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Research article

Human fetal kidney cells regenerate acellular porcine kidneys via upregulation of key transcription factors involved in kidney

development

Running title: Regeneration of porcine kidneys

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Supplement Methods

1. Decellularization of porcine kidneys

The kidneys were decellularized by perfusion of 4% sodium deoxycholate, 4% Triton X-100 and 40 IU/ml DNase at 10 ml/min through renal artery and ureter until satisfactory cell removal was noticed by histology. The renal artery and ureter were cannulated for 5 kidneys and perfused with decellularization solutions using peristaltic pump (Oina, Sweden) at 10 ml/min flow rate. The kidney was first perfused with distilled water for 72 h followed by 4% sodium deoxycholate (SDC) (30970, Sigma, Germany), 4% Triton X-100 (902-93-1, Alfa Aesar, Germany) and 40 IU/ml deoxyribonuclease I (DNase I) (LS002007, Worthington, USA) for 4 h with each solution. After treatment with each detergent solution, the kidney was washed over night with distilled water to remove cell debris. The cycle from SDC to DNase was repeated until seemingly satisfactory

decellularization was observed by histology. At the end of decellularization, the kidneys were washed for 72 h with distilled water. Distilled water used throughout protocol contained 0.02% SA and 5 mM EDTA while Triton X-100 contained 0.02% SA, 5 mM EDTA and 0.4 mM poly methyl sulfonyl fluoride (PMSF) (P7626, Sigma, Germany). The detergent SDC contained only 0.02% SA and 0.4 mM PMSF. The enzyme DNase was prepared in Dulbecco's phosphate buffer saline containing CaCl₂ and MgCl₂ (D8662, Sigma, Germany).

To test whether same protocol could be used for decellularization of kidney pieces, one whole kidney was sliced at 3 mm thickness (Histocenter, Gothenburg) and each slice was decellularized individually by agitation at 100 rpm following the same protocol as above. The characterization procedure for decellularized kidneys is explained in supplement methods.

2. Characterization of decellularized kidneys

After complete decellularization, biopsies were collected and processed for histology, DNA quantification, collagen quantification and elastin quantification (all n = 5) while for glycosaminoglycans (GAGs) quantification (n = 4). For comparison, biopsies from normal porcine kidneys (n = 3) were used in all quantifications. DNA was extracted using DNeasy blood and tissue DNA kit (69506, Qiagen, Germany) and was quantified with Nanodrop (Implen, Germany) at 260 nm wavelength. Collagen (insoluble and soluble), elastin and GAGs were quantified using Biocolor kits following manufacturer's recommendation. For histology, biopsies were fixed in 4% formaldehyde (Histolab, Sweden) for 48 h and tissue processed in ascending series of ethanol (Histolab, Sweden), Xtrasolv (a clearing agent made of isoparaffins, Solveco, Sweden) and embedded in paraffin (Histolab, Sweden) finally. Sections of 5 µm thickness were cut using microtome (ThermoFisher Scientific, USA) and rehydrated in xylene, descending series of ethanol and finally in water. Hematoxylin and eosin (HE) and Masson's trichrome (MT) stainings were done to demonstrate the presence of nuclei and collagen respectively. Using immunohistochemistry, major ECM proteins fibronectin (1:500, ab23751, Abcam, UK) and (1:100, BS-8561-R, Bioss, USA) laminin were stained. The detailed immunohistochemistry protocol is explained in supplements. For evaluating decellularization in kidney slices, biopsies were collected from 3 random pieces and stained by HE to check the presence of nuclei.

