METHODS ARTICLE

Dual-Purpose Bioreactors to Monitor Noninvasive Physical and Biochemical Markers of Kidney and Liver Scaffold Recellularization

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Analysis of perfusion-based bioreactors for organ engineering and a detailed evaluation of physical and biochemical parameters that measure dynamic changes within maturing cell-laden scaffolds are critical components of *ex vivo* tissue development that remain understudied topics in the tissue and organ engineering literature. Intricately designed bioreactors that house developing tissue are critical to properly recapitulate the *in vivo* environment, deliver nutrients within perfused media, and monitor physiological parameters of tissue development. Herein, we provide an in-depth description and analysis of two dual-purpose perfusion bioreactors that improve upon current bioreactor designs and enable comparative analyses of *ex vivo* scaffold recellularization strategies and cell growth performance during long-term maintenance culture of engineered kidney or liver tissues. Both bioreactors are effective at maximizing cell seeding of small-animal organ scaffolds and maintaining cell survival in extended culture. We further demonstrate noninvasive monitoring capabilities for tracking dynamic changes within scaffolds as the native cellular component is removed during decellularization and model parenchymal cells are introduced into the scaffold during recellularization and proliferate in maintenance culture. We found that hydrodynamic pressure drop (ΔP) across the retained scaffold vasculature is a noninvasive measurement of scaffold integrity. We further show that ΔP , and thus resistance to fluid flow through the scaffold, decreases with cell loss during decellularization and correspondingly increases to near normal values for whole organs following recellularization of the kidney or liver scaffolds. Perfused media may be further sampled in real time to measure soluble biomarkers (e.g., resazurin, albumin, or kidney injury molecule-1) that indicate degree of cellular metabolic activity, synthetic function, or engraftment into the scaffold. Cell growth within bioreactors is validated for primary and immortalized cells, and the design of each bioreactor is scalable to accommodate any three-dimensional scaffold (e.g., synthetic or naturally derived matrix) that contains conduits for nutrient perfusion to deliver media to growing cells and monitor noninvasive parameters during scaffold repopulation, broadening the applicability of these bioreactor systems.

Introduction

NEARLY 124,000 PATIENTS are wait-listed for an organ transplant in the United States, yet fewer than 29,000 transplants are performed each year.¹ The development of new laboratory-derived tissues may one day improve the outcome of patients waiting for an organ transplant by both reducing the

wait time and—given the potential to use patient-derived cells through induced pluripotent stem cell technology—eliminating the need for lifelong immunosuppression.

Bioengineered tissue combines three-dimensional (3D) extracellular matrix (ECM) scaffolds with tissue-specific cells, but matrix size, complexity, and high cell number necessitate growth of recellularized scaffolds within perfusion

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bioreactors that deliver essential nutrients (e.g., oxygen and glucose) to cells deep within developing tissue by infusion through retained vascular conduits within the scaffold matrix.² Bioreactor systems recapitulate the niche microenvironment leading to cell and tissue development; therefore, a high degree of attention to detail in bioreactor construction is crucial for successful long-term development of functional engineered tissues and organs.

Despite the importance of perfusion bioreactors to the engineering of complex 3D tissue, a complete detailed evaluation of bioreactor development and a thorough analysis of noninvasive physical and biochemical markers indicative of tissue maturation and function are deficient in the current bioengineering literature. Decellularization of livers and kidneys through perfusion of detergent solutions through the vasculature has been well documented^{3,4}—with technical feasibility demonstrated using both large animal and human-scale organs^{5–8} as well as small animal organs for repopulation with stem or immature progenitor cells.^{7,9–13} The current challenges and limitations to organ and tissue engineering have shifted to the cellular repopulation phase of tissue development where donor cells are reintroduced into whole-organ ECM scaffolds; however, very little detail is typically provided describing bioreactor construction despite the identification of specific design features that are critical to optimize scaffold recellularization and overall function.¹⁴

Herein, we discuss the evolution of two bioreactors for liver and kidney scaffold recellularization and address specific design considerations that not only allow these devices to achieve optimal cell delivery to each scaffold but also allow for noninvasive monitoring of cell growth. Specifically, given the inherent complexity of the renal architecture, kidney scaffold recellularization has been particularly challenging.^{7,10,15,16} To overcome this limitation, we provide new detail on bioreactor features that allow for comparison of alternative seeding strategies and demonstrate improved renal epithelial cell dispersion throughout the tubular compartment of decellularized scaffolds.

Measurement of incremental cell growth and tissue maturation by histological evaluation or gene expression analysis requires premature termination of maturing tissue within bioreactors. A more efficient approach is to monitor tissue development noninvasively through soluble proteins (e.g., biomarkers) released from cells and/or by sensors that measure physical parameters, such as perfusion pressure. To develop a nondisruptive tracing of dynamic changes associated with scaffold preparation by perfusion decellularization followed by cell maturation within the matrix, we adapted a strategy currently used to assess graft organ quality in clinical renal transplantation. In particular, cadaveric donor kidneys are perfused on a peristaltic pump before transplantation and flow rate and resistance to flow through the organ correlate with clinical outcomes.¹⁷ Using our improved bioreactor systems, we show that noninvasive measurements of pressure drop (ΔP) during fluid perfusion across an organ scaffold provides insight into (1) initial cellular distribution following recellularization, (2) extent of parenchymal repopulation (e.g., initial seeding efficiency and cell proliferation), and (3) may indirectly indicate ECM remodeling while tissues develop during maintenance culture.

