

A Patient-Derived Tumoroid Platform for NF1 Low-Grade Glioma shows Myeloid Evolution and Shifting Metabolic Dependencies in vitro

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Abstract

About 10–15% of individuals with Neurofibromatosis Type 1 develop a low-grade glioma (LGG) within the optic pathway, and an additional 3–5% of individuals develop LGG outside of the optic pathway. In recent years molecular analysis has greatly enhanced our understanding of what drives these NF1 LGG; however, to date *in vitro* and *in vivo* models are rare. Here, we present the first *in vitro* tumor-derived spheroid cultures (tumoroids) of 3 NF1-associated LGGs grown for up to 4 weeks that can be passaged and banked. To determine how accurately the NF1 LGG tumoroid cultures reflect human disease, and how much and how quickly they drift from the primary tumors, we performed single nuclear RNAseq (snRNA-seq) analysis on primary tumor and 1, 2 and 4 weeks in culture for one of these NF1 LGG tumoroid lines. Observations were confirmed with immunofluorescence staining on all 3 lines. As expected, the few CD3 + T-cells present in the primary tumor are lost by day 14 (IF) and not detected on scRNAseq; macrophages/microglia remain present for at least 14 days but are largely depleted by week 4. Additionally, we observe a shift from more homeostatic microglia in the primary tumor to CD163 positive tumor-associated macrophages/microglia (TAM) *in vitro*. Neoplastic clusters uniquely associated with the primary tumor had clear upregulation of genes associated with neuronal communication. Our analysis also shows that primary NF1 LGGs rely on the metabolism of fatty acids (FA) for energy, while *in vitro*, glycolysis is the primary energy source. Additionally, instead of metabolizing FA, both neoplastic and immune cells switch to FA synthesis *in vitro*. Finally, we observe a slightly increased proliferative index *in vitro* versus *in vivo*, both in neoplastic and immune cells. Given the paucity of *in vitro* NF1 models, patient-derived tumoroids open opportunities for screening drug sensitivity and other studies.

Introduction

Neurofibromatosis type I (NF1) is one of the most common tumor predisposition syndromes with an incidence of about 1/3000.[6] Throughout their lifetime, patients can develop benign and malignant tumors, mostly of the peripheral and central nervous system. Between 10–15% of children with NF1 will develop a low-grade glioma (LGG) within the optic pathway (optic pathway glioma, OPG), with an additional 3–5% arising outside of the optic pathway[4, 5, 13, 14, 17], in the brainstem, cerebellum, or cerebral hemispheres. Besides homozygous inactivation of the NF1 tumor suppressor, these gliomas rarely exhibit other pathogenic mutation, though mutations in FGFR1 or ATRX,[7] have been identified in a small subset of patients. Management of NF1 LGG is individualized based on symptoms and tumor location. Many tumors are followed by neuroimaging as the clinical behavior of these tumors is heterogeneous. Some NF1 LGG may not evolve or cause symptoms, while others do progress and become symptomatic. In rare instances, spontaneous regression has been observed[12]. Up to half of children with NF1 LGG will ultimately require treatment, with chemotherapy remaining the first line of management for inoperable tumors. Targeted therapies such as MEK inhibitors and pan-Raf inhibition often serve as second line treatments. Treatment with radiation is avoided due to the elevated risk of secondary malignancy and cerebral vasculopathy in this population[3, 16]. Notably, tumors outside of the optic pathway are both more likely to require treatment and to harbor additional pathologic mutations[7].

Due to the clinical variability in tumor growth, the high prevalence of tumors in regions less amenable to safe biopsy (optic pathway and brainstem), and infrequent nature of clinically actionable mutations outside of NF1 loss, biopsy of LGG in children with NF1 is infrequent. Biopsies are often reserved for tumors with atypical neuroimaging features or lack of response to frontline chemotherapy.[11] The relative rarity of primary tumor samples underscores one of the many challenges in improving our understanding of the pathophysiology of these tumors. To date, generation of NF1 LGG cell lines had been challenging, with only one published cell line available. Additionally, while there multiple OPG GEMM[9] non-optic pathway glioma models do not exist. The paucity of model systems severely hinders our ability to perform preclinical testing and to mechanistically dissect the pathways driving NF1 LGG formation.

In recent years the importance of the tumor microenvironment for tumor growth has become evident. Specifically, NF1 LGG are dependent on the NF1 heterozygous microenvironment to form and Gutmann *et al.* showed an intricate interplay between neurons, T-cells and macrophages to stimulate OPG formation. To more accurately model human tumors *in vitro*, 3-dimensional tumor organoid or tumoroid models, which are essentially tumor explants maintained *in vitro*, can be used to capture the tumor microenvironment and phenotypic heterogeneity of the tumor. Recent advances have allowed for generation of these tumoroid cultures directly from adult glioma samples without dissociation to single cells[1, 8]. Excitingly, these tumoroid models are very successful in establishing *in vitro* cultures of both high-grade glioma (HGG) and LGG raising the possibility that this system would also be effective in pediatric brain tumors. What makes these cultures unique is the absence of most growth factors (except insulin) and a maintained reliance on the tumor microenvironment. Moreover, these cultures can be propagated for several passages or banked for later use. Eventually, these tumoroid cultures will evolve and lose their microenvironment. Here, we describe the first tumoroid cultures from pediatric gliomas and the first NF1 LGG tumoroid models and performed single cell RNAseq analysis to investigate tumoroid composition and signaling.

