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Supplemental Information

Fine-Needle Aspiration-Based Patient-Derived Cancer Organoids

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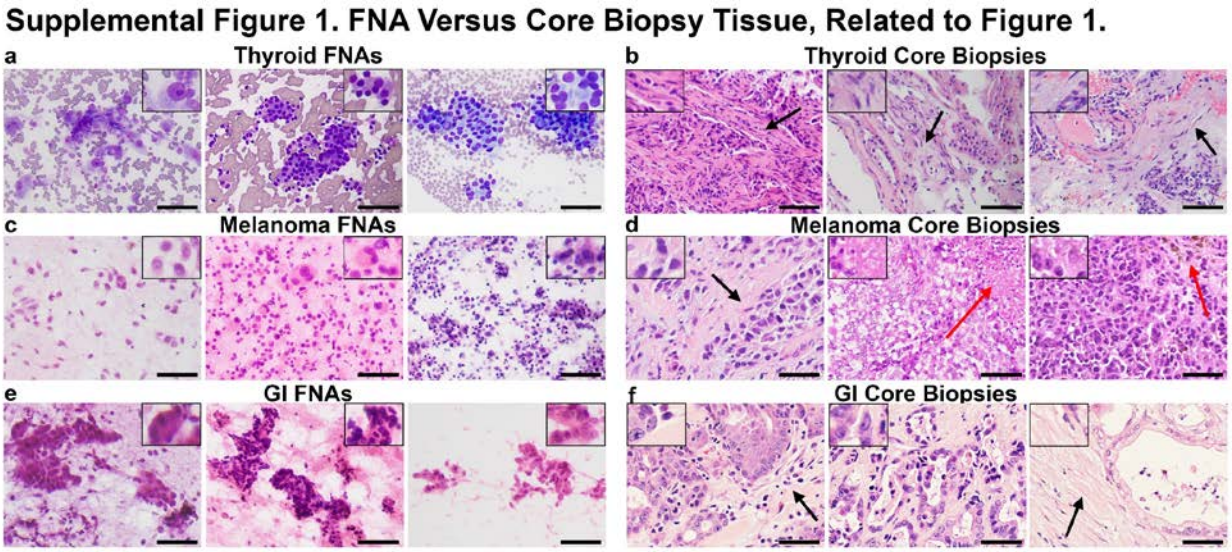
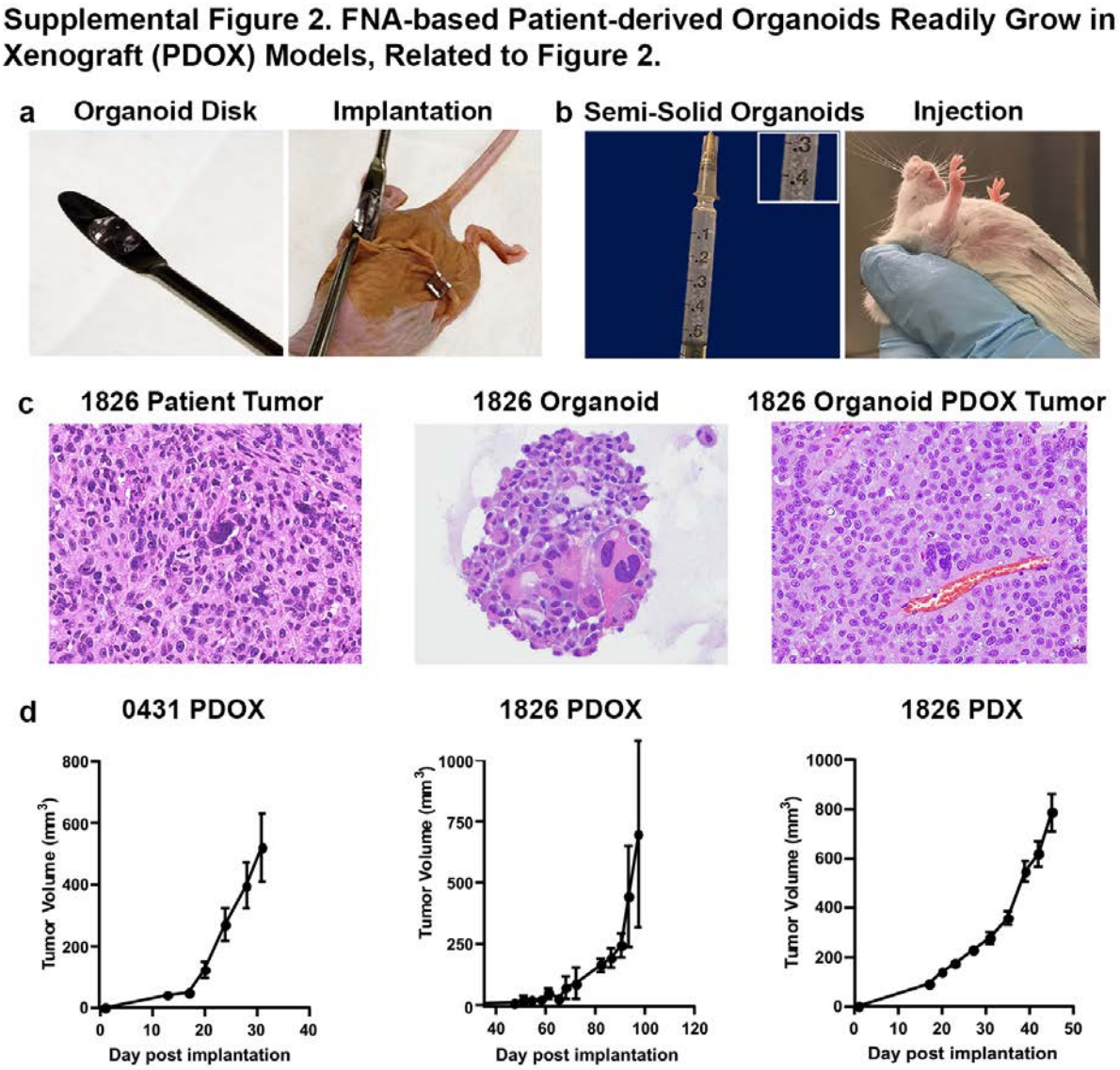
SUPPLEMENTAL FIGURES AND LEGENDS