To provide a complete real-time analysis of tissue growth, these bioreactor systems also allow for sampling of soluble

biomarkers within media (e.g., reduced resazurin, kidney injury molecule-1 [KIM-1], and albumin) that indicate specific elements of cellular functionality. Because strict consideration to optimal bioreactor construction and assessment of noninvasive markers of tissue maturation is critical for optimal *ex vivo* tissue development, we provide granular detail and analysis of bioreactor construction and assessment of noninvasive markers to improve dissemination of bioreactor technology to a broader group of investigators to address this particularly challenging area of 3D tissue development.

Materials and Methods

Organ recovery

All procedures involving animals were performed according to the guidelines approved by the Institutional Animal Care and Use Committee of the Northwestern University. Kidneys were recovered from male Sprague Dawley rats (200–250 g) as described.¹³ Briefly, 24G catheters (No. 381412, BD Insyte Autoguard; BD Biosciences) were inserted into the renal artery and ureter of each kidney. The renal vein was retained and left open for media to drain during bioreactor operation. The liver was recovered while removing kidneys. The portal vein was cannulated with an 18G catheter (No. 381447, BD Insyte Autoguard; BD Biosciences). The vena cava was resected and left open to allow media to drain from the scaffold during perfusion in the bioreactor. Once organs were isolated, cold phosphate-buffered saline (PBS) was perfused until all blood was cleared. All organs were stored in PBS at -20°C .

Perfusion bioreactor assembly

Kidney bioreactor. The bioreactor chambers for both the kidney and liver scaffolds are made of borosilicate laboratory glass. The kidney chamber measures 5.5×11 cm and is designed as a vertical suspension vessel to provide extra headspace to allow suspension of kidney scaffolds within an adjustable atmosphere over the culture medium (Fig. 1). The design principle is adapted from early studies in cardiac physiology^{18,19} and more recent investigations using negative pressure gradients across renal scaffolds during recellularization.⁷ Here, we have further refined the system by improving long-term culture capacity, providing utility for comparative evaluation of seeding techniques and validation of noninvasive measurements of cell growth within the scaffold. The top (head) and bottom (body) sections are outfitted with flanges containing an O-ring groove (a). When clamped together, both sections create a gas-tight seal. The body of the bioreactor vessel contains a media reservoir. Vacuum is provided through an adjustable valve near the apical portion of the vessel body (b). The head of the bioreactor consists of five parts: a valve (c), cap (d), two Luer acceptors (e, f), and a tubing connection (g). The valve (c) is closed to seal the chamber during vacuum application to the intravessel environment and may later purge the system through a sterile filter to ventilate the chamber and aerate perfused medium during maintenance culture. The screw cap (d) can be opened to sample or replenish perfusion medium under aseptic conditions by detaching the perfusion tubing from the pump head and transferring the bioreactor,

FIG. 1. Detailed schematic representation of kidney recellularization bioreactor. Photographs of the kidney bioreactor (A, C–E) and cross-sectional schematics (B) illustrate the geometry and components of the bioreactor vessel: (a) flange, metallic clamp, and O-ring to seal the head and body of the bioreactor, (b) adjustable vacuum valve, (c) adjustable vent connected to sterile filter for gas exchange and pressure equilibration with the surrounding environment, (d) cap for media addition/removal and sampling in a biological safety cabinet, (e) inlet Luer acceptor to a cannula within the renal artery to perfuse the vasculature, (f) ureter Luer acceptor, (g) media perfusate outlet, (h) kidney scaffold, and (i) silicone rubber tubing to withdraw media into the bioreactor outlet and back to the recirculation pump. The reactor dimensions are shown in (B). Up to four replicate perfusion circuits can be run in parallel for each pump, as shown in (D). High-magnification image (E) shows where the medium flows into the kidney scaffold through the renal artery and may freely flow out from the scaffold (h) through the open renal vein (data not shown). Color images available online at www.liebertpub.com/tec

with its attached perfusion circuit, to a biological safety cabinet. The screw cap is removed, and the medium may be withdrawn using a small-volume pipette (10 mL or less). The renal scaffold is connected to two Luer (e, f) ports—the primary Luer adapter (e) attaches to a catheter within the scaffold's renal artery and a secondary catheter may be inserted into the ureter (f). During operation, the medium is withdrawn from a silicone rubber tube (i) submerged in the media reservoir and travels through the media perfusate outlet (g) to a continuous peristaltic pump (No. HV-07523-80, Masterflex L/S) and is returned to the developing scaffold through the inlet Luer acceptor leading to the primary catheter (e) (Fig. 1C).

Liver bioreactor. Liver bioreactor chambers measure 10.5 × 8 cm (Fig. 2). Both head and body sections are constructed with the same flange and groove (a) described for

the kidney bioreactor. Stopcock valves are incorporated into the head and body (b, c) to ventilate and drain the bioreactor chamber, respectively. The cap (d) is used for aseptic sampling, withdrawing, and replacing media, using the same method described for the kidney bioreactor. During operation, the medium is withdrawn directly from the media perfusate outlet (f) to the peristaltic pump and returned to the developing scaffold (g) through the inlet Luer acceptor leading to the primary catheter (e) (Fig. 2D). All glass vessels were manufactured by Wilmad-LabGlass.

