Supporting Information for: Primary Liver Cells Cultured on Carbon Nanotube Substrates for Liver Tissue Engineering and Drug Discovery Applications

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1. Cell Culture Methods

1.1 Cell Culture

The plateable, cryopreserved primary rat hepatocytes (from male Sprague-Dawley rats, Lot No: Rs619 and Rs660) were purchased from Invitrogen (Paisley, UK). This primary cell line was maintained in complete DMEM medium supplemented with 10% FBS, 1% (v/v) penicillin-streptomycin, 1% (v/v) L-glutamine) and 1% (v/v) non-essential amino acids. The ampoules containing cryopreserved rat hepatocytes were firstly thawed quickly (less than 2 minutes) in the 37 °C water bath and thawed hepatocyte suspension was gently poured into a centrifuge conical tube containing pre-warmed DMEM medium, and then centrifuged for 3 minutes at 50 g. The supernatant was then poured off and fresh DMEM medium was added into the centrifuge conical tube and the tube was inverted completely a few times to resuspend the pellet. Cells were firstly checked for viability (70 to 90% viable) prior to seeding at the desired density. A cell suspension was diluted to the desired cell seeding concentration (1 x 10^6 cells per mL) and then seeded on test and control substrates pre-coated with collagen Type I solution.

The human hepatoma cells (Huh7) were also cultured in DMEM supplemented with 10% FBS, 1% (v/v) of antibiotics solution (penicillin and streptomycin) and 1% (v/v) non-essential amino acids. Huh7 cells were passaged every three days, and for experiments, only cells up to passage number 19 were used. Cells were seeded on test and control substrates and cultured under humidified atmosphere at 37 °C with 5% CO₂. Huh7 cells were seeded at a density of ~5 x 10^4 cells per mL in the case of aligned MWNT sheets and on glass coverslip, and at 5 x 10^4 cells per mL for MWNT yarns.
1.2 Fixing and fluorescent staining of F-actin, albumin and the nuclei of cells on substrates

The cell-seeded substrates were washed with PBS and fixed for 20 minutes with 4% (v/v) paraformaldehyde prepared in PBS. After fixation, the samples were rinsed gently with PBS and then permeabilized with 0.1% non-ionic surfactant, Triton X (Sigma) prepared in PBS (PBST) for 5 minutes. Samples were then thoroughly washed with PBS and stained. Samples were incubated with AlexaFluor phalloidin (Molecular Probes) at a concentration of 22 nM for 30 minutes at room temperature to stain F-actin.

The procedure for staining the albumin secreted by the cells is as follows. After fixation and permeabilization the samples were then blocked in blocking buffer (5% bovine serum albumin (BSA) in PBS) for 60 minutes. Samples were then washed three times with PBS and incubated with the primary antibody, anti-albumin antibody produced in chicken (1:1000; Sigma). This was done overnight at 4 °C. Next, samples were washed twice with PBS followed by wash buffer, PBST (PBS + 1% Triton X-100) and then incubated with secondary antibody, rabbit anti chicken IgY (IgG) conjugated to the fluorescein isothiocyanate (FITC) (1: 500; Sigma) for 60 min at 37 °C. After rinsing with PBS, samples were subsequently incubated with DRAQ5 (BD Biosciences Limited) at a concentration of 5 µM for 10 minutes at room temperature. Stained cells were then mounted with a coverslip in mounting medium (Vectorshield, Vector Laboratories, Inc., Burlingame, CA), and sealed with nail varnish. Samples were examined via confocal fluorescence microscopy using a Zeiss LSM 510 META.