Supplemental Figure 1: FNA Versus Core Biopsy Tissue, Related to Figure 1. a, FNA smears of thyroid cancer (Diff-Quik stain). Left panel demonstrates predominantly discohesive tumor and immune cells aspirated from an anaplastic thyroid carcinoma. Middle and right panels demonstrate clusters of tumors cells isolated from well-differentiated papillary thyroid carcinomas. **b,** Core biopsy specimens (hematoxylin and eosin, H&E stain) of thyroid carcinoma from two lung metastases (left and middle panels) and one bone metastasis (right panel). Black arrow indicates fibrous stroma in core biopsies. **c,** FNA smears of three melanomas (H&E stain) demonstrate largely discohesive, single malignant cells. **d,** Core biopsy specimens (H&E stain) demonstrate numerous malignant cells with surrounding stroma (black arrow) and necrosis (red arrows). **e,** FNAs from gastrointestinal cancers (H&E stain) demonstrate clusters of malignant epithelial cells in colorectal adenocarcinoma (left panel), gastric adenocarcinoma (middle panel), and pancreatic adenocarcinoma (right panel). **f,** Core biopsy specimens (H&E stain) demonstrate malignant glands with intervening stroma in colorectal adenocarcinoma (left panel), gastric adenocarcinoma (middle panel), and the classic dense desmoplastic stroma of

pancreatic adenocarcinoma (right panel). Black arrow indicates fibrous stroma in core biopsies. All images taken at 20X magnification, 50μm scale bar.



