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Supplemental Information

Human liver organoids for disease modeling of fibrolamellar carcinoma

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Tumor processing

Following resection, tissue was transferred to cold PBS in a petri dish and placed on ice, then cut into pieces (2 cm x 0.5 cm) and transferred to 50ml conical tubes in Roswell Park Memorial Institute (RPMI) media supplemented with 2% penicillin/streptomycin. Some samples were fixed in formaldehyde and paraffin embedded for histological analysis or pieces were flash frozen or frozen in Optimal Cutting Temperature (OCT) for RNA and protein analysis. Connective tissue, blood clots, and necrotic tissue was cut away prior to fixing or freezing the samples.

Fresh patient tissue was cut into 2x2 mm pieces and placed into 50 ml Falcon tubes with RPMI + 2% penicillin/streptomycin, collagenase 4 (Worthington) and DNase 0.1 mg/mL (Sigma, St. Louis, MO) for digestion. The tubes were rotated at 37°C (Benchmark Scientific Roto-therm) and digestion assessed by ease of pipetting the tissue solution with a 10 ml serological pipet. Digestion time ranged from 30 minutes to 2.5 hours, longer if there were dense collagen bands in the tumor. The dissociated tissue was then passed sequentially through 200 μm and 100 μm nylon cell strainers (Fisher). The flow-through was centrifuged at 300 x g for 5 minutes at 4°C and the supernatant was aspirated. The cells were counted, resuspended in BME-2 (Basement Membrane Extract, Type 2, Pathclear, Trevigen, Gaithersburg, MD), and seeded on tissue culture treated 24-well plates (~30 μl per well). Plates were incubated at 37°C for 10 minutes until BME-2 polymerized and culture media was added.

RNA isolation, generation of cDNA and PCR

Organoid RNA was extracted after harvesting from BME-2. Tissue RNA was extracted from OCT. RNA was harvested with RNeasy Mini Kit (Qiagen) and concentrations measured wiht Nanodrop 2000c (Thermofisher) and quality was evaluated by the 260/280 ratio. All RNA samples were diluted to an equal concentration for the reverse transcription reaction. The LunaScript RT SuperMix Kit (New England Biolabs) was used to convert RNA into cDNA according to the manufacturer's instructions. Platinum PCR SuperMix High Fidelity (Invitrogen) was used for PCR with the following conditions for each reaction: 22.5 μL of supermix, 1.5 μL of cDNA, and 0.5 μL of each primer at 10 μM (final primer concentration: 200 nM). Reactions were performed on a C1000 Thermal Cycler (Bio-Rad) as follows: 2 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 20 seconds at 68°C. The PCR product was run on a 2% agarose gel with SYBR Safe (Invitrogen) powered with a PowerPac (Bio-Rad) set at 100V for 60 minutes. Gel was imaged using Gel Doc EZ imager (Bio-Rad). Primer sequences: DNAJB1-PRKACA forward – GCCGAGGAGAAGTTCAAGGA, reverse – CTGTGTTCTGAGCGGGACTT, expected amplicon – 160 kb. PRKACA forward – GAGCAGGAGAGCGTGAAAGAA, reverse – TCATGGCATAGTGGTTCCCG, expected amplicon – 184 kb.

RNA Sequencing and bioinformatics

Total RNA concentrations and 260/280 ratios were measured using the Nanodrop 2000c (Thermofisher) and RNA quality was evaluated by RNA Integrity Number (RIN) values (Agilent BioAnalyzer and TapeStation). RNA sequencing libraries were prepared by Genewiz using the SMARTer Stranded Total RNA-Seq Kit v2- Pico input Mammalian (Takara Bio #634411) with Ribo-Zero Gold ribosomal RNA depletion (Illumina). The libraries were sequenced on an Illumina HiSeq 4000 with 2 x 150bp paired-end reads. Quality assessment and trimming were performed using FastQC v0.11.7 and BBDuk (included in BBMap v38.22). Reads were mapped to the human reference genome hg38 supplemented with the EMSEMBL GRCh38.92 gene annotations using STAR v2.6.1 (Dobin et al., 2013). Differential expression analysis was

conducted in R version 4.0.2 and Rstudio 1.3.959 using DESeq2, excluding rRNA and mt-rRNA genes as well as the immune and stromal signature genes (Yoshihara et al., 2013). t-Distributed Stochastic Neighbor Embedding (t-SNE) plots and heatmaps were generated using a FLC transcriptomic profile that we established (supplemental table 1) as a set of 509 genes we found consistently dysregulated in FLC primary tumors vs. adjacent non-tumor ("normal") liver tissue (FDR <0.05) (283 upregulated, 226 downregulated genes) (Simon *et al.*, 2015).

Protein isolation and immunoblotting

Normal and tumor organoid total protein was extracted from organoid pellets after harvesting from BME-2. FLC patient tumors and non-tumor liver was extracted from tissue frozen in OCT. Organoids and tissues were lysed with RIPA buffer (Sigma) and supplemented with protease inhibitors (cOmplete EDTA-free, Roche, Indianapolis, IN) and phosphatase inhibitors (PhosSTOP, Roche, Indianapolis, IN). Tissue samples were sonicated on ice for complete lysis. All samples were incubated on ice for 20 minutes and centrifuged at max speed for 10 minutes at 4°C. The lysate was collected and protein concentrations were measured using the DC protein assay (Bio-Rad), and samples were diluted to an equal protein concentration. 4x NuPAGE LDS sample buffer and 10x NuPAGE sample reducing agent (Invitrogen) were added to the samples. Samples were heated at 100°C for 5 minutes, and then loaded on 4–12% Bis-Tris gels (NuPAGE) and run in MOPS buffer for 50 minutes at 200V. Transfer was performed using the iBlot 2 (Life Technologies). Membranes were blocked for 1 hour at room temperature with blocking buffer (5% milk in TBS-T) and then probed overnight at 4°C with primary antibodies in blocking buffer. After washing in TBS-T, membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated appropriate secondary antibodies in blocking buffer. The membranes were washed again, incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific), and exposed to Amersham Hyperfilm (GE Healthcare).

Antibodies: PKA C-α (D38C6) Rabbit mAb (Cell Signaling Technology) at 1:3000, and goat anti-rabbit IgG A0545 (Sigma) at 1:50,000.

Histology and Immunohistochemistry

Patient FLC tumor and adjacent normal tissue was formalin-fixed and paraffin embedded. Normal and tumor organoids were also fixed in formalin after harvesting from BME-2 and embedded in paraffin. Paraffin embedded tissue and organoid blocks were sectioned (5 μm) and stained with hematoxylin and eosin for morphologic evaluation and/or probed with CD68 (Dako, KP-1 clone, 1:2000) and CK7 (Dako, OV-TL 12/30 clone, 1:1000) for immunohistochemical analysis using the Discovery XT IHC staining platform (Roche). The slides were pre-treated with CC1 reagent (Roche) for 32 minutes followed by primary antibody incubation for 60 minutes and anti-mouse secondary antibody (Roche, biotinylated, 1:200) for 60 minutes. The Discovery ChromoMap DAB kit (Roche) was used for visualization.

Imaging

Live organoid cultures and H&E/IHC slides of both patient normal and tumor tissues and normal and tumor organoids were imaged on an Olympus IX83 microscope. Live organoids were imaged at 10x with a Hamamatsu Flash-4.0 camera and slides were imaged at both 10x and 60x using an Olympus DP26 camera and cellSens software (Olympus).