Immunohistochemistry and Immunofluorescence: The sections were rehydrated in xylene, ascending series of ethanol, water followed by antigen retrieval for 20 min in boiling 10 mM sodium citrate buffer (pH 6) and washing with distilled water and PBS. Only for DLK-1 staining, antigen retrieval was done for 2 min as the regular 20 min of boiling destroyed the DLK-1 protein. Incubation with 3% hydrogen peroxide blocked endogenous peroxidases activity. After washing in PBS, sections were blocked. For immunohistochemistry, the protein blocking solution (X0909, Dako, USA) was used while for immunofluorescence goat or donkey serum was used. Later, slides were incubated with primary antibodies diluted in antibody diluent (S0809, Dako, USA) for overnight at 4 °C. Next day, slides were washed with PBS and incubated with species-specific secondary antibody (1:500, 111-036-047, Jackson Immunoresearch, USA) for 30 min or for 10 min with broad-spectrum secondary antibody Super Picture HRP Polymer Conjugate (878963, Life Technologies, USA) at room temperature in immunohistochemistry. Species specific secondary antibody was used for staining of fibronectin, laminin and DLK-1 while Super Picture HRP Polymer Conjugate was used for rest stainings. For immunofluorescence, secondary antibodies used were either A11031 or A11057 (1:100,

Life Technologies, USA). After secondary antibody incubation, slides were washed in PBS followed by incubation with 3,3'-diaminobenzidine (K3468, Dako, USA) and counter staining with hematoxylin. Finally, sections were dehydrated and mounted with coverslip using Pertex (00840, Histolab, Sweden) medium. Sections with only antibody diluent and secondary antibody served as negative control. The slides were scanned using Leica SCN400 microscope (Leica Microsystems, Germany) and photographs were taken with Leica SlidePath Gateway LAN software.

3. Isolation and culture of human fetal kidney cells (hFKC)

The hFKC were isolated from 3 fetal kidneys. The isolated fetal kidneys were placed on a 70 µm nylon mesh (352350, Falcon, USA) and then disintegrated with the plunger of a 1 ml syringe (309659, BD, USA) into small pieces. The cells passed through the 70 µm mesh by adding 3 ml Dulbecco's modified eagles medium (BE12-707F, Lonza, Belgium) on the mesh while the tissue debris that is big in size remained in the mesh. The flow through is pipetted multiple times to dissociate if any cell clusters were present. The cell suspension was centrifuged at 200 xg for 10 min to pellet the cells. Isolated hFKC were seeded on cell culture flasks pre-coated with placental collagen. The cells were enriched for endothelial and epithelial cells using a commercially available endothelial cells medium (EnCM) (10372019, Gibco, USA) and renal epithelial cells medium (EpCM) (PCS-400-040, ATCC®, USA or C26001, Promo Cell, Germany) respectively. The EpCM was supplemented with 10% heat inactivated human AB serum (H3667, Sigma, USA), 1% L-glutamine (25030-024, Gibco, UK), 1% penicillin streptomycin (151-40-122, Gibco, USA) while the EnCM was supplemented with 10% heat inactivated human AB serum, 1% L-glutamine, 1% penicillin streptomycin and EGM-2 single quotes kit except FBS (cc-4176, Lonza, USA). The EpCM and EnCM media in addition with supplements are recommended to maintain the culture of primary renal cortical epithelial, proximal tubule epithelial and microvascular endothelial cells.

Flow cytometry: Approximately 4×10^5 cells were transferred to each tube and centrifuged at 400 xg for 5 minutes to pellet cells and washed twice with phosphate buffer saline (PBS). The cells were stained with primary antibodies for 30 min at 4 °C. After washing, the cells were further stained with isotype control (20 µl/reaction, 342409, BD, USA) for FITC conjugated primary antibodies or respective FITC-conjugated goat anti-mouse (1 µl/reaction, F0257, Sigma, USA) or goat anti-rabbit (1 µl/reaction, F2765, Invitrogen, USA) secondary antibodies for 30 min at 4°C. For staining of EpCM cells except EPCAM, the cells membrane was permeabilized by treatment using 0.5% saponin (47036, Sigma, Germany) before staining with primary antibodies. By the gating of cells from histogram overlay plots, the percentage of cells expressing the marker was noted for each. From these values, the mean percentage of cells from all three fetuses expressing the respective marker and the standard error mean was calculated.

