Recellularization of Well-Preserved Acellular Kidney Scaffold Using Embryonic Stem Cells

Barbara Bonandrini, BiolSciD,^{1,*} Marina Figliuzzi, BiolSciD,^{1,*} Evangelia Papadimou, PhD,¹ Marina Morigi, PhD,¹ Norberto Perico, MD,¹ Federica Casiraghi, Chemistry Dipl,¹ Fabio Sangalli, BSc,¹ Sara Conti, BSc,¹ Ariela Benigni, PhD,¹ Andrea Remuzzi, EngD,^{1,2} and Giuseppe Remuzzi, MD^{1,3}

For chronic kidney diseases, there is little chance that the vast majority of world's population will have access to renal replacement therapy with dialysis or transplantation. Tissue engineering would help to address this shortcoming by regeneration of damaged kidney using naturally occurring scaffolds seeded with precursor renal cells. The aims of the present study were to optimize the production of three-dimensional (3D) rat whole-kidney scaffolds by shortening the duration of organ decellularization process using detergents that avoid nonionic compounds, to investigate integrity of extracellular matrix (ECM) structure and to enhance the efficacy of scaffold cellularization using physiological perfusion method. Intact rat kidneys were successfully decellularized after 17 h perfusion with sodium dodecyl sulfate. The whole-kidney scaffolds preserved the 3D architecture of blood vessels, glomeruli, and tubuli as shown by transmission and scanning electron microscopy. Micro-computerized tomography (micro-CT) scan confirmed integrity, patency, and connection of the vascular network. Collagen IV, laminin, and fibronectin staining of decellularized scaffolds were similar to those of native kidney tissues. After infusion of whole-kidney scaffolds with murine embryonic stem (mES) cells through the renal artery, and pressure-controlled perfusion with recirculating cell medium for 24 and 72 h, seeded cells were almost completely retained into the organ and uniformly distributed in the vascular network and glomerular capillaries without major signs of apoptosis. Occasionally, mES cells reached peritubular capillary and tubular compartment. We observed the loss of cell pluripotency and the start of differentiation toward meso-endo-dermal lineage. Our findings indicate that, with the proposed optimized protocol, rat kidneys can be efficiently decellularized to produce renal ECM scaffolds in a relatively short time, and rapid recellularization of vascular structures and glomeruli. This experimental setup may open the possibility to obtain differentiation of stem cells with long lasting *in vitro* perfusion.

Introduction

CHRONIC KIDNEY DISEASE (CKD) is a progressive condition marked by deteriorating kidney function over time. Eventually, end stage renal failure ensues and renal replacement therapies with dialysis and/or kidney transplantation are required.¹ Patients with some degree of CKD represent at least 8% (~40 million people) of the European population with a progressive trend to increase each year.¹ To cope with these increases, national governments can expect an expenditure of almost 3–5% of their annual healthcare budgets for renal replacement therapies. Although these therapies are still affordable in industrialized nations, the vast majority of world's population has little

chance to access either dialysis or transplantation.² Moreover, there is yet no adequate alternative with curative intent than renal transplantation, but the approach is seriously impaired by eventually limited graft survival and by the scarce availability of donors.

Tissue engineering technologies for regeneration of damaged kidney would help to simultaneously overwhelm the reduced access to dialysis, the need of organs for transplantation, and the avoidance of patient exposure to immunosuppressive drugs. The concept is to use a scaffold to grow precursor renal cells and obtain a new template for kidney transplantation. Due to the complexity of the scaffold required for engineering a whole organ, a naturally occurring scaffold made of decellularized kidney extracellular matrix

¹IRCCS—Istituto di Ricerche Farmacologiche Mario Negri, Bergamo, Italy.

²Department of Industrial Engineering, University of Bergamo, Bergamo, Italy.

³Unit of Nephrology and Dialysis, Ospedale Giovanni XXIII, Bergamo, Italy.

*These authors equally contributed to this work.

(ECM) is a key step for this regenerative approach.³ The scaffold must retain intact the three-dimensional (3D) structure of the organ, including vasculature, and the innate matrix components needed for promoting progenitor cell adhesion, migration, proliferation, and differentiation.