1.3 Fixing and drying techniques of cells on substrates and substrates coated with collagen for SEM
Following the allotted incubation time, cells were washed once with culture media and rinsed twice with PBS. Cells were then immersed for 10-15 minutes in 2.5% glutaraldehyde in phosphate buffer saline solution. Cells were then rinsed twice with phosphate buffer saline solution followed by rinsing twice with deionized water before being allowed to dry in air, at room temperature, until all the liquid had evaporated from the substrates. They were then dried using critical point drying (CPD) where cells were dehydrated through a graded series of ethanol 30%, 50%, 70%, 85%, 95% and 100% (10 minutes in each). Fixed dried cells were stored at 4 °C until ready for SEM analysis. In addition, for SEM observation of substrates coated with collagen, a similar fixing and drying protocol was followed in order to preserve the collagen.

1.4 XTT cell proliferation assay

The reduction of the colourless second generation tetrazolium dye, XTT (sodium 2, 3, -bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt) by metabolically active cells to the water soluble, bright orange compounds of formazan, was determined using the Cell Proliferation Kit II (Promega, UK). The reduction of XTT is an indicator of the metabolic activity due to the fact that it is a substrate for the intracellular and plasma membrane oxidoreductases (PMOR). The assays were performed after culturing cells on scaffolds for 0, 24, 48, 72 and 96 hours, according to the manufacturer’s instructions. Briefly, following growth on scaffolds, cells were exposed to XTT substrate for 4 hours at 37 °C. After incubation, production of the reduced formazan metabolite was quantified at the test wavelength of 490 nm and using the reference wavelength of 650 nm.

1.5 MRP2 drug transporter efflux assay
The formation of functional bile canaliculi networks was examined using 5 (and 6)-carboxy-2', 7'-dichlorofluorescein diacetate (CDFDA), the hydrolysed product of which is fluorescent and a substrate for the MRP2 efflux transporter localised on the canalicular membrane of correctly polarized hepatocytes. Primary rat hepatocytes in sandwich culture configuration were rinsed twice with 2 mL of a standard buffer (1X Hank’s balanced salt solution (HBSS) with calcium and magnesium). Cells bathed in HBSS were returned to the 37 °C incubator for 10 minutes and the buffer was then replaced with HBSS containing 10µM CDFDA dissolved on 0.1% DMSO. Cells were incubated for 30 minutes at 37 °C to allow uptake of CDFDA into cells through passive transport processes. Next, uptake buffer was removed and replaced with standard buffer. Both cell morphology and bile canalicular network formation were imaged with an inverted Nikon Eclipse TS 100 microscope in phase contrast and fluorescence mode (using green (FITC) filter cube).

1.7 Cytochrome-P450 activity assay

CYP1A and CYP3A activity in both immortalized (Huh7) and primary (rat hepatocyte) cell lines was measured using a P450-Glo assay kit (Promega) according to the manufacturer’s instructions. Briefly, cells were allowed to attach and grow on each substratum for the indicated time, and then the culture medium was replaced with medium containing the P450 luminogenic substrates, 200 µM Luciferin-ME (CYP1A) or 50 µM Luciferin-PPXE (CYP3A) in DMEM. Cells were incubated with the substrate for 4 hours at 37 °C, and then the medium was mixed with luciferin detection reagent, incubated for 20 minutes, and CYP1A or CYP3A enzyme activity detected using a luminometer (LumiCount, Packard) and expressed in relative luminescent units (RLU).

For induction experiments, cells were exposed to either 10µM β-Naphthoflavone (β-NF; CYP1A inducer), or 10µM dexamethasone (Dex; CYP3A) for 48 hours. Control cultures
were exposed to vehicle (0.1% DMSO) alone. After treatment with inducers, CYP activity was determined as described previously.

Activity measurements of CYP1A and CYP3A induction were normalized to the number of viable cells using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Cat No: G 7570), according to the manufacturer’s instructions.

1.8 ELISA assay

Albumin production within the cultured hepatocytes was assessed using the Rat Albumin ELISA Quantitation kit from Bethyl Laboratories Inc. Cell culture medium was analyzed for albumin secretion following 3, 5, and 7 days of culture on different substratum, with the quantity of albumin interpolated from a standard curve of rat albumin.

References
