interpreted by Gene Set Linkage Analysis (GSLA)[11]. GSLA has been shown to anticipate the collective functional consequence of a set of changed genes, rather than solely annotating them to biological processes. The criteria for significant biological processes were set to a density of > 0.01 (criterion for the strength of functional coupling) and $P \leq 1 \times 10^{-5}$ (criterion for biological significance).

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6 Xin J, Ding W, Hao S, Jiang L, Zhou Q, Wu T, *et al.* Human bone marrow mesenchymal stem cell-derived hepatocytes express tissue inhibitor of metalloproteinases 4 and follistatin. Liver Int 2015;35:2301-10.

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SUPPLEMENTARY FIGURES

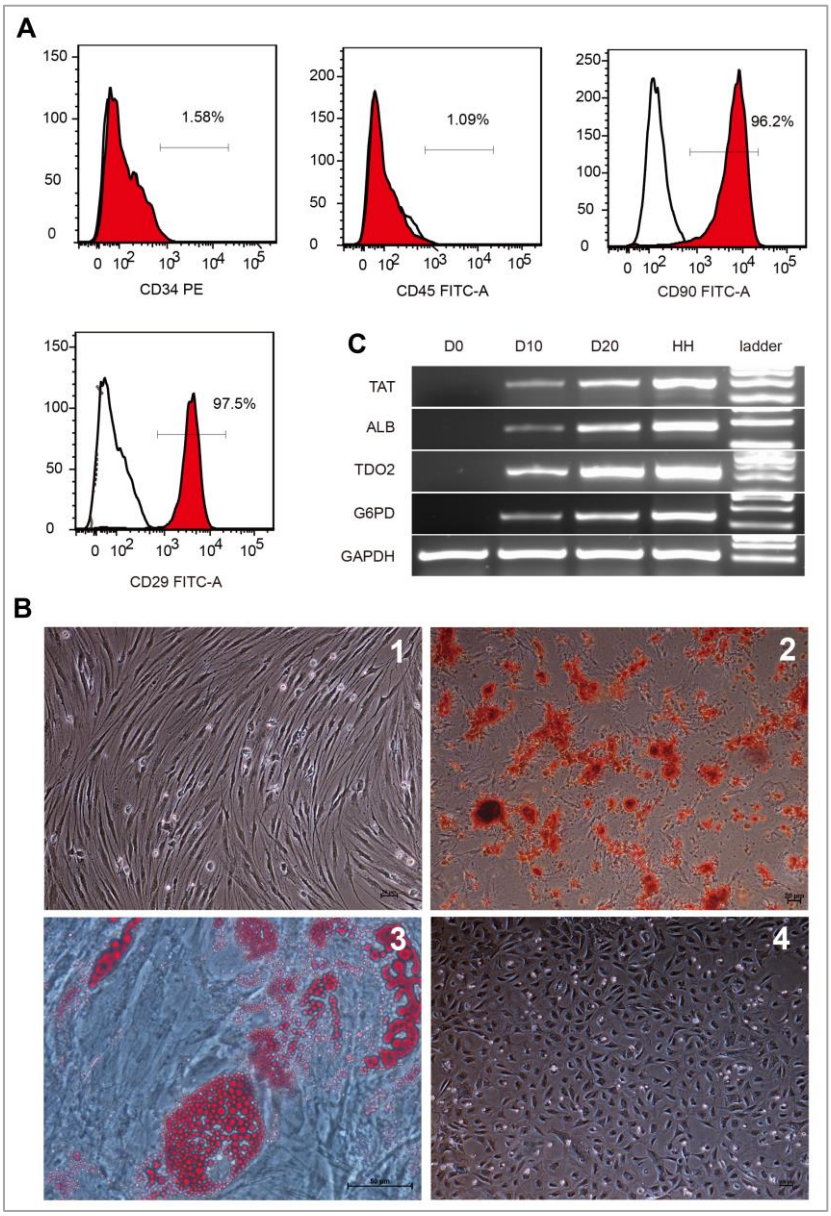

Figure S1. Characterization of hBMSCs and hBMSC-derived hepatocytes. (A) The immunophenotypic properties of hBMSCs were analysed by flow cytometry. hBMSCs (7^{th}-passage) were positive for CD29 and CD90 but negative for CD34 and CD45. (B) Multipotent differentiation of hBMSCs (1) into osteocytes (2), adipocytes (3) and hepatocytes (4). Magnification of ×10, ×10, ×20, and ×4. (C) Detection of hepatocyte-specific markers by RT-PCR. The lanes, from left to right, represent the hepatic differentiation of hBMSCs at day 0 (D0), D10 and D20; HHs; and a ladder. TAT, tyrosine aminotransferase; TDO2, tryptophan 2,3-dioxygenase; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde phosphate dehydrogenase; HH, human hepatocyte. n=5/group.