Decellularization of biological tissues for engineering approach has traditionally been limited to thin tissue structures such as intestines,⁴ heart valve,⁵ and blood vessels.^{6,7} More recently, however, complex organs from rodents such as heart, liver, and lung have also been successfully used for generation of bioscaffolds by decellularization to preserve the intact organ structural organization.^{8–12}

Ross and collaborators¹³ first extended this approach to kidney regeneration and reported the feasibility to obtain an acellular ECM scaffold from rat kidney. The 5-day stepwise decellularization protocol allowed to progressively remove cells from the whole kidney using nonionic and ionic detergents and enzymatic degradation of nuclear cell material. Nonionic detergents, however, could be traumatic to the tissue and may disrupt collagen structure of the ECM. Pluripotent murine embryonic stem (mES) cells were then manually infused via the renal artery and ureter to seed the whole-kidney scaffold. Nevertheless, mES cell proliferation and differentiation were mainly assessed by *in vitro* culture of thin sections of the scaffold in static conditions, and only preliminary feasibility studies of intact kidneys undergoing pulsatile perfusion of growth media were reported.¹³ Thus, this approach does not provide robust evidence for cell seeding and proliferation in a setting that more closely mimics the *in vivo* exposure of innate ECM scaffold to renal precursors. More recently, renal ECM scaffolds were also successfully produced from porcine,¹⁴ monkey¹⁵, and human¹⁶ kidneys as a platform for renal bioengineering investigations, but no attempt to repopulate the whole-kidney scaffolds with cells was pursued in this investigation.

The aim of our present study was to improve the protocol of producing decellularized rat renal ECM scaffolds and repopulating them with mES cells under physiologic *in vitro* perfusion conditions. In particular, our efforts focused on shortening the time of decellularization process of the kidney using more gentle detergents, investigating the integrity of ECM components and organ structure, and finally testing the suitability and efficacy of dynamic perfusion method to seed and repopulate the whole-kidney scaffold and to induce stem cell differentiation.

Materials and Methods

Kidney retrieval

Twenty-four adult male Sprague-Dawley rats (Charles River Laboratories International, Inc., Wilmington, MA) weighing 250–400 g were used. After anesthesia with isoflurane, a longitudinal abdominal incision was made and the left kidney, aorta, vena cava, and ureter were identified. The kidney was freed from surrounding fat and mobilized. The left renal artery was then cannulated with a PE50 catheter and secured with a 4/0 silk suture. Then, the kidney was perfused using a saline solution (NaCl 0.9%; SALF SpA, Bergamo, Italy) containing a vasodilator (nitroprusside, 10^{-4} M; Sigma-Aldrich Co. LLC, St Louis, MO) to remove blood. After blanching, the kidney was harvested and maintained immersed in saline solution until processing.

Animal care and treatment were conducted in conformity with the institutional guidelines, in compliance with national (DL n. 116/1992, Circ. 8/1994) and international (EEC Dir. 86/609, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, US NRC, 1996) laws and policies.

Organ decellularization

An *ad hoc* device has been designed and constructed to perfuse kidney under controlled conditions with different solutions for the decellularization process. The device consists of a glass chamber to hold the kidney, connected to a perfusion system designed to minimize air entrapment. The kidney was transferred at room temperature into the chamber containing the solution used for the organ decellularization. The cannula previously inserted in the renal artery was connected to a peristaltic pump. The kidney was perfused with a solution of 1% sodium dodecyl sulfate (SDS; Sigma-Aldrich Co. LLC) in PBS for 17 h at a flow rate of 0.4 mL/min. Hydraulic pressure was continuously recorded by a pressure transducer (WPI, Inc., Sarasota, FL), a digital data acquisition system MP150, and AcqKnowledge software (BIOPAC Systems, Inc., Goleta, CA). Perfusion pressure was maintained in physiological range (from 62 ± 16 to 107 ± 23 mmHg) during the entire duration of the decellularization process. A compliance was used in the circuit to dump pressure oscillation generated by the pump and to avoid air circulation in the circuit. Finally, the kidney was perfused with distilled water to wash out the detergent. Decellularized kidney scaffolds were either used for histologic examination or recellularization experiments.

Whole-kidney bioscaffold characterization

Decellularized kidneys ($n=11$) were cut into six transversal sections, three were maintained in Duboscq-Brazil fixative (Diapath S.p.A., Bergamo, Italy) overnight, dehydrated in ascending concentrations of alcohol, and embedded in paraffin for histological analysis. For each paraffined block of tissue, 3 μ m sections were obtained and stained with hematoxylin and eosin to assess matrix morphology. The other three transversal sections were fixed in periodate (Merck KGaA, Darmstadt, Germany) -lysine (Sigma-Aldrich Co. LLC) -paraformaldehyde (Electron Microscopy Science, Hatfield, PA) fixative (PLP) at 4°C overnight, immersed in 30% sucrose overnight, frozen in liquid nitrogen, and cut into 3 μ m sections. The frozen sections were stained with Fluorescein Wheat Germ Agglutinin (diluted 1:400; Vector Laboratories, Inc., Burlingame, CA) and DAPI 1 μ g/mL (Sigma-Aldrich Co. LLC) to assess both morphology of the matrix and the absence of residual nuclei in the scaffold. Immunofluorescence stainings for collagen IV, fibronectin, and laminin were performed on frozen sections to evaluate matrix composition of the decellularized kidney. Sections were incubated with rabbit polyclonal anti-collagen IV (diluted 1:50; Thermo Fisher Scientific, Inc., Waltham, MA), rabbit polyclonal anti-rat fibronectin (diluted 1:40; Chemicon International, Inc., Billerica, MA), or rabbit anti-laminin (diluted 1:100; Sigma-Aldrich Co. LLC). Secondary antibodies were goat anti-rabbit IgG FITC conjugate (diluted 1:25; Jackson ImmunoResearch Europe Ltd., Suffolk, United Kingdom) for collagen IV and fibronectin