Methods

Human Subjects

Patient tumor tissue and peripheral blood samples were collected following ethical and technical guidelines from the Children's Hospital of Philadelphia on the use of human samples for biomedical research. As part of their clinical care tumors were analyzed to identify mutations, copy number variations and fusions using the Comprehensive Solid Tumor Panel that includes the V2.2 Solid Tumor Panel for sequence and copy number analyses of 239 cancer genes and genotyping of two genes associated with cancer pharmacogenomics, and V3 Fusion Panel which targets over 700 exons of 117 cancer genes (Supplementary table 1). Nucleic acid is extracted from the patient's sample following standard DNA and RNA extraction protocols. Extracted DNA is fragmented and tagged using SureSelect QXT target enrichment to generate adapter-tagged libraries. Biotin-labeled probes specific to the targeted regions are used for capture hybridization. Libraries are enriched for the desired regions using

streptavidin beads. Enriched libraries are then indexed and pooled for sequencing. Libraries are subject to sequence analysis on Illumina NovaSeq 6000 system for 150 bp paired end reads. All coding exons and the flanking intron sequences of targeted genes in the panel are sequenced, and selected promoter regions and known intronic variants are also evaluated. Sequence data are analyzed using the home brew software ConcordS V4.0.0 and NextGENe V2 NGS Analysis Software. Sequence variants within exons and 5 bp flanking intron sequences are annotated. Copy number variation (CNV) analysis for gross deletions and duplications are evaluated using NGS data. RNA sequencing libraries are prepared using Archer Universal RNA Reagent Kit with CHOP fusion panel custom-designed primers with target specific molecular barcode. Sequencing data are analyzed using ArcherTM Analysis for fusion genes. Clinically significant variants including single nucleotide variants (SNVs), indels, CNVs and fusion genes are confirmed by Sanger sequencing, MLPA, Real-Time PCR, or ddPCR only when necessary. Tumor tissue and peripheral blood were collected at the Children's Hospital of Philadelphia as part of the Children's Brain Tumor Network biobank following informed consent per a protocol approved by the Institutional Review Board. All tumors were diagnosed and graded in accordance with the 2021 WHO Classification of Tumors of the Central Nervous System, 5th edition by a board-certified neuropathologist. Patient samples were de-identified prior to processing. A total of 3 patients were included in the present study.

Tumoroid culture

Glioma tissue was obtained from the operating room, suspended in Hibernate A medium (Gibco, A1247501) maintained at 4°C. The tissue was processed within an hour of surgical resection to maximize tumoroid formation using a previously described protocol (Jacob et al., 2020). In short, resected tumor tissue was dissected into segments of approximately 0.5 to 1mm in diameter using dissection scissors (Fine Science Tools, 15044-08) in dissecting media containing Hibernate A, 1% GlutaMax (Thermo Fisher Scientific, 35050061), and 1% Anti-Anti (Thermo Fisher Scientific, 15070063) under a stereomicroscope (Zeiss, 495015-0030-000) housed in a laminar flow biosafety cabinet. After micro-dissection, tumor pieces were rinsed with DMEM/F12 (Gibco, 11320033) medium for removal of cellular debris. Red blood cell contamination was removed by incubating tumor pieces in 1X RBC lysis buffer (Thermo Fisher Scientific, 00433357) on a conical shaker. Several tumor pieces were snap frozen for single cell RNA sequencing.

All residual tumor segments were placed in ultra-low attachment 6-well culture plates (Corning, 3471) in tumoroid culture medium comprised of 47% DMEM:F12 (Gibco, 11320033), 47% Neurobasal (Gibco, 21103049), 1% GlutaMax (Thermo Fisher Scientific, 35050061), 1% NEAAs (Thermo Fisher Scientific, 11140050), 1% PenStrep (Thermo Fisher Scientific, 15070063), 1% N-2 supplement (Thermo Fisher Scientific, 17502048), 2% B-27 without vitamin A supplement (Thermo Fisher Scientific, 12587010), 0.1% β -mercaptoethanol solution (Thermo Fisher Scientific, 21985023), and 0.025% human insulin solution (Sigma-Aldrich, I9278) per well. Plates were incubated on an orbital shaker (Thermo Fisher Scientific, 88881102) at 120 rpm within a tissue culture incubator at 37°C and 5% CO₂. Tumoroid media was

replenished every 48 hours following removal of 75% of conditioned media. Cultures 7316–10565, 7316–10741 did not require additional passaging through day 28 in culture. Culture 7316–12943 was passaged by sequential bisection of tumoroids at day 14 *in vitro* following the processes described above.

Tissue processing and immunofluorescence

For histological studies, several tumor pieces were placed in 4% paraformaldehyde (Thermo Fisher Scientific) for 30 minutes at room temperature then washed in PBS and underwent cryoprotection overnight via incubation in 30% sucrose (Sigma-Aldrich, S0389) diluted in TBS at 4°C. Tumor segments were embedded in optimal tissue freezing media (TFM) (General Data, 15148-031) and flash frozen on powdered dry ice. Samples were maintained at -80°C until processing, at which point they were cryosectioned at 10µm thickness onto charged slides (VWR, 48311-703). Slides were dried at room temperature for two hours before being processed for immunofluorescence (IF).

TFM was washed off with 1x TBS (Fisher Scientific, BP152-1) and cells were subsequently permeabilized with 1% Tween 20 (BioRad, 170–6531) in 1X TBS (1x TBST). Sections were blocked for 1 hour at room temperature in blocking solution (0.1% gelatin (Sigma, G7041), 2.25% glycine (Sigma, G8790), 1% bovine serum albumin (BSA) (Sigma, A7906) 10% normal goat serum (NGS) (Invitrogen, 16210-072), 0.5% Triton X 100 (Sigma, T8787) in 1x TBST). Primary antibodies were diluted according to manufacturer's recommendations in antibody solution (5% NGS, 0.1% Triton X 100 in 1x TBST) and incubated overnight at 4 °C The following day, slides were washed and incubated in secondary antibodies in antibody solution for 1.5 hours at room temperature. TrueBlack Lipofuscin Autofluorescence Quencher (Biotium Inc., 23007) was used according to manufacturer's instructions prior to slide mounting with Prolong Gold antifade mountant (Invitrogen, P36934).

Antibodies

CD45 (Cell Signaling, 86532S), CSF1R (Cell Signaling, 67455S), CD163 (Invitrogen MA5-54106), LDHA (cite), Ki67 (Millipore MAB342), CD3 (BioLegend 344802), AF 568 goat anti-rabbit (Life Technologies A11001), AF 488 goat anti-mouse (Life Technologies A11006), DAPI (Sigma D9542).