Kidney and liver decellularization

All organs were gradually thawed at room temperature. Kidneys were decellularized using a Triton X-100 mixture followed by a sodium dodecyl sulfate solution as described.¹³ Whole livers were decellularized using a protocol

FIG. 2. Detailed schematic representation of liver recellularization bioreactor. Photographs of the liver bioreactor (A, C–E) and cross-sectional schematics (B) illustrate the cylindrical geometry of the vessel: (a) flange, metallic clamp, and O-ring to seal the bioreactor, (b) adjustable vent connected to sterile filter for gas exchange and pressure equilibration with the environment, (c) adjustable valve for draining media, (d) cap for media addition/removal and sampling in a biological safety cabinet, (e) inlet Luer acceptor connected to a cannula in the portal vein used to perfuse hepatic vasculature, (f) media perfusate outlet, (g) liver scaffold, and (h) restraint to hold the liver scaffold below the liquid level. The reactor dimensions are shown in (B). High-magnification image (C) shows the catheterized hepatic portal vein where media flows into the liver scaffold (g); media may then freely flow out from the scaffold through the open vena cava where it mixes with the surrounding medium. The liver scaffold is submerged by the restraint (h), as shown in (D). Perfusion into the liver scaffold is controlled by a peristaltic pump, as shown in (E). Color images available online at www.liebertpub.com/tec

similar to that described by Baptista *et al.*⁹ After decellularization, the left and medial lobes were isolated, ligated, and resected from the scaffold leaving only the right and caudate lobes (referred to hereafter as the “right lobe”) to limit the number of HepG2 cells needed for recellularization. All decellularized scaffolds were stored in PBS at 4°C for a maximum of 2 weeks before use.

Recellularization

Kidney scaffolds. Model parenchymal cells are used to repopulate rodent-derived scaffolds with the long-term goal of evaluating interactions between organ-specific ECM components and parenchymal cells in a small-scale model system that can eventually be scaled up to larger-sized scaffolds for future clinical translation. Decellularized

scaffolds were placed in the bioreactor chamber (Fig. 1), connected to the primed perfusion circuit through the renal artery catheter, and sterilized by perfusion with 50 mL 0.1% peracetic acid/4% ethanol for 1 h at 5 mL/min.²⁰ The acidified ethanol was aspirated, and pH was balanced by three sequential 1 h washes with 50 mL 1 × PBS (pH = 7.4) at 5 mL/min. PBS was exchanged for 50 mL DMEM/F-12 (No. 11320-082, Gibco; Life Technologies) with 10% fetal bovine serum (FBS; No. 35-010-CV, Corning) and 1% penicillin/streptomycin. Bioreactors were transferred to an incubator and perfused at 5 mL/min for at least 1 h. For seeding, perfusion was halted, and 40 million immortalized Madin-Darby canine kidney (MDCK) cells^{21,22} or 6.25 million primary human renal papillae-derived CD133/1⁺ cells from anonymous cadaveric donors²³ were loaded into a syringe (2 mL volume) and manually injected at ~2 mL/min

through a three-way stopcock into the ureter (Fig. 1, marker f) or the renal artery (Fig. 1, marker e). MDCK cells are a well-established immortalized line of renal epithelial cells. They are characterized by a polygonal appearance in two-dimensional culture, typical of renal epithelial cells, and become polarized to form primary cilia.^{21,22} Before ureteral seeding, vacuum was applied to the chamber, lowering the gauge pressure to -40 mmHg. After injection into the ureter, cells were allowed to attach for 10 min before flow through the bioreactor circuit was resumed at 4 mL/min under normal atmospheric pressure. For arterial seeding, the scaffold was immediately perfused at 25 mL/min for 15 min through the bioreactor circuit to perfuse cells further into the scaffold. The flow rate was subsequently decreased to 4 mL/min. The medium was exchanged every 2 days.

Liver scaffolds. Liver bioreactors were fitted with perfusion circuit tubing (Fig. 2) and autoclaved. The system was transferred to a biological safety cabinet, and the bioreactor reservoir was filled with 200 mL of 0.1% peracetic acid/70% ethanol.²⁰ After priming the circuit, the right lobe scaffold was connected (by portal vein catheter) to the chamber's inlet Luer acceptor (Fig. 2, marker e) and perfusion started at 6 mL/min for 1 h to sterilize the scaffold. The acidified ethanol was drained, and the organ was perfused three times at 6 mL/min for 1 h each with 200 mL fresh sterile $1 \times$ PBS. PBS was exchanged for 200 mL DMEM/F-12 with 10% FBS and 1% penicillin/streptomycin. Due to the difficulty of culturing primary human hepatocytes, and their loss of functionality upon cryopreservation and transport, we use a well-established model hepatocyte line to evaluate recellularization within the bioreactor and measure noninvasive indices of cell growth and function during maintenance culture. A total of 50 million human HepG2 hepatocarcinoma cells (immortalized line; No. HB-8065, ATCC) were injected into the portal vein cannula in two separate inoculations of 10 mL each at 2 mL/min through a three-way stopcock placed directly upstream of

the inlet Luer acceptor (Fig. 2, marker e). The bioreactor was moved to a 5% CO₂ incubator at 37°C and after 10 min flow was resumed at 6 mL/min. The medium was exchanged every 2–3 days.