Immunocytochemistry: The cells were seeded into each well of an 8 chamber slide and cultured for 1 day. The cells were fixed and permeabilized using 30% acetone-70% methanol for 2 min, washed thrice with PBS and blocked in 1% Bovine Serum Albumin (BSA). The cells were incubated overnight with primary antibody at 4 $\$, washed with PBS thrice and stained with isotype control (1:20) or goat-anti-mouse (1:200, A11001, Life Technologies, USA) secondary antibody for 30 min at 4 $\$. This was followed by further washing with PBS thrice, counterstaining with 4',6-diamidino-2-phenylindole (DAPI, D1306, Life Technologies, USA) followed by placing coverslip with a drop of mounting medium (S3023, Dako, USA).

Double Immunofluorescence staining: Antigen retrieval was done in rehydrated sections for 2 min in boiling 10 mM sodium citrate buffer followed by washing with distilled water and PBS. After washing in PBS, sections were blocked with 5% goat serum followed by incubation with DLK-1 primary antibody overnight at 4 °C and washed thrice with PBS. Later, sections were incubated with secondary antibody (1:100, F2765, Invitrogen, USA) for 45 min at room temperature in dark and washing thrice with PBS. The same protocol was followed again using EphA7 primary antibody (1:50, LS-C321138, Nordic Biosite, Sweden) and secondary antibody (1:100, A11011, Life Technologies, USA) followed by counter staining with DAPI and cover slipped with mounting medium (S3023, Dako, USA). The slides were viewed and pictures were captured with fluorescence microscope (Leica Microsystems, Germany).

4. Recellularization of kidney slices

Decellularized whole kidneys (n = 2) were cut into slices of 1 mm thickness and a piece of 1 X 1.2 cm were cut. The two pieces were sterilized by agitation in 0.1% peracetic acid in PBS for 2 h at 37 $^{\circ}$ C and then washed in PBS containing 1% penicillin streptomycin for 48 h. Since the ECM of the kidney is dense, we hypothesized that treatment of the kidney pieces with metalloproteinases (MMP) would open up the collagen bundles and enable easy migration of cells into tissue. We therefore treated the decellularized tissue pieces with 40 mM 4-aminophenylmercuric acetate activated 2.5 µg MMP-2 (42002, Peprotech, UK) and 2.5 µg MMP-9 (550504, Biolegend, USA) in buffer containing 150 mM tris-HCl (pH 7.5), 150 mM sodium chloride (13423, Sigma, Germany), 5 mM calcium chloride (102382, Merck, Germany) and 0.025% Brij L23 (B4184, Sigma, USA) for 24 h at 37 °C and washed with distilled water containing 20 mM EDTA followed by PBS. The washed tissue pieces were placed on 12-well transwell membrane (0.4 µm pore size, Costar, USA) and a mixture of cells grown in EpCM (passage 4 °) and EnCM (passage 9 $^{\circ}$ -11 $^{\circ}$) from one fetus were seeded at approximately 30×10^{6} cells per piece. The cells were suspended in 200 μ l medium and seeded using 10 μ l pipette tip and then incubated for an hour to allow cell attachment. Later, 2 ml medium was added gently along the sides of the well and cultured for 2 weeks in transwell plates. The medium (50% EnCM and 50% EpCM supplemented with 5 ng/ 500 ml Activin-A (120-15, Peprotech, USA)) was changed once a week. A similar experiment was performed without treating the decellularized tissue with MMPs.

RNA Extraction: RNA was extracted from the samples using Qiagen RNeasy Mini Kit (Qiagen, Germany). Each sample was added to a 2 ml tube with a stainless steel bead and 600 μ l RLT buffer and homogenized in a TissueLyser (Qiagen, Germany) at 25 Hz for 2 x 5 min. The samples were centrifuged 16,000 xg for 3 min at 4 °C and the supernatants were added to the kit columns. After binding and washing the samples were DNase treated on column by adding 80 μ l DNase I solution and incubated at room temperature for 15 min. After washing the samples were eluted in 30 μ l RNase free water.