Imaging

All slides were imaged with 10X and 40X objective lenses on the Leica DM4000B upright imaging scope with Leica DFC7000 T Camera.

Nuclei isolation and single-nucleus RNA sequencing

Nuclei were isolated using Chromium Nuclei Isolation Kit: Single Cell Gene Expression & Chromium Fixed RNA Profiling protocol (10X Genomics, 1000494). Organoid samples (6-10mg) were placed in media (DMEM) on ice and moved into a pre-chilled sample dissociation tube in 200µL of lysis buffer for nuclei isolation. The tissue was homogenized 20–60 times using a pre-chilled pestle (10X Genomics, 2000561) on ice, and 300µL of lysis buffer was added. The dissociated tissue was then incubated on ice for 10 minutes. Homogenized tissue was transferred to a nuclei isolation column in a collection tube and centrifuged at 16000g for 20 seconds at 4°C. After discarding the nuclei isolation column, flowthrough was vortexed and centrifuged at 500g for 3 minutes at 4°C. Following centrifugation, the pellet was resuspended in 500µL of debris removal buffer and centrifuged at 700g for 10 minutes at 4°C. The pellet was then resuspended in 1 mL of wash and resuspension buffer (1X PBS + 1% BSA+ RNase Inhibitor) on ice. The pellet went through centrifugation at 500g for 5 minutes at 4°C and then resuspended in 50µL of ice-cold wash and resuspension buffer twice. Nuclei were counted manually using a hemocytometer, with library preparation conducted thereafter. Single nuclei suspensions were pooled with ~ 2000 nuclei per sample. Approximately 8000 nuclei were loaded on to the on-chip multiplexing 3' chip per well and processed using GEM-X OCM 3' Chip Kit v4 4-plex, PN-1000747 and GEM-X Universal 3' Gene Expression v4 4-plex, PN-1000957. The pools were loaded in quadruplicates. Fragment size of libraries was determined using the Agilent high-sensitivity DNA kit (Agilent Technologies, 5067 – 4626) on Bioanalyzer 2100 (Agilent Technologies). Libraries were quantified using the qPCR-based KAPA quantification kit (Roche, 07960140001). Gene expression libraries were sequenced on NovaSeqX series, 10B, 100 cycles kit (Illumina) using sequencing parameters 28:10:10: 90::R1:i5:i7:R2.

Processing and integration of snRNA-seq data

Read count matrices from snRNA-seq data were generated from raw FASTQ files using CellRanger v9.0.1, with alignment to the GRCh38 transcriptome reference and demultiplexing using the CellRanger multi function. Raw read count matrices were cleaned of ambient RNA contamination using SoupX[19]. Briefly, each sample was log-normalized, 2000 variable features computed and scaled, PCA was computed, and the cells were clustered at a resolution of 0.5. The contamination fraction was set to 20% and the read count matrices were adjusted using the adjustCounts function. The resulting count matrices were processed and analyzed using Seurat v5²⁸. Quality control filtering was applied to each cell, using filters of nFeature_RNA > 500, nFeature_RNA < 10000, and mitochondrial read percentage < 10. The cells were log-normalized and visualized on a UMAP representation to assess for batch effect, which was found to be present between time points. Thus, the data were split by time point and integrated using reciprocal principal component analysis (RPCA). Briefly, the Seurat object for each time point was log-normalized, the top 2000 variable features were selected and scaled and a PCA was computed. These objects were then integrated using the FindIntegrationAnchors and IntegrateData functions with default parameters. Cells were clustered using the FindNeighbors function with the top 30 principal components (PCs) followed by the FindClusters function and visualized on a UMAP with the top 30 PCs. Clusters representing clear artifacts were removed—one cluster of dying/necrotic cells and a

cluster of tumor/immune doublets. After removal of these clusters, the integration procedure was repeated as above to generate the final UMAP and clusters.

Annotation of pathway analysis of snRNA-seq data

The snRNA-seq data were uploaded in the BbrowserX portal and further analyzed. Differential gene expression between cell populations were identified using a Pseudobulk analysis. The tumor cells and immune cells were identified based on differentially expressed genes and AUCell gene scores, a method to determine the level of expression of a gene set within a cell, of clusters (AC-like, OPC-like, NPC-like, MES-like and Neurogranin-neuron like (NEU-NRGN))[10]. These populations were then re-clustered, subclusters were annotated based on differentially expressed genes. For violin plots, pathway scores were computed using AUCell with default parameters.[2] For the quadrant plots, gene sets were scored using the AddModuleScore function and were plotted based on relative pathway scores as described previously.[10]

Data Availability

Singe nucleus RNA-seq data was uploaded to GEO (GSE320279).

Results

NF1 LGG tumoroid cultures

We successfully established patient-derived tumoroid cultures of 3 out of 3 distinct NF1 LGG cases. Tumoroid models were bio-banked while material for the study was collected at 0, 2 and/or 4 weeks. Table 1 details the epidemiological data for each subject, the histological diagnosis for individual tumors, and the testing results for molecular sequencing. Besides mutations in or LOH of *NF1*, no additional alterations were identified. DNA methylation analysis, as part of the clinical diagnosis, showed that all 3 NF1 LGG are Pilocytic Astrocytomas.