Pressure drop measurement at defined flow rates

The ΔP is defined as the pressure drop across the scaffold (where ΔP = absolute inlet pressure – ambient hydrostatic pressure). The ΔP testing system was configured as shown in Figure 3. Scaffolds were immersed in PBS in the reservoir of a liver bioreactor, as shown in Figure 2. PBS was circulated by external silicone rubber tubing from the outlet of the reservoir through a positive displacement pump (Fig. 3, marker a, STRH Pump Drive Module, H1CTCLF Pump Head, and SCST-01 Stepper Controller Kit; Fluid Metering, Inc.; set to 0–20 mL/min) to a 0–260 mmHg pressure transducer (Fig. 3, marker b, Honeywell FP2000), through the bioreactor inlet and into the cannulated scaffold, and finally drained into the bulk fluid maintained at atmospheric pressure. A LabView (National Instruments) program was created to control pump speed and collect data from the pressure transducer through an internet hookup. The transducer signal was interpreted by a chassis (cRIO-9076 with NI9205 and NI9263 modules, National Instruments, Fig. 3, marker c) connected to the pressure transducer amplifier (Honeywell GM-A, Fig. 3 marker e). To measure ΔP at discrete flow rates, the pump was programmed to perform a series of 1 mL/min step changes lasting 30 s each until a maximum flow rate of 20 mL/min was reached. Data were collected once per second. The pressure transducer (Fig. 3, marker b) was housed in a custom polycarbonate flow cell machined at the Rehabilitation Institute of Chicago machine shop.

For further information on histological staining methods, biochemical assessment of soluble factors, and statistical analysis, see the Supplementary Materials and Methods section (Supplementary materials are available online at <http://www.liebertpub.com/tec>).

FIG. 3. Pressure drop (ΔP) measurement system. The perfusion circuit used to measure ΔP in kidneys or livers is shown in a photograph (A) and diagram (B). Various components of the perfusion flow circuit and data acquisition apparatus are shown: (a) Fluid Metering, Inc. controllable step pump, (b) Honeywell pressure transducer (0–260 mmHg), (c) National Instruments chassis used to collect data from the amplifier, (d) custom glass vessel to contain the organ/scaffold within a reservoir containing physiologically equilibrated fluid phosphate-buffered saline that is perfused during evaluation, (e) Honeywell amplifier to convert transducer signal and transport data to chassis.

Results and Discussion

Kidney bioreactor design

Bioreactors were constructed from glass to allow sterilization by autoclaving, chemical treatment, or irradiation. Unlike polyvinyl chloride (PVC) or metal composites, glass does not contain plasticizers or oils that may leach into the culture medium.^{24,25} We previously described a horizontal perfusion bioreactor in which the kidney scaffold is immersed in the culture medium.¹³ However, to allow for more effective media sampling and establishment of negative trans-scaffold pressure by applying vacuum to the environment surrounding the scaffold, this bioreactor was redesigned in a vertical configuration with media sampling through the chamber head (Fig. 1A). As a further design improvement, two Luer adapters were added to the vessel head for direct delivery of cells into either the scaffold renal artery or collecting duct system through the decellularized ureter. The scaffold is suspended by these catheters that are configured to prevent kinking of the renal artery or ureter while the scaffold is held in the air space above the gas-liquid interface (Fig. 1E). Stopcocks incorporated into previous design were removed to decrease the risk of contamination within small pockets of stagnant media that may accumulate inside the valve stem during sampling, which is now completed aseptically through screw caps on the vessel head after transfer to a biological safety cabinet (Fig. 1, marker d).

This design improves upon other bioreactors described briefly in the literature⁷ by permitting both seeding under vacuum and extended perfusion culture within the same vessel, thus decreasing the risk for contamination as the recellularized scaffold does not require transfer to a maintenance bioreactor chamber. The use of two Luer acceptors for catheters further allows for direct comparison between infusion of cells through either the renal artery or the ureter.

Characterization of native, decellularized, and recellularized renal scaffolds

Change in hydrostatic pressure across the kidney scaffold (ΔP) was measured using the system illustrated in Figure 3. PBS was perfused through a cannula placed in the arterial system of the renal scaffold over the range of 0–20 mL min⁻¹, and the resulting pressure was recorded by a pressure transducer (0–260 mmHg) within the perfusion circuit. We assessed ΔP throughout a range of perfusion flow rates to obtain raw ΔP profiles (Supplementary Fig. S1); however, for consistency and ease of comparison at different stages in the decellularization and recellularization processes, we compared ΔP at the normalized physiological rodent renal blood flow rate of 1.35 mL · min⁻¹ · g⁻¹.²⁶ Furthermore, to determine the true ΔP across the scaffold, the system contribution (i.e., resistance to flow contributed by tubing, catheters, and pump) was determined (Supplementary Fig. S1A, System-Run 1 and System-Run 2) and subtracted from raw ΔP to obtain the true corrected value at a flow rate of 1.35 mL · min⁻¹ · g⁻¹ for direct comparison of scaffolds at each stage (Fig. 4A).

