Cell and Tissue Engineering

http://www.aimspress.com/journal/CTE

AIMS Cell and Tissue Engineering, 3(1): 26–46.
DOI: 10.3934/celltissue.2019.1.26
Received: 24 March 2019
Accepted: 08 July 2019
Published: 23 July 2019

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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Abstract: Interaction between organ-specific stem cells and extracellular matrix (ECM) is crucial for regeneration. We therefore, investigated novel stem cells markers in human kidney and verified the potential of human fetal kidney cells (hFKC) to repopulate decellularized porcine kidneys. Adult and fetal human kidneys were stained by immunohistochemistry for putative stem cell markers. In addition, hFKC were isolated and characterized phenotypically and by gene expression. Furthermore, whole porcine kidneys were decellularized using detergents, cut into 1 mm slices, seeded with hFKC, cultured for 14 days and characterized by histology and qPCR. We found that, decellularized porcine kidneys showed significant loss of DNA but preserved some ECM components. Human fetal kidneys including hFKC expressed stem cell markers CD133, DLK-1, EPCAM and ephrin receptor EphA6. Interestingly, EphA7 and SIX2 were markedly expressed only in fetus. Furthermore, in fetal kidneys EphA7 was co-expressed with DLK-1. Recellularized kidney pieces showed cell infiltration, growing in orchestrated fashion distributed around the scaffold. These pieces also demonstrated cells expressing CK8, CK18, DLK-1, CD133, EphA7, EphB3, PCNA, podocin and increased levels of transcriptional factors in kidney development (SIX2, EYA1, CITED1, LHX1, SALL1, DLK-1 and WT1). We conclude that decellularized porcine kidneys support the culture, proliferation of hFKC and regenerate by upregulation of transcription factors. We suggest that expanded hFKC may be the

ideal cell source for whole kidney regeneration in the future. We also postulate EphA7 might be a novel stem cell marker in kidneys.

Keywords: decellularization; recellularization; tissue engineering; kidney; fetal kidney cells; ephrin

1. Introduction

Chronic kidney disease is a major public health problem [1]. The incidence and prevalence is increasing. Prevalence is estimated to be 8–16% worldwide [2]. Current therapies for end stage kidney disease mainly include dialysis and renal transplantation. However, due to organ donor shortage, the demand for organs has reached a level that far exceeds the static supply. This disparity has contributed to a rapidly expanding waiting list and burdensome waiting times that accrue mortality and morbidity for patients [3]. These grim statistics underscore the urgent need to find new, potentially inexhaustible, sources of transplantable organs.

Emerging technologies in regenerative medicine have demonstrated the advantages of preserving innate extracellular matrix (ECM) for organ bioengineering [4–6]. Researchers are exploring the possibility to use intact ECM from animal or human whole organs for organ bioengineering purposes generated through detergent-based decellularization [4,7]. Decellularized tissue scaffolds provide a platform for developing functional tissue replacements by offering mechanical, structural and biological properties similar to the native tissues from which they were derived. Current decellularization protocols are capable of removing DNA, cellular material and cell surface antigens from the ECM scaffold while preserving attachment sites, structural integrity and vascular channels [8]. Successful recellularization of ECM scaffolds has been reported in several organ systems [9–14].

Strategies for repopulation of porcine or human kidneys and optimal cell sources for kidney recellularization is incompletely explored, as only few studies to date report partial repopulation of decellularized kidney ECM [15–18]. Identification of multi-potential progenitor populations in mammalian tissues is important both for therapeutic potential and an understanding of developmental processes and tissue homeostasis. Progenitor populations can be used for several regenerative approaches such as cell transplantation, gene therapy, and tissue engineering of bioartificial organs [19,20]. It has been reported [21,22] that renal stem/progenitor cells appear in the 5th week of gestation in the metanephros and are induced to form all cell types of the nephron till 34th week of gestation. Kidney stem cells were mostly described in literature from their expression of transcription factors like Sine Oculis Homeobox Homolog 2 (SIX2), Wilms tumor 1 (WT1), Cbp300/P300 Interacting Transactivator with Glu/Asp Rich Carboxy-Terminal Domain 1 (CITED1) and cell surface markers like FZD2, ACVRIIB, NTRK2 [23]. Some other traditional stem cell markers known from staining of adult and diseased kidneys and *in vitro* multiplication studies include CD133, CD24, Nestin, CD106, CD105, CD44 and vimentin [24]. Studies reporting the successful differentiation of these cells either *in vitro* or use in tissue engineering applications is limited. Thus, identification of useful surface stem cell markers is crucial for isolation of these important cell types.

To determine whether human fetal kidneys expressed some putative cell surface progenitor markers, we focused on the expression of ephrin (Eph) receptors as markers of stem/progenitor cells. Eph receptors constitute the largest family of tyrosine kinase receptors in mammals and are widely

expressed during embryogenesis and regulate developmental processes [25]. Eph receptors and ephrins are commonly expressed in adult stem cell niches, most studies showed their importance in the nervous system, the intestine [26–29] and in developmental functions [30]. Very little is known about the expression of these markers in the developing human kidney. In addition, we also examined the expression of some well-known stem cell markers known to be expressed in human fetal kidneys such as cluster of differentiation (CD) 133, delta-like 1 homolog (DLK-1) and epithelial cell adhesion molecule (EPCAM) [23,31].

We then tested whether human fetal kidney cells (hFKC) isolated in gestation week 8-11 continued to express these putative stem cell markers when grown *in vitro*. Furthermore, we tested the hypothesis that decellularized porcine kidney ECM can influence the behavior of hFKC, possibly directing their differentiation towards a more mature phenotype. The development of a bioengineered kidney by appropriate combination of cells, biomaterial scaffolds and biologically active molecules could provide an alternative avenue for renal therapy and toxicological studies.

2. Materials and methods

All experiments and experimental protocols involving animals in present study are in accordance with approved ethical guidelines at Gothenburg University.

2.1. Porcine kidney retrieval

Porcine kidneys (n = 6) were retrieved from pigs weighing 40–50 kg at the animal facility from Gothenburg University. The kidneys were dissected keeping ureter, renal artery and renal vein intact. The renal artery was cannulated with 4 mm arteriotomy cannula (31104, Medtronic, USA) and perfused with distilled water containing 0.02% sodium azide (SA) (S2002, Sigma, Germany) and 5 mM ethylenediaminetetraacetic acid (EDTA) (15161, Alfa Aesar, Germany) to remove the blood and either decellularized immediately or freeze thawed at −20 ℃ up to three times before decellularization. Fresh biopsies from 3 other porcine kidneys were collected to use as controls in histology and ECM characterizations. Biopsies for histology were immediately fixed in formalin while the rest were snap frozen in liquid nitrogen and stored at −80 ℃.

2.2. Decellularization of porcine kidneys

The kidneys were decellularized using repetitive treatments of 4% sodium deoxycholate (SDC), 4% Triton X-100 and 40 IU/ml deoxyribonuclease I (DNase) until satisfactory cell removal was noticed by histology. At the end kidneys were washed for 72 h in distilled water. The detailed decellularization and characterization procedure for kidneys is explained in supplement methods.

2.3. Retrieval of human fetal kidneys

Human fetal kidneys (n = 9) were isolated from aborted fetuses at 8–11 weeks of gestation after informed consent and ethical approval from the ethical committee of Gothenburg University. Gestational age is given as menstrual age. All women donating fetal tissue had been serologically screened for syphilis, toxoplasmosis, rubella, HIV-1, cytomegalovirus, hepatitis B and C, parvovirus and herpes simplex types 1 and 2. The detailed procedure for isolation and culture of human fetal kidney cells (hFKC) is explained in supplement methods.

2.4. Characterization of hFKC

Flow cytometry: Single color fluorescence was used to phenotypically characterize hFKC (n = 3) grown in endothelial cells medium (EnCM) and epithelial cells medium (EpCM) respectively. The cells grown in EnCM (passages between 2°–16°) were stained using primary antibodies against von Willebrand factor (vWF, 5 µl/reaction, AHP062F, AbD Serotec, Germany), Ulex Europaeus (5 µl/reaction, L-9006, Sigma, Israel) and CD133 (5 µl/reaction, AMB-7558, Nordic Biosite, Sweden). The cells grown in EpCM (passages between 1°–8°) were stained using primary antibodies against cytokeratin (CK) 8 (5 µl /reaction, SC-8020, Santa Cruz, USA), CK18 (10 µl /reaction, SC-51583, Santa Cruz, USA), EphA6 (5 µl/reaction, LS-B14564, Nordic Biosite, Sweden), EphA7 (12 µl/reaction, LS-C321138, Nordic Biosite, Sweden), DLK-1 (2 µl/reaction, LS-C169429, Nordic Biosite, Sweden) and EPCAM (5 µl/reaction, ab7504, Abcam, UK). The detailed protocol is explained in supplements.

