GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Single-Cell RNA Sequencing Identifies Yes-Associated Protein 1—Dependent Hepatic Mesothelial Progenitors in Fibrolamellar Carcinoma

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Accepted for publication
September 26, 2019.

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Fibrolamellar carcinoma (FLC) is characterized by in-frame fusion of DnaJ heat shock protein family (Hsp40) member B1 (DNAJB1) with protein kinase cAMP-activated catalytic subunit α (PRKACA) and by dense desmoplasia. Surgery is the only effective treatment because mechanisms supporting tumor survival are unknown. We used single-cell RNA sequencing to characterize a patient-derived FLC xenograft model and identify therapeutic targets. Human FLC cells segregated into four discrete clusters that all expressed the oncogene Yes-associated protein 1 (YAP1). The two communities most enriched with cells coexpressing FLC markers [CD68, A-kinase anchoring protein 12 (AKAP12), cytokeratin 7, epithelial cell adhesion molecule (EPCAM), and carbamoyl palmitate synthase-1] also had the most cells expressing YAP1 and its proproliferative target genes (*AREG* and *CCND1*), suggesting these were proliferative FLC cell clusters. The other two clusters were enriched with cells expressing profibrotic YAP1 target genes, *ACTA2*, *ELN*, and *COL1A1*, indicating these were fibrogenic FLC cells. All clusters expressed the YAP1 target gene and mesothelial progenitor marker mesothelin, and many mesothelin-positive cells coexpressed albumin. Trajectory analysis predicted that the four FLC communities were derived from a single cell type transitioning among phenotypic states. After establishing a novel FLC cell line that harbored the DNAJB1-PRKACA fusion, YAP1 was inhibited, which significantly reduced expression of known YAP1 target genes as well as cell growth and migration. Thus, both FLC epithelial and stromal cells appear to arise from DNAJB1-PRKACA fusion in a YAP1-dependent liver mesothelial progenitor, identifying YAP1 as a target for FLC therapy. (*Am J Pathol* 2020, 190: 93–107; <https://doi.org/10.1016/j.ajpath.2019.09.018>)

Fibrolamellar carcinoma (FLC) is a rare liver cancer occurring predominantly in adolescent and young adult patients with no history of liver injury.¹ This is in marked contrast to the much more common hepatocellular carcinoma, which arises mainly in older adults with chronically injured livers, particularly patients with advanced fibrosis or established cirrhosis.

FLC is strongly associated with a specific chromosomal microdeletion that leads to fusion of the DnaJ heat shock protein family (Hsp40) member B1 (*DNAJB1*) and protein kinase cAMP-activated catalytic subunit α (*PRKACA*) genes, leading to a spliced mRNA that directs expression of

a fusion of *DNAJB1* exon 1 to *PRKACA* exons 2 to 10.² This fusion is thought to lead to aberrant activation of the cAMP-dependent protein kinase, protein kinase A (PKA).² Most patients with FLC have this genomic deletion/gene fusion in tumor cells, but adjacent normal tissue or other cells in the tumor lack this.^{2–8} Presumed FLC cases that lack this deletion/fusion event are often reclassified as

Supported by the Fibrolamellar Carcinoma Foundation (A.M.D. and C.G.) and the Duke Endowment (A.M.D.).

M.L.J. and J.R.G. contributed equally to this work.

Disclosures: None declared.

unusual presentations of hepatocellular carcinoma, although there are reports of distinct genetic events associated with FLC, such as mutation of *PRKARIA*, a PKA regulatory subunit that restricts PKA activity.⁹ Curiously, genomic and transcriptomic analysis of FLC tumors reveals a striking paucity of accumulated genetic errors, compared with cancers that increase in prevalence with age,^{3–5,7,8} although this conclusion has been contested by others who found p53 loss and genetic instability in FLC and particularly in its metastases.¹⁰

Despite progress toward understanding the molecular basis of FLC, numerous fundamental questions remain unanswered. An important feature of FLC is that, despite primarily developing in young patients, it is nearly always detected at an advanced stage because of the absence of symptoms or tumorigenic biomarkers. Therefore, the speed of tumor growth and basis for its association with young and otherwise healthy-appearing liver are unknown. Another striking feature of FLC is that, although it does not arise within fibrotic/cirrhotic liver, the tumor itself is associated with its own lamellar stroma. That is, the tumor generates its own fibrotic microenvironment, but the reasons for this remain unknown. Attempts to discern a direct causative role for the *DNAJB1-PRKACA* gene fusion in FLC have suggested that elevated PKA activity is an important driver of tumorigenesis.¹¹ Mouse models that used clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9)-mediated *DNAJB1-PRKACA* fusion in hepatocytes after hydrodynamic injection of plasmids found that liver tumors arose slowly, over the course of a year.^{12,13} Interestingly, transposon-based overexpression of the fusion protein also produced such tumors,¹³ suggesting that the loss of the seven genes lying between *DNAJB1* and *PRKACA* is not a key driver of FLC. Nevertheless, all of the mouse liver tumors from these studies lack the characteristic fibrotic lamellae of FLC, as well as expression of some known tumor marker genes, particularly CD68.^{12,13} These findings suggest that mature hepatocytes might not be the proper cell of origin to target to recapitulate the human tumorigenic event.

The Reid laboratory has previously generated an FLC model that uses a patient-derived explant grown in immunodeficient mice to recapitulate all of the features of the originating tumor.¹⁴ In this model, human tumor cells are injected into mice to generate tumors that also contain mouse stroma.¹⁴ This endogenous drive to generate tumor-associated stroma allowed us to ask how the FLC tumor recruits and maintains its own fibrotic stroma, as a step toward understanding the functional role of the stroma in supporting FLC tumorigenesis. This knowledge will also illuminate the general cause of liver tumorigenesis in the context of liver injury because normal liver repair requires the cooperation of liver epithelial and stromal cells, whereas dysfunctional repair generates a chronic fibrotic microenvironment that promotes hepatocarcinogenesis, making liver cirrhosis a major risk factor for hepatocellular carcinoma.

