Fluid Flow Regulation of Revascularization and Cellular Organization in a Bioengineered Liver Platform

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Supplemental Methods:

Bioreactor Assembly: The perfusion bioreactor is composed of a spinner flask (Bellco Glass, Vineland, NJ, USA) with a magnetic stirring bar, platinum-cured silicone tubing (Cole-Parmer Instrument Company, Vernon Hills, IL, USA), tube fittings and 4 way valves (Cole-Parmer Instrument Company, Vernon Hills, IL, USA), Masterflex L/S computer-compatible/programmable peristaltic pump (Cole-Parmer Instrument Company, Vernon Hills, IL, USA), magnetic spinner unit (Fisher Scientific, Pittsburgh, PA, USA), pulse dampener (Cole-Parmer Instrument Company, Vernon Hills, IL, USA) and a luer-lock injection valve (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). After set up, the bioreactor was primed with EGM-2 culture medium (Lonza BioResearch, Basel, Switzerland) + 5% FBS (Thermo Scientific HyClone Logan, UT, USA) before connecting to the catheter on the liver bioscaffold. A needle-guided transducer tipped catheter (Millar SPR-524 [3.5F], Millar Instruments Inc., Houston, TX, USA) and MPVS-400 signal conditioning hardware (Millar Instruments, Houston, TX, USA) with integrated DAQ technology (Powerlab, ADInstruments, Colorado Springs, CO, USA) was used to measure and record system pressure. The probe was inserted into the tubing distally from the portal vein prior to seeding of the scaffold and pressure was recorded until the scaffold was removed from the bioreactor. Pressure measurements were sampled at a rate of 1kHz and the measurement reported in this paper is the average pressure over the entire 24 hours.

Cell Seeding: HepG2 and MS1 cells were mixed at a 1:1 ratio in 10ml of culture medium, and loaded in a 30ml syringe (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were gently injected into the bioreactor vessel by the luer lock injection port and

kept in suspension with magnetic stirring at ~40rpm. Since for every flow condition tested most cells were already deposited inside the scaffold after 2-3 hours, loading of a new bolus of cells into the bioreactor was executed every 4 hours until completing the total of 60 million cells per scaffold. Twelve hours after cell seeding, medium was changed with fresh EGM-2 + 5% FBS and continuous medium perfusion was performed for either 1 or 7 days after seeding, with the different flow rates tested. In the case of the 7 days experiments, medium was changed every 72 hours. Glucose concentrations and pH were monitored every 24h, beginning after the 4^{th} day post-seeding. At any point, when glucose concentrations were detected below 50mg/dl or pH below 7.10, medium was changed immediately.