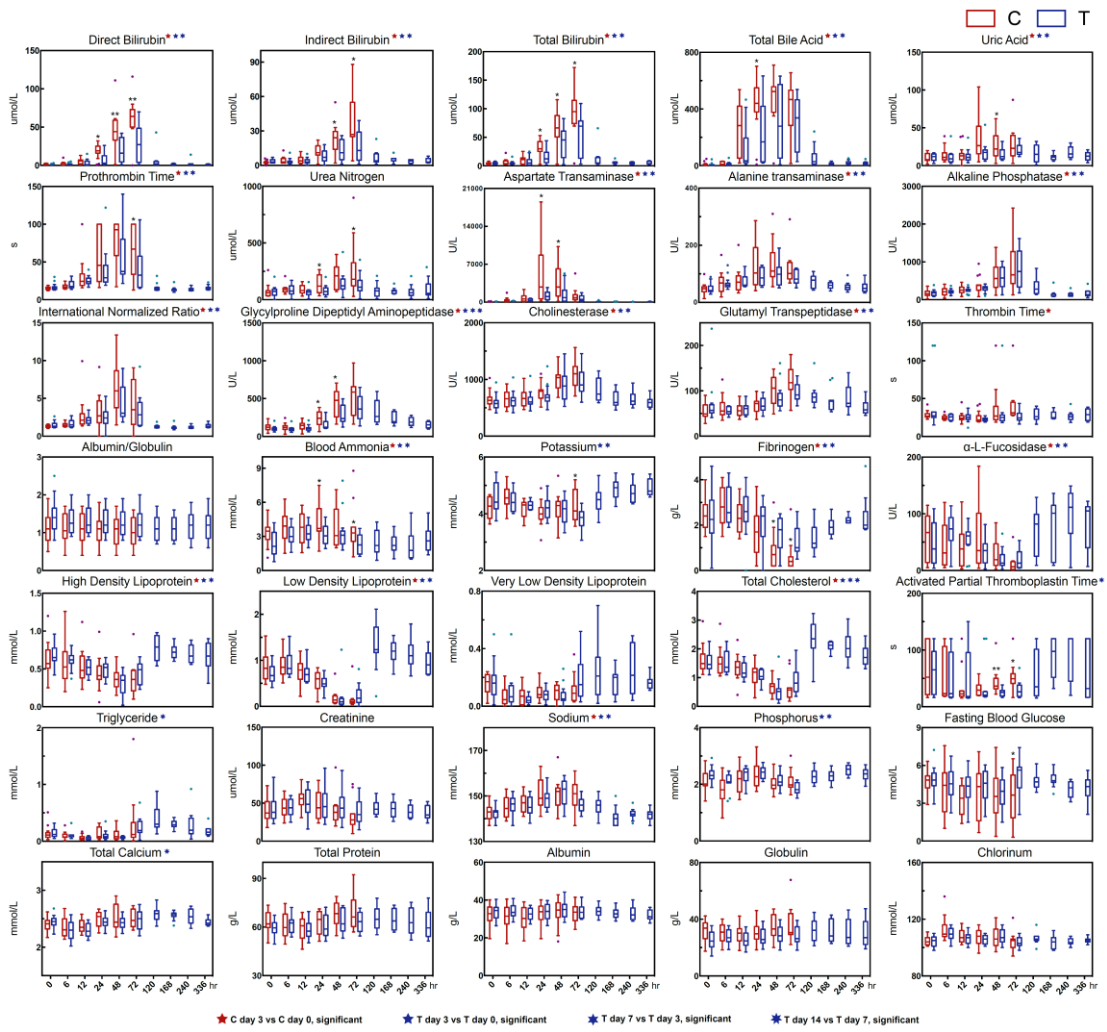

Figure S2. Serum levels of 35 biochemical markers. Among the 20 biomarkers that showed significant changes at day 3 (D3) compared with D0 in both the control (C) and the transplantation (T) groups, changes in 18 biomarkers were attenuated in the T group. Aspartate transaminase (AST), prothrombin time (PT) and international normalized ratio (INR) are the most commonly used clinical measures to quantify extent of hepatic injury and synthetic impairment. There is significant difference in the level of AST/PT rises between the control and transplantation group (PT at 72h; AST at 24, 48h). The difference between the control and transplantation group INR was not significant. Error bars, SEM; Mann-Whitney U test, $^{**}P<0.01$, $^{*}P<0.05$. n=5-15/group.