Table 1
Summary of Molecular and Clinical Characteristics of Patient Samples

Tumor #	Age	Sex	Tumor location	Histopathologic diagnosis	Additional molecular alterations	Prior Treatment	Clinical status
7316–10565	15y	Male	Posterior fossa	Pilocytic Astrocytoma	No	No	Alive
7316–10741	10y	Male	Posterior fossa	Pilocytic Astrocytoma	No	No	Alive
7316–12943	12y	Male	Posterior fossa	Pilocytic Astrocytoma	No	No	Alive

Characterization of tumoroid culture

We performed snRNA-seq on NF1 LGG 7316–12943 tumoroids at multiple timepoints in culture (Day 0 = primary tumor, n = 5; Day 7, n = 4; Day 14, n = 4; Day 28, n = 3). In total, 86,065 cells passed quality control (Supplementary Fig. 1A-C), with a median of 3,328 genes detected. Subsequently, independent clustering of all sampled cells was performed (UMAP, Fig. 1A) and we were able to distinguish neoplastic and immune cell clusters over the different time points. Because NF1 LGG do not have major copy number variations, we were unable to use inferCNV to confirm neoplastic cellular identity and instead used the AUCell scores and marker gene expression to identify the different clusters (Fig. 1B-C, Supplementary Fig. 1D). We used the AUCell score for AC-like (Astrocyte-like), OPC-like (oligodendrocyte precursor cell-like), NPC-like (neural progenitor cell-like), and MES-like (mesenchymal-like) gene set and expression of neuronal markers like *SOX6* (Fig. 1B-C) to identify neoplastic clusters. The immune cell cluster scored high for genes associated with immune cells and is positive for *CD45* (*PTPRC*), as well as myeloid makers like *CSF1R*, *P2RY12*, and *CD163*. The endothelial cell cluster scores high for an endothelial geneset and expresses endothelial markers like *PECAM1*, *CLDN5* and *FLT1*. Of note, the endothelial cluster has some *SOX6* expression as well and scores for neoplastic associated geneset using AUCell; this is likely due to some ambient RNA contamination. However, this contamination did not preclude us from drawing pertinent conclusions. Figure 1D and E show a breakdown of the UMAP and the composition of the clusters by timepoint.

Immune cells

We observe a substantial presence of myeloid immune cells in the primary tumor and in week 1 and 2 cultures (17–34% of all cells), with a dramatic loss of immune cells by week 4 (Fig. 2A). The immune cell cluster is comprised of 3 subclusters: (a) homeostatic microglia marked *P2RY12* and *CSF1R*, (b) tumor associated macrophages/microglia (TAM) marked by *CD163* and (c) proliferating TAM additionally marked by *KI67* expression and a high cell cycle gene score (Fig. 2B-C, Supplementary Fig. 2A). Almost all immune cells present in the primary tumor were classified as homeostatic microglia. This cluster gradually disappeared in culture and was replaced with TAMs, suggesting an activation and differentiation of these microglia in culture. (Fig. 2D). These observations were confirmed by IF staining for *CD45*, *CSF1R*, and *CD163* on the 3 NF1 LGG tumoroid cultures (Fig. 2E-G and supplementary Fig. 2B). Intriguingly, almost all immune cells have migrated to the edge of the tumoroid by day 14. This is especially clear in tumoroid culture 7316–10471. This ring formation is less clear in culture 7316–12943, potentially because these tumoroids were passaged prior to day 14. The few immune cells that were present on day 28 are again spread throughout the tumoroid. T-cells were not identified in any of the timepoints using snRNA-seq; however, they were detected in extremely low numbers in the primary tumor and not detected later in culture by IF staining for *CD3* (Supplementary Fig. 2C). About 10% (day 7) and 8.6% (day 14) of immune cells are dividing TAMs (snRNA-seq). Strikingly, we also observe an upregulation of *NT5E* and an increase in the number of cells that express *NT5E* (Fig. 2H). *NT5E* converts AMP to adenosine, which is an important factor that drives TAM formation.

Neoplastic cells

The neoplastic cells did not cleanly cluster in AC-like, OPC-like, NPC-like, GPC-like, MES-like or NEU-NRGN-like clusters, which are mainly used for high-grade glioma and glioblastoma analysis. Therefore, we decided only to maintain two clusters (Proliferating neoplastic cells and non-proliferating neoplastic cells) defined by the expression of proliferative markers (MKI67, TOP2A and CENPF, Fig. 3A-B). As expected, the primary NF1 LGG are more quiescent, and stimulating the growth of cells *in vitro* increases the percentage of neoplastic cells that are in the “proliferating” cluster (Fig. 3C). Proliferation of neoplastic cells has largely halted by day 28.

When we compare the differentiation states of all the neoplastic cells across timepoints (gene score, AUCell), we find that NPC, GPC and OPC differentiation states are largely maintained over 2 weeks in culture. We see a gradual decrease in AC-like features and a slight increase in MES-like features. The increase in NEU-NRGN-like features, however, is more profound. Overall, these trends are more accentuated by week 4 (Fig. 3D). Not every cell cleanly and uniquely expresses genes of one of the 4 main cell states (AC-like, OPC-like, MES-like and NPC-like); therefore, we also compared the relative contribution of each program in each cell and plotted this in a single graph (Supplementary Fig. 3). The AUCell score of NEU-NRGN gene sets is represented by the color of each cell.

Differential gene expression analysis between the primary tumor and the tumoroids showed expression changes in 2593 genes (Pseudobulk analysis, FDR < 0.01, > 2-fold change, Supplementary Table 2). Subsequently, we wondered if there was any evolution in the expression of receptor-tyrosine-kinases and other signaling receptors. Intriguingly, we observed a gradual and significant decrease in EGFR and ERBB4 expression and an increase in FGFR1 and IGF2R. IGF2R is noteworthy as insulin is the only growth factor that is supplied in the media. The ERBB4 ligand NRG3 is downregulated as well. (Fig. 3E)

In recent years, the electrical integration of gliomas in neuronal circuitry was shown to support glioma proliferation. As expected, we found significant reduced expression of postsynaptic machinery and genes involved in neuronal integration in culture (GRIA1, GRIN2A, SPARCL1, etc; Fig. 4A, Supplementary Table 2).