Materials and Methods

FLC Patient-Derived Tumor Explants

FLC patient-derived tumor explants (FLC-PDXs) were generated in mice, as previously detailed by Oikawa et al,¹⁴ using some of the same human FLC sample (generously provided by Lola M. Reid and Eliane L. Wauthier, whose laboratories at University of North Carolina at Chapel Hill had generated the FLC-PDX described by Oikawa et al¹⁴). Briefly, approximately 1×10^6 tumor cells were suspended in Kubota media supplemented with 1 mg/mL hyaluronans and 50 ng/mL of hepatocyte growth factor and vascular endothelial growth factor and then the cells were transplanted into the flanks of new recipient severe combined immunodeficiency–beige immunocompromised mice (Taconic Biosciences, Rensselaer, NY). Recipient mice were monitored, and tumor explants were measured $2 \times$ / week until they reached a size of 1000 mm³. At that time, each new FLC-PDX was harvested. For passage, harvested tumors were minced into pieces <0.1 cm, transferred to Kubota media supplemented with hyaluronan/hepatocyte growth factor/vascular endothelial growth factor, and injected subcutaneously into the flanks of additional severe combined immunodeficiency–beige mice. A portion of each passaged FLC-PDX was reserved for histologic and gene expression characterization. Tumors were passaged five generations over the course of this study. A portion of the cell suspension from a third-generation FLC-PDX was used for single-cell RNA sequencing (scRNA-seq). Another aliquot of that FLC-PDX suspension was used to generate a new FLC cell line [partially reprogrammed FLC (prFLC); *Partially Reprogrammed FLC-PDX Cell Line*]. A tumor cell suspension from a fifth-generation FLC-PDX was characterized by fluorescence-activated cell sorting (*Partially Reprogrammed FLC-PDX Cell Line*). Presence of transcripts for *DNAJB1*, *PRKACA*, and *DNAJB1-PRKACA* fusion was detected in both the FLC-PDX and prFLC line using primers described in Table 1 and product bands confirmed by Sanger sequencing.

Immunohistochemistry and Immunocytochemistry

Formalin-fixed, paraffin-embedded tissue sections from each FLC-PDX were cut (4 to 5 μ m thick). The slides were warmed to 60°C for 1 hour and then deparaffinized. The following antibody manufacturer, dilution, incubation time, and detection systems were used: HepPar-1 (carbamoyl palmitate synthase-1): Cell Marque 264R-15 (Cell Marque, Rocklin, CA), 1:200, 60 minutes, Cell Marque Hi Def Detection Kit; CD68: Cell Marque 168M-95, 1:500, 60 minutes, Cell Marque Hi Def Detection Kit; keratin 7: Dako M7018 (Dako, Santa Clara, CA), 1:1000, 30 minutes, Biocare Mach 2 Detection Kit; Yap1: Cell Signaling number 4912 (Cell Signaling, Danvers, MA), 1:20, 60 minutes; mesothelin (MSLN): Rockland 200-301-A88 (Rockland,

Table 1 Primer Sequences

Target	Forward	Reverse
<i>DNAJB1</i>	5'-GAGGAGATCAAGCGGGCTAC-3'	5'-AGCCAGAGAATGGGTCAATG-3'
<i>PRKACA</i>	5'-ACGCCGCCGCCCAAGAA-3'	5'-GAGGAACGGAAAGTTGACAGCT-3'
<i>YAP1</i>	5'-CCGACTCCTTCTTCAAGCC-3'	5'-CGAACATGCTGTGGAGTCA-3'
<i>CTGF</i>	5'-GAAATGCTGCGAGGAGTG-3'	5'-GCTCTAATCATAGTTGGGTCTG-3'
<i>CYR61</i>	5'-GAGGTGGAGTTGACGAGAAAC-3'	5'-TGGCCTTGTAAGGGTTGTATAG-3'
<i>MSLN</i>	5'-CTGGACAAGACCTACCCACAA-3'	5'-CTGGTGAGGTCACATTCCACT-3'
<i>Fusion</i>	<i>DNAJB1</i> forward	<i>PRKACA</i> reverse

Limerick, PA), 1:500, overnight at 4°C, Mach 2 Detection Kit or Liquid DAB + Substrate Chromogen system (Dako; K3468). Before the application of chromogen, the tissue sections were treated with hydrogen peroxide to block endogenous peroxidase activity. The bound immune complex was visualized with application of diaminobenzidine and subsequently counterstained with hematoxylin. Completed slides were dehydrated with alcohol, cleared with xylene, and coverslipped with permanent mounting media. Immunostained slides from the FLC-PDXs were compared with similarly immunostained deidentified slides from five FLC patient primary tumors in the Duke Department of Pathology archives. Human tissues were used in accordance with the Duke Institutional Review Board—approved protocol. Representative photomicrographs from the FLC-PDX and human FLC samples are shown (Supplemental Figure S1).

Immunocytochemistry

To generate cell suspensions, FLC-PDXs were minced and enzymatically processed in digestion buffer consisting of Kubota medium (PhoenixSongs Biologicals, Branford, CT) supplemented with 600 unit/mL collagenase IV (Gibco, Gaithersburg, MD) and 0.3 mg/mL DNase (Sigma, St. Louis, MO) at 37°C for 45 minutes with frequent agitation. After the initial digestion, cell suspensions were spun at $400 \times g$ for 5 minutes, supernatant was removed and stored at 4°C, and the pellet was resuspended in fresh digestion buffer for an additional 45 minutes at 37°C. Suspensions from the secondary digestion were combined with supernatant from initial digestion, spun, resuspended in Kubota medium, and seeded on tissue culture—treated cell culture plates for a 45-minute attachment to remove mesenchymal cells. After attachment, the supernatant was collected and spun, and cells were resuspended in Kubota medium and seeded on ultralow attachment plates (Corning, Tewksbury, MA). Cell suspensions were maintained in Kubota medium at 37°C and 5% CO₂. Isolated explant tumor cells were removed from ultralow attachment plates and cultured in 8-well chamber slides (ThermoFisher-LabTek II; Thermo Fisher, Grand Island, NY) or immobilized by cytospin (500 μ L of 1×10^6 /mL suspension). For immunofluorescence staining, cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature, rinsed with phosphate-buffered saline (PBS)/100 mmol/L

glycine, permeabilized with 0.5% Triton X-100/PBS, and blocked with 1% bovine serum albumin/PBS for 2 hours. Fixed cells were incubated with primary antibody solution at 4°C for 12 hours, followed by washing with PBS/0.1% bovine serum albumin, incubation with isotype-specific secondary antibodies, and counterstaining with 0.5 ng/mL DAPI.