Supplemental Figure 2: FNA-based Patient-derived Organoids Readily Grow in Xenograft (PDOX) Models, Related to Figure 2. a, FNA-based patient-derived organoid disc cultures can be surgically implanted into immune compromised mice for creation of an FNA-PDOX model. **b,** For slower-growing tumors, organoids can be cultured in the semi-solid format using enriched media and then injected into immune compromised mice. **c,** FNA-PDOX tumors from

melanomas display morphology similar to both the original patient tumor and the organoid culture. **d,** PDOX tumors grow robustly in both nude and NSG mice with similar tumor growth characteristics to a standard PDX model follow disc implantation. Images representative of 3 replicates; error bars represent standard deviation.

SUPPLEMENTAL TABLES AND LEGENDS

Supplemental Table 1: FNA cellular yield per tumor type, related to figure 1.

	Melanoma (Xenograft) (n=3)	**Thyroid** (Patient and xenograft) (n=4)	**Renal** (Patient) (n=4)
Average Number of Cells Extracted/ Pass	1.3 $x10^6$	1.0 x 10^6	1.7 $x10^5$
Range of cells collected/ Pass	$8x10^5$-$1.8x10^6$	1x 10^5-$3x10^6$	$1.1x10^5$-$2.2x10^5$
Range of tumor sizes	1.5-1.6 cm	1.0-3.0 cm	5.3-10 cm

Legend Supplemental Table 1: Cell counts from representative tissues following FNA.

Supplemental Table 2: Success rate of organoid culture following FNA of patient and xenograft specimens, related to figure 1.

	Melanoma Xenograft (n=24)	**Colorectal** (n=8)	**Pancreatic** (n=5)	**Other GI (appendix, hepatobiliary, gastric)** (n=4)	**Thyroid** (n=45)	**Renal** (n=15)*
Organoid formation in culture (≥3 weeks)	19/24 (79%)	7/8 (88%)	5/5 (100%)	4/4 (100%)	44/45 (98%)	12/15 (80%)
Successful propagation/ cryopreservation	13/24 (54%)	2/2	N/A	2/2	12/12 (100%)	N/A

Legend Supplemental Table 2: Organoid culture success rates following FNA. *= rates reflect organoid growth in enriched media containing noggin, R-spondin, and Wnt3A.

TRANSPARENT METHODS

Fine-needle aspiration technique for tumors

Fine-needle aspiration was performed on patient tumors using a sterile 25-gauge beveled needle attached to a sterile 10ml syringe with a syringe holder used for gentle aspiration (**FNA Procedure Demonstration Video**). The target was immobilized with one hand with the syringe holder held in the other. The procedure can be performed without the use of a syringe holder, allowing for the sample to be drawn into the needle through capillary action. However, the concentration of tumor cells in the sample may be higher with the use of aspiration from a syringe holder. Of note, for highly vascular or bloody lesions, a higher gauge needle and capillary action may yield a more concentrated sample. The target was immobilized with one hand and the needle was inserted perpendicular to the mass. Once the needle was within the

lesion, backward pressure was applied on the syringe using the syringe holder. The needle was then quickly agitated within the lesion using long needle excursions at a rate of 2-3 strokes per second, for a total of 10-20 excursions. This rapid cutting motion was essential for the collection of a generous sample. Once cellular material was identified in the hub of the needle, the syringe holder suction was released and the needle was removed from the patient or specimen. One to three needle passes were collected from each tumor. During each aspiration, the excursions were performed in a fan-shape to ensure a larger region of sampling. In addition, particularly for larger lesions, each needle pass was performed in a separate area of the tumor for the most heterogeneous and cellular tissue collection. Insertion of the needle in along the same path as a prior pass led to increased blood collection and a more dilute sample. Following each needle pass, the needle was rinsed in either sterile RPMI 1640 or DMEM. The FNA-acquired cells were then centrifuged and immediately plated for FNA-PDO culture or stained for flow cytometry. All FNA training for this study was carried out by an experienced cytopathologist (VW).