We also observed expression changes in genes involved in metabolism. For example, genes associated with fatty acid oxidation are higher expressed in the primary tumor samples, where genes associated with lipid biosynthesis, glycolysis, and oxidative phosphorylation are upregulated in culture (Fig. 4B). These results were confirmed when we performed scGSEA (using MSigDB_Hallmark, MSigDB_Oncogenic_signatures and WikiPathways_human) and found a marked switch from fatty acid oxidation to upregulation of genes associated with lipid synthesis, oxidative phosphorylation and glycolysis *in vitro* (Fig. 4B-C). IF staining for LDHA, a marker of glycolysis, confirms this switch towards glycolysis *in vitro* (Fig. 4D). It is noteworthy that we observe the same switch in metabolic markers in immune cells (Fig. 4B).

Discussion

One of the main challenges in establishing any *in vitro* NF1 LGG culture is the limited sample availability, due to scarcity of NF1-LGG biopsies or resections, and the low proliferative index of benign tumors. Moreover, the amount of primary tissue samples obtained from many surgeries are low in volume and after meeting all the clinical needs limited material remains for research, including for *in vitro* modeling. Therefore, there is a high unmet need for accurate NF1 glioma models to perform mechanistic studies and preclinical testing. We established 3 pediatric, NF1 LGG tumoroid cultures with 100% success rate and performed a detailed analysis of the exact changes induced by culture over time. These tumoroid lines can be passaged and viably bio-banked for later use, which makes them valuable tools for mechanistic and preclinical studies. It is important to note that in general, sampled NF1 lesions may represent more aggressive tumors that require a better understanding of their pathophysiology. As such, tumoroid cultures may provide molecular insight on resistance mechanisms to current therapies, which are understudied in this population. Given these are slow growing tumors, NF1-LGG tumoroids also do not possess the same expansion capacity of glioblastoma tumoroids. It remains uncertain if NF1-LGG tumoroids will form xenografts and expand in murine models, which could potentially mitigate the limitations in expansion *in vitro*.

Based on our data, these NF1 LGG tumoroid cultures most accurately represent the primary tumor during the first 2 weeks in culture, with a similar percentage of immune cells and neoplastic cell differentiation states. Despite the high degree of similarity to primary tumors, our culture conditions did induce some changes, including an increase in proliferation both in the neoplastic and the immune cells. This is expected as we stimulate these cultures to grow. Proliferation had all but halted by day 28, potentially coinciding with the loss of the immune microenvironment.

When we determined if the neoplastic cells aligned with differentiation states observed (NPC-like, OPC-like, AC-like, MES-like) in high-grade glioma and glioblastoma, we could not identify clear distinct clusters that mainly aligned with one of those states and instead subclustered the neoplastic cells based on proliferation state. Further analysis on the relative contribution of the transcriptional programs within the neoplastic NF1 LGG cells however, showed a strong bias to OPC-like and AC-like signatures. Those signatures gradually evolved and became more MES-like as well as an upregulation of NEU-NRGN programs in culture (Supplementary Fig. 3). The presence of AC-like and OPC-like programs in LGG has been shown before[15, 18].

Neuronal connectivity has been shown to drive sporadic and NF1-associated LGG (Citation needed). Because we are growing these tumoroids *ex-vivo* and are not supplying excitatory neurons, we observe a loss in genes associated in the post-synaptic machinery by day 7. NRG3 is in this context very intriguing, as it is typically secreted by excitatory neurons and plays a role in establishing the connectivity between glioma tumor cells and the brain neuronal circuitry when it binds to ERBB4 on glioma cells. ERBB4 activation by NRG3 can also stimulate glioma proliferation and migration. Whether loss of the neuronal

integration of tumor cells in the brain circuitry can be mitigated by co-culture with excitatory neurons or supplementation of the media with relevant ligands remains to be seen.

We also observed metabolic shifts in our cultures. While the primary tumor mainly relies on fatty acid oxidation (FAO) as a source of building blocks and energy, in culture the tumoroid metabolism shifts to glycolysis, fatty acid synthesis, and oxidative phosphorylation. Notably, the protocols for deriving glioma tumoroids were optimized in HGGs, which are more dependent on glycolysis for energy compared to LGG. Of note, pediatric HGG have been shown to more frequently use glycolysis when compared to adult HGG (Sviderskiy 2025 *Acta Neuropathologica* communications). Our observations suggest that further optimization may help enhance this protocol for LGG tumoroids as it was initially optimized for adult HGG tumoroid cultures. For example, use of Palmitic and Oleic acid and the reduction of glucose and insulin levels could reduce the metabolic switch to glycolysis.

We also noted that the immune cells in the primary tumor are mostly homeostatic microglia and that these microglia become activated and differentiate in true CD163 positive TAMs within 7 days in culture. What drives this abrupt shift in culture remains unclear; however, it is known that immune cells are very dependent on FAO, and that glycolysis stimulates TAM formation. Intriguingly, adenosine is a potent stimulant of TAM formation, and we observe a profound upregulation during culture of NT5E, a critical enzyme in adenosine synthesis.

In conclusion, our tumoroid models relatively accurately represent the primary NF1 LGG for the first 2 weeks in culture. Afterwards more profound shifts are observed. However, our findings, especially the metabolic ones, also suggest further optimization of the protocols towards maintaining FAO and reducing glycolysis and oxidative phosphorylation and preventing rapid *in vitro* TAM differentiation. These observations are not only important for establishing NF1 LGG tumoroid cultures but have consequences for the establishment of sporadic LGG and potentially other benign tumoroid cultures as well.

Declarations

Ethics approval and consent to participate

This study was approved by the Children's Hospital Philadelphia Institutional Review Board.

Consent for publication

Informed consent/assent was obtained from all patients and guardians included in this study.

Competing interests

The authors declare no competing interests.