Gene Expression by Real-Time Quantitative PCR

Total RNA was prepared from fresh or frozen tumor or cells using the AllPrep kit (Qiagen, Germantown, MD), according to the manufacturer's instructions. First-strand cDNA was synthesized using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Gene expression was quantified after amplification using gene-specific intron-spanning primers, using SYBR Green PCR Master Mix in the StepOnePlus cycler (both from ABI, Grand Island, NY), compared with expression of the ribosomal S9 gene using the $\Delta\Delta$ Ct method. Primers used are listed in Table 1.

Single-Cell RNA-Seq

Cells were disassociated from a freshly excised third-generation FLC-PDX, washed, and resuspended in a $1 \times$ PBS/0.04% bovine serum albumin solution, at a concentration of 1000 cells/ μ L. After size selection ($<50 \mu$ m), the cell suspension was washed with a $1 \times$ PBS/0.04% bovine serum albumin solution to remove debris, clumps, dead cells, and contaminants; and a Countess (Thermo Fisher Scientific, Waltham, MA) was used to determine the cell viability and concentration to normalize to 1×10^6 cells/mL. Titered cells were combined with a master mix that contains reverse transcription reagents. Gel beads carrying an Illumina (San Diego, CA) R2 primer sequence, a 16-bp $10 \times$ barcode, a 10-bp randomer (unique molecular identifiers), and a poly-dT primer were loaded onto the chip, together with oil for the emulsion encapsulation. The chromium controller partitions the cells into nanoliter-scale gel beads in emulsion within which reverse transcription occurs. After the reverse transcription reaction, gel beads in emulsion were broken and the cDNAs cleaned with Silane DynaBeads MyOne Silane (Thermo Fisher Scientific). The cDNA was amplified and cleaned with SPRIselect beads (Beckman Coulter, Brea, CA), followed by a run on an Agilent 4200 TapeStation High Sensitivity D5000

Figure 1 Human fibrolamellar carcinoma (FLC) xenograft model. **A:** Human FLC cells were injected subcutaneously into immunodeficient mice to generate patient-derived explants (PDXs) that expressed the human FLC signature in-frame fusion of *DNAJB1* and *PRKACA*. **B:** PDX histology recapitulates typical features and immune reactivity of the desmoplastic human cancer, with positive staining for HepPar-1, cytokeratin 7 (KRT7), and CD68. **Arrowheads** indicate positively stained cells; **asterisk**, stromal cells. **C:** PDX cell suspensions grow as spheroids when cultured in serum-free conditions that prevent cell attachment. **Arrowheads** indicate nuclear heterogeneity. **D:** Cell suspensions express classic FLC markers, KRT7 (or CK7) and CD68, and the mesenchymal marker, vimentin. **E:** Differential staining of FLC cells that become adherent and those that remain unattached when cultured under serum-containing conditions. Original magnification: $\times 200$ (**B**); $\times 40$ (**C**, **D**, right panels, and **E**, left and right panels); $\times 20$ (**D**, left panels, and **E**, middle panel). H&E, hematoxylin and eosin; SCID-bg, severe combined immunodeficiency—beige; α -SMA, α -smooth muscle actin.

ScreenTape (Agilent, Santa Clara, CA) for qualitative and quantitative analysis. The cDNA was then enzymatically fragmented to a target size of approximately 200 bp, and sequencing libraries were constructed following the 10 $\times$ Genomics (Pleasanton, CA) Single Cell 3' Reagent Kits version 2 User Guide. The final libraries contain P5 and P7

primers used in Illumina bridge amplification. Sequence was generated using paired end sequencing on an Illumina HiSeq 2500 sequencing platform with a target of 50,000 reads/cell. This entailed end repair and A-tailing, adapter ligation, SPRIselect bead cleanup, a sample index PCR, and further SPRI select bead cleanups.

Figure 2 Single-cell RNA sequencing of the patient-derived explant reveals heterogeneity of human fibrolamellar carcinoma (FLC) cells. **A:** Single-cell transcripts align to either mouse or human transcriptomes, allowing discrete analysis of human cells from a representative explant. **B:** tSNE plot of the explant isolate showing four discrete communities (clusters 0 to 3) of human FLC cells. **C:** Distribution of a proliferation marker, cyclin D1 (CCND1), human FLC markers, carbamoyl phosphate synthase-1 (CPS1; HepPar-1), EPCAM, cytokeratin (KRT) 7, KRT18, KRT19, CD68, and mesenchymal genes, collagen 1a1 (COL1A1) and α -smooth muscle actin (ACTA2), among the four human FLC cell clusters. UMI, unique molecular identifiers.

Single-Cell RNA-Seq Analysis

The primary analytical pipeline for the single-cell analysis followed the recommended protocols from 10 $\times$ Genomics. Briefly, raw base call (BCL) files generated by Illumina sequencers were demultiplexed into FASTQ files, on which

alignment to the appropriate reference transcriptome, filtering, barcode counting, and unique molecular identifiers counting were performed using Cell Ranger software version 2.2 (10 $\times$ Genomics). Cell Ranger uses the chromium cell barcode to generate feature-barcode matrices encompassing all cells captured in each library. The

secondary statistical analysis was performed using the R package Seurat version 2.3.4 (Satija Lab, New York City, NY), which performs quality control and subsequent analyses on the feature-barcode matrices produced by Cell Ranger.¹⁵ In Seurat, data were first normalized and scaled after basic filtering for minimum gene and cell observance frequency cutoffs. Further filtering was then performed on the basis of a range of metrics to identify and exclude possible multiplets. The removal of further technical artifacts was performed using regression methods to reduce noise. After quality control procedures were complete, linear dimensional reduction was performed by calculating principal components using the most variably expressed genes in our data set. Genes underlying the resulting principal components were examined to exclude genes involved in cell division or other standard cellular processes.¹⁶ Significant principal components for downstream analyses were determined through methods mirroring those implemented by Macosko et al,¹⁶ and these principal components were carried forward to perform cell clustering and to enhance visualization. Cells were grouped into an optimal number of clusters for de novo cell type discovery using Seurat's Find Neighbors and Find Clusters functions, graph-based clustering approaches with visualization of cells being achieved through the use of t-SNE5, which reduces the information captured in the selected significant principal components to two dimensions.¹⁷ Differential expression of relevant cell marker genes was visualized on t-SNE plot to reveal specific individual cell types. Additional downstream analyses include examining the cellular distribution of a priori genes of interest, closer examination of genes associated with cell clusters, and the refined clustering of cells to identify further resolution of cell types, in addition to comparing differences between experiments of different states.