Immunocytochemistry: The cells grown in EnCM and EpCM (n = 2, respectively) were also characterized by immunocytochemistry. The cells grown in EnCM (passages between 10°–13°) were stained using vWF (1:50, SC73268, Santa Cruz, USA), Ulex Europaeus (1:20) and CD133 (1:100) antibodies, while the cells grown in EpCM (passages between 3°–6°) were stained with CK8 (1:50, ab9023, Abcam, UK), CK18 (1:50), EphA6 (1:100, MCA4602Z, AbD Serotec, Germany) and EphA7 (1:100, Orb69120, Biorbyt, UK) using same antibodies as in flow cytometry unless specified. The detailed protocol is explained in supplements.

2.5. Determination of stem cell markers in human fetal and adult kidneys by immunohistochemistry

To identify the stem cells markers expressed and their distribution in human fetal kidneys, the paraffin sections from 5 human fetal kidneys were stained by immunohistochemistry using primary antibodies to EphA6 (1:30, MCA4602Z, AbD Serotec, Germany), EphA7 (1:50, Orb69120, Biorbyt, UK), EphB2 (1:30, MCA3090Z, AbD Serotec, Sweden), EphB3 (1:30, MCA3091Z, AbD Serotec), DLK-1 (1:500, ab21682, Abcam, UK), CD133 (1:300, AMB7558, Nordic Biosite) and EPCAM (1:50, SC25308, Santa Cruz, Germany). The expression of these markers for fetuses in capsule, mesenchyme and interstitium was recorded. To compare, biopsies of human adult kidneys (n = 2) and a needle biopsy of ischemia reperfused kidney (n = 1) were used. The expression of these markers in tubules, ducts and glomeruli was recorded. Based on intensity and distribution of cells expressing markers, all observations were graded as strong (+++) to absent (-) and the average was represented as strong, moderate, weak and absent. All tissues were obtained after informed consent and ethical approval from the ethical committee of Gothenburg University have been obtained. The

co-expression of DLK-1 and EphA7 in fetal kidneys was stained by double immunofluorescence as explained in supplement methods.

2.6. Recellularization of kidney slices

From two decellularized whole kidneys, pieces of 1 X 1.2 X 1 cm were cut, sterilized in 0.1% Peracetic acid, washed in PBS and treated with matrix metalloproteinases (MMP)-2 and -9. Later, pieces were kept on 0.4 μm transwell membranes, seeded with approximately $30x10^6$ hFKC, cultured for 2 weeks in incubator with 50% EnCM and 50% EpCM. A similar experiment with non-MMP treated tissue was also performed. The detailed recellularization protocol is explained in supplement methods.

2.7. Characterization of recellularized tissue by histology

The recellularized tissue was fixed in formalin and characterized by histology using HE staining and by immunohistochemistry or immunofluorescence for expression of CK8 (1:100, SC-8020, Santa Cruz, USA), CK18 (1:100, SC-51583, Santa Cruz, USA), CD133 (1:300, AMB7558, Nordic Biosite), EphA7 (1:50, Orb69120, Biorbyt, UK), DLK-1 (1:1000, ab21682, Abcam, UK), EPhB3 (1:75, MCA3091Z, AbD Serotec), podocin (1:150, AF1658, R&D systems, UK) and PCNA (1:800, ab184660, Abcam, UK) using antibodies. The immunohistochemistry and immunofluorescence protocols were explained in supplements.

2.8. Characterization of recellularized tissue by gene expression

These experiments were performed by TATAA Biocenter, Gothenburg. RNA from cells cultured in EnCM and EpCM (n = 1, passage 9 ° and 11 ° respectively), recellularized porcine kidney tissue (n = 2) and a negative control (water) were extracted. The extracted RNA for all 5 samples and a commercial human kidney total RNA sample (AM7976, Invitrogen, USA) to use as experimental positive control were reverse transcribed. The cDNA was preamplified for 19 of 20 genes (the primers for the 18S assay was not included to avoid preamplification of 18S rRNA due to the natural high abundance). Later, cDNA was quantified by qPCR for 20 genes using the same assays as in preamplification and analyzed. The detail protocols for RNA extraction, reverse transcription, preamplification, qPCR and data analysis are explained in supplements.

2.9. Statistics

The values and graphs represented were the mean of the group and the error bars were standard error mean. Using GraphPad Prism software, version 7.02, the normality for each group was first confirmed using Shapiro-Wilk test and then significant changes between groups were calculated using Welch's t-test and the p-value less than 0.05 was considered significant. The graphs were plotted using GraphPad Prism software.

3. Results

3.1. Kidney decellularization and characterization

Compared to normal kidney (Figure 1A), the decellularized kidney appeared pale and white (Figure 1B) and retained kidney gross morphology. By perfusion of Triton X-100, SDC and DNase the kidneys were decellularized for either 4 cycles (n = 2) or 6 cycles (n = 2) or 4 cycles with 120 h of additional washing (n = 1). We found on detailed examination that kidneys decellularized by these protocols did not show notable differences in relation to any of the tested parameters of decellularization efficiency. The kidney slices were decellularized by agitation in same detergents for 4 cycles.

Histological staining using hematoxylin and eosin (HE) showed presence of nucleated cells (blue) in the normal porcine kidney (Figure 1C) but no basophilic staining of the nuclear material in tubules, glomeruli and blood vessels of decellularized kidney (Figures 1D–G). However, as known and expected an occasional damage in structure and orientation of glomerular and tubular structures because of detergent treatments was seen. Though varying between kidneys and locations, in few sections insufficient removal of cytoplasmic remnants was also noticed. Masson's trichrome staining of normal kidney showed black nuclei and blue staining for collagen (Figure S1A), while the decellularized tissue showed loss of nuclei but retained collagen (Figure S1B). Immunohistochemistry staining of normal kidneys showed staining of fibronectin in blood vessels and around the glomeruli (Figure S1C), which was also retained in the decellularized kidney (Figure S1D). Similarly, laminin was detected in both the normal (Figure S1E) and decellularized kidney (Figure S1F). The negative control showed no staining (Figure S1G).

DNA quantification of normal and decellularized samples showed significant removal of DNA in decellularized samples (Figure 1H), from 1.3 ± 0.02 μg/mg tissue in normal to 0.03 ± 0.002 μg/mg tissue in decellularized ($p = 0.0003$). The quantification of collagen showed a significant increase in decellularized kidneys (80.9 ± 9.6 μg/mg) in comparison to normal (40.5 ± 8.9 μg/mg; $p = 0.0236$) while glycosaminoglycans (GAGs) decreased significantly from 3.7 ± 0.04 μg/mg in normal to 0.2 ± 0.04 μg/mg in decellularized kidneys ($P < 0.0001$). The amount of elastin was not affected when compared between normal (21.2 ± 0.6 μg/mg) and decellularized (23.9 ± 1.9 μg/mg) kidneys (Figure 1H).