Semi-solid FNA-PDO culture

FNAs were collected from patient tumors (1 needle pass) and rinsed in 20ml of DMEM. FNA-collected cells were then mixed with ice-cold complete media containing DMEM/Hams F12/MCDB105 (2:1:1 ratio), 12% FBS (Gibco), B27-supplement (Gibco), and 5% Matrigel (Corning). Cells were then immediately plated into 12 wells of a 24-well ultra-low attachment plate (Corning 3473). 200µl of 5% Matrigel in complete media was added drop-wise to each well weekly to compensate for volume loss through evaporation.

Disc FNA-PDO culture

FNAs were collected from patient tumors (1 needle pass) and rinsed in 20ml DMEM. FNA-collected cells were then gently mixed with ice-cold complete media containing DMEM/Hams F12/MCDB105 (2:1:1 ratio), 10% FBS, B27-supplement, and 75% Matrigel. The ice-cold

Matrigel and cells were then plated in 50µl discs in the bottom of each well (3 discs per well) of pre-warmed 6-well tissue culture plate and placed in the incubator for 10 min to allow the Matrigel to solidify. Once solidified, the Matrigel discs were covered with pre-warmed complete media containing 10% FBS plus B27 supplement and this media was changed every other day.

H&E and Immunohistochemical staining of FNA-PDOs

FNAs were collected from patient tumors and plated in disc FNA-PDO culture as described above. Following FNA-PDO growth (1-2 weeks in culture), organoids were centrifuged 1200rpm for 10 minutes. Media was aspirated, and the organoids were resuspended in 10% neutral buffered formalin (NBF) for 30 minutes. The organoids were then centrifuged at 1200rpm for 10 minutes. The NBF was aspirated and the organoids were resuspended in 70% alcohol for 15 minutes three times, organoids were centrifuged at 1200rpm for 10 minutes between each alcohol wash. After the final alcohol wash cells were resuspended in 1.5% heated UltraPure™ Agarose Invitrogen/Thermo (# 16500100) and transferred to a cryomold for 30 minutes. The agarose-organoid block was processed and embedded in paraffin using a two-hour processing run.

Immunofluorescent staining of FNA-PDOs

Organoids were fixed in 4% formaldehyde prior to permeabilization with 0.5% TBST. Following a 0.1% TBST rinse, organoids were rotated in Abdil (0.1% TBST, 2% BSA) for 1 hour at RT. Organoids were stained with anti-smooth muscle actin (rabbit anti-SMA, 1:250, Abcam) and anti-cytokeratin 8/18 (guinea pig anti-CK8/18, 1:250, Abcam) in blocking buffer overnight at 4˚C. After incubation, organoids were washed in 0.1% TBST and secondary stained with AlexaFluor488-conjugated anti-guinea pig antibody and Alexa-Fluor647-conjugated anti-rabbit antibody (both 1:250, Abcam) for 2-3 hours at RT. Organoids were stained with Hoechst

(Abcam) washed with 0.1% TBST, resuspended in PBS, and mounted onto glass slides with 50µl ProLong Gold Antifade Reagent (Invitrogen). After a 15-minute covered incubation at RT, coverslips were sealed with nail polish. Images were acquired using a Nikon Spinning Disk microscope with Andor DU-897 EMCCD camera and 647nm, 488nm, and 405nm lasers. Images were processed using ImageJ (Fiji, Build: 269a0ad53f).