Single-Cell Trajectory Analysis

RNA trajectory analysis was performed using the R package Monocle2 version 2.12,¹⁸ which uses reversed graph embedding to describe multiple cell fate decisions in a fully unsupervised manner. Data were imported from the existing Seurat object and/or Cell Ranger-generated gene-barcode matrix. Basic filtering for minimum gene and cell observance frequency cutoffs was then performed, as well as size and dispersion estimates on count data. Dimensionality was reduced by performing a principle components analysis,

followed by t-Distributed Stochastic Neighbor Embedding (t-SNE) to project cells onto two dimensions. Density peak clustering, based on each cell's local density and the nearest distance (Δ) of a cell to another cell with higher distance, identified cell clusters in two-dimensional t-SNE space.¹⁸ Differential gene expression testing was performed to extract the genes that distinguish clusters. Top significant genes across all clusters were selected as input for the regions graph extraction algorithm that was used to define progress through the trajectory. The regions graph extraction algorithm DDRTree was used to learn a principal tree on the population of single cells that describes changes to global gene expression as a cell progresses through the biological process under study (pseudotime) as well as identify branch points that describe significant divergences in cellular state. Genes with significant branch-dependent expression were identified using branch expression analysis modeling.¹⁸

Protein Interactions and Gene Ontology Analysis

Using STRING database version 11.0,¹⁹ the protein-protein interactions and biological processes of genes that are highly expressed in clusters 2 and 3 were analyzed (Supplemental Table S1).

Partially Reprogrammed FLC-PDX Cell Line

Single-cell suspensions isolated from a third-generation FLC-PDX were cultured to select for cells that maintained decreased epithelial differentiation and high proliferative capacity in the absence of feeder cells. Briefly, cells were seeded atop irradiated rat fibroblast feeder cells and grown in reprogramming media, according to Liu et al.²⁰ Reprogramming F media consisted of a 3:1 mix of Ham F12 media and Dulbecco's modified Eagle's media, supplemented with 5% fetal calf serum, insulin, epidermal growth factor, cholera toxin to elevate cAMP, and the p kinase inhibitor Y-27632. The resultant partially reprogrammed FLC-PDX cells can be maintained in the absence of feeder cells for several passages.²⁰

Fluorescence-Activated Cell Sorting of FLC-PDX Cells

Single-cell suspensions from a fifth-generation FLC-PDX were generated as described above. Cells were incubated with FcR

Figure 3 YAP1 is highly expressed and active in human fibrolamellar carcinoma (FLC) cells. **A:** YAP1 immunostaining of a representative FLC patient-derived explant (**left panel**) and distribution of YAP1 transcripts among human FLC cell communities (**right panel**). **B:** Many human FLC cells that express the FLC marker, AKAP12, coexpress YAP1. **C:** Heat map of single-cell gene expression demonstrates enrichment of YAP1 and its target genes in cluster 0. **D:** FLC cells with capacity for autocrine self-renewal in culture [partially reprogrammed FLC (prFLC) cells express the FLC signature in frame fusion of *DNAJB1* and *PRKACA*]. **E:** prFLC cells express YAP1 and YAP1 target genes that are sensitive to YAP1 inhibition by verteporfin (VP), including connective tissue growth factor (CTGF), CYR61, and mesothelin (MSLN). **F:** VP inhibits prFLC cell growth, monolayer colony formation, and migration. **Dotted lines** show the noncell area. Data are expressed as means $\pm$ SEM (**E**). $n = 4$ (**E**). * $P < 0.05$, ** $P < 0.01$ versus vehicle (t -test). Original magnification: $\times 200$ (**A**); $\times 40$ (**F**). IHC, immunohistochemistry; MW, molecular weight.

Figure 4 Human fibrolamellar carcinoma (FLC) cell communities reflect different phenotypic states of a single cell type. **A:** All FLC communities express the *YAP1* target gene and mesothelial cell marker, mesothelin (*MSLN*); expression of the *MSLN* ligand, *MUC16*, and hepatic epithelial markers [*HNF4A*, *HNF1B*, and albumin (*ALB*)] is highest in clusters 0 and 1, whereas clusters 2 and 3 are enriched with elastin (*ELN*), a portal fibroblast marker. **B** and **C:** RNA trajectory analysis shows epithelial (**B**) and mesenchymal (**C**) genes are reciprocally regulated over pseudotime. *ACTA2*, actin alpha 2; *AGR2*, anterior gradient protein 2 homolog; *AREG*, amphiregulin; *COL1A1*, collagen 1a1; *CPS1*, carbamoyl phosphate synthetase I; *CTGF*, connective tissue growth factor; *CYR61*, cysteine-rich angiogenic inducer 61; *EPCAM*, epithelial cellular adhesion molecule; *KRT19*, keratin 19; *KRT7*, keratin 7; *YAP1*: yes-associated protein 1.

block (Miltenyi Biotech, Bergisch Gladbach, Germany), labeled with 10 μ g/mL mouse IgG (MOPC-21) or anti-CD63 (H5C6) and sorted on a BD DiVa (BD Biosciences, Franklin Lakes, NJ) selecting for phosphatidylethanolamine.

Results

FLC-PDX Model Faithfully Recapitulates Typical Human FLC

The FLC-PDX model of Oikawa et al¹⁴ was established in our laboratory by passaging samples of the human tumor that

Oikawa et al¹⁴ had used into new severe combined immunodeficiency—beige recipient mice (Figure 1A). The newly generated FLC-PDXs retained the histology expected for FLC, with large nucleoli-dense epithelial-like cells and small stromal-like cells within bands of fibrous matrix. Tumors stained positively for the FLC markers HepPar-1, cytokeratin 7 (KRT7), and CD68 (Figure 1B) and expressed the characteristic gene fusion of *DNAJB1* to *PRKACA* (Figure 1A). Therefore, this FLC-PDX model faithfully recapitulates the FLC-PDX model of Oikawa et al¹⁴ from which it was derived, as well as typical human FLC, particularly with regard to the abundant stromal element.