and anti-rabbit IgG Cy3 conjugate (diluted 1:50; Jackson ImmunoResearch Europe Ltd.) for laminin. Counterstaining with DAPI 1 µg/mL, for 20 min at 37°C was performed for cell nuclear staining. Sections were finally washed and mounted using DAKO fluorescent mounting medium (Dako Italia S.p.A., Milano, Italy). Then, samples were examined with a fluorescence microscope (Carl Zeiss, Inc., Göttingen, Germany). Two samples fixed in 2.5% glutaraldehyde (Sigma-Aldrich Co. LLC) were analyzed with transmission electron microscopy (TEM) and scanning electron microscopy (SEM), using methods previously described.¹⁷ Cortex and medulla samples of decellularized kidney were observed at TEM using a Morgagni FP5005 electron microscope (FEI Company, Eindhoven, The Netherlands) and at SEM using a field emission scanning electron microscope (1540 EsB; Carl Zeiss GmbH, Oberkochen, Germany).

Micro-computerized tomography imaging of the vasculature

To investigate the structural integrity of the vascular tree, two decellularized kidneys were imaged by micro-computerized tomography (micro-CT 1076; Skyscan, Kontich, Belgium) during arterial perfusion with contrast agent. Briefly, at the end of decellularization process the kidney was placed on the micro-CT stage and a radiopaque solution (Microfil, MV-122; Flow Tech., Carver, MA) was injected through the renal artery using a syringe pump (Harvard Apparatus, Holliston, MA) at a rate of 0.2 mL/min. Projected images were taken every second at 18 µm/pixel (2000 × 1336 pix) resolution using an Al filter. When Microfil flowed out from the renal vein, both vein and artery were closed and resin polymerization was allowed for 3 h. The kidney was then placed again on the stage of the micro-CT and scan was performed by using an Al filter (0.5 mm thickness), rotation step 0.4°, and image resolution of 9 µm/voxel. Projection images were then reconstructed by NRecon software (Skyscan) and visualized by CTvox software (Skyscan).

R1 mES cell culture

The R1 pluripotent mES cells¹⁸ were obtained upon MTA agreement from A. Nagy lab at Mount Sinai Institute, Toronto, Canada. R1 mES cells were cultured and passaged every two days on mitotically inactivated mouse embryonic fibroblast (MEFs; E13.5; strain 129) in proliferation medium DMEM supplemented with 1 mM sodium pyruvate, 100 µM nonessential amino acids, 2 mM L-glutamine, 100 U/mL penicillin–100 µg/mL streptomycin, 0.1 mM β-mercaptoethanol, 10% ES cell qualified FBS (all products were obtained from Life Technologies Italia, Monza, Italy), and 1000 U/mL leukemia inhibitory factor (LIF ESGRO; Merck Millipore, Billerica, MA). When needed, R1 mES cells cultures were treated with 2 mg/mL collagenase IV (Life Technologies Italia) for 10 min at 37°C to avoid the presence of MEFs.

Recellularization of kidney scaffolds with mES cells

Eleven acellular kidney scaffolds were used for recellularization experiments. Before cell seeding, the scaffold was washed in open circuit with 1% (v/v) penicillin/streptomycin (Life Technologies Italia) in PBS for 2 h. The

kidney was perfused with the same medium used for cell culture devoid of LIF at 37°C for 30 min to provide nutrients and establish the right pH in the scaffold. For cell seeding, 12×10^6 mES cells were infused antegrade through the arterial cannula connected to a syringe pump (Harvard Apparatus) at 0.2 mL/min. The seeded scaffold was perfused with recirculating medium for 24 or 72 h. Culture conditions were maintained placing the entire system in the incubator.