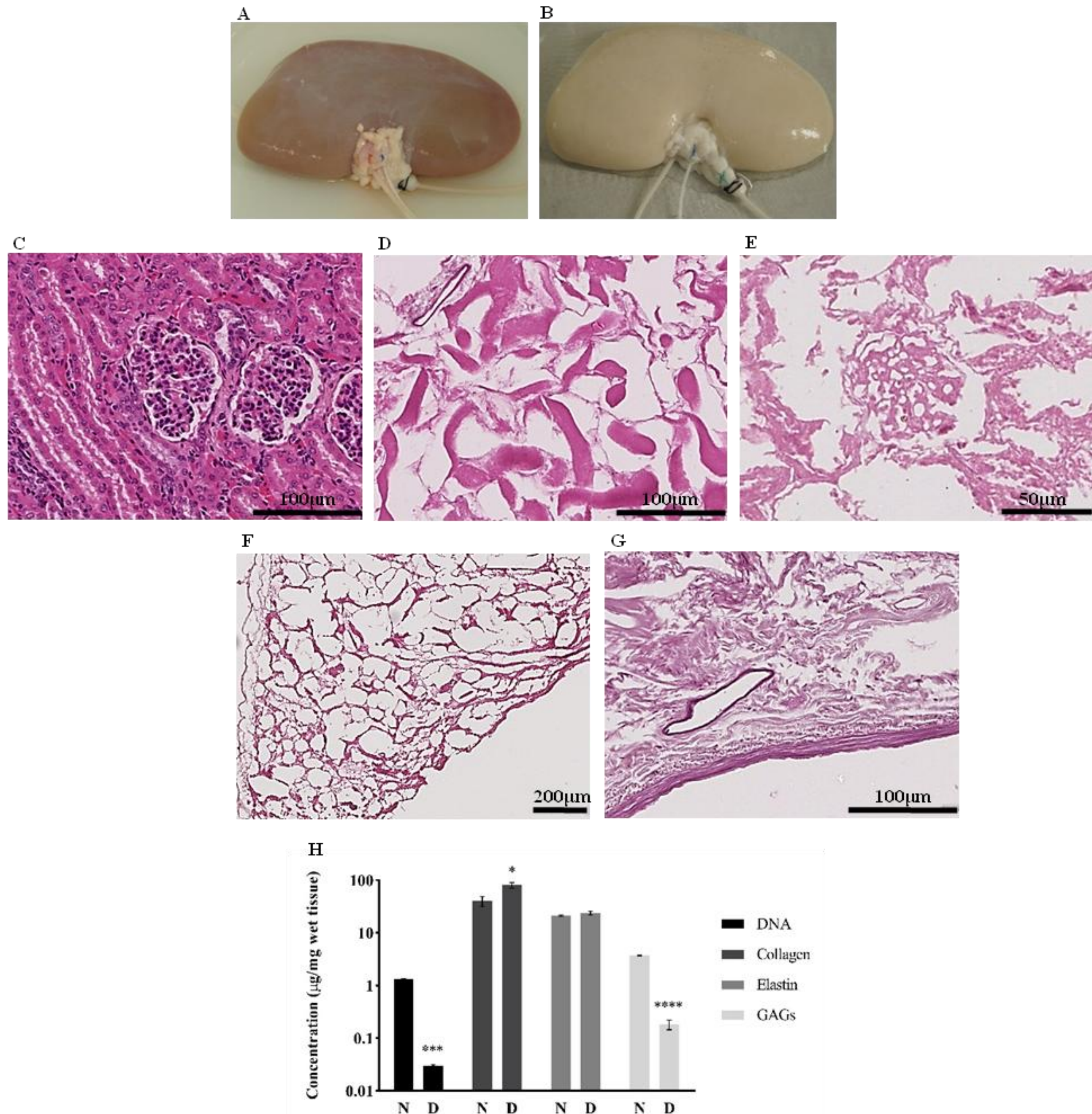


Figure 1. The gross morphology of (**A**) normal porcine kidney showing brown color, (**B**) decellularized porcine kidney showing a pale-white color. (**C**) HE staining of normal kidney showing nuclei in blue and ECM in pink. (**D–G**) HE staining of decellularized kidney sections showing removal of nuclei in the parenchyma, glomerulus and vasculature. (**H**) Quantification of proteins in decellularized ECM showing decrease in DNA and GAGs, increase in collagen and no change for elastin in decellularized kidneys as compared to normal.

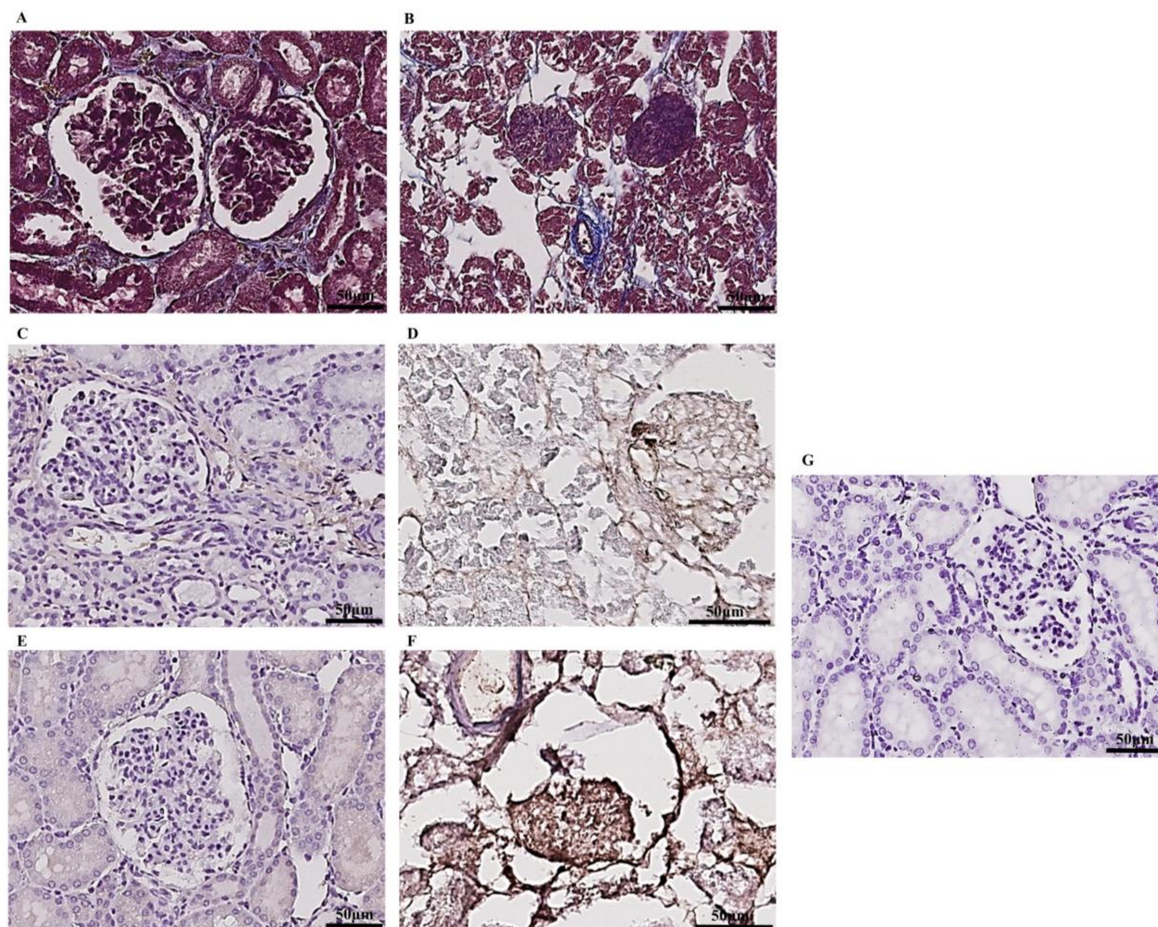
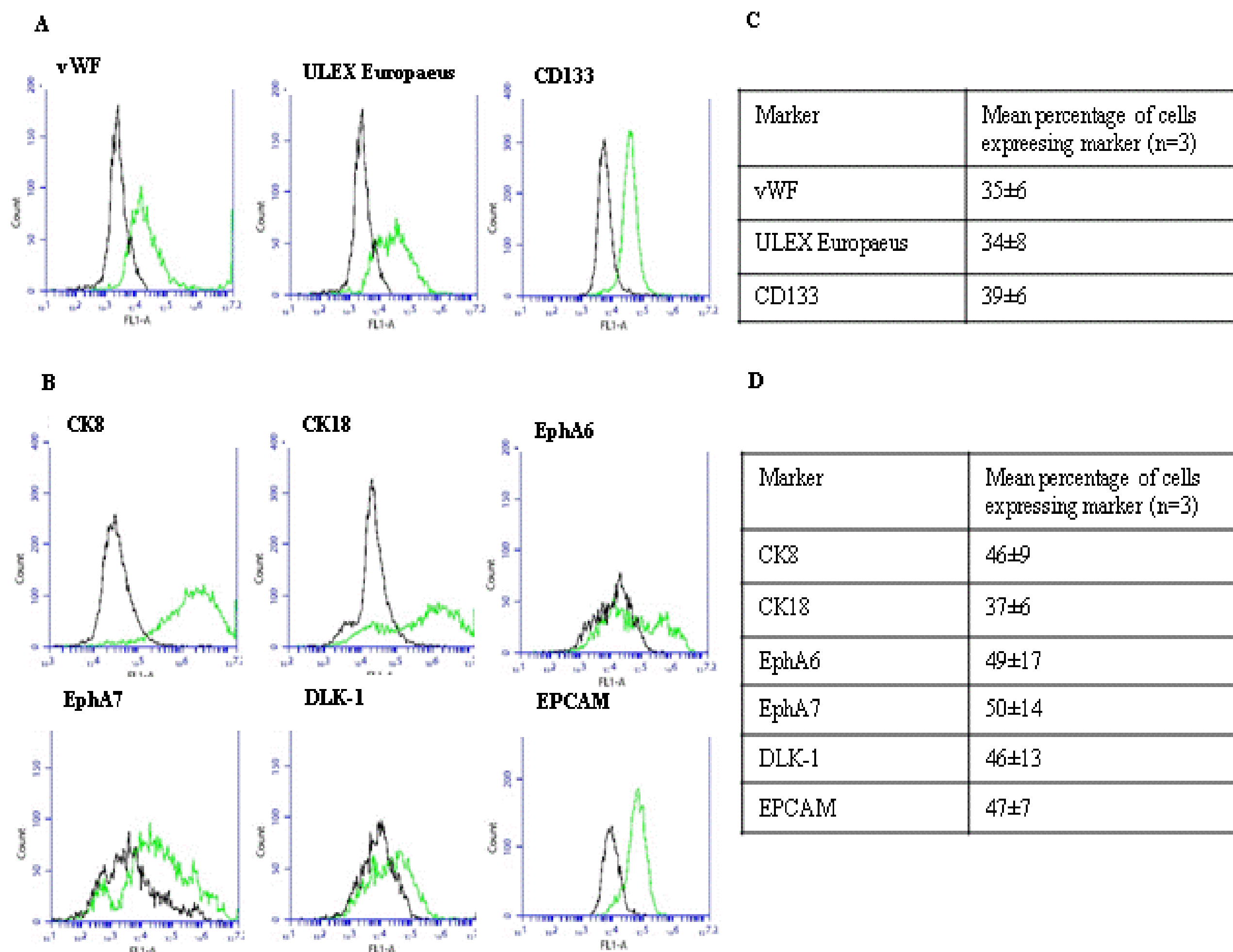