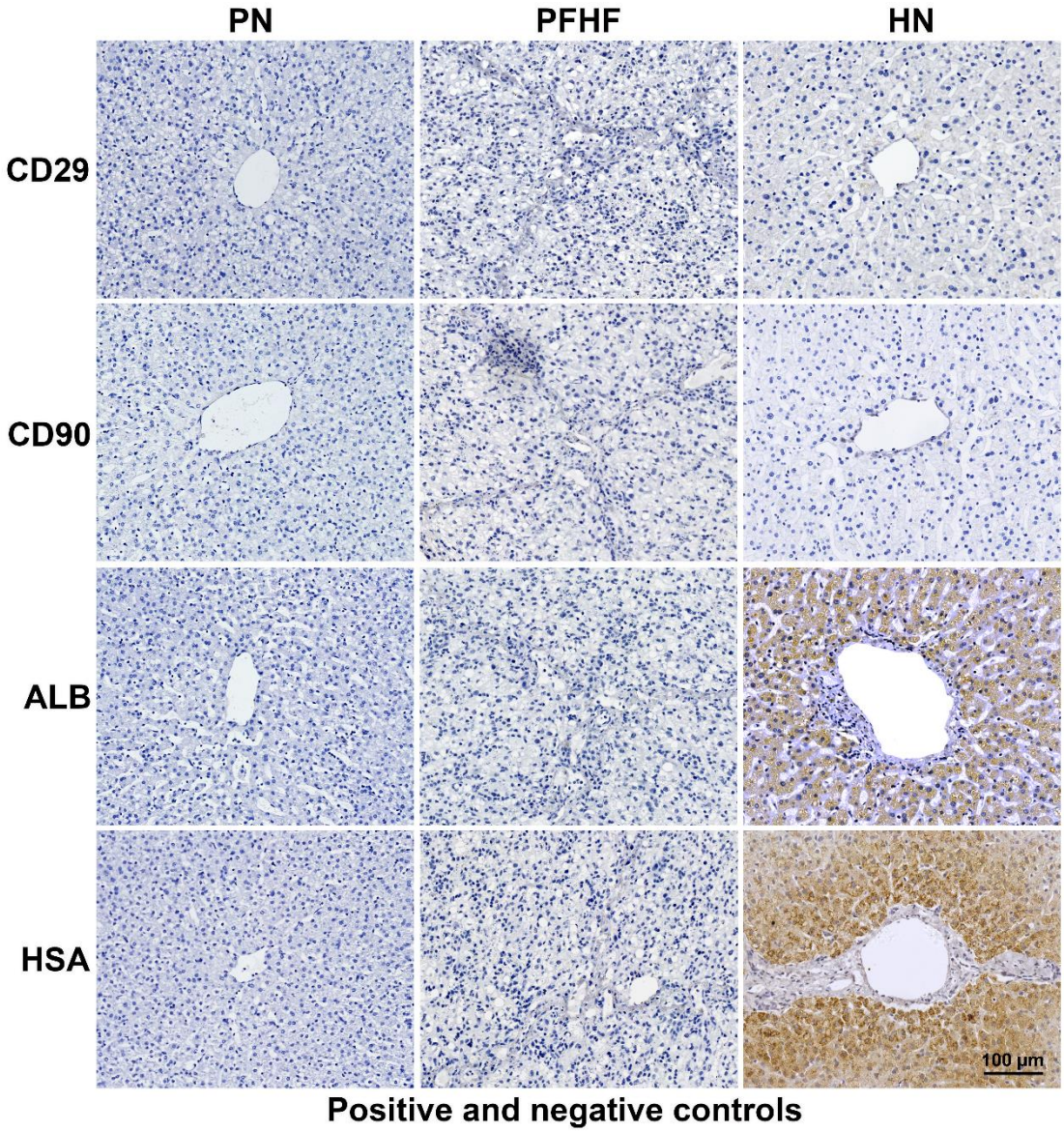

Figure S3. Positive and negative controls in the immunohistochemical staining of liver tissues. Brown staining indicates positive expression. PN, pig normal liver tissue; PFHF, pig FHF liver tissue; HN, human normal liver tissue. Scale bar, 100 μm. n=5/group.