Histologic and histochemical characterization

At the end of perfusion the recellularized kidney was processed for histologic and immunofluorescence analysis, as previously described. Tissue sections of hematoxylin and eosin staining were examined on Axiovision light microscope (Zeiss, Jena, Germany). For each sampling section, the area was scanned with a 20× objective using a MosaiX program. Analysis was performed on the obtained images by ImageJ (National Institutes of Health, Bethesda, MI) image analysis program. Cell repopulation of each glomerulus was estimated by scoring the level of recellularization (0=no cells, 1=cells covering 25% of tuft area, 2=cells covering 50% of tuft area, and 3=cells covering 100% of tuft area). Immunohistochemic analysis was performed on paraffin sections to stain proliferating cells using a proliferating cell nuclear antigen (PCNA) antibody. Dubosq-Brazil fixed, paraffin-embedded sections (3 µm) were deparaffinized, hydrated, and incubated with 0.3% H₂O₂ in methanol to quench endogenous peroxidase. Antigen retrieval was performed by boiling sections using microwave (twice for 5 min in citrate buffer 10 mM, pH 6.0 at operating frequency of 2,450 MHz and 600 W power output). Sections were then incubated with mouse monoclonal anti-PCNA antibody (diluted 1:250; Sigma-Aldrich Co. LLC). After incubation with horse biotinylated anti-mouse IgG (diluted 1:200; Vector Laboratories, Inc.), staining was developed with diaminobenzidine (DAB; Merck KGaA) substrate solution. Slides were counterstained with hematoxylin, dehydrated in graded alcohols, and mounted with coverslips and observed by light microscopy. Immunofluorescence staining for cleaved caspase-3, octamer-binding transcription factor-4 (Oct-4), neuronal cell adhesion molecule (NCAM), Tie-2, and CD31 was performed on frozen sections to evaluate apoptosis and expression of differentiation markers of the cells repopulating the scaffolds at 24 and 72 h after perfusion. Sections were incubated with rabbit monoclonal anti-caspase-3 (diluted 1:200; Cell Signaling Technology, Inc., Danvers, MA), mouse monoclonal anti-Oct-3/4 (diluted 1:50; Santa Cruz Biotechnology, Inc., Dallas, TX), rabbit polyclonal anti-NCAM (diluted 1:100; Millipore Corporation, Billerica, MA), goat polyclonal anti-Tie-2 (diluted 1:100; Santa Cruz Biotechnology, Inc.), and rabbit polyclonal anti-CD31 (diluted 1:100; Abcam plc, Cambridge, United Kingdom). Secondary antibodies were goat anti-rabbit IgG Cy3 conjugate (diluted 1:50; Jackson ImmunoResearch Europe Ltd.), donkey anti-mouse IgG Cy3 conjugate (diluted 1:100; Jackson ImmunoResearch Europe Ltd.), and rabbit anti-goat IgG Cy3 conjugate (diluted 1:50; Jackson ImmunoResearch Europe Ltd.). Counterstaining with DAPI 1 µg/mL was performed for cell nuclear staining. Tissue sections were examined with a fluorescence microscope (Carl Zeiss, Inc.)

Results

Kidney decellularization

Whole-organ decellularization was achieved by arterial perfusion with SDS, an anionic detergent that lyses cells and solubilizes cytoplasmatic components. Detergent perfusion and washing of rat kidney was maintained with physiological perfusion pressure for 17 h. The recorded pressure ranged 62 to 107 mmHg for all the duration of decellularization process. At the end of the perfusion, a white acellular scaffold was obtained, which retained the overall shape and the macroscopic structure of kidney (Fig. 1A, B). Histologic evaluation with hematoxylin and eosin staining showed pink eosinophil staining typical of collagen, whereas no basophilic staining typical of cellular nuclear material was observed (Fig. 1D, E), as compared to normal rat kidney staining (Fig. 1C). Moreover, the cortical ECM microstructure appeared totally preserved. Similar results were found using Fluorescein Wheat Germ Agglutinin and DAPI, showing total absence of cell nuclei (Fig. 1F). The scaffolding architecture of vessels, glomeruli, and tubuli was preserved.

To evaluate whether the scaffold ultrastructure was preserved after the decellularization process, kidney scaffolds were fixed in glutaraldehyde and examined by TEM and SEM. TEM analysis documented the complete removal of cellular components and uniform preservation of basement membrane integrity (Fig. 2A, B). The 3D ultrastructure of glomerular and tubular matrix after decellularization was assessed by SEM. It was confirmed that the whole-kidney scaffolds were completely denuded of cells, with a continuous 3D ECM microstructure of glomerular capillaries, including capillary segments and the Bowman's capsule (Fig. 2C–F). Integral ultrastructure was also observed for tubular basement membrane and vascular structures (Fig. 2C, D).