Figure S1. MT staining of (**A**) normal kidney showing nuclei (black) and distribution of collagen (blue) and (**B**) decellularized tissue showing removal of cells but preservation of collagen. Immunohistochemistry staining in normal kidney showed brown staining of (**C**) fibronectin and (**E**) laminin. Similarly, the immunohistochemistry staining showed preservation of (**D**) fibronectin and (**F**) laminin in decellularized tissue. The (**G**) negative control showed no staining.

3.2 Characterization of hFKC

The hFKC grown in endothelial cells medium (EnCM) showed an elongated growth (Figure S2A). Characterization of these cells by flow cytometer showed the expression of endothelial specific markers von Willebrand factor (vWF) and endothelial progenitor/precursor cell markers Ulex Europaeus and CD133 (Figure 2A). The mean percentage of cells expressing vWF, ULEX Europaeus and CD133 markers were 35%, 34% and 39% respectively (Figure 2C). Characterization of the same cells by immunocytochemistry gave reproducible results demonstrating the expression of endothelial cells marker vWF and progenitor endothelial cell markers Ulex Europaeus and CD133 (Figure S2B). The cells grown in epithelial cells medium (EpCM) showed the characteristic polygonal shape of epithelial cells (Figure S2C). When characterized by flow cytometer, they expressed phenotypic epithelial cell markers cytokeratin (CK) 8 and CK18, the ephrin receptors EphA6 and EphA7 and the stem cell markers DLK-1 and EPCAM (Figure 2B). The calculation of

percentage cells expressing the marker showed that cells expressing CK8, CK18 and EPCAM were 46%, 37% and 47% respectively where cells expressing EphA6, EphA7 and DLK-1 were 49%, 50% and 46% respectively (Figure 2D). Once again the immunocytochemistry staining gave reproducible results demonstrating the expression of CK8, CK18, EphA6 and EphA7 (Figure S2D).



Marker	Mean percentage of cells expressing marker (n=3)
vWF	35±6
ULEX Europaeus	34±8
CD133	39±6

Marker	Mean percentage of cells expressing marker (n=3)
CK8	46±9
CK18	37±6
EphA6	49±17
EphA7	50±14
DLK-1	46±13
EPCAM	47±7

Figure 2. (**A & C**) Flow cytometric analysis of human fetal kidney cells (hFKC) grown in endothelial cell medium showing positive staining for endothelial cells marker vWF and progenitor endothelial cells markers Ulex Europaeus and CD133. (**B & D**) Flow cytometric analysis of hFKC grown in epithelial cell medium showing positive staining for epithelial cell markers CK8, CK18, ephrin receptors EPhA6 and EphA7 and stem cell markers DLK-1 and EPCAM.

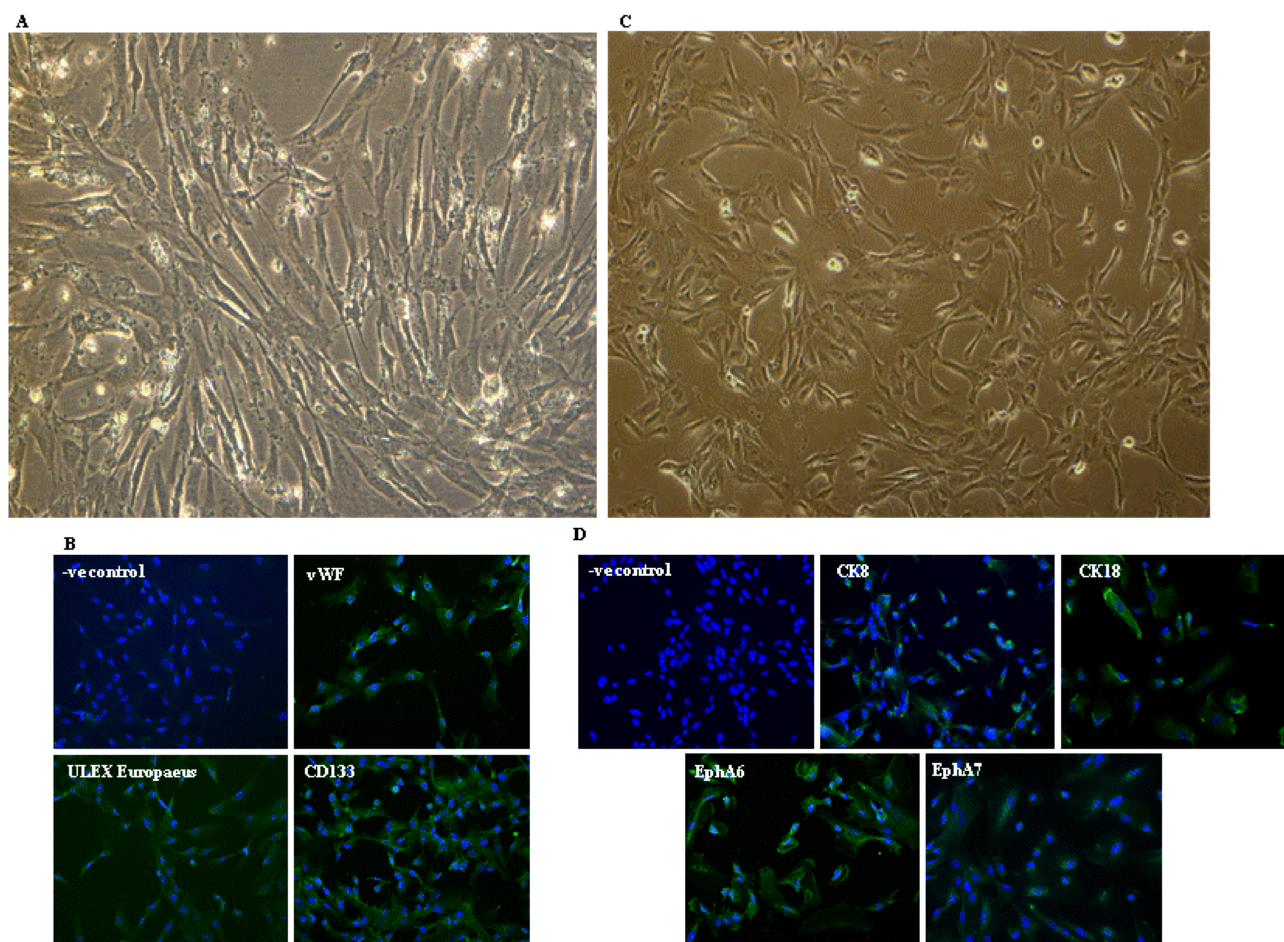


Figure S2. (**A**) Phase contrast micrograph of cultured human fetal kidney cells (hFKC) grown in EnCM. (**B**) Further test by immunocytochemistry showed cells positive for vWF, Ulex Europaeus and CD133. (**C**) Phase contrast micrograph of hFKC grown in EpCM. (**D**) The further confirmation of same cells by immunocytochemistry also showed expression of, CK8, CK18, EphA6 and EphA7.