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Authorship:

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Figures

Figure 1

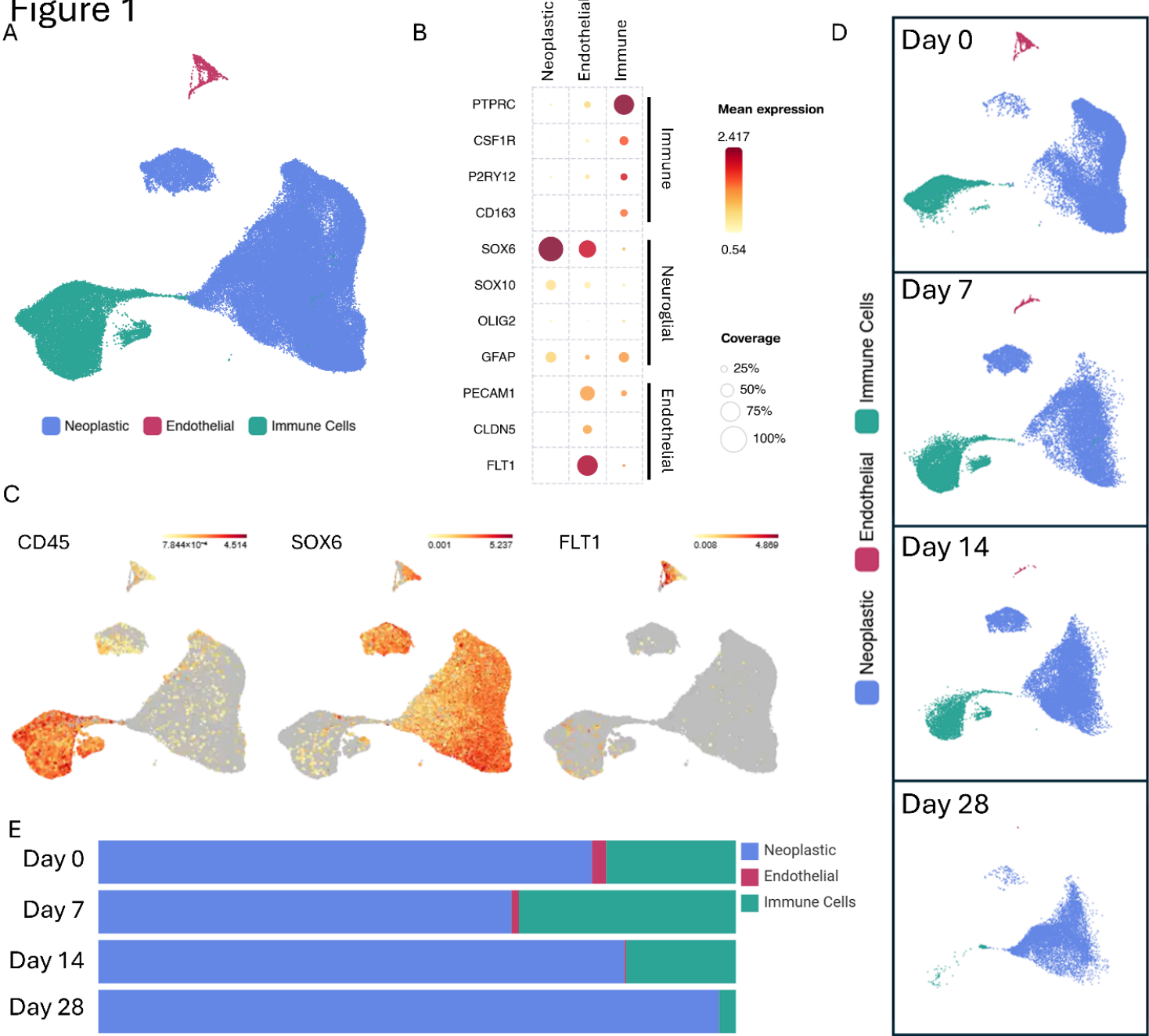


Figure 1

Characterization of NF1 LGG tumoroid cultures: A. Uniform Manifold Projection and Approximation (UMAP) representation depicting clusters of all analyzed cells with clusters identified as neoplastic cells, immune cells and endothelial cells. B. Dot plot of markers used to identify different cell lineages. C. Projection of the expression of marker genes for specific cell lineages on UMAP. D. UMAPs of the same

cellular clusters comparing the primary NF1 LGG to the tumoroid culture at day 7, 14 and 28. E. Overview of the composition of the different clusters within each timepoint.