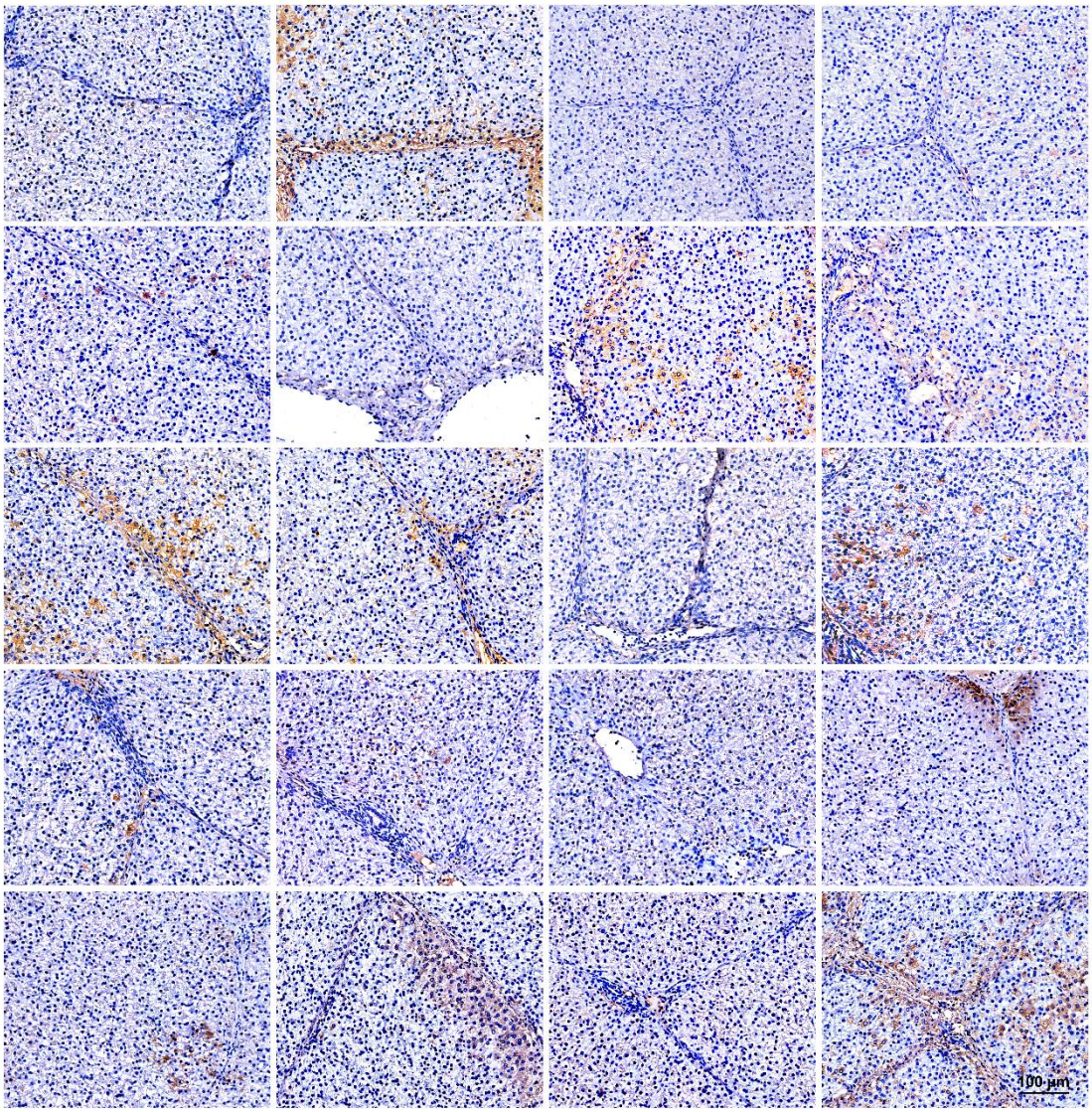

Figure S4. Immunohistochemical staining for human ALB at day 7 (D7). Twenty images were randomly chosen from the full set of scan images and were used to calculate the percentage of positively stained cells. The results are representative of 5 independent samples. Brown staining indicates positive expression. Scale bar, 100 μm.