FNA-PDO MTT assay

Gastric signet ring organoids were harvested from a 6-well plate (one 200ul Matrigel disc per plate with near confluent organoids) in ice-cold PBS, subjected to partial mechanical dissociation by pipetting up and down approximately 10 times with a glass Pasteur pipette and then centrifuged for 10 min at 8,000rpm at 4°C. Residual Matrigel was removed with a pipette, and pelleted organoids were resuspended in fresh Matrigel. 50ul of Matrigel containing FNA-PDOs were plated into a black 96-well plate, ensuring that the bottom of each well was covered. Organoids were fed with 100ul of media (Advanced DMEM, Gibco) containing 5% FBS (Gibco), 1X B-27 (Gibco), 100mg/ml EGF (Life Technologies), 10mg/ml FGF (Life Technologies), 1X Insulin-Transferrin-Ascorbic Acid (Life Technologies) every 3-4 days. Organoids were allowed to grow for approximately 2 weeks and drugs then administered in triplicate or quadruplicate every 3-4 days for an additional 2 weeks. Drug concentrations were calculated for 150ul (100ul media+50ul Matrigel). At the end of the trial, 20ul CellTiter-96 (Promega) was added to 100ul of fresh media and the absorbance (492nm) measured on a microplate reader (Molecular Devices). Background readings (Matrigel + media without organoids) were subtracted from each measurement, and data normalized by dividing each measurement by the largest value obtained on the plate.

FNA-PDO fluorescent viability assay

Cell suspensions were prepared from organoids by trypsinization. Cells were seeded in semisolid media containing 5% Matrigel (Corning) supplemented with 15% FBS, penicillin/streptomycin (Corning; 1X) and B27 (Thermo Fisher Scientific) at an equal number of organoids per well in ultra-low attachment 96 well plate. Drugs were added directly to culture wells for 72 hours in triplicate including vehicle treated control. After 48 hours of drug dosing, propidium iodide was added to the wells at a final concentration of 50 µg/ml. After 24 hours of culturing, Hoechst 33342 and Calcein AM (Thermo Fisher Scientific) were added to the wells with organoids at final concentration of 10 µg/ml and 5 µM, respectively. Organoids were incubated in standard cell culture conditions at 37C with 5% CO2 cultured for two hours. Imaging were taken on inverted fluorescent microscope (EVOS™ M7000, Thermo fisher). The intensity of red (dead cells) and green (live cells) fluorescent signal in individual organoids was quantified using Image J. Ratios of red/green fluorescence signal in individual organoids were plotted and statistical comparison was performed using Prism software (GraphPad).

High-throughput 384-well FNA-PDO assay

Organoids were washed and resuspended in 5ml TrypLE for dissociation. Organoids were incubated at 37°C for 30 min, with vigorous pipetting every 10 min to prevent clumping. Cells were spun down and resuspended in complete DMEM + 2% Matrigel at a concentration of 9500 cells/ml. 30 µl of cell suspension was plated per well in black 384-well cell culture microplates with a cell-repellant surface (Greiner Bio-One #781976). Organoids were allowed to form for 24 hours prior to treatment with doxorubicin (25-10,000nM) or vehicle (DMSO) as a control. Following 72 hours of treatment, wells were imaged using an ImageXpress Micro XL automated high-content microscope (Molecular Devices) in the Vanderbilt High-throughput Screening (VHTS) core facility. To assess viability, CellTiter-Glo 3D (Promega) was added in equivalent volume to wells and mixed with the Bravo automated pipette liquid transfer system (Velocity

11/Agilent). Per the CellTiter-Glo 3D protocol, plates were placed on a shaker for 25 min before luminescence was quantified using a Synergy NEO (BioTek), in the VHTS core.

Disc implantation for FNA-PDOX generation

Organoids were grown in solid 75% Matrigel disc cultures. When organoid confluency in the disc reached approximately 20-40%, disks were lifted with cell scraper and gently rinsed with 1X PBS. Female Foxn1/nu mice (Jackson Laboratories) mice were anesthetized with isoflurane and small skin incision is made on the mouse flank. The disk was then lifted with a spoon-shaped microspatula and inserted subcutaneously at the incision site. The skin incision was then closed with a surgical clip. Mice were monitored weekly for tumor development and tumor volume is estimated as $V=0.5*(\text{length} \times \text{width}^2)$ based on weekly measurements of tumor dimensions with digital calipers.