Following decellularization, kidney scaffolds became translucent with intact vasculature clearly visible (Fig. 4B). We previously characterized decellularized renal scaffolds

to show that ~95% DNA is removed along with complete native cell removal with the maintenance of microstructural features (glomeruli, tubules) and a porous architecture (Fig. 4B, native vs. decellularized [decell]).¹³ Despite perfusion of PBS through the scaffold at elevated pressure to determine ΔP , and thus scaffold mechanics, the structural integrity of kidney scaffolds was maintained and not damaged by elevated perfusion pressures as assessed by both histology (Fig. 4B, native vs. decell) and an analysis of hysteresis curves that describe sequential variable-flow rate tests for ΔP that yield identical pressure-flow profiles for normal kidneys or freshly decellularized scaffolds (Supplementary Fig. S1A, Native-Run 1 vs. Native-Run 2 and Decell-Run 1 vs. Decell-Run 2).

This system allows for noninvasive pressure tracing during the entire course of the decellularization process, and the ΔP was found to decrease over time during decellularization but is dependent on the flow rate during decellularization (Supplementary Fig. S2A). We then compared ΔP at the conclusion of the 26-h decellularization process to evaluate the effect of full decellularization on ΔP (Fig. 4A, Decell). After decellularization, ΔP decreased from 74.4 ± 33.3 mmHg (average ± standard deviation) in normal kidneys to 29.1 ± 12.2 mmHg in decellularized scaffolds ($p < 0.001$, Fig. 4A), tracking with the change in matrix histology as cells are removed during decellularization (Fig. 4B, Native vs. Decell). On a per-kidney basis, decellularization resulted in a maximum decrease in ΔP by 73%. Of the 19 kidneys evaluated, ΔP decreased by 25–75% in 15 scaffolds, while three did not change and a singular scaffold was an outlier, increasing by 80% and removed from further analysis (Supplementary Fig. S3). This observed reduction in ΔP following decellularization is in line with previous observations by Orlando *et al.* for human kidney decellularization, providing proof-of-concept for this smaller-scale model system for rodent scaffolds, which are more advantageous for future repopulation with stem or progenitor cells due to the limited availability of these cell populations.⁸

We then went further to evaluate the influence of scaffold recellularization on ΔP . We first assessed a subset of acellular scaffolds perfused within the bioreactor at 37°C for 7 days without cells to evaluate matrix architecture and the effect on ΔP during maintenance culture. We refer to these as “process” scaffolds as a control to evaluate changes in acellular scaffold integrity over time. In comparison to freshly decellularized scaffolds, we found that ΔP in process scaffolds increased from 29.1 ± 12.2 to 52.7 ± 10.6 mmHg after 7 days of perfusion culture without cells (Fig. 4A, Decell vs. Process, $p = 0.370$). It was evident from the histological sections of 7-day process scaffolds that the ECM degraded in the absence of a cellular component, resulting in the formation of ECM aggregates embedded within the scaffold. This increasing trend in ΔP is likely due to the obstruction of microfluidic flow (Fig. 4B, Decell vs. Process scaffolds), as others have suggested that emboli of cell culture components may increase trans-scaffold resistance.⁷

Furthermore, unlike earlier stages in scaffold preparation, we observed a small, but consistent, degree of hysteresis caused by perfusion of PBS through 7-day process scaffolds during evaluation of matrix mechanics, suggesting that the matrix is more fragile upon interrogating the 7-day process

FIG. 4. Comparison of pressure drop and morphological characteristics of kidney scaffolds during decellularization and recellularization. **(A)** The mean ΔP across the organ (mean $\pm$ standard deviation) at a constant normalized flow rate of $1.35 \text{ mL} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ for native ($n=17$), decellularized (Decell; $n=18$), decellularized process control kidneys perfused with medium for 7 days without cells (Process; $n=4$), kidney scaffolds recellularized through the ureter after 3 days (day 3; $n=8$) or 7 days (day 7; $n=3$), or kidneys scaffolds recellularized through the renal artery after 3 days (day 3; $n=6$) or 7 days (day 7; $n=4$). #Over each bar indicates a significant difference ($p < 0.05$) as determined by the Tukey HSD compared to Decell. *Indicates a significant difference ($p < 0.05$) as determined by the Tukey HSD between the groups specified. **(B)** Macroscopic images of native and decellularized kidneys, and representative images of hematoxylin and eosin (H&E)-stained histological sections from each condition. Macroscopic image scale bars represent 5 mm, while H&E scale bars represent 100 μm . **(C)** The mean metabolic activity in artery-seeded ($n=3$) or ureter-seeded ($n=3$) scaffolds at days 1, 3, 5, or 7 after seeding. #Indicates a significant difference in metabolic activity in artery-seeded scaffolds compared to all other time points evaluated. Calculated number of cells, based on a standard curve as described in the Supplemental Materials and Methods section, is shown in the secondary vertical axis. HSD, honestly significant difference. Color images available online at www.liebertpub.com/tec

scaffold with our pressure-testing method. This observed hysteresis leads to a lower ΔP in sequential runs of the same scaffold (Supplementary Fig. S1A, Process-Run 1 vs. Process-Run 2). Together with histological data showing matrix deterioration, these findings suggest that this method may provide an indirect indicator of matrix degradation over time (Fig. 4B, Process).