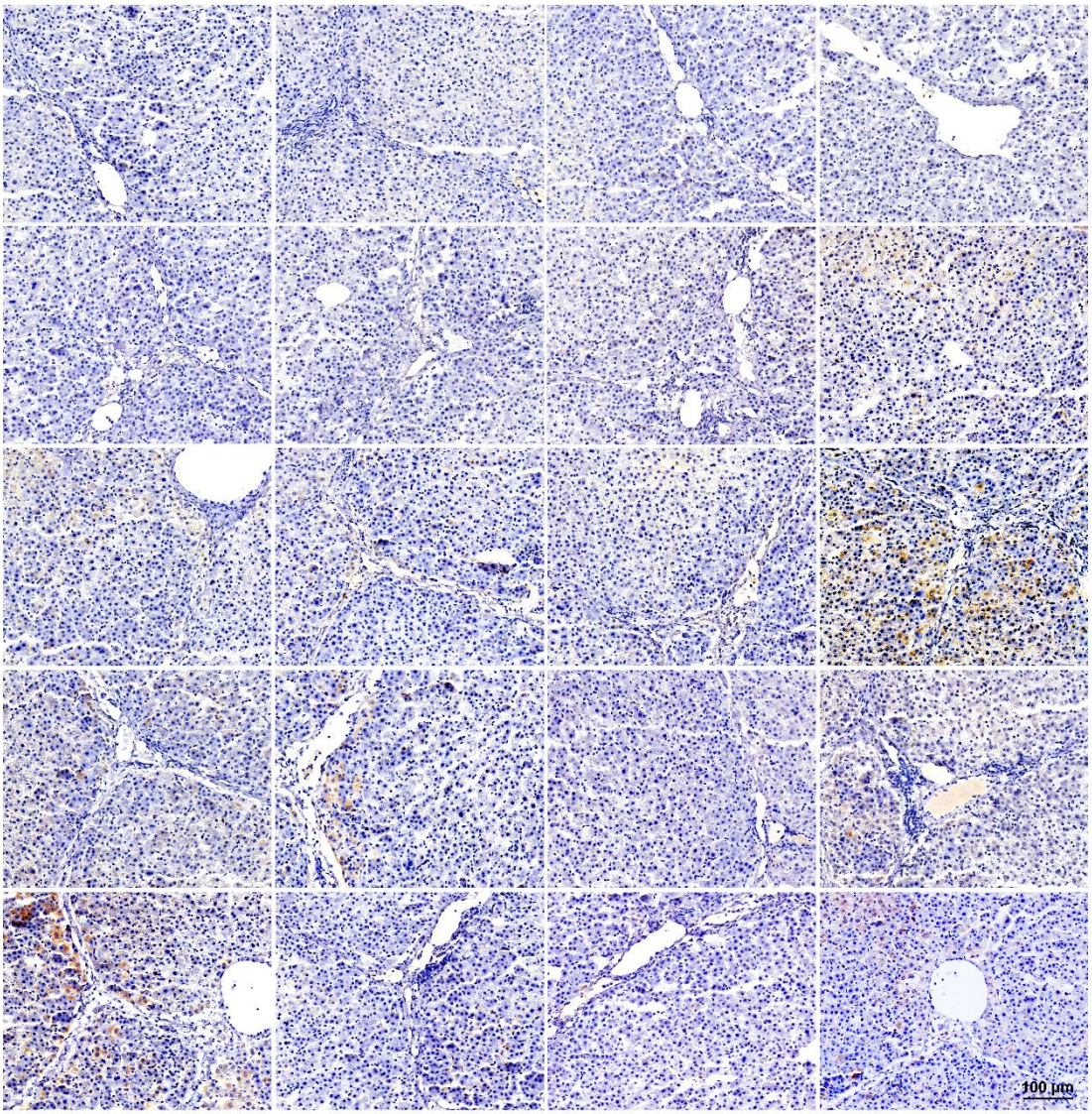

Figure S5. Immunohistochemical staining for human ALB at day 14 (D14). Twenty images were randomly chosen from the full set of scan images and were used to calculate the percentage of positively stained cells. The results are representative of 5 independent samples. Brown staining indicates positive expression. Scale bar, 100 μm.