Figure 5 Differential expression of cell surface receptors distinguishes human fibrolamellar carcinoma (FLC) cell populations with differential expression of the signature *DNAJB1-PRKACA* fusion. **A:** Distribution and relative expression levels of anterior gradient-2 (AGR2) and CD63 in human FLC cells. Clusters 0 and 1 are enriched with cells that express the highest levels of both receptors. **B:** Fluorescence-activated cell sorting separates human FLC cells into CD63-low and CD63-high populations. **C:** The CD63-high cell population is enriched with the FLC signature in-frame fusion of *DNAJB1* and *PRKACA*. **D:** CD63 and its ligand, tissue inhibitor of metalloproteinase-1 (TIMP1), are coexpressed by most human FLC cells, and levels of TIMP1 transcripts correlate with CD63 expression ($r = 0.56$). MW, molecular weight.

Cells from FLC-PDX Tumors Exhibit Subpopulations

To further characterize the FLC-PDX, cell suspensions were generated from explanted tumor samples, subjected to cytopsin, and stained for epithelial/tumor markers [eg, cytokeratin 7 (CK-7), CD68, HepPar-1] and for mesenchymal markers [eg, vimentin, α -smooth muscle actin (α -SMA), and fibroblast activation protein (FAP)]. Tumor cells isolated from previous FLC-PDXs have been reported to be maintained *in vitro* under serum-free conditions, where the cells cannot attach to a culture dish.¹⁴ It was confirmed that when cultured in these conditions, the cells from the FLC-PDX also aggregated into floating (non-adherent) spheroids. DAPI staining demonstrated nuclear size variation among spheroid-forming cells (Figure 1C), as well as variable staining for tumor markers and mesenchymal markers (Figure 1D). Tumor and mesenchymal markers were sometimes coexpressed, suggesting that a subpopulation of cells in the spheroids may have been undergoing epithelial-to-mesenchymal/mesenchymal-to-epithelial transitions. Indeed, some cells that expressed α -SMA or FAP quickly attached and spread when cultured

on plastic in the presence of serum. These adherent cells generally lacked tumor/epithelial markers. In contrast, α -SMA (+) or FAP (+) cells that maintained coexpression of the tumor/epithelial markers (HepPar-1, CK-7, and CD68) tended to remain nonadherent (Figure 1E).

Single-Cell RNA-Seq of FLC-PDX Cells Confirms Tumor Cell Heterogeneity

The FLC-PDX model generated by Oikawa et al¹⁴ was reported to consist of human tumor cells interspersed among nonmalignant host (mouse) stromal cells, with mouse cells comprising up to 70% of the FLC-PDX. In that study, cell suspensions from four different FLC-PDX passages were subjected to bulk RNA-seq transcriptional profiling after enriching for human cells by immunodepleting cells that expressed mouse H2K^d. However, we were interested in establishing how epithelial tumor cells recruit stromal cells to generate the characteristic fibrotic environment within FLC tumors to maintain the FLC cancer stem cells. Therefore, a cell suspension was generated from a fresh FLC-

PDX and the entire (ie, nonselected) cell suspension was subjected to single-cell RNA-seq. The single-cell transcripts were aligned to the human and mouse transcriptomes. This approach revealed few barcodes that contained mixed mouse and human doublets, establishing that most aligned data represent single cells aligning to one specific species and thereby allowing discrete analysis of human versus mouse cells within the explant (Figure 2A). Hereafter, FLC-PDX tumor cells that were human, as defined by exclusive expression of human transcripts, were studied further.

The human cell population in the FLC-PDX was heterogeneous and sorted into four distinct clusters based on their gene expression profiles (Figure 2B). The two most abundant human cell clusters (0 and 1) were transcriptionally similar and characterized by high expression of genes in metabolic and biosynthetic pathways involved in cell proliferation, including cyclin D1 (*CCND1*). Multiple FLC marker genes were also most abundant in these two largest clusters, including HepPar-1 (carbamoyl phosphate synthase-1), EPCAM, cytokeratins (*KRT*) 7, 18, and 19, and CD68 (Figure 2C). The presence of these markers identifies these cells as the epithelial, and presumed malignant, proliferative tumor cells. Surprisingly, the two smaller human clusters (2 and 3) were not enriched with classic FLC markers. Rather, they were characterized by expression of extracellular matrix components, including collagen 1- α -1 and multiple other forms of collagen (Supplemental Figure S2). Mesenchymal and stromal marker genes, such as α -SMA (actin alpha 2), were also most abundant in clusters 2 and 3 (Figure 2C). Therefore, these clusters are human tumor-associated stromal cells that have been maintained in explant passaging. Hence, scRNA-seq analysis of this FLC-PDX revealed unexpected heterogeneity within the human cell population, demonstrating that only a subgroup of the human cells in this tumor express classic FLC markers, whereas many other human cells lack these markers and appear to be stromal.

High YAP1 Activity in Subpopulation of Human FLC Cells

Previous analysis of bulk RNA-sequencing data from the mouse FLC-PDX model led to the conclusion that malignant FLC cells resemble multipotent stem-like cells that are localized in the peribiliary glands of the adult extrahepatic biliary tree.¹⁴ Biliary stem-like cells exhibit high activity of a stem cell–associated transcriptional regulator, Yes-associated protein 1 (YAP1), and are thought to provide a source of cells that ultimately differentiate into pancreatic ductal cells, cholangiocytes, or hepatocytes.^{21,22} When YAP1 protein is regulating gene transcription, it localizes in cell nuclei and modulates expression of various YAP1 target genes that control cell viability, growth, and differentiation.²³ Therefore, immunohistochemistry was used to screen primary human FLCs (Supplemental Figure S3) and the FLC-PDX mouse model (Figure 3A) for expression of YAP1 protein. Many cell nuclei in both primary human

FLCs and the FLC-PDX model were strongly positive for YAP1, suggesting that high YAP1 activity might occur in FLCs. Subsequent scRNA-seq analysis of an FLC-PDX cell suspension demonstrated that human clusters 0 and 1 were enriched with cells that expressed *YAP1* mRNA (Figure 3A) and confirmed that many cells in those clusters coexpressed the FLC marker A-kinase anchoring protein 12 (*AKAP12*) and YAP1 (Figure 3B). Furthermore, a heat map of single-cell mRNA expression of *YAP1* and YAP1-regulated genes showed that expression of YAP1 and its target genes was particularly robust in cluster 0 (Figure 3C). Evidence that YAP1 transcriptional activity was greatest in a discrete subpopulation of the human cancer cells that expressed both high mRNA levels of *YAP1* (Figure 3A) and known FLC markers (Figure 2C) suggested that this tumor cell community harbored FLC stem cells.