Vascular architecture preservation and patency

To assess integrity and patency of the vascular segments, and of the entire vasculature, we acquired X-ray projection of decellularized kidney using micro-CT during infusion of a contrast agent (Microfil). The image sequence reported in

FIG. 1. Perfusion decellularization of whole rat kidneys and preservation of kidney matrix architecture. Photograph of rat kidney at the beginning (A) and after (B) the decellularization protocol. Hematoxylin and eosin staining of sections of renal cortex before (C, original magnification 20 $\times$) and after (D, original magnification 20 $\times$ and E, original magnification 40 $\times$) SDS decellularization shows the absence of any cellular content and the integrity of the architecture of glomerular and tubular structures within the scaffold. WGA agglutinin and DAPI staining of decellularized kidney (F, original magnification 40 $\times$). No blue staining resulted in the scaffold, as indirect proof of complete cell clearance. SDS, sodium dodecyl sulfate.

FIG. 2. Ultrastructural analysis of the acellular bioscaffold. (A, B) Transmission electron microscopy images of decellularized kidney demonstrating the absence of cellular components and the preservation of basement membranes integrity. Scanning electron microscopy images (C–F) of the scaffold confirm complete removal of cellular material and maintenance of well-organized 3D architecture of kidney matrix. Arrow indicates glomerular basement membrane and asterisks indicate the glomerular capillary lumen. 3D, three-dimensional.

Figure 3A and B shows a uniform distribution of contrast agent through the hierarchical branching structures from renal artery, to interlobular arteries and to arcuate arteries and small arterioles, without extravasation into the surrounding tissue. After the arterial phase, the contrast media was evident later in the venous side of the renal vasculature (Fig. 3C) and finally in the renal vein (Fig. 3D). These findings confirmed the integrity of the vascular network, its patency, and the connection of the arterial tree with the venous tree that must take place through glomerular and peritubular capillaries. As shown in Figure 3E and F by 3D digital reconstruction, both the arterial and the venous sys-

tem are well preserved, and they must have connection through the microcirculation.

Localization of scaffold ECM proteins

To evaluate the degree of preservation of renal ECM, we compared the distribution of specific ECM proteins in the scaffold and in native rat kidney tissue. Collagen type IV, laminin, and fibronectin displayed similar expression patterns in the decellularized kidney and native renal tissue (Fig. 4). Immunofluorescence staining showed a completely contiguous network of collagen IV and laminin in tubuli and

FIG. 3. Micro-CT sequence of a representative rat kidney acellular scaffold during infusion of Microfil (**A–D**) and 3D digital reconstruction of the scaffold after Microfil infusion (**E–F**) demonstrating uniform connection of the renal artery to the renal vein through the capillary bed. Black arrow in panel **A** indicated renal artery. Gray arrow in panel **D** indicated renal vein.

FIG. 4. Immunohistochemistry of extracellular matrix proteins in control (**A**, **C**, **E**, original magnification 20 $\times$) and decellularized kidney (**B**, **D**, **F**, original magnification 20 $\times$). Decellularized tissues display similar distribution of collagen IV (**B**) and laminin (**D**) in tubular and glomerular basement membranes, as control (**A**, **C**). Fibronectin staining shows intense signal in glomeruli of control (**E**) and decellularized tissue (**F**).

glomerular basement membrane (Fig. 4A–D), while fibronectin expression was intense in glomeruli, but almost absent in the tubuli (Fig. 4E, F).

Scaffold recellularization with mES cells

mES cells were infused via the renal artery with a syringe pump at the constant perfusion rate. Preliminary experiments with 4 and 8×10^6 mES cells resulted in low repopulation of the whole-kidney scaffold (data not shown). Significant improvement was, however, achieved by infusing 12×10^6 mES cells. Therefore, all experiments in the present study were performed with this number of mES cells infused in 6 mL volume over 30 min. Approximately 2.5% of the 12 million cells were recovered in the culture medium after the 30 min infusion, indicating that most of mES cells were seeded in the acellular whole-organ scaffold. After seeding, the perfusion system with the recellularized scaffold was moved into the incubator and the kidneys perfused for 24 or 72 h with recirculating medium. Of note, at the end of the perfusion time, mES cells in the circulating medium were only 2.5% of the initial 12 million, suggesting that the seeded scaffold re-

tained most of the cells even after long-term exposure to perfusion-related shear stress.