3.3 Identification of stem cells markers in human fetal and adult kidneys

Immunohistochemistry staining in fetal kidneys showed that DLK-1 was strongly expressed in capsule, while in adult kidneys it was mainly expressed by tubules and ducts. The ephrin receptor EPhA7 showed a strong expression in capsule and weak expression in mesenchyme for fetal kidneys while no staining was seen in adult kidneys. SIX2, was expressed in capsule and strongly in the mesenchyme of fetal kidneys, while no staining was seen in adult kidneys. The negative control showed no staining (Figure 3).

The expression of CD133 was found throughout in capsule, mesenchyme and interstitium of fetal kidneys while no expression was seen in adult kidneys. The ephrin receptors EPhA6 and EphB3 were weakly expressed in the capsule of fetal kidneys while in adult kidneys, no specific staining was seen. Positive staining for EPCAM was found in the epithelium of tubules and ducts for fetal kidneys while only in ducts for adult kidneys (Figure S3). The ephrin receptor EphB2 was not expressed in fetal and adult kidneys. See also table 1A for expression of all markers in fetal and adult kidneys.

To test whether EphA7 was co-expressed with a known cell surface expressed stem cell marker in the kidney, we performed double immunofluorescence staining for DLK-1 (green) and EphA7 (red) in fetal kidneys. We found a co-expression of this marker with DLK-1 (yellow) in the capsule region (Figure S4A). The negative control showed no staining for DLK-1 and EphA7 (Figure S4B).

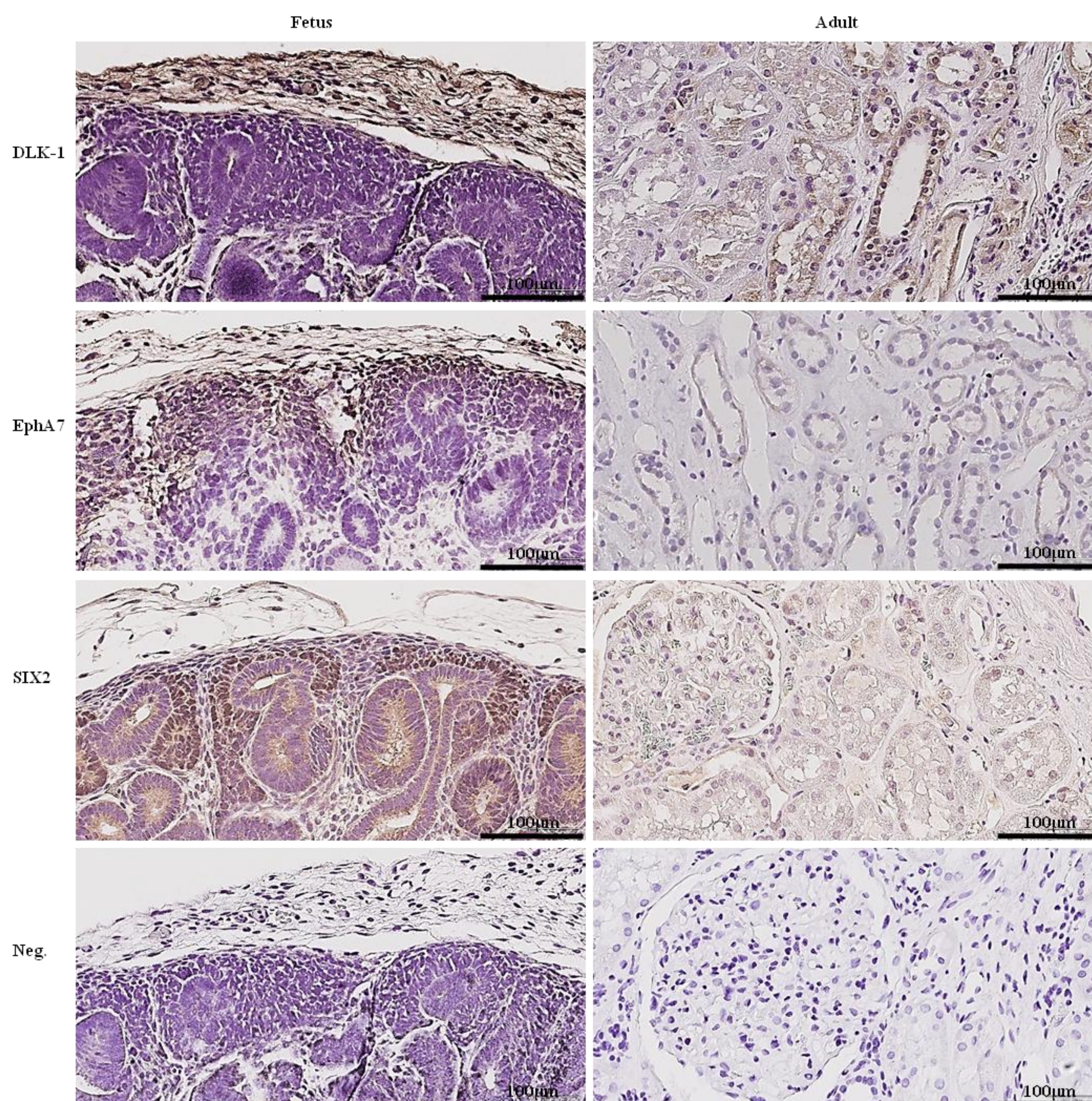


Figure 3. Immunohistochemistry staining for fetal (left panel) and adult kidneys (right panel) for expression of DLK-1, ephrin receptor EphA7 and stem cell marker SIX2. DLK-1 was mainly seen in capsule for fetal kidneys and in ducts and tubules for adult kidneys. The ephrin receptor EphA7 was only seen in capsule and mesenchyme of fetal kidneys. The SIX2 was also only found in mesenchyme and capsule of fetal kidneys. The negative control showed no staining in both adult and fetal kidneys.