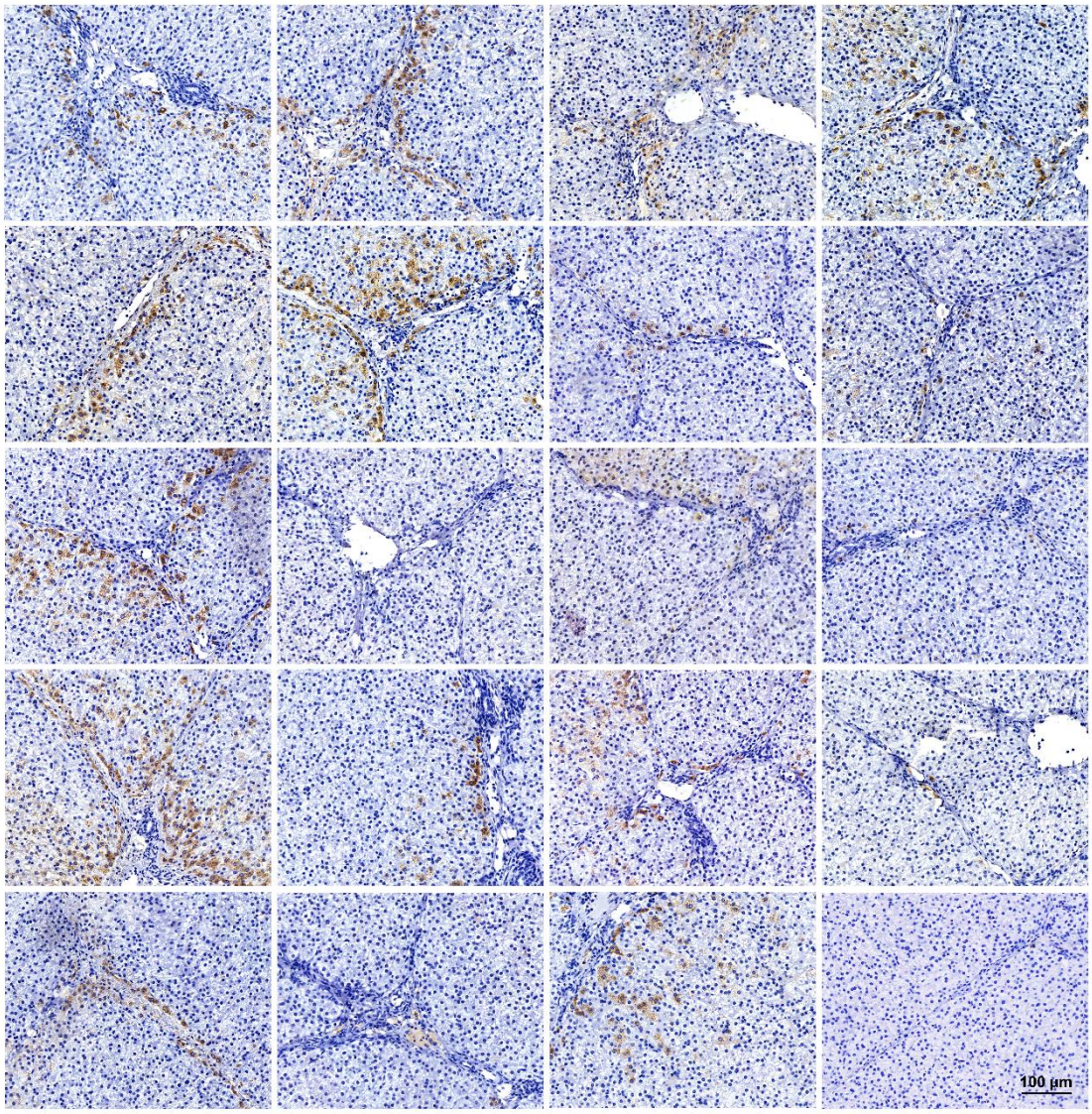

Figure S6. Immunohistochemical staining for human HSA at day 7 (D7). Twenty images were randomly chosen from the full set of scan images and were used to calculate the percentage of positively stained cells. The results are representative of 5 independent samples. Brown staining indicates positive expression. Scale bar, 100 μm.