Histologic staining with hematoxylin and eosin showed uniform distribution pattern of cells in the vascular network and in the glomeruli at 24 h (Fig. 5A, C, E) and 72 h (Fig. 5B, D, F). Uniform cell distribution at glomerular level was confirmed by Fluorescein Wheat Germ Agglutinin and DAPI staining that documented cellular nuclei in the scaffold (Fig. 5G, H). Transversal cross-section of recellularized whole-organ scaffold further demonstrated the almost complete cell repopulation of glomeruli in the entire kidney cortex (Fig. 6A). Semiquantitative analysis of individual glomeruli showed that mES cells covered all the tuft area (score 3) in 61% of the glomeruli, and more than half of this area (score 2) in 25% of the glomeruli. In few glomeruli (9%) cells were localized only in more than 25% of the tuft area (score 1), while no cells were counted in the remaining 5% of glomeruli (score 0). Moreover, despite the high number of seeded cells, most glomerular capillaries were patent. Interestingly, into the medulla some mES cells were located in the peritubular capillary (Fig. 5E). More specific localization of these cells is reported in Figure 6B–E. As shown by serial sections (3 μ m thickness), infused cells

FIG. 5. Repopulation of kidney scaffolds with mES cells. Hematoxylin and eosin staining at 24 h (A, C, E) and 72 h (B, D, F) and immunohistochemistry to WGA agglutinin and DAPI at 24 h (G) and 72 h (H) of kidney scaffold seeded with mES cells show homogeneous distribution of cells into glomerular, vascular structures, peritubular capillaries, and tubules. mES, murine embryonic stem.

repopulated peritubular capillaries that appear interposed between adjacent tubules.

Immunohistochemical analysis of recellularized kidney

As assessed by PCNA staining, the majority of mES cells seeded in the scaffolds appeared to proliferate after at least 72 h of perfusion with recirculating culture medium (Fig. 7A, C). The mean percentage of proliferating cells (obtained counting from 2300 to 3400 cells) was $77.0\% \pm 7.9\%$ and $86.0\% \pm 7.5\%$ at 24 and 72 h, respectively. To evaluate vi-

ability of cells, we investigated cell apoptosis. As shown in Figure 7B and D, we observed few apoptotic cells, both at 24 and 72 h after perfusion. The mean percentage of apoptotic cells (obtained counting from 1000 to 1200 cells) was $3.2\% \pm 2.7\%$ and $1.3\% \pm 1.3\%$ at 24 and 72 h, respectively. These results suggest that the scaffold maintained innate matrix components that promote viability of progenitor cells.

To investigate whether mES cells undergo differentiation when seeded in the kidney scaffold, we analyzed the expression of pluripotency and mesoderm-derived endothelial

FIG. 6. Mosaic view of a transversal cross section of recellularized kidney stained with hematoxylin and eosin (A) demonstrating a homogeneous repopulation of glomeruli by mES cells. Hematoxylin and eosin image sequence of serial sections of recellularized kidneys (B–E) showing that mES cells are located in peritubular capillaries between adjacent renal tubules. A single tubular loop around a peritubular capillary is marked by asterisks.

precursor markers. We studied, at different time points of cell perfusion, the expression of Oct-4, a marker of embryonic stemness, NCAM, a marker of mesoderm precursors, and the endothelial cell markers Tie-2 and CD31. As shown in Figure 8, cultured mES cells expressed high levels of Oct-4 before perfusion. After 24 h, cells showed a reduction of Oct-4 expression that was almost completely lost after 72 h, indicating a progressive lack of cell pluripotency during this time.

NCAM, absent in cultured mES cells, was slightly expressed at 24 h and markedly expressed at 72 h, suggesting that cell contact with 3D renal matrix induced progressive cell differentiation toward mesoderm-derived endothelial precursors. The membrane markers Tie-2 and CD31 were expressed in cells within the glomerular capillaries already at 24 h and the staining was maintained for Tie-2 and increased for CD31 at 72 h. The staining for these markers was negative in cultured cells before perfusion in kidney scaffold.

FIG. 7. Immunohistochemistry of PCNA at 24 h (A, original magnification 20 $\times$) and 72 h (C, original magnification 20 $\times$), of mES cells in kidney scaffold. Positive immunoreactive cells are colored brown. Immunofluorescence of caspase-3 at 24 h (B, original magnification 20 $\times$) and 72 h (D, original magnification 20 $\times$), of mES cells in kidney scaffold. Positive apoptotic cells are colored red. PCNA, proliferating cell nuclear antigen.

FIG. 8. Immunofluorescence staining for Oct-4, NCAM, Tie-2, and CD31 (original magnification $40\times$) in mES cells in culture and maintained in kidney scaffolds for 24 and 72 h (scale bars represent $20\mu\text{m}$). NCAM, neuronal cell adhesion molecule; Oct-4, octamer-binding transcription factor-4.