Reverse transcription: Reverse transcription was done using TATAA GrandScript cDNA Synthesis Kit (A103, TATAA Biocenter, Sweden). Ten μ l RNA of each sample was used in a total volume of 20 μ l in a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, USA) using a temperature protocol of 5 min at 22 °C, 30 min at 42 °C, 5 min at 85 °C and hold at 4 °C.

Preamplification: Four μ l of each cDNA sample and 4 μ l of a negative PreAmp control (water) were preamplified in a total volume of 20 μ l using TATAA Probe GrandMaster Mix (TA02, TATAA Biocenter, Sweden) and 50 nM of each of 19 of the 20 primer pairs of the analyzed assays (the primers for the 18S assay was not included to avoid preamplification of 18S rRNA due to the natural high

abundance). Amplification was performed in a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, USA) using a temperature protocol of enzyme activation 1 min at 95 $^{\circ}$ C followed by 17 cycles of 1 min at 95 $^{\circ}$ C, 2 min at 60 $^{\circ}$ C and 1 min at 72 $^{\circ}$ C. Samples were flash frozen directly from 72 $^{\circ}$ C after the last cycle. The preamplification protocol using the same qPCR assays had previously been verified to have a good efficiency and reproducibility as described previously [1]. The assay identity numbers and context sequences were provided in Table 1.

qPCR: The preamplified samples were thawed by adding 140 μ l RNase free water to each sample and mixing thoroughly by pipetting. The diluted samples and no-template control (water) were analyzed in duplicates for 20 genes, using same assays as in preamplification, in a Bio-Rad cfx384 Real-Time System (Bio-Rad Laboratories, USA). Each reaction contained 2 μ l sample in a total volume of 10 μ l using TATAA Probe GrandMaster Mix (TA02, TATAA Biocenter, Sweden) with 400 nM primers and 200 nM probe. For amplification, samples were enzyme activated for 30 seconds at 95 °C followed by 40 cycles of 5 seconds at 95 °C, 30 seconds at 60 °C and 10 seconds at 72 °C.

Data analysis: All negative controls were negative or had Cq-values at least 5 times higher than the highest Cq for a positive sample. Samples were evaluated for excessive amount of genomic DNA using the ValidPrime approach [2]. The 12 candidate reference genes (TATAA Human Reference Gene Panel, A101p, Tataa Biocenter AB, Sweden) were evaluated with the GeNorm and Normfinder algorithms to find the most stable reference genes. Both methods found TBP and YWHAZ to be the most stable. For remaining genes, Cq-values for qPCR replicates were averaged, normalized with the two reference genes (delta-Cq were calculated for each sample using the average of Cq-values for TBP and YWHAZ), relative quantities (2^delta-delta-Cq) were normalized to the average of the two renal fetal cell samples, and log2 values were calculated. All data analyses were performed using GenEx version 6.1.0.757 software (MultiD Analysis AB, Sweden).

Gene	Unique ID No.	MIQE context sequence
WT1	qA-01-0756	CTCAGTGAAATGGACAGAAGGGCAGAGCAACCACAGCACAGGGTACG
		AGAGCGATAACCACACACGCCCATCCTCTGCGGAGCCCAATACAGA
		ATACACACGCACGGTGTCTTCAGAGGCATTCAGGATGTGCGACGTGTG
		CCTGGAGTAGCCCCGACTCTTGTACGGTCGGCATCTGAGACCAGTGAG
		AAACGCCCCTTCATGTGT
SIX2	qA-01-0758	GAGGAGGCCAAGGAAAGGGAGAACAACGAGAACTCCAATTCTAACA
		GCCACAACCCGCTGAATGGCAGCGGCAAGTCGGTGTTAGGCAGCTCC
		AG
EYA1	qA-01-0685	CAACAAGCTACAGCCTATGCCACGTACCCACAGCCAGGACAGCCGTA
		CGGCATTTCCTCATATGGTGCATTGTGGGCAGGCATCAAGACTGAAGG
		TGGATTGTCACAGTCTCAGTCACCTGGACAGACAGGATTTCTCAGCTA
		TGGCACAAGCTTCAGTACCCCTCAACCTGGACAGGCACCATACAGCTA
		CCAGATGCAAGGTAGCAGTTTTAC
SALL1	qA-01-0759	GTGAGATTCACGAGAGAACTCACACTGGAGAGAAACCCTTTGCTTGC
		ACTATTTGTGGAAGAGCTTTCACGACTAAAGGCAATCTTAAGGTACAC
		ATGGGCACTCACATGTGGAATAGCACCCCTGCACGAAT