We then evaluated two distinct strategies of introducing cells into renal scaffolds and their effect on recellularization efficiency, as assessed by scaffold histology and corresponding ΔP . MDCK cells were injected into the collecting system of decellularized renal scaffolds through the cannulated ureter while the scaffold was exposed to a brief -40 mmHg vacuum. Upon establishing the negative pressure gradient, the scaffold was noted to swell, which may facilitate the entry of cells into tubules through the collecting duct as suggested by Song *et al.*⁷ Having established that application of a temporary negative pressure environment does not alter ΔP (29.1 ± 12.2 vs. $27.9 \pm 7.0 \text{ mmHg}$ after application of -40 mmHg for 10 min, $p=0.831$), we recellularized kidney scaffolds with MDCK cells at -40 mmHg for 10 min before purging the bioreactor vessel. Cells were

cultured within the scaffold at normal-pressure maintenance culture within the bioreactor at 37°C .

After 3 days, ΔP slightly increased from $29.1 \pm 12.2 \text{ mmHg}$ (Fig. 4A, Decell) to $44.4 \pm 14.6 \text{ mmHg}$, but this change was not significant (Fig. 4A, Ureter-Seeded Day 3, $p=0.566$). MDCK cells injected through the ureter traveled through the collecting ducts to line more proximal tubular structures adjacent to glomeruli (Fig. 4B, Ureter-Seeded Day 3), although cells were sparsely distributed across the scaffold suggesting low seeding efficiency. However, after 7 days, ΔP increased significantly to $71.7 \pm 7.0 \text{ mmHg}$ (Fig. 4B, Decell vs. Ureter-Seeded Day 7, $p=0.024$). At this time, cells clearly outlined tubular structures to form more defined structures than noted earlier on day 3 (Fig. 4B, Ureter-Seeded Day 7) and were histologically similar to ureteral seeding methods used by Ross *et al.*¹⁰ and Song *et al.*⁷

We then considered an alternative approach to recellularize renal scaffolds by infusing MDCK cells through the renal artery at elevated pressure (25 mL/min or 232 mmHg).¹³ Unlike the ureteral method, we found ΔP to be significantly higher at 3 days (Fig. 4A, $29.9 \pm 12.2 \text{ mmHg}$ Decell vs. $68.4 \pm 13.5 \text{ mmHg}$ Artery-Seeded Day 3, $p=0.002$) and 7

days (Fig. 4A, 29.9 ± 12.2 mmHg Decell vs. 73.4 ± 16.0 mmHg Artery-Seeded Day 7, $p = 0.004$). MDCK cells infused through the renal artery formed hollow tubule-like structures throughout the scaffold, as noted with cells infused through the ureter, but arterial infusion resulted in improved cellular coverage (Fig. 4B, Artery-Seeded Day 7 vs. Ureter-Seeded Day 7), at a level consistent with our previous results.¹³ Taken together, both seeding strategies led to a return of ΔP to values approaching those of normal rodent kidneys at 7 days ($p = 1.000$, both cases compared to normal kidneys), but perfusion of cells into the arterial tree led to a quicker return of ΔP to near normal levels at 3 days and improved cell distribution within tubules, possibly due to enhanced translocation of cells from vessel matrices into the peritubular space.

The bioreactor design allows for sampling of endogenous or exogenous markers of cell functionality. As an example, we added resazurin to the culture media at different time points. Resazurin is a marker of cellular metabolism and indicates cell viability and proliferation without damaging the cells.^{27,28} This reagent is reduced intracellularly and leads to a color change in the perfusion media that is monitored by spectrophotometry. Consistent with the histological differences in cell density between arterial and ureteral seeding techniques, total metabolic activity (as determined by the resazurin perfusion assay) and calculated cell number were significantly higher at all time points evaluated (Fig. 4C, $p < 0.05$) in artery-seeded scaffolds compared to ureter-seeded scaffolds despite the return of ΔP to normal levels in ureter-seeded scaffolds at 7 days. This trend indicates that ΔP is a more complex measurement than cell number alone and also reflects dynamic matrix properties as well, such as matrix structure, and possibly remodeling, as suggested by experiments with process control scaffolds.

A significant increase in metabolic activity was observed in arterially recellularized scaffolds after 5 ($p < 0.001$) and 7 ($p < 0.001$) days, indicative of cell proliferation, whereas cell number in ureter-seeded scaffolds was stable (ANOVA, $p = 0.469$) (Fig. 4C). Placing this finding in context of other

methods of cell delivery into the scaffold, this method of tubular repopulation is more efficient compared to infusion of cells through the ureter or artery at flow rates (e.g., < 1 mL/min) which others have shown to preferentially restrict cells to glomeruli and nearby arterioles.^{7,10,15,16}

We additionally infused primary CD133/1⁺ human renal progenitor cells recovered from the renal papillae²³ using the arterial seeding method to validate the bioreactor system using primary cells. We observed similar dispersion of the cells throughout the renal cortex over a 3- and 7-day culture period (Fig. 5A). We found a sustained level of resazurin reduction indicating cellular viability with limited proliferation over the 1-week culture period (Fig. 5B). To demonstrate the ability to monitor endogenous biomarkers shed into the media from developing cells within the scaffold, we assessed media for KIM-1, a urinary biomarker of renal injury.²⁹ Production of KIM-1 was high on day 1, early in the culture period, and then decreased significantly at days 3, 5, and 7 (Fig. 5C, $p < 0.05$ in all cases compared to day 1). In conjunction with stable metabolic functionality indicating viability by resazurin reduction, the decline in production of this injury biomarker over time suggests cellular recovery after seeding into the scaffold and engraftment within the matrix during maintenance culture.