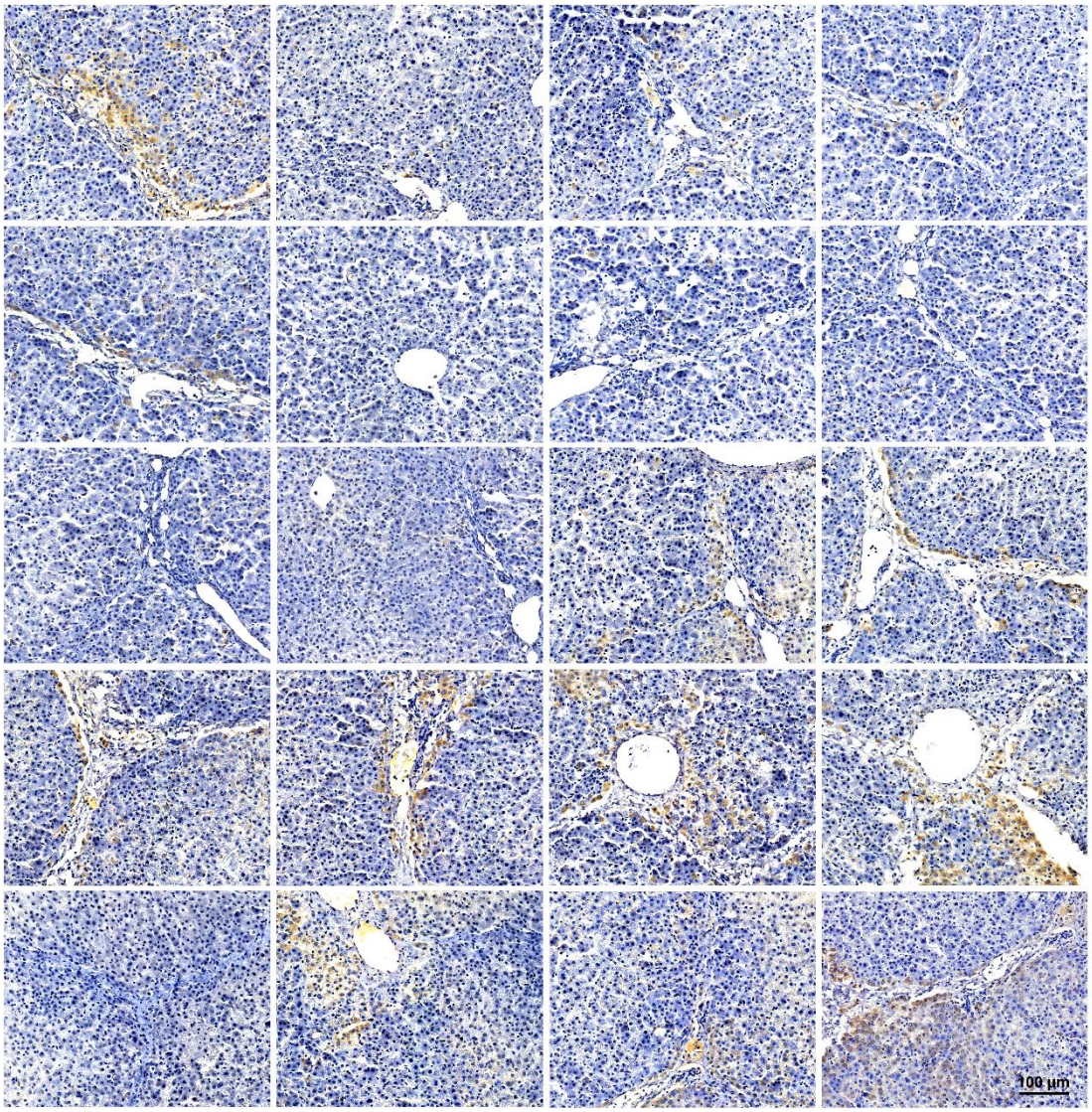

Figure S7. Immunohistochemical staining for human HSA at day 14 (D14). Twenty images were randomly chosen from the full set of scan images and were used to calculate the percentage of positively stained cells. The results are representative of 5 independent samples. Brown staining indicates positive expression. Scale bar, 100 μm.

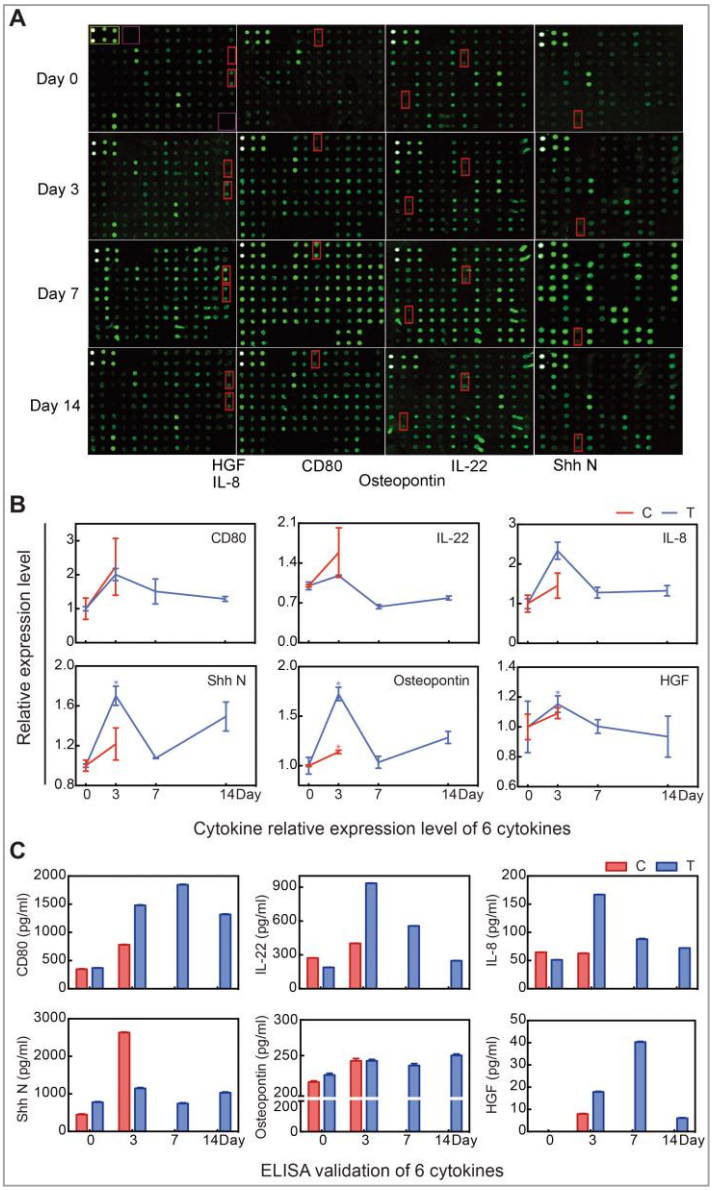

Figure S8. Results of the cytokine arrays and ELISA validation. (A) Duplicate spot sites corresponding to cytokines in the cytokine arrays. Yellow box, positive controls (upper left corner); pink boxes, negative controls (upper left and lower right corners, no spots); red boxes, location of the detected cytokines. (B) Relative serum levels of six random cytokines at day 3 (D3), D7 and D14 after D-Gal administration and at D0, as measured by antibody array. (C) Concentration of six cytokines in the serum of both groups at D0, D3, D7 and D14, as measured by ELISA. C, control group; T, transplantation group. Error bars, SEM. At D3, $*P<0.05$ versus D0, Student's t-test. B, n=3/group; C, n=11/group.