Table 1. The assay identity numbers and context sequences.

Continued on next page

Gene	Unique ID No.	MIQE context sequence
CITED1	qA-01-0760	CACCTGCGAAGGAGGATGCCAACCAAGAGATGAGCTCCGTGGCCTAC
		TCCAACCTTGCGGTGAAAGATCGCAAAGCAGTGGCCATTCTGCACTAC
		CCTGGGGTAGCCTCAAATGGAACCAAGGCCAGTGGGGCTCCCACTAG
		TTCCTCGGGATCTCCAATAGGCTCTCCTACAACCACCCCTCCCACTAA
		ACCCCCATCCTTCA
PAX2	qA-01-0689	CCAGCGTCTCTTCCATCAACAGAATCATCCGGACCAAAGTTCAGCAGC
		CTTTCCACCCAACGCCGGATGGGGCTGGGACAGGAGTGACCGCCCCT
		GGCCACACCATTGTTCCCAGCACGGCCTCCCCTCCTGTTTCCAGCGCCT
		CCAATGACCC
LHX1	qA-01-0762	TTCTACGGAGATTACCAGAGCGAGTACTACGGGCCCGGGGGGCAACTA
		CGACTTCTTCCCGCAAGGCCCCCGTCCTCGCAGGCCCAGACACCAGT
		GGACCTACCCTTCGTGCCGTCATCTGG
DLK1	qA-01-0761	CCGCCCTTTGTGACCAGTGCGTGACCTCTCCCGGCTGCCTTCACGGAC
		TCTGTGGAGAACCCGGGCAGTGCATTTGCACCGACGGCTGGGACGGG
		GAGCTCTGTGATAGAGATGTTCGGGGCCTGCTCATG

Continued on next page

Gene	Unique ID No.	MIQE context sequence
YWHAZ	qA-01-0105	CATCTTGGAGGGTCGTCTCAAGTATTGAACAAAAGACGGAAGGTGCT
		GAGAAAAAACAGCAGATGGCTCGAGAATACAGAGAGAAAATTGAGA
		CGGAGCTAAGAGATATCTGCAATGATGTACTGTCTCTTTTGGAAAAGT
		TCTTGATCCCCAATGCTTCACAAGCAGAGAGCAAAGTCTTCTATTTGA
		AAATGAAAGGAGATTACTACCGTTACTTGGCTGAGGTTGCCGCTGGTG
		ATGACAAGAAAGGGATTGTCGATCAGTCACAACAAGCATACCAAGAA
		GCTTTTGAAATCAGCAAAAAGGAAATGCAACCAACACCTATCAG
		ACTGGGTC
TBP	qA-01-0109	GGCACCACTCCACTGTATCCCTCCCCATGACTCCCATGACCCCCATC
		ACTCCTGCCACGCCAGCTTCGGAGAGTTCTGGGATTGTACCGCAGCTG
		CAAAATATTGTATCCACAGTGAATCTTGGTTGTAAACTTGACCTAAAG
		ACCATTGCACTTCGTGCCCGAAACGCCGAATATAATCCCAAGCGGTTT
		GCTGCGGTAATCATGAGGATAAGAGAGCCACGAACCACGGCACTGAT
		TTTCAGTTCTGGGAAAATGGTGTGCA

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