Partially Reprogrammed FLC Cell Line Identifies Yap1 as Possible FLC Therapeutic Target

To evaluate the functional relevance of YAP1 signaling in FLC stem-like cells, a partial reprogramming protocol²⁰ was used to select cells isolated from a FLC-PDX for the ability to maintain autocrine self-renewal in attached culture (a defining feature of stem-like cells). These partially reprogrammed FLC cells maintain expression of the FLC signature *DNAJB1-PRKACA* fusion (Figure 3D). As previously reported, such prFLC cells express YAP1 and YAP1 target genes,²⁴ and thus prFLC cell line and the YAP1 inhibitor drug verteporfin²⁵ were used to test whether inhibiting YAP1 alters tumor-associated activities (Figure 3, E and F). Compared with vehicle, verteporfin treatment significantly reduced the rate of cell growth, colony formation, and migratory capacity of prFLC cells (Figure 3F). mRNA expression of YAP1 target genes [eg, connective tissue growth factor (*CTGF*), *CYR61*, and *MSLN*] was also inhibited by verteporfin (Figure 3E).

FLC Cells with High Yap Activity May Derive from Hepatic Mesothelial Progenitors

The scRNA-seq data demonstrate that virtually all of the human FLC cells in clusters 0 and 1 strongly express the YAP1 target gene *MSLN* (Figures 3C and 4A), and immunostaining of primary human FLCs and our FLC-PDXs confirmed expression of MSLN protein in the cancer (Supplemental Figure S4). MSLN is highly expressed during embryonic development and down-regulated in adulthood, but strongly reexpressed in certain cancers, including cholangiocarcinomas.²⁶ Interestingly, MSLN is also strongly induced in adult portal fibroblasts when bile ducts are injured.²⁷ The source of portal fibroblasts in adult livers is uncertain, but it has been suggested that they may derive from injury-activated hepatic mesothelial progenitors; and studies in MSLN-depleted portal fibroblasts

proved that MSLN critically regulates the proliferative activity of those cells by engaging its ligand, mucin 16 (MUC16).²⁷ Many of the MSLN-expressing cells in human cell clusters 0 and 1 of the FLC-PDX express MUC16 (Figure 4A). In addition, these communities are enriched with CCND1-expressing cells (Figure 2C), suggesting that MUC16 also increases the proliferative activity of MSLN-positive human FLC cells.

More important, the vast majority of human MSLN-positive cells in clusters 0 and 1 express KRT7 (Figure 2C). Other markers of hepatocyte and cholangiocyte progenitors [eg, hepatocyte nuclear factor (HNF)4 α and HNF1 β] (Figure 4A), cholangiocytes (KRT19) (Figure 2C), and hepatocytes (albumin) (Figure 4A) are also enriched in these clusters. Therefore, the aggregate data suggest that these two communities of malignant human FLC cells are enriched with proliferative progenitors of mature liver epithelial cells. Interestingly, a subset of cells in clusters 0 and 1 express factors that promote epithelial-to-mesenchymal transitions [eg, snail family zinc finger (SNAIL)1, SNAIL2, or zinc finger E-box binding homeobox 1 (ZEB1); data not shown] and a few express α -SMA (ACTA2) or elastin (ELN; a marker of portal fibroblasts²⁸), raising the possibility that some of the FLC cells in clusters 0 and 1 are transitioning into (or out of) a mesenchymal state. Clusters 2 and 3 may also harbor some transitioning cells on the basis of low-level expression of epithelial-to-mesenchymal transition—promoting factors (eg, TWIST or ZEB1; data not shown), MSLN, and ACTA2. However, clusters 2 and 3 are predominately composed of fibrogenic, ACTA2- and ELN-expressing human cells (Figures 2C and 4A), suggesting enrichment with fibrogenic myofibroblasts. The myofibroblastic cells in clusters 2 and 3 might be portal fibroblasts as the scRNA-seq data demonstrate that collagen 1 α 1 (COL1 α 1) is the second most highly expressed gene in FLC communities 2 and 3 (Supplemental Figure S2) and Msln (+)/Acta2 (+)/Eln (+) portal fibroblasts are major collagen-producing myofibroblasts in mice after biliary injury.²⁸

RNA-trajectory analysis (Figure 4, B and C) was performed to plot gene expression in pseudotime to further characterize relationships among the clusters of human cells in the FLC-PDX. Unexpectedly, the results suggest that the four human clusters (Figures 2, B and C, and 4A) reflect a single cell type in different states, with some clusters being relatively more (or less) epithelial than others. Cells that are the most epithelial, as evidenced by expression of liver epithelial cell genes (eg, albumin, carbamoyl palmitate synthase-1, EPCAM, KRT19, and KRT7), coexpress amphiregulin (AREG) (Figure 4B), a YAP1 target gene that encodes an epithelial growth factor.²⁹ Cells that are the most mesenchymal, as evidenced by expression of mesenchymal genes (eg, ACTA2, ELN, and COL1 α 1), coexpress CTGF and CYR61 (Figure 4C), YAP1 target genes that are profibrogenic.^{30,31} Interestingly, expression of anterior gradient-2 (AGR2), a gene that others have also found to be selectively up-regulated

in human FLC versus other types of primary liver cancer³² and that promotes mesenchymal to epithelial transitions and inhibits epithelial to mesenchymal transitions,³³ is fairly ubiquitously expressed (Figure 4B).