Organoid injection for FNA-PDOX generation

FNA-derived organoids were expanded to confluency in 12 wells of semi-solid culture in a low-attachment 24-well plate. Organoids were spun down, washed, and resuspended in 400µl of PBS. Per IACUC-approved protocol, female NOD.*Prkdc*^{scid}*Il2rg*$^{-/-}$ (NSG) mice received 150µl of organoid suspension subcutaneously in each flank using a 1ml Sub-Q syringe and 22 gauge needle. On average, 1,000 organoids were injected per site (range 800-1,400 organoids) with an average organoid area of $15mm^2$ (range 10-$30mm^2$ area per organoid, varying based on tumor type and patient sample). Tumor growth was monitored and volume measured every other day until an endpoint of tumor size $1cm^3$. At this time, mice were euthanized and tumors removed. Fine-needle aspiration was performed on the excised tumor using a 25 gauge needle and collected cells were washed and cultured in complete DMEM to create a stable PDOX line.

Analysis of the FNA-PDO tumor microenvironment

Organoid disc cultures were generated following FNA extraction using the method described above. At the time of FNA, a portion of the sample was briefly incubated for 5 min with collagenase (Sigma C2674-1G) and DNAse (Sigma D5025-150kU) at 37°C prior to evaluation of immune cell presence and viability by flow cytometry using a MacsQuant Analyzer 10 (Miltenyi Biotec). Following FNA, the tumor was digested according to our previously published standard digestion protocol (Siska et al., 2017). Approximately 100,000 cells were plated from either FNA or digested tumor samples in each 300µL Matrigel disc according to the methods described above. Wnt3A-enriched media was created as previously described by Neal et al.[2]. Briefly, L-WRN cells (ATCC CRL-3276) were grown to confluence prior to collection of Wnt3A, R-spondin 3, and Noggin conditioned media. Cells were removed using centrifugation, and the supernatant was frozen to ensure no L-WRN cell contaminant. Wnt3A-enriched culture media was subsequently composed of DMEM supplemented with 50% L-WRN conditioned media, 10% FBS, 1mM HEPES (Sigma), 10mM nicotinamide (Sigma), 1mM N-acetylcysteine (Sigma), 50ng/mL EGF (Thermofisher), B-27 without vitamin A (Fisher Scientific), L-glutamine (VWR), and 1X penicillin-streptomycin (Sigma). Recombinant human IL-2 (TECIN (Teceleukin) Bulk Ro 23-6019) was added (100IU/mL) to some discs and was provided by the National Cancer Institute (NCI). Following 2 weeks in disc culture, organoids were released from Matrigel using Cell Recovery Solution (BD Biosciences) per manufacturer's protocol. Once isolated, organoids were enzymatically digested using collagenase (Sigma C2674-1G) and DNAse (Sigma D5025-150kU) at 37° C for 5 minutes, and passed through a 70um strainer to ensure cells stained were in single cell suspension. Recovered single cells were then washed with FACS buffer (2% FBS in PBS) and transferred into a 96-well round bottom plate for immune profiling by flow cytometry on on the MacsQuant Analyzer 10. Immune cell evaluation was performed following staining with the following antibodies: Human BD fc Block (BD Biosciences), anti-human CD3 (BioLegend, clone UCHT1), anti-human CD45 (BioLegend, clone HI30), anti-human CD8a (BioLegend, clone RPA-T8), anti-human CD19 (BioLegend, clone SJ25C1), anti-human CD11b,

BioLegend, clone ICRF44), anti-human CD14 (BioLegend, clone M5E2), anti-human HLA-DR (BioLegend, clone L243), anti-human CD163 (BioLegend, clone GHI/61), and anti-human CD4 (BioLegend, clone OKT4).

SUPPLEMENTAL REFERENCES

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