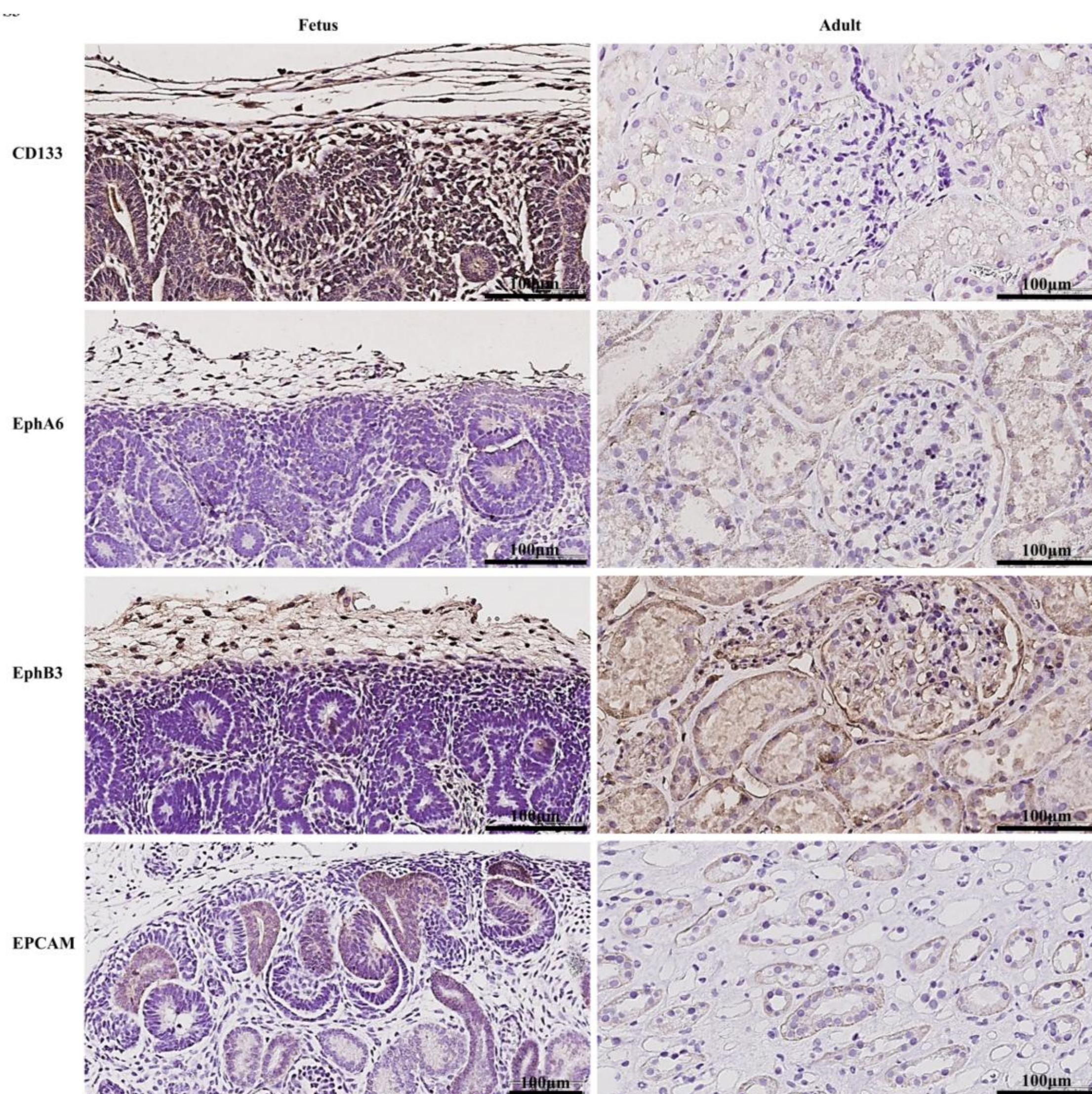


Figure S3. Immunohistochemistry staining for fetal (left panel) and adult kidneys (right panel) for expression of EPCAM, CD133, ephrin receptors EphA6 and EphB3. CD133 was found in capsule, mesenchyme and interstitium for fetal kidneys. The ephrin receptors EphA6 and EphB3 were only seen in capsule and mesenchyme of fetal kidneys. The EPCAM was found in tubules and ducts for both fetal and adult kidneys.

The Table 1A shows the average expression visualized for each marker in different regions of fetal kidneys. Eph receptors (EphA6, EphA7 and EphB3) were expressed in capsule and mesenchyme. SIX2 was mainly expressed in mesenchyme and weakly in capsule. DLK-1 was strongly expressed in capsule, weakly in mesenchyme while in tubules and ducts of adult kidneys. CD133 was expressed in all regions of fetal kidneys and absent in adult kidneys. In fetal kidneys, EPCAM was seen in tubules and ducts while only in ducts in adults. EphB2 was not expressed in fetal and adult kidneys.

Table 1A. Expression of stem cell markers and ephrin receptors in human fetal and adult kidneys.

	Fetus			Adults		
Marker	Capsule	Mesenchyme	Interstitial cells	Tubules	Ducts	Glomeruli
EphA6	++	-	-	-	-	-
EphA7	+++	++	-	-	-	-
EphB3	++	+	-	-	-	-
SIX-2	++	+++	-	-	-	-
DLK-1	+++	+	-	+++	+++	-
CD133	+++	++	++	-	-	-
EphB2	-	-	-	-	-	-
EPCAM	Tubules (++) and Ducts (+++)			-	++	-

Note: "+++ = Strong", "++ = Moderate", "+ = Weak" and "- = Background like or absent".

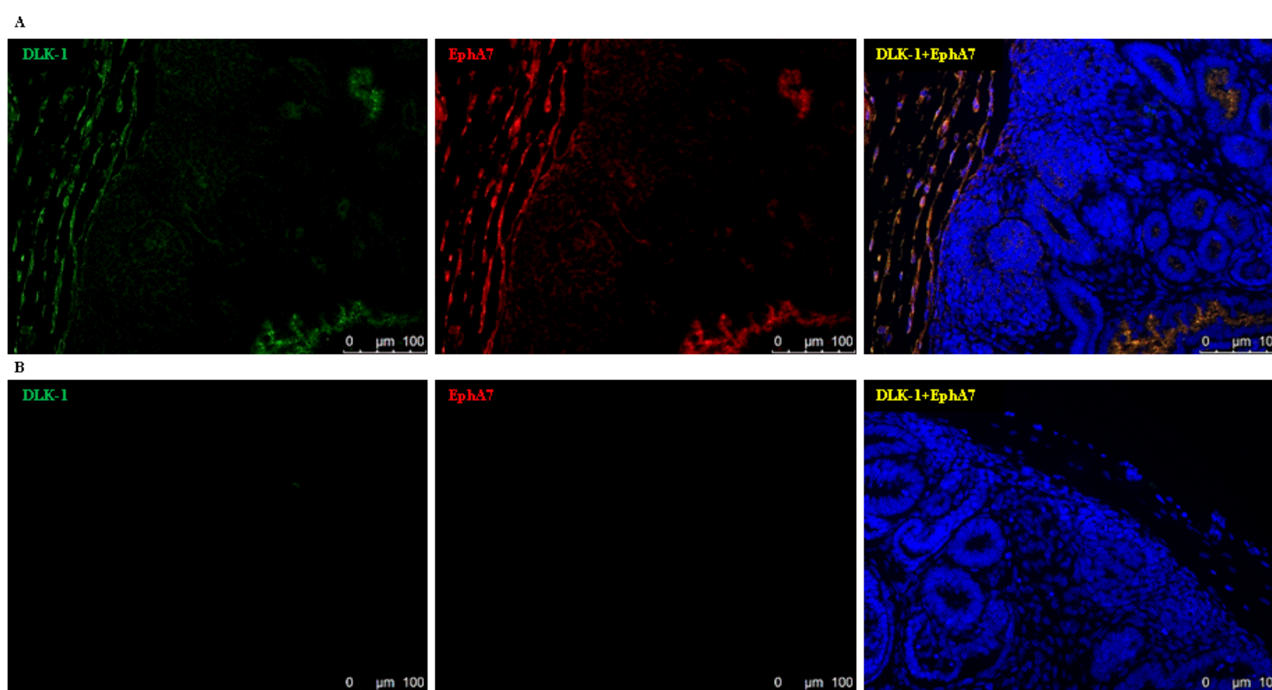


Figure S4. The (S4A) double immunofluorescence staining of fetal kidneys showed strong expression of DLK-1 (green) and EphA7 (red) in capsule. A co-expression of DLK-1 and EphA7 (yellow) was also seen in cells of capsule. The (S4B) negative control showed no expression of both markers.

3.4 Recellularization of sliced decellularized kidney scaffolds with human fetal kidney cells.

After decellularization, the ECM fibers were found to be still compact (Figure 4A) but after treatment with metalloproteinase (MMP)-2 and -9 the ECM appeared less compressed in some areas (Figure 4B). HE staining of the recellularized kidney pieces cultured in transwell for 2 weeks showed several colonies of cells spread throughout the tissue, in the parenchyma and occasionally in tubules (Figure 4D and 4E respectively). In some areas of the recellularized kidney slices around the scaffold, large numbers of cells were found attached to the ECM and growing around in orchestrated fashion

(Figure 4F). Interestingly, at two weeks the cells in these areas were found to form glomeruli-like (Figure S5A) and tubule-like structures (Figure S5B). In recellularized kidney pieces without treatment with MMPs, no or rare attachment of cells was noticed (Figure 4C). The immunohistochemical analysis of the recellularized kidney pieces also showed some cells positive for the epithelial cell markers CK8 (Figure 5A), CK18 (Figure 5B), endothelial progenitor cell marker CD133 (Figure 5C), ephrin receptor EphA7 (Figure 5D) and the stem cell marker DLK-1 (Figure 5E). Immunofluorescence staining showed some cells expressing ephrin receptor EphB3 (Figure 5G) and many for the kidney specific marker-podocin (Figure 5H). Few cells were also positive for proliferating cell nuclear antigen (PCNA) (Figure 5I) indicating the presence of dividing cells. No or minimal positive staining was seen in negative controls for immunohistochemistry and immunofluorescence (Figure 5F).