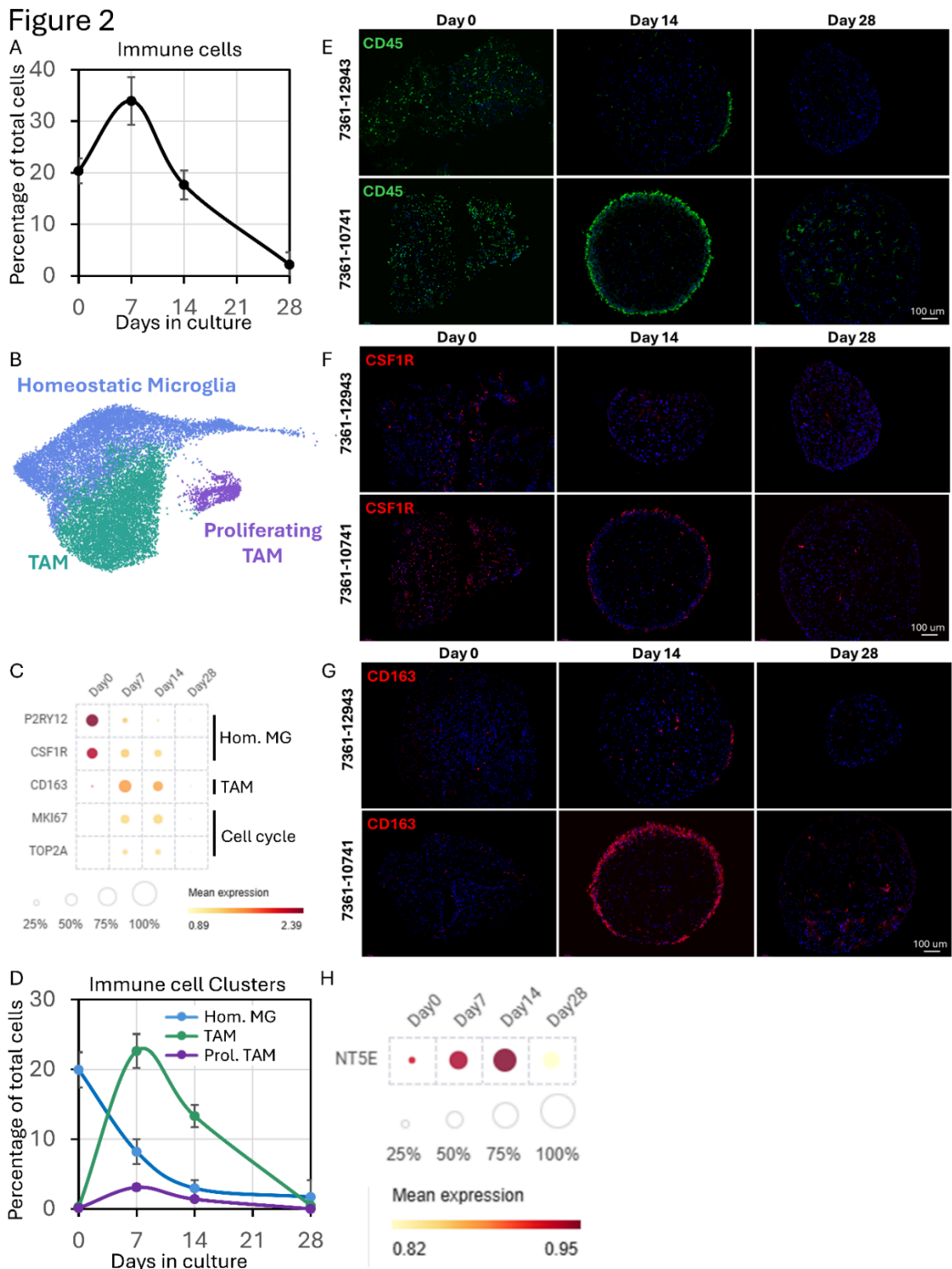


Figure 2

Immune cells: A. Graph depicting the evolution of the percentage of immune cells present in each tumoroid culture across different timepoints. B. UMAP showing the different immune cell subclusters

present in the immune cells. C. Dot plot of markers for homeostatic microglia (Hom. MG), tumor-associated macrophages (TAM) and cell cycle-related genes across different timepoints showing a loss in homeostatic microglia markers and a gain in TAM and cell cycle markers. D. Graphical representation of the evolution of the proportion (percentage of total cells present) of immune cell subclusters across timepoints. E-G. IF staining for CD45, CSF1R, and CD163 in 2 NF1 LGG tumoroid cultures shows a decrease in homeostatic microglial markers and an increase in TAM markers at 7 and 14 days in culture. At 28 days, very few immune cells are present. H. Dot plot for *NT5E* across different timepoints on all cells combined shows a marked increase in *NT5E* expression at day 7 and 14 in culture.