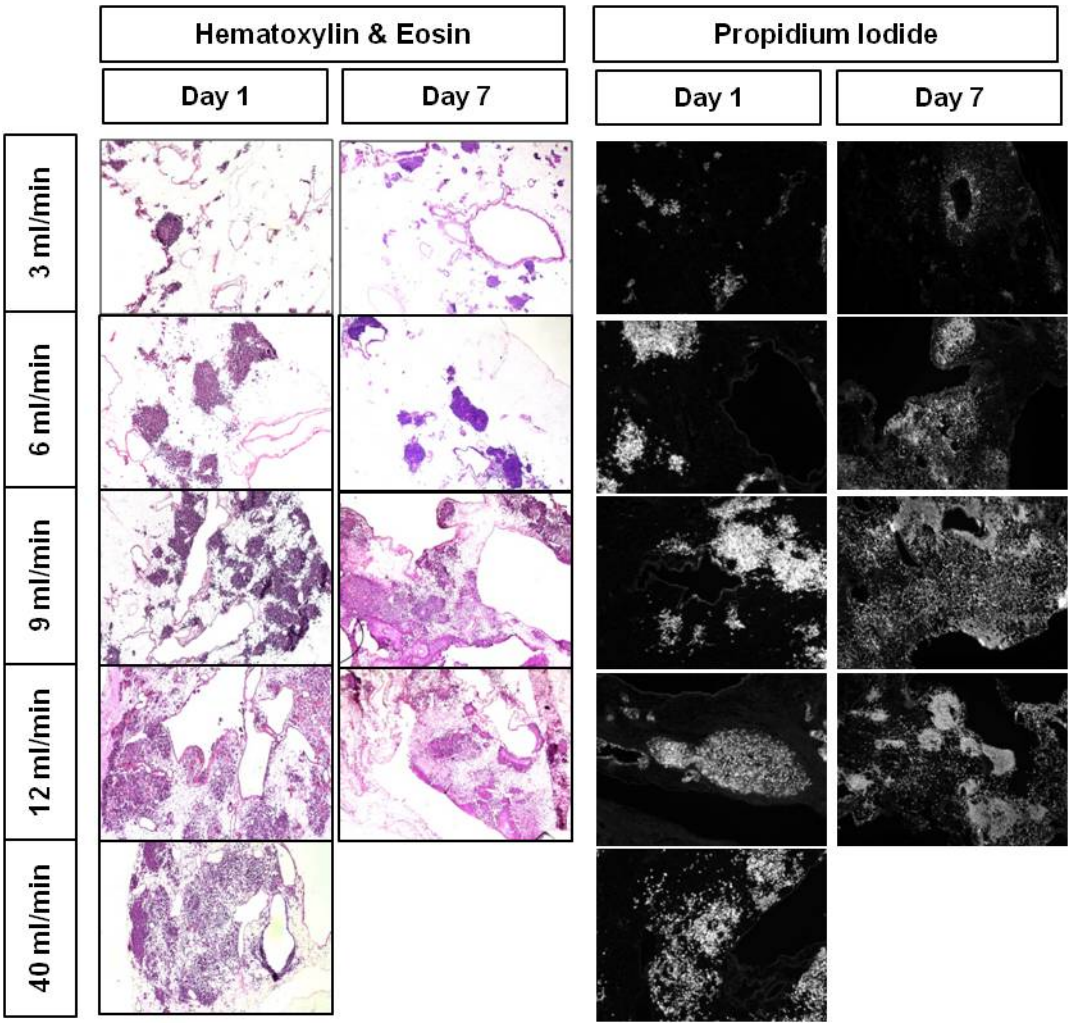
Fixation and Tissue Processing: After 1 or 7 days in continuous perfusion in the bioreactor (post-seeding), the seeded scaffolds were retrieved for fixation. Prior to fixation, each scaffold was washed with 200 ml PBS (Invitrogen, Carlsbad, CA, USA) perfused with the identical flow rate used in the bioreactor. After washing, the scaffold was perfused overnight with 10% buffered formalin (Fisher Scientific, Pittsburgh, PA, USA) at room temperature. Following fixation, the scaffold was processed for paraffin embedding in a Shandon Citadel Tissue Processor (Thermo Fisher Scientific, Waltham, MA, USA).

Static Culture Controls: A total of 12 different conditions were applied to 6-well culture controls to analyze the effects of the drugs on static cultures. Cells were seeded at a density of 25,000 cells/cm^2 in the following conditions: MS1 cells only, HepG2 cells only, or transwell inserts (PET membrane (1.0 μm), Becton Dickinson, Franklin Lakes, NJ, USA) with MS1 cells on top of the membrane and HepG2 cells on the bottom. Cells

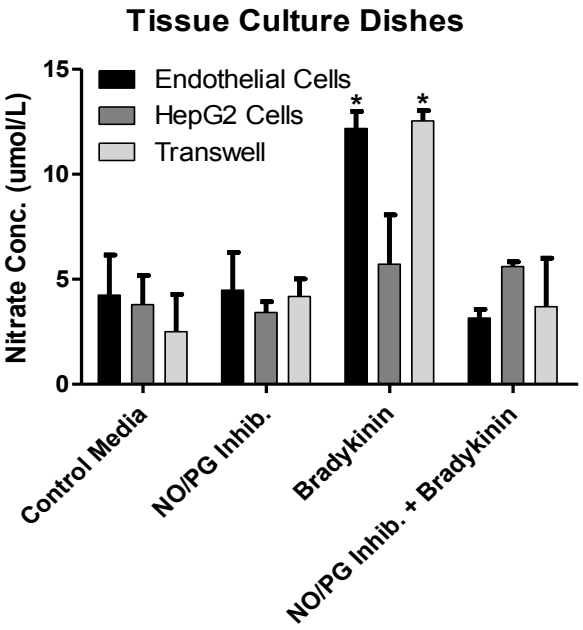
were then exposed to one of four types of media: EGM-2 with 5% FBS (standard media), standard media supplemented with 1mM L-NAME and 50μM Indomethacin, standard media supplemented with 1μM Bradykinin (to stimulate NO and PGI2 production), and standard media supplemented with 1mM L-NAME, 50μM Indomethacin and 1μM Bradykinin. A total of n=3 was used for each conditions and media was collected and cell numbers were quantified on day 3 and day 7.