Cell Surface Receptors Differentiate FLC Cells with High and Low YAP1 Activity

The aggregate findings in the FLC explant and prFLC cell line reveal that YAP1 is a potential therapeutic target in FLC, and thus the scRNA-seq data were reexamined to identify surface markers that might be used either to identify FLC cells with high YAP1 activity or as novel tools to manipulate the fate of such cells therapeutically. The top two differentially expressed genes encoding cell surface receptors were AGR2 and CD63, and cells that expressed the highest levels of AGR2 and CD63 localized to clusters 0 and 1 (Figure 5A). Furthermore, the mRNA abundances of these two genes were highly correlated with each other ($r = 0.81$), indicating that most AGR2-expressing cells also expressed CD63 and vice versa. Therefore, anti-human CD63 antibodies and fluorescence-activated cell sorting were used to isolate human cells with strong human CD63 expression (CD63-high cells) from an FLC-PDX cell isolate, and gene expression in this CD63-high human cell population was compared with that of the CD63-low cell population (Figure 5B). The FLC signature *DNAJB1-PRKACA* fusion was significantly enriched in the CD63-high population (Figure 5C). Furthermore, CD63 expression strongly correlated with expression of several YAP1 target genes that were preferentially expressed in the high YAP1 activity human FLC cluster 0 (Figure 3C), including thioredoxin ($r = 0.88$), MSLN ($r = 0.84$), baculoviral IAP repeat containing 2 (BIRC2; $r = 0.63$), and AREG (0.51). These results confirm that differential expression of cell surface receptors (eg, CD63) can be exploited to distinguish malignant human FLC cells with high YAP1 activity (presumably cancer stem cells) from other cells in the tumor with lower YAP1 activity. This advance will facilitate future efforts both to develop targeted therapies to eliminate the cancer stem cell compartment in FLC and to evaluate treatment response by monitoring depletion of the CD63-high community.

CD63 itself might be a useful therapeutic target to deplete putative FLC stem cells because CD63 is known to transduce fate-regulating signals in other stem cells. CD63 is the receptor for tissue inhibitor of metalloproteinase-1 (TIMP1),³⁴ a factor that is mostly known for its ability to promote fibrosis progression by blocking matrix degradation.³⁵ Intratumor fibrosis is a defining characteristic of FLC and, thus, evidence that the TIMP1 gene is expressed by some cells in each of the human FLC clusters (Figure 5D) was not surprising. However, this finding also raised the possibility that TIMP1, generated by CD63-low cells, might support some function in CD63-high stem-like FLC cells.

Interestingly, mRNA levels of *TIMP1* and *CD63* correlated with each other at the single-cell level ($r = 0.56$), suggesting that CD63-high FLC cells themselves produced TIMP1 to autoregulate CD63 signaling. TIMP1-CD63 interaction is known to launch an autocrine mechanism that regulates fate decisions in human mesenchymal stem cells (hMSCs). In those cells, binding of endogenous TIMP1 to CD63 promotes accumulation of axis inhibition protein 2 (AXIN2), a negative regulator of β -catenin signaling, to suppress hMSC differentiation and maintain hMSC stemness.³⁶ Interestingly, a subpopulation of stem-like cells with high Axin2 transcriptional activity was recently identified in adult mouse livers, and lineage tracing showed that progeny of these cells gradually replenish adult hepatocyte populations in healthy livers.³⁷ The scRNA-seq data demonstrate that CD63-high FLC cells express *MSLN*, an acknowledged marker of hMSC-like cells,^{38–40} raising the intriguing possibility that similar to hMSCs, stem-like human FLC cells may become less stem-like when TIMP1-CD63 signaling is disrupted.

Discussion

Taken together, our findings demonstrate unexpected complexity in both the epithelial and stromal components of FLC and support the novel hypothesis that FLC may arise from malignant transformation of a multipotent hepatic mesothelial progenitor that is able to generate both hepatic epithelial and stromal cells. On the basis of a previous bulk analysis of human versus mouse cells isolated from a mouse FLC-PDX model,¹⁴ it has been assumed that malignant human FLC epithelial cells recruit normal mouse stromal cells to generate the characteristic fibrous matrix associated with FLC. In the case of FLC-PDXs grown in mice, this stroma would be composed of nonmalignant normal mouse stromal cells. Extrapolating from this model to the human tumor *in situ*, it was therefore expected that the stroma in primary human FLCs would also be composed of normal human stromal cells recruited to the malignant tumor epithelium. However, our single-cell RNA-seq analysis of a mouse FLC-PDX model unequivocally identifies human cell subpopulations with stromal characteristics, as well as the expected mouse stromal cells. These data suggest that spontaneously occurring FLCs in humans harbor a stem cell population that gives rise to both human tumor epithelium and stromal cells, in addition to recruiting other stromal cells from nontumor tissue. This new evidence that a stem cell in FLCs is capable of differentiating into either hepatocytic/biliary epithelial cells or stromal cells suggests that FLCs arise from mutations in some multipotent progenitor rather than in a mature hepatocyte. This may explain why hydrodynamic targeting of mature hepatocytes with Cas9 gene editing machinery to recreate the *DNAJB1-PRKACA* fusion, or with a transposon to direct overexpression of

the fusion, fails to recapitulate some of the features of FLC, particularly the lamellar stroma.^{12,13}

Further research is necessary to determine whether any of the stromal cells in FLCs are malignant, or at least contain the signature FLC gene fusion. This merits consideration because low expression of the *DNAJB1-PRKACA* fusion product was detected in the subpopulation of FLC-PDX cells that express high levels of fibroblast-associated genes and low levels of human CD63. However, that finding might simply reflect contamination of the CD63-low fraction with rare CD63-high malignant human epithelial cells despite cell sorting. Unfortunately, single-cell RNA-seq is not helpful for directly detecting the hybrid mRNA arising from the *DNAJB1-PRKACA* fusion in FLC as it is biased to 3' untranslated region sequences. Therefore, the presence of this fusion in cell clusters cannot be directly measured. Also, these approaches do not exclude the possibility that other mutations that are known to occur in FLC¹⁰ might have occurred in the human stromal cells of this FLC-PDX. Finally, it is conceivable that the human fibroblastic cells in the FLC-PDX are residual nonmalignant stromal elements that remain closely associated with the malignant CD63-high human FLC cells, despite many passages, and this should be tested using sorted human cell populations (CD63-high versus CD63-low) to generate explant tumors. Regardless of their cellular origin, however, these human stromal cells exhibit gene expression, indicating that they contribute to the desmoplastic stroma in FLC.