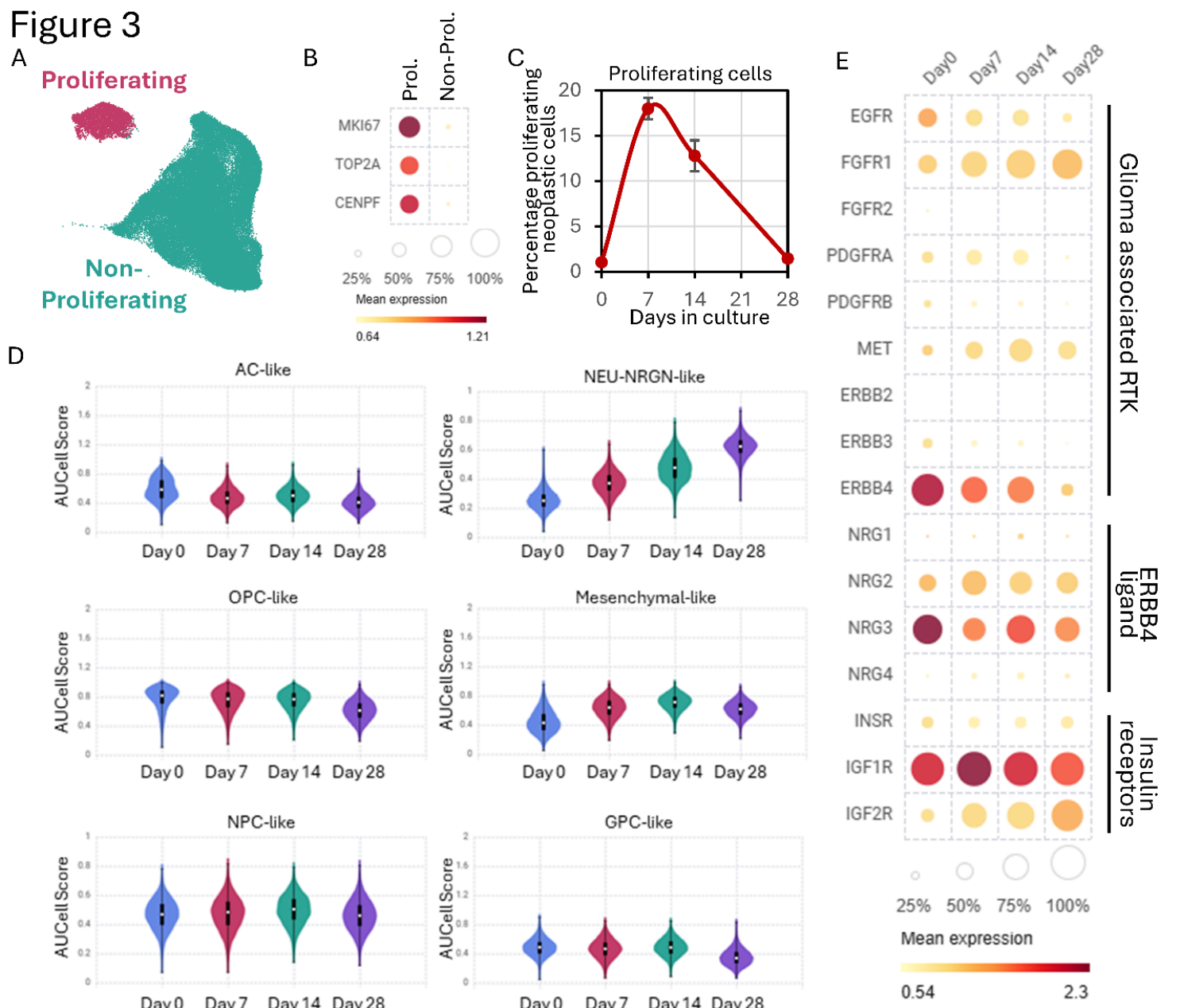


Figure 3

Neoplastic cells: A. UMAP showing the two neoplastic cell subclusters present (proliferating and non-proliferating neoplastic cells). B. Dot plot of cell cycle markers shows active cell division in the proliferative subcluster. C. Graph depicting the percentage of proliferating cells within the neoplastic clusters. D. AUCell score for gene sets associated with AC-like, OPC-like, NPC-like, NEU-NRGN-like, GPC-like, and mesenchymal-like transcriptional programs shows a gradual decrease in AC-like programs and an increase in NEU-NRGN-like and mesenchymal-like programs. E. Dot plot of cell receptor tyrosine kinases and ERBB4 ligands across the different timepoints.

Figure 4

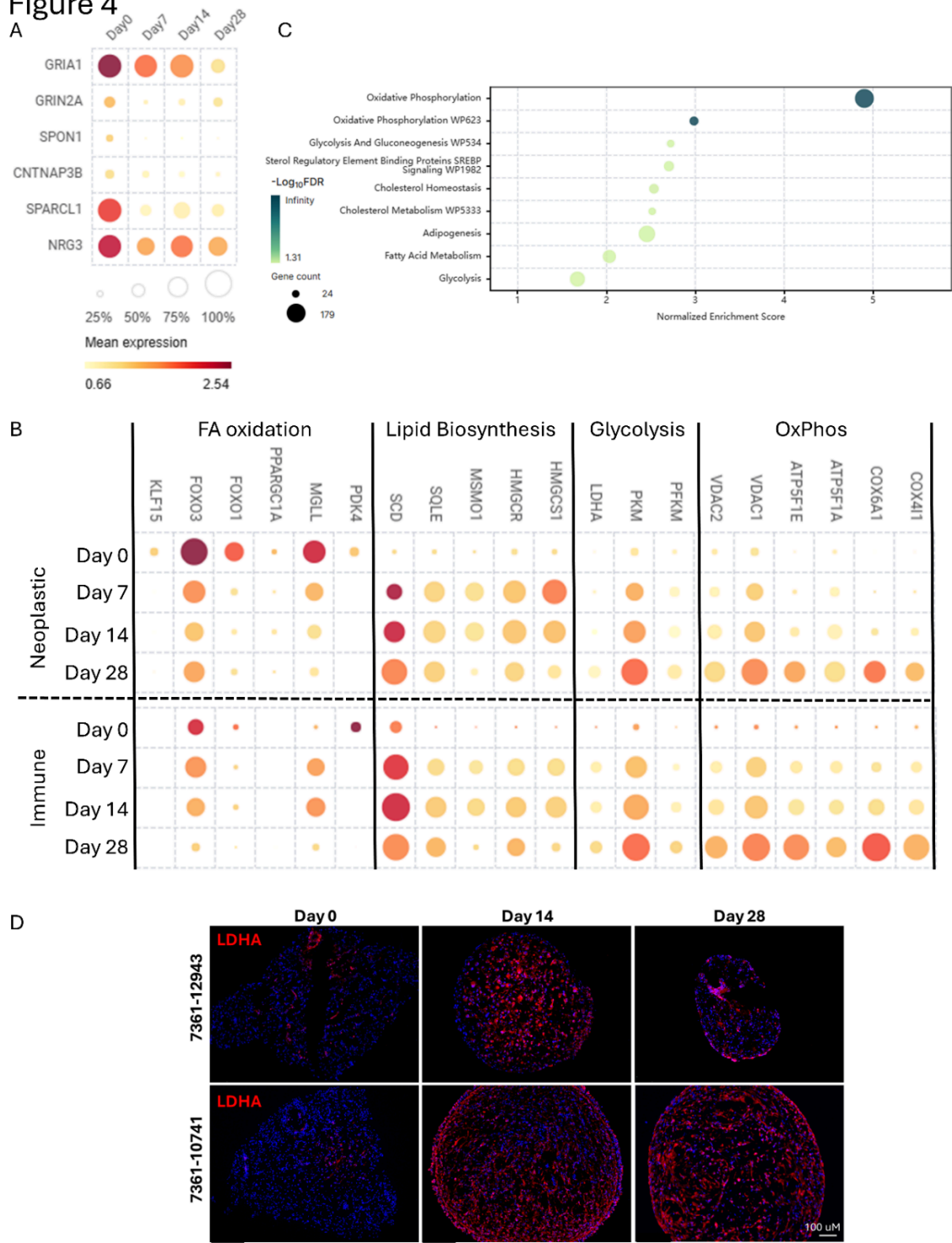


Figure 4

Functional analysis: A. Dot plot of genes involved in the post-synaptic machinery show a marked decrease in expression in culture. B. Dot plot of genes involved in different metabolic programs showing a decrease in fatty acid oxidation and an increase in lipid biosynthesis, glycolysis and oxidative phosphorylation in culture compared to the primary tumor (Day 0). C. scGSEA shows enrichment of different metabolic pathways enriched in tumoroid cultures versus primary tumor. D. IF staining for LDHA in 2 NF1 LGG tumoroid cultures shows a clear metabolic shift to glycolysis *in vitro*.

Supplementary Files

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