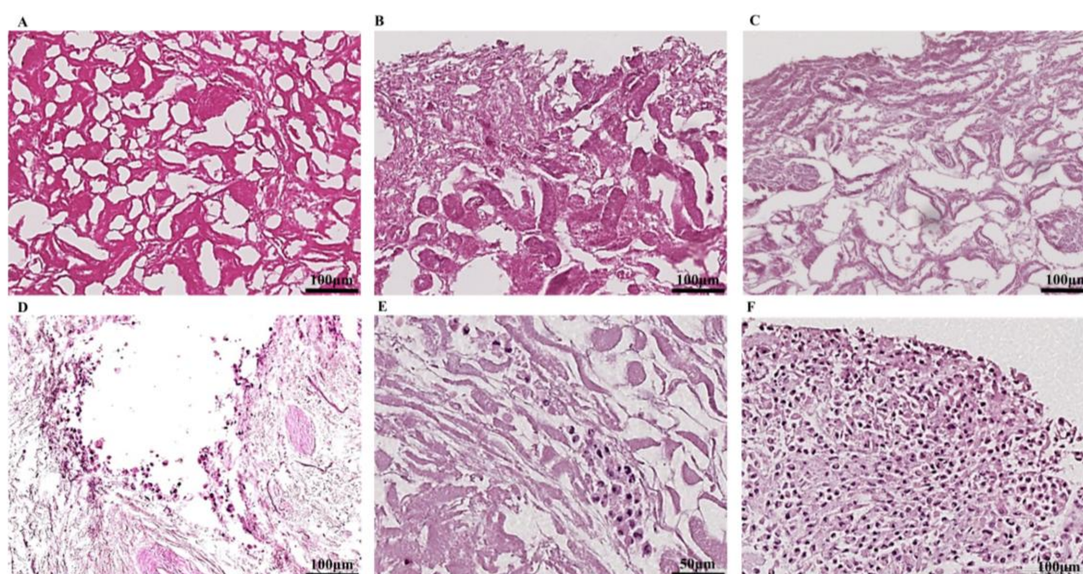


Figure 4. HE staining of decellularized porcine kidney ECM (**A**) before and (**B**) after treatment with MMP-2 and -9. HE staining of recellularized kidney pieces showing absence of cells in pieces not treated with MMPs (**C**). In the HE staining of recellularized kidney pieces treated with MMPs, presence of cells scattered in the (**D**) parenchyma and (**E**) a tubule, while in certain areas (**F**) abundant cells were found on the surface of the decellularized ECM.

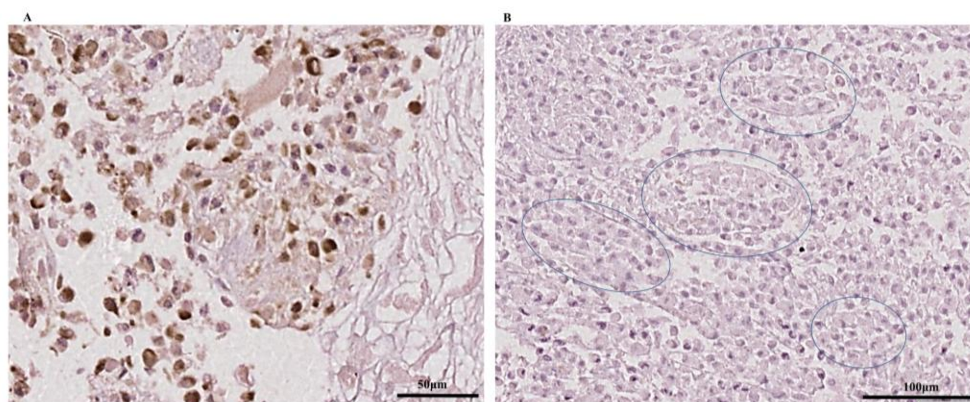


Figure S5. In recellularized kidney, immunohistochemical staining for CK18 showed that in the areas where cells were growing in orchestrated fashion, the cells themselves formed (**A**) glomerular-like structures and (**B**) tubular like structures (in blue circles) in its negative control.

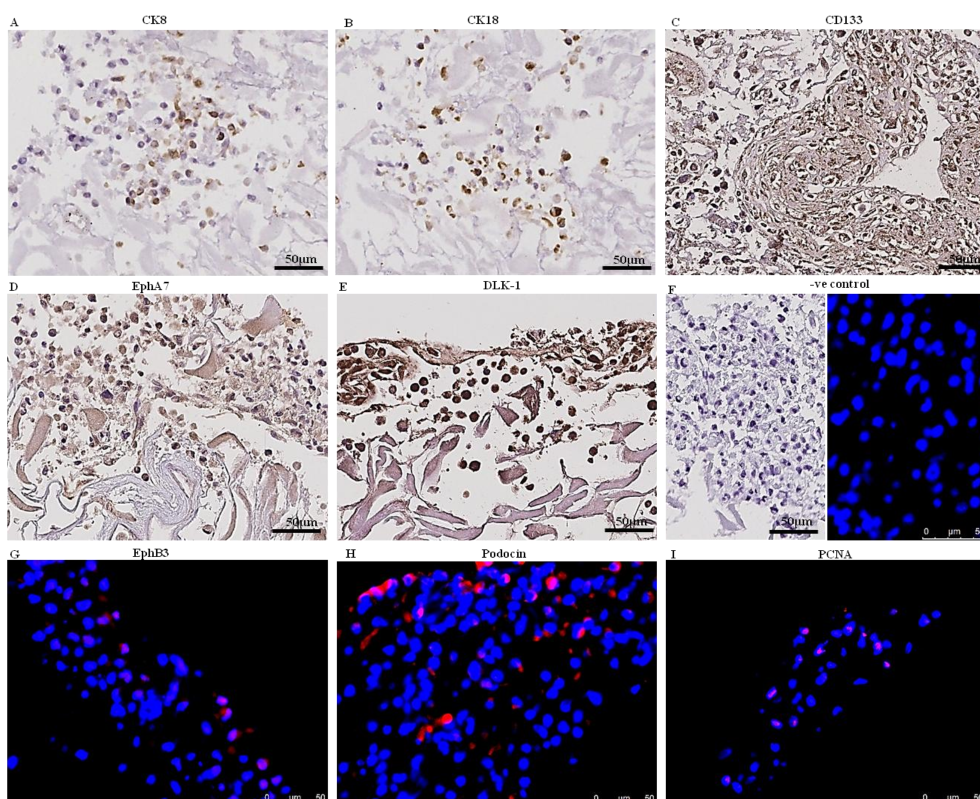


Figure 5. Immunohistochemical analysis of recellularized porcine kidneys showing positive staining for the epithelial cell markers (**A**) CK8 and (**B**) CK18. Staining for progenitor endothelial cells marker (**C**) CD133 showed positive in parenchyma and blood vessels, while most of the cells were found to be also positive for the ephrin receptor (**D**) EphA7 and the stem cell marker (**E**) DLK-1. Immunofluorescence staining showed that few of the cells were also positive for (**G**) EphB3, functional protein of kidney glomeruli (**H**) podocin and cells proliferative marker (**I**) PCNA. The (**F**) negative controls for immunohistochemistry and immunofluorescence showed weak or no staining.

3.5. Gene analysis of recellularized tissue

The qPCR analysis of recellularized tissue showed an increased expression of transcription factors SIX2 and Eyes Absent Homolog 1 (EYA1) that are involved in kidney development when compared to the seeded cells alone. A few other transcriptional factors involved in kidney development such as WT1, Spalt Like Transcription Factor 1 (SALL1), CITED1, and LIM Homeobox 1 (LHX1) were also slightly increased in comparison to cells alone but less in comparison to adult kidney. The transcription regulator Paired Box 2 (PAX2) was less expressed in recellularized tissue as compared to cells. In addition, an increased expression of DLK-1 the transcriptional factor important in growth and differentiation of epithelial cells was seen in recellularized tissue in comparison to cells alone. The expression of DLK-1 and EYA1 in recellularized and adult kidney were similar. All these results together show the upregulated expression of transcription factors involved in proliferation and differentiation of cells in the recellularized kidney tissue (Figure 6).

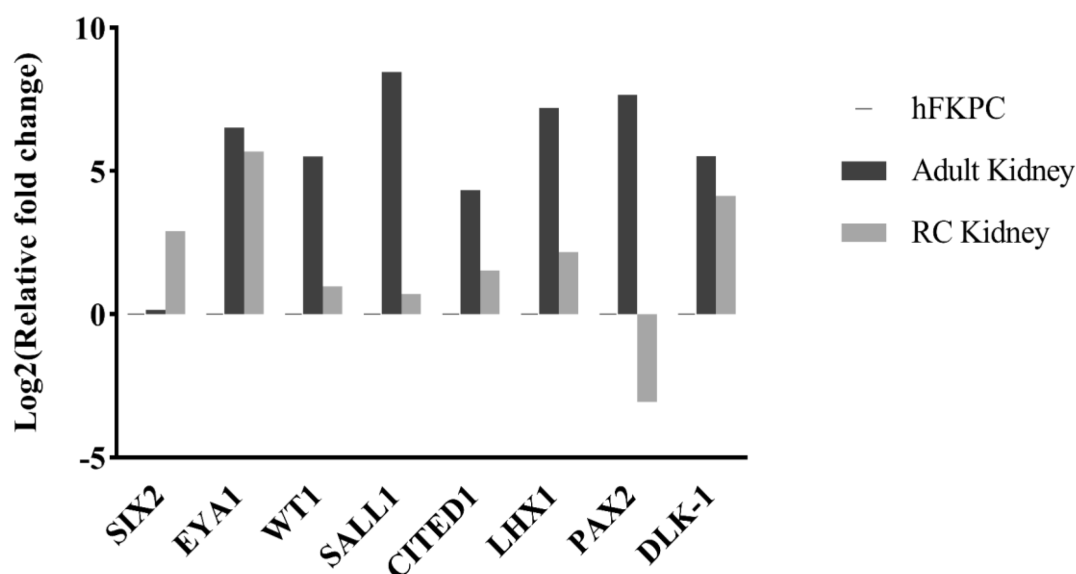


Figure 6. Gene expression analysis of the recellularized porcine kidneys showed in general an increased expression of important transcription factors in kidney development such as SIX2, EYA1, WT1, SALL1, CITED1, LHX1 and DLK-1 as compared to only seeded cells. PAX2 was expressed low as compared to cells and adult kidney. If compared between adult and recellularized kidneys, similar expression was seen for genes EYA1 and DLK-1, increased expression for SIX2 while WT1, SALL1, CITED1 and LHX1 are less expressed.