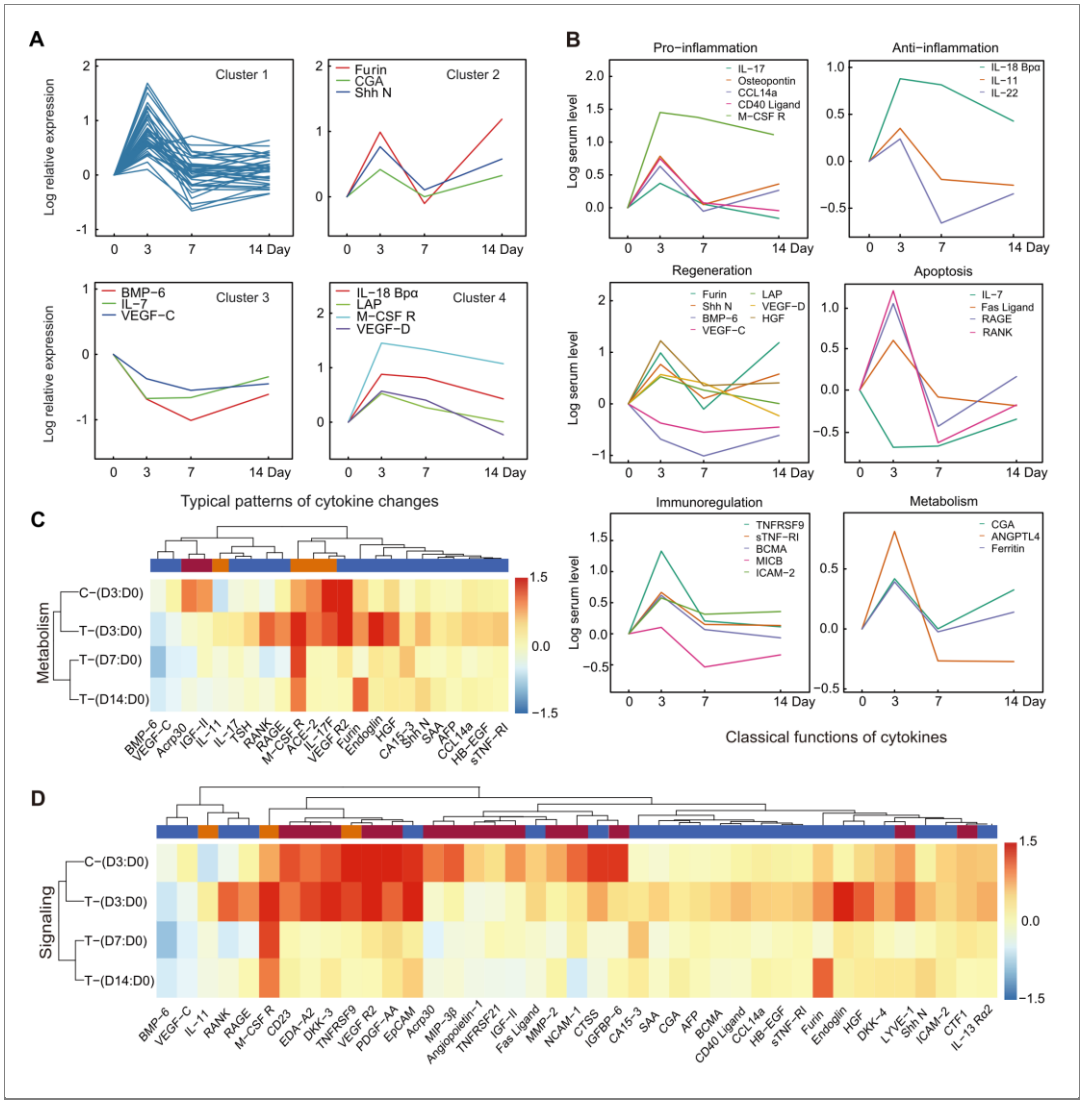

Figure S9. Functional classification of differentially regulated cytokines. (**A**) Four typical patterns of temporal cytokine changes, as identified by hierarchical clustering. (**B**) Relative serum levels of typical cytokines representing six classical functional categories. (**C, D**) Heat maps of differentially regulated cytokines involved in the functional themes of signalling and metabolism. C, control group; T, transplantation group; D3:D0, D7:D0, D14:D0, days 3, 7, and 14 versus day 0. Three colours above the heat map: red, C-group-specific differentially regulated cytokines; blue, T-group-specific differentially regulated cytokines; and orange, differentially regulated cytokines shared by the two groups. n=3/group.