Discussion

Recent advances in tissue engineering have lead to the production of simple tissues such as vessels, urethras, tracheas, and bladders,^{19–21} where thin layer of cells and a supporting scaffold can be implanted without reconstituting the tributary vasculature.²² In these engineered tissues, host cell infiltration is usually obtained. Reconstitution of more complex organs, however, requires a scaffold with a complete vasculature, to provide nutrients and oxygen for supporting the cellular compartment,^{22,23} and the possibility to repopulate different cell populations along the entire organ structure. Despite these difficulties, some promising attempts have been made for whole organ engineering²⁴ and particularly in kidney engineering. Several aspects distinguish our present approach from previously reported studies.^{13,25,26} We simplified the decellularization setup with the use of only one detergent (SDS) that maintained the 3D structure and composition of ECM, avoiding the toxicity due to prolonged use of other chemicals. We shortened the time required for cell removal (17 h) that better preserved the integrity of the kidney matrix. Our perfusion experiments allow to obtain efficient and uniform intrarenal cell engraftment achieved by controlled perfusion conditions. At

variance to previous reports, we used ES cells to repopulate kidney matrix and we obtained evidence of embryonic cell differentiation toward vascular phenotype.

Our present study builds on previously reported techniques for production of renal ECM scaffold from rat kidneys.¹³ Here, we show that the cellular compartment of rat kidneys was completely removed, leaving behind an acellular ECM scaffold, which retains its complex 3D structure, by perfusing the organ with only SDS anionic detergent, instead of a combination of ionic and nonionic substances such as Triton X-100, as in the previously published protocols.¹³ As compared to the 5 day protocol previously reported,¹³ our shorter infusion of SDS detergent under physiologic pressure conditions allowed complete cell removal. SDS is an anionic detergent that allows rapid cell disruption and clearance of nuclear and cytoplasmic residues.^{25,27} Similarly, Triton X-100 was used to solubilize hydrophobic cell membranes, lyse cells, and remove cytoplasmic contents,^{8,13} but it may also damage the collagen structure.²⁸ Indeed, there is evidence that treatment of rat tendon with Triton X-100 disrupted the collagen structure, an effect not seen with SDS.²⁸ Moreover, cell removal was greater with SDS than with Triton X-100.²⁸ These results, however, do not match those obtained with Triton treatment

of both pericardial and heart valve tissues^{29,30} and of animal liver and kidneys in organ decellularization studies.^{13,31,32} It appears that the effects observed using decellularization protocols with Triton are dependent on the type of tissue. Nevertheless, our findings indicate that complete decellularization of rat kidneys can be safely achieved using SDS alone as detergent, as documented by the histologic and immunofluorescence findings that confirmed complete cell removal without loss of structural ECM proteins.

We perfused kidneys using a peristaltic pump, and we controlled perfusion conditions during the entire decellularization process to ensure monitoring of perfusion pressure. This approach differs from the gravity-based perfusion system adopted by Ross *et al.*,¹³ and eventually may have contributed to faster cell removal. The controlled perfusion-based decellularization procedure was effective also in maintaining kidney architecture. This is demonstrated by findings of intact, well organized, and uniform structure and ultrastructure of glomerular and tubular basement membranes at TEM and SEM. Further, as shown by micro-CT imaging of decellularized kidneys obtained during Microfil infusion, the acellular scaffold maintained the integrity of the renal vascular network. Actually, the arterial compartment was linked to venous vessels by preserved capillary bed, and this is the basic requirement for successful organ cellularization.

The innate ECM scaffold structure is considered critical to direct cell seeding and the reconstitution of the cellular compartment, and eventually organ regeneration.²⁴ It can be anticipated that the persistence of the intact glomerular, tubular, and vascular structures within the renal ECM scaffold with native chemical signals would facilitate the recognition by precursor cells of their natural niche along the nephron and vascular segments during the recellularization process. Thus, another aim of our study was to assess the ability of the obtained renal ECM scaffold to drive proper seeding and adaptation of repopulating cells. Given the complexity of kidney structure, which includes over thirty different cell types, we used ES cells to seed the whole-kidney scaffolds. ES cells are available as established cell lines; they have the ability to grow indefinitely while maintaining pluripotency, and they have the potential to develop into and form any embryonic organ *in vivo*.^{18,33} Moreover, ES cells are a potential source of renal cells for regenerative therapy thanks to their capacity to differentiate into any of the adult renal cell type.³⁴ High efficiency intrarenal cell engraftment was achieved by infusing mES cells via renal artery at low pressure. At variance with previous scaffold repopulating protocols in static culture conditions,¹³ we choose to maintain the cellularized scaffolds under pressure-control perfusion setting with recirculating cell medium, eventually allowing more adequate oxygen delivery into the entire scaffold volume and avoiding cells entering apoptotic process. With this approach we were able to obtain a uniform distribution of the seeded mES cells into the vasculature up to glomerular capillaries, as confirmed by morphometric analysis of cell seeded inside individual glomeruli, with some cells reaching a peritubular capillary localization. Despite the massive presence of cells, the lumen of capillary segments was still patent, and perfusion of culture media allowed adequate delivery of nutrients and removal of metabolic waste products. The