4. Discussion

Organ formation requires not only stem cells but also participation from the surrounding stem cell niche or microenvironment and ECM. In fact, 3D tissue scaffold has been demonstrated to be very critical for organ regeneration [32]. Our present study builds on previously reported techniques

for production of renal ECM scaffold from porcine kidneys [8,10]. However, to our knowledge this is the first study reporting the use of human fetal kidney cells for recellularization. Here, we show that the cellular components of porcine kidneys were removed, leaving behind an acellular ECM scaffold, which retains its complex 3D structure using a milder protocol involving the use of ionic and non-ionic detergents as compared to protocols involving the use of harsh detergents such as sodium dodecyl sulphate [17,33]. The acellular scaffold retained structural ECM proteins with a significant loss of DNA. Our results are in agreement with those of Bonandrini et al [34] that a perfusion based decellularization protocol using a peristaltic pump results in decellularization with effective cell removal. We believe perfusion of decellularization solutions through artery and ureter is important since our optimization experience showed inefficient cell removal when perfusion was only through artery. Though we cannot explain the reason for increased amount of collagen in decellularized kidney than normal, we speculate the collagen weight had compensated for the loss of water in decellularized tissues.

Considering the complex cellular nature of human kidney, the morphological and functional heterogeneity of renal cell types, we used human fetal kidney cells as the precursor population since they possess a high proliferative and differentiation capacity into renal specific cell types. A study on tissue engineering with monkey kidneys also suggested a mixture of glomerular, tubular and mesenchymal cell fractions as efficient for kidney recellularization [35]. We isolated hFKC and grew them in epithelial and endothelial media in order to enrich for these cell types. The characterization by flow cytometer and immunocytochemistry verified the presence of lineage specific cells and respective progenitor cells.

The staining in fetal kidneys show that compared to EphA6 and EphB3, intense and reproducible staining for EphA7 was observed in the capsule and mesenchyme of only fetal but not adult kidneys. Recent experiments have established that the progenitor cells in the metanephric mesenchyme fulfills the criteria of a true committed stem cell that is capable of self-renewing and of differentiating towards different types of nephron epithelia [36,37]. Very little is known about the role of EphA7 in kidneys, but interestingly, the kidney has been reported to have the highest amounts of EphA7 mRNA of all the investigated tissues in humans [38]. A recent study showed that a truncated form of secreted EphA7 receptor as important regulator for reprogramming mouse embryonic fibroblasts and the maintenance of pluripotency by increased expression of Nanog and Oct3/4 genes [39]. Based on the literature and expression pattern of EphA7 in human fetal kidneys, it is tempting to postulate that EphA7 might be a new interesting renal stem/precursor cell population. Our postulation is strengthened by the finding that in all fetuses, EphA7 was co-expressed with DLK-1, a known cell surface expressed stem cell marker in kidneys. However, understanding the function of EphA7 requires studies that compare current observations in large sample numbers and testing it's potential in diseased models.

In addition, we were able to confirm the expression of previously suggested renal stem cell markers SIX2, DLK-1, CD133 and EPCAM in human fetal kidneys [23,31,37]. However, it is also reported that CD133 mostly qualifies as a marker for identification of differentiated tubular cells and will not enrich for a progenitor phenotype [23]. In adult kidneys no expression of CD133 was observed but, DLK-1 expression was strong in tubules and ducts. In both fetuses and adults, EPCAM was mainly found only in the epithelia of tubules and ducts. In addition, cells isolated and cultured from fetal tissues (hFKC) demonstrated the continued expression of all the stem cell markers tested such as DLK-1, CD133, EPCAM, EphA6 and EphA7 as evidenced by flow cytometric and immunocytochemical analysis.

The recellularization experiments without MMP treatment and transwell showed poor or no attachment of cells. We speculated that the complex ECM structure or less porosity are inhibiting cell attachment. Since human cells produce MMPs to breakdown surrounding tissue to facilitate migration during tissue remodeling, we treated the tissue with MMP-2 and -9 to open up the compact ECM microstructure. We noticed that, MMP treatment together with incubation of the tissue in a transwell improved infiltration of cells under static culture conditions as presence of cells in the parenchyma was noticed in sections used for various stainings up to 300 μm depth. With this approach we were able to obtain a good though not uniform distribution of the seeded cells into the porcine renal tissue, as confirmed by immunohistochemical analysis of cells found inside glomeruli and renal parenchyma. The cells expressed epithelial, podocytic, and endothelial lineages. This finding is not surprising since the isolated hFKC were already committed toward these lineages. Though the reason was not explained, a recent study also used collagenase as a last step in decellularization [40].

We also studied the gene expression that was up-regulated in the recellularized grafts, as these have been suggested to characterize the "progenitor" population genes. Among these were the transcription factors specifying the kidney (nephron) progenitor cells, intermediate and metanephric mesenchyme including WT1, PAX2, SIX2, EYA1, SALL1, and CITED1. We found an increased level of many tested genes in the recellularized tissue pieces as compared to seeded cells which may implicate the additive effect of intrinsic cues present in decellularized ECM on kidney regeneration by hFKC. To our surprise, the genes SIX2 and CITED1 not usually expressed in normal adult kidneys were detected in the normal adult kidney obtained from a commercial vendor. However, we are currently unable to explain this finding. Further experiments using a larger number of normal adult kidney donors will be required to address this issue. Expression of SIX2, SALL1, and WT1 are considered to be markers of the metanephric cap mesenchyme [41]. Thus, the hFKC that recapitulate the *in vivo* metanephric development are most likely to efficiently generate mature kidney cells. PAX2 though is expressed by nephron progenitors in metanephric mesenchyme, its expression is downregulated as the cells differentiate to progenitors of podocytes, proximal and distal tubules [42]. The decreased expression of PAX2 in recellularized tissue compared to cells in culture might indicate differentiation of hFKC to podocytic and tubular progenitors.

Even though, characterization of cells and recellularized tissue showed cells were not differentiated and continued expressing stem cell markers, upregulation of transcription factors in kidney development and arrangement of cells as tubule, glomeruli like structures show these tissues if cultured longer might facilitate cell differentiation and form complete nephrons and kidney.

5. Conclusion

To conclude, we showed that the implemented decellularization protocol removed most cellular components from porcine kidneys while preserving some ECM components and architecture. The rapid recellularization and characterization of acellular porcine scaffolds with hFKC showed cell attachment, proliferation and supported regeneration by increased expression of various kidney developmental genes. This technique of recellularization with hFKC paves the ground for extensive experimental investigations to achieve uniform recellularization and possible differentiation into mature human renal cells. The efficient differentiation of hFKC toward mature cell populations will be beneficial in future for studying cell biology and drug screening for toxicity testing applications.

Acknowledgements

We would like to thank Erik Elebring for research suggestions and proof reading of manuscript. We would like to also thank Clary Björkholm for help in obtaining porcine kidneys.

Author contributions

Vijay Kumar Kuna: Collection and data assembly, data analysis and interpretation, manuscript writing and final approval of manuscript.

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Suchitra Sumitran-Holgersson: Conception and design, financial support, data analysis and interpretation, manuscript writing and final approval of manuscript.

Data availability

The data sets generated during this study are available from the corresponding author on reasonable request.

Conflicts of interest and Source of Funding

SSH holds shares in Verigraft AB, a company developing tissue engineered blood vessels for diagnostic and therapeutic purposes. The other authors have no conflicts of interest. This study was financed by the Swedish Government LUA ALF grant to SSH.

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