Having identified subpopulations of human cells within the FLC-PDX model, those markers and functions that associate with one or another subpopulation can be clearly differentiated. These analyses reveal that only some human cells in the tumors express known FLC-associated markers, including HepPar-1 (carbamoyl palmitate synthase-1), EPCAM, KRT7, KRT18, and KRT19,^{6,41} and are highly proliferative [as indicated by strong cyclin D1 (*CCND1*) expression].⁴ Cells in this tumor community harbor the FLC signature *DNAJB1-PRKACA* fusion product and also strongly express CD63, and the latter marker was used as a cell surface tag to sort them. Intriguingly, these CD63-high cells also highly express YAP1, which has been associated with growth of multiple epithelial tumor types, including cholangiocarcinoma^{21,22} and pediatric primary liver cancers, including FLC.⁴² YAP1 appears to be critically involved in FLC biology on the basis of several lines of emerging evidence. First, YAP1 is directly phosphorylated by PKA; PKA activity is increased by the FLC signature *DNAJB1-PRKACA* fusion, but the transcriptional activity of the canonical PKA-activating transcription factor, cAMP-regulated binding protein, is not increased. The FLC gene expression is likely controlled by alternative PKA targets, possibly including YAP1. This concept is supported by results of an early study that compared the transcriptomes of seven primary human FLCs with those of the respective nontumor livers. The cancers were consistently found to

overexpress AREG, a direct YAP1 target gene that activates the epidermal growth factor/ErbB receptor to induce a mitogenic signaling pathway that is strongly up-regulated in human FLC.⁷ Second, subsequent profiling of miRNAs and long noncoding RNAs in these same seven human FLCs and adjacent nontumor livers identified the Hippo-Yap pathway as one of the most selectively targeted oncogenic signaling pathways in FLC. For example, 74 of the miRNAs were found to be uniquely down-regulated in FLC target 117 genes involved with the Hippo-Yap signaling pathway that are differentially induced in FLC relative to nontumor liver. Conversely, 68 of the up-regulated miRNAs in FLCs target another 113 genes involved in Hippo-Yap signaling that are differentially suppressed in FLC. Furthermore, simply manipulating one of the differentially expressed miRNAs in a hepatocellular carcinoma cell line (Huh-7) was sufficient to alter oncogenic signaling in those cells, leading the authors to suggest that in FLCs, miRNAs and long noncoding RNAs help promote the activity of oncogenic pathways, such as Hippo-Yap.⁴³ Third, additional evidence supports the functional significance of miRNA-YAP1 interaction in FLC. miR-375, an miRNA that directly binds to and destabilizes YAP1 transcripts, is known to be suppressed by PKA and is one of the most down-regulated miRNAs in human FLC. Introducing miR-375 mimics into prFLC cells significantly suppressed YAP1 expression, reduced the expression of YAP1 target genes, and inhibited growth and migration of the cancer cells.²⁴ Finally, in the present study, similar responses were achieved by treating prFLC cells with verteporfin, a well-accepted pharmacologic inhibitor of YAP1.²⁵ YAP1 is known to maintain the proliferative and migratory capabilities of multipotent progenitors and, thus, antagonizes the terminal epithelial differentiation of such cells during development.²³ Herein, it was demonstrated that FLC cells with high YAP1 activity and CD63 expression strongly express MSLN, a gene that is known to mark portal fibroblasts²⁷ and their progenitors, including mesenchymal stem-like cells.^{39,44} Co-expression of these markers at the single-cell level suggests that the CD63-high malignant tumor cells may be derived from some hybrid-type multipotent progenitor cell that co-expresses epithelial and mesenchymal genes. Hence, such cells might be the direct mutagenesis targets in FLC patients, rather than hepatocytes, as thought.

More important, both human epithelial and stromal compartments in FLCs express CD63 and MSLN (albeit to different degrees), identifying these two receptors as novel therapeutic targets that are likely to be broadly relevant in this malignancy. Blocking the interaction of CD63 with its endogenous ligand TIMP1³⁴ might be exploited to deplete FLC stem cell populations by disrupting stem cell maintenance mechanisms.³⁶ Drugs that inhibit YAP1,^{37,45} AREG,^{46,47} CTGF,^{30,31} and/or cysteine-rich angiogenic inducer 61 (CYR61)^{48,49} could be engineered to bind to CD63, thereby targeting their delivery to FLC cells to reduce tumor growth, metastasis, and desmoplasia. Finally,

MSLN has been successfully targeted by CAR-T cells and other immunotherapies in ovarian cancer,^{50–52} suggesting another novel, and more personalized, therapeutic strategy that might be applied to FLC.

In summary, we generated a novel reagent (ie, prFLC cell line) that can be exploited for high-throughput screening of putative therapeutic agents for FLC and are the first to demonstrate the utility of scRNA-seq analysis for defining FLC biology, resulting in the unexpected discovery that a multipotent hepatic mesothelial cell is likely the cell of origin for this cancer. Furthermore, we provide proof of concept that this approach can identify novel targets, such as CD63 and MSLN, which could be leveraged to improve diagnosis and treatment of FLC. Nevertheless, our work has some limitations. The scRNA-seq analysis is based on findings in <300 cells from a single FLC explant. More important, both the prFLC cell line and FLC-PDX mouse model were derived from cancer cells from a single FLC patient. Thus, further research is needed to determine how relevant our findings are to cancer cells from other FLC patients. Indeed, a library of ubiquitous, and patient-specific, FLC targets may be developed by broadly applying the methods we used in this study.

Acknowledgments

We thank Lola M. Reid and Eliane L. Wauthier (University of North Carolina at Chapel Hill, Chapel Hill, NC) for providing the primary human fibrolamellar carcinoma cells for the patient-derived explant mouse (PDX) model and providing help with establishing the PDX model at Duke; and Emily Hocke and Karen Abramson (Molecular Genomics Core, Duke Molecular Physiology Institute) for generating the single-cell libraries.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.09.018>.

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