Liver bioreactor design

In designing the bioreactor vessels for both organs, we utilized a cylindrical model (Fig. 2) to facilitate mixing of the effluent media from the scaffold with residual media within the reservoir. This allows nonadherent cells to recirculate through the media circuit and into the scaffold to maximize overall recellularization. In the course of testing our bioreactor system, we found that smaller reservoirs restrict the volume of reserve media held within the perfusion circuit, leading to rapid nutrient depletion and alteration in cell metabolism. Our final bioreactor design therefore employs a cylindrical, round-bottom reservoir (to prevent

FIG. 5. Characterization of morphological adaptation, sustained viability, and decrease in injury biomarker expression by CD133/1⁺ cell-repopulated kidney scaffolds. (A) CD133/1⁺ cells repopulated renal cortical tubules but were not found in glomeruli (g) 3 days after seeding. After 7 days of culture, CD133/1⁺ cells aggregated to form tubule-like structures (arrows). (B) One hour resazurin perfusion assay performed every 2 days indicates that CD133/1⁺ cells remained viable within the kidney scaffold over 7 days with no statistically significant deviation during this time course ($p > 0.05$ by ANOVA). (C) Injury biomarker kidney injury molecule-1 (KIM-1) release from CD133/1⁺ cells decreases over 7 days suggesting adaptation and engraftment into renal scaffolds. #Denotes a significant difference as determined by the Tukey-HSD relative to all other time points ($p < 0.05$). Data are presented as mean $\pm$ standard deviation ($n = 3-4$ replicates per time point). Color images available online at www.liebertpub.com/tec

settling of cells in corners) with a maximum capacity of ~ 300 mL of medium (Fig. 2). The inlet and outlet of the vessel are placed $\sim 120^\circ$ apart to prevent channeling, such that the effluent from the liver scaffold mixes with the reserve media to distribute metabolites (e.g., glucose) rather than flow directly out of the reservoir (Fig. 2A, B, D).³⁰

A key design feature is a concave-shaped downward-facing glass restraint (Fig. 2, marker h) added to the head of the bioreactor to fully submerge the buoyant liver scaffold within the media reservoir and prevent the formation of bubbles within the scaffold at the gas–liquid interface that otherwise accumulate during fluid perfusion. Entrapped bubbles may damage the ECM and prevent cells or medium from reaching distal areas behind obstructed vessels. In contrast, scaffolds did not entrap bubbles when the restraint was used to maintain the scaffold below the liquid surface, and slots were added to the top of the restraint to prevent bubbles from accumulating under it. To our knowledge, no other published bioreactor incorporates a similar scaffold

restraint. The liver bioreactor is easy to operate and can be used for decellularization, cell seeding, and extended perfusion for tissue development.

Characterization of native, decellularized, and recellularized livers

Portal hypertension after liver transplant has been linked to poor graft function with increased patient morbidity and mortality.^{31,32} Portal resistance (proportional to ΔP) is therefore an important parameter that can be measured noninvasively in developing engineered liver tissue. For consistency, we restrict comparisons of ΔP at different stages in the decellularization and recellularization process to a normalized volumetric flow rate of $0.72 \text{ mL} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, which corresponds to the physiologic hepatic portal flow rate of $16 \text{ mL} \cdot \text{min}^{-1}$ normalized by the mass average of the native rodent liver.³³ An equivalent volumetric flow rate of $6 \text{ mL} \cdot \text{min}^{-1}$ (determined by multiplying $16 \text{ mL} \cdot \text{min}^{-1}$ by

FIG. 6. Comparison of pressure drop and morphological characteristics of liver scaffolds during decellularization and recellularization. **(A)** The mean ΔP across the organ (mean $\pm$ standard deviation) for native whole ($n = 16$), decellularized whole ($n = 11$), decellularized right lobe ($n = 14$), and recellularized right lobe livers at 3 days (day 3, $n = 4$) and 7 days (day 7, $n = 4$) at a constant normalized flow rate of $0.72 \text{ mL} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$. Asterisk indicates a significant difference between native and decellularized whole livers as determined by the Student's t -test ($p < 0.05$). [†]Indicates a significant difference as determined by one-way ANOVA ($p = 0.05$). **(B)** Macroscopic images of native and decellularized whole livers, and representative images of H&E-stained histological sections from each condition. Macroscopic image scale bars represent 15 mm, while H&E scale bars represent 100 μm . **(C)** The mean metabolic activity in recellularized scaffolds at days 1, 3, 5, or 7 after seeding. [#]Indicates a significant difference in metabolic activity between time points as indicated. Calculated number of cells, based on a standard curve as described in the Supplemental Materials and Methods section, is shown in the secondary vertical axis. **(D)** Cumulative albumin production by HepG2 cells within recellularized scaffolds during 1 week of perfusion culture. [#]Indicates a significant difference relative to all other time points as determined by Tukey-HSD ($p < 0.05$). Color images available online at www.liebertpub.com/tec

the mass ratio of the native right lobe to the native whole liver) was used to obtain ΔP values of the decellularized right lobe at the same normalized flow rate.