uniform mES cell distribution within the kidney scaffolds was achieved within 24 h and was maintained until 72 h of perfusion. Proliferation of mES cells was observed during 72 h of scaffold perfusion. These findings further confirm the integrity of the underlying renal ECM scaffolds, since for cells to function they must be properly supported, having contact with neighboring cells and ECM. Our results provide evidence for the feasibility of accelerating the whole-kidney scaffold cell repopulation using controlled perfusion conditions.

Occasionally, we observed mES cells also in the tubular compartment of the scaffolds, specifically in peritubular capillaries, suggesting that potential repopulation of tubular nephron segments may effectively take place even with perfusion of cells through the renal artery and after only 72 h. Since it is known that cells respond to the mechanical and biochemical changes in ECM through the crosstalk between integrins and actin cytoskeleton,³⁵ the possibility exists that the perfusion pressure maintained in peritubular capillary during the recellularization process can promote interaction of mES cells and ECM, inducing changes in cell shape and motility, and eventually migration to repopulate the peritubular capillary bed.

It is well accepted that cell-ECM interaction and 3D microenvironment play a role in cell morphology, migration, proliferation, and differentiation. The basement membrane matrix, which underlies epithelial and endothelial cells, has been shown to facilitate the differentiation and formation of tissue-like structures of many cell types. As shown by Cortiella *et al.*,³⁶ acellular lung allowed for better retention of cells with more differentiation of mESCs into epithelial and endothelial lineages.³⁷ In addition, 3D cultures have been shown to improve differentiation of ES cells through increased cell-cell and matrix association.³⁸ Cultured mES cells within a 3D scaffold can achieve higher hematopoietic differentiation efficiency than traditional 2D suspension cultures.³⁹ Here, we have documented that embryonic stem cells, when perfused in a decellularized renal vascular compartment, were induced by 3D ECM to alter their phenotype and to differentiate into meso-endodermal lineage. The results of our immunohistochemical analysis suggest that mES cells infused into kidney scaffold, progressively lost their stemness during time. Moreover, we demonstrated an increase in glomerular labeling for NCAM, an adhesive molecule that represents a marker of mesodermal-derived renal progenitors and endothelial precursors.⁴⁰ A positive staining for Tie-2 and CD31, markers of endothelial lineage, was also observed after infusion in kidney scaffold. Altogether these data suggested a positive role of signals contained in kidney ECM and of 3D structure in influencing the commitment of infused cells.

Very recently, Song and co-workers²⁶ reported that perfusion with differentiated cells, endothelial cells, and neonatal kidney cells of a decellularized kidney matrix resulted in a recellularization of the kidney scaffold, very similar to that obtained in our present experiments. Of interest, these authors did perfuse cells in the kidney scaffold not only through the renal artery, but also by the ureter maintaining, during cell perfusion, a negative pressure outside the kidney. Despite the double infusion, even after several days of culture, most cells were observed, as in our present investigation, at the level of glomerular capillaries; very few cells

have been observed in the tubular compartment. Altogether, these results suggest that complete kidney repopulation is a process that has to be still improved. The way to obtain this important goal may likely depend upon the use of pluripotent stem cells in combination with more favorable pressure and flow distribution along the complex structure of the kidney matrix.

In conclusion, we showed that the implemented decellularization protocol we set up successfully removed all cellular components from rat kidneys in short time, preserved ECM, vasculature architecture, glomerular capillaries, and tubular membrane. The successful rapid recellularization of vascular structures, glomerular capillaries, and occasionally peritubular capillaries with dynamic perfusion at physiologic pressure of the acellular scaffolds paves the ground for more extensive experimental investigations with prolonged culture to achieve uniform cellularization within the tubular compartments of the whole-kidney scaffold, and it possibly induces differentiation of embryonic cells into renal somatic cell.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Andrea Remuzzi, EngD

Department of Biomedical Engineering

IRCCS—Istituto di Ricerche Farmacologiche Mario Negri

Via Stezzano, 87

24126 Bergamo

Italy

E-mail: andrea.remuzzi@marionegri.it

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