Image Analyses: *PI, Ki-67, and TUNEL Images:* Images were taken at 10x magnifications in 6 separate areas of each section, with three sections per bioreactor (picked randomly from the first slides, middle, and last 10 slides out of the 50 slides cut per each liver construct). Image analyses were completed using a custom Mathematica (Wolfram Mathematica 8.0, Wolfram Research, Campaign, IL, USA) program. Briefly, thresholding was applied to the images to calculate the number of pixels that a particular stain occupied. PI stain analysis determined the percentage of the scaffold occupied with cells. Ki-67 and TUNEL staining determined the percentage of cells that were either proliferating or undergoing apoptosis, respectively. *H&E Images to quantify penetration of cells into the parenchyma:* H&E images were taken at 5x magnifications, with five images per section and three sections per scaffold. These images were then imported into ImageJ to quantify the distance that cell clusters travel from a blood vessel into the parenchyma of the tissue. The average of all of these measurements for a single experiment is referred to as cell penetration. *Albumin and ENOS staining to analyze organization of HepG2 and EC:* Double staining of Albumin (HepG2 cells) and eNOS (MS1 cells), along with a DAPI nuclear stain, reveals the organization of cells within the scaffolds. Three images were taken on each section at 20x, with two sections

per bioreactor. The albumin (green) and eNOS (red) images were merged into a single image and imported into ImageJ and two separate analyses were performed. The first analysis quantified the average size of albumin positive, therefore HepG2, clusters. The ImageJ loop tool was used to segment the albumin positive clusters and measure the internal area in pixels. The second analysis evaluated all of the vascular structures within an image. Every vessel was binned into one of three groups: no cells, a combination of HepG2 and EC, and EC only. The vascular structures that were grouped into the latter two groups had to be more than 50% coated with cells. These groups were compiled for each scaffold, and then normalized by dividing them by the total number of vessels for that particular scaffold.



Supplementary Figure 1: Effect of Flow Rate on Cellular Distribution. Panel of representative images of liver ECM scaffolds after 1 and 7 days in the perfusion bioreactor across all flow rates. H&E images were used to quantify cell penetration and PI (propidium iodide) analysis quantified the amount of cells in the scaffold.



Supplementary Figure 2: Nitrate (NO) static culture control. Nitrate (NO) concentrations in the media of HepG2 and MS1 cells grown separately in tissue culture dishes or together in "transwell" plates. Some cells were cultured in the presence of bradykinin and a combination of bradykinin and NO and PG synthesis inhibitors. *$p<0.05$

	Flow Rate				
	3ml/min	**6ml/min**	**9ml/min**	**12ml/min**	**40ml/min**
Cell Delivery Rate into Bioscaffold	360,000 cells/min	360,000 cells/min	385,000 cells/min	360,000 cells/min	750,000 cells/min
Medium Volume in Bioreactor Vessel	250ml	250ml	350ml	400ml	400ml
Number of Total Cells per Injection	$30x10^6$ cells	$15x10^6$ cells	$15x10^6$ cells	$12x10^6$ cells	$7.5x10^6$ cells
Number of Injections	2	4	4	5	8
24 Hour Bioreactors	n = 4	n = 4	n = 4	n = 4	n = 2
7 Day Bioreactors	n = 2	n = 3	n = 4	n = 3	None

Supplementary Table 1: Seeding and maintenance flow rates, volumes and cell numbers.