As we previously evaluated in kidneys, ΔP values across liver scaffolds at various stages of scaffold development were tested twice in sequence (Supplementary Fig. S1B). After whole livers were decellularized, the right lobe was isolated and used for recellularization; therefore ΔP was evaluated in normal whole livers and whole right lobe decellularized scaffolds (Fig. 6A). The overlap of the ΔP versus flow rate curves for each condition illustrates that high ΔP (up to 25 mmHg) at 20 mL $\cdot$ min⁻¹ did not disrupt matrix structure or alter flow mechanics (Supplementary Fig. S1B).

After decellularization, liver scaffolds were transparent and cell-free with >98% of DNA removed (Fig. 6B). Scaffolds retained the native hepatic extracellular architecture and vascular conduits. Histological evaluation confirmed that scaffolds became highly porous with complete removal of both hepatocytes from the parenchyma and endothelial and smooth muscle cells from vascular channels (Fig. 6B, Native vs. Decell). We correlated this histological finding with a significant decrease in ΔP as whole livers are decellularized (Fig. 6A, 16.1 $\pm$ 9.0 mmHg Native vs. 6.6 $\pm$ 4.4 mmHg Decell Whole Liver, $p=0.003$). This is consistent with Moran *et al.*,³⁴ who assessed liver parenchymal fluid pressure (PFP) in native and decellularized ferret right lobes using a microneedle inserted into the scaffold parenchyma and measured a reduction in PFP after decellularization. Following decellularization of the whole liver, we resected and isolated the right lobe to decrease the number of cells needed for recellularization.

As with kidney scaffolds, we assessed “process control” acellular liver scaffolds that were maintained in the bioreactor and perfused with media for 7 days at 37°C. Unlike process control kidney scaffolds, process control liver scaffolds completely and uniformly deteriorated after 7 days at 37°C, and therefore, pressure tests could not reliably be conducted. However, recellularization of liver scaffolds with HepG2 hepatocytes infused through the portal vein maintained overall scaffold architecture and stabilized the scaffold matrix without deterioration (Fig. 6B, day 3 and 7), suggesting that the cellular component maintains ECM architecture, possibly due to ECM remodeling.

Slow infusion (2 mL/min) of HepG2 cells followed by a brief stagnation period to allow cell adhesion led to uniform cell dispersion within the decellularized liver right lobe after 3 days (Fig. 6B). After 7 days of maintenance culture, an increase in cell density was apparent, indicating substantial proliferation within the hepatic ECM, and overall tissue morphology that approached native liver parenchyma (Fig. 6B, day 7) in agreement with other reports using different sources of hepatocytes.^{5,12} An increasing trend in overall HepG2 metabolic activity was observed, and resazurin reduction (and calculated cell number) was significantly higher after 5 ($p=0.002$) and 7 days ($p<0.001$) of perfusion culture compared to 3 days (Fig. 6C). Moreover, as a noninvasive indicator of cellular synthetic function, albumin produced by HepG2 hepatocytes grown within decellularized scaffolds could be measured within the media and accumulated over time (Fig. 6D).

ΔP measurements at each time interval correspondingly increased during recellularization from 5.1 $\pm$ 2.9 mmHg (de-

cellularized right lobe) to 9.1 $\pm$ 4.2 mmHg at 3 days and 10.9 $\pm$ 7.8 mmHg at 7 days. However, despite the results from ANOVA suggesting a significant difference in ΔP among these groups ($p=0.050$), no statistically significant differences were observed using *post hoc* analyses. This may be due to the low ΔP of the portal system and corresponding error associated with pressure measurement at this low range (Fig. 6A). Alternatively, the absence of a consistent increase in ΔP during recellularization may be characteristic of a distensible portal venous system, compared to the renal arterial network, with higher compliance and less change in ΔP as a function of flow rate.

Conclusions

Both kidney and liver bioreactors described here represent organ-specific refinements to improve recellularization and monitoring of cell growth and function based on the original cardiac perfusion model described by Langendorff^{18,19} and later modified for the kidney^{7,10,15,16} and liver^{2,9,11,12,30} bioengineering. The modified kidney bioreactor design described here improves upon these models by incorporating both ureteral and arterial routes for recellularization allowing for a direct comparison, and we show improved recellularization using high-pressure arterial infusion compared to ureteral infusion of renal epithelial cells. Moreover, we show that rodent scaffolds may be recellularized with xenogeneic model cell lines or primary renal progenitor cells that grow, remain viable, and can be monitored noninvasively in long-term culture within the same bioreactor chamber that also allows for *in situ* sampling of media perfusate to measure biomarkers in a time-dependent manner to characterize cell metabolism, adaptation into the scaffold, and synthetic functionality.

Additionally, physical markers, such as ΔP , correlate with scaffold properties such as increased porosity in both kidney and liver scaffolds as cells are removed following decellularization and return to normal levels with increased cellularity and formation of multicellular tubule-like structures over time leading to preservation of matrix structure, possibly through remodeling during the maintenance culture phase after recellularization. As perfusion and flow mechanics are commonly used to predict the suitability of clinically transplantable organ grafts, this similar methodology using noninvasive measurement of ΔP profiles can be obtained continuously during organ decellularization or scaffold recellularization, allowing for these parameters to be used in conjunction with other noninvasive biochemical methods for an improved characterization of tissue maturation during long-term bioreactor maintenance culture.

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