



## Research paper

# A human ACTH-secreting corticotroph tumoroid model Novel Human ACTH-Secreting Tumor Cell *in vitro* Model

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## ABSTRACT

**Background:** Cushing disease (CD), although rare, is a life-threatening disorder caused by an adrenocorticotrophic hormone (ACTH)-secreting pituitary adenoma, which leads to excess adrenal-derived cortisol. Efficacious and safe medical therapies that control both hormonal hypersecretion and pituitary corticotroph tumor growth remain an unmet need in the management of CD. Translational research in pituitary tumors has been significantly hampered by limited quantities of surgically resected tissue for *ex vivo* studies, and unavailability of human pituitary tumor cell models.

**Methods:** To characterize human corticotroph tumors at the cellular level, we employed single cell RNA-sequencing (scRNA-seq) to study 4 surgically resected tumors. We also used microarrays to compare individualized paired consecutive culture passages to understand transcriptional shifts as *in vitro* cultures lost ACTH secretion. Based on these findings, we then modified our *in vitro* culture methods to develop sustained ACTH-secreting human corticotroph tumoroid cultures.

**Findings:** scRNA-seq identified 4 major cell populations, namely corticotroph tumor (73.6%), stromal (11.2%), progenitor (8.3%), and immune cells (6.8%). Microarray analysis revealed striking changes in extracellular matrix, cell adhesion and motility-related genes concordant with loss of ACTH secretion during conventional 2D culture. Based on these findings, we subsequently defined a series of crucial culture nutrients and scaffold modifications that provided a more favorable trophic and structural environment that could maintain ACTH secretion in *in vitro* human corticotroph tumor cultures for up to 4 months.

**Interpretation:** Our human corticotroph tumoroid model is a significant advance in the field of pituitary tumors and will further enable translational research studies to identify critically needed therapies for CD.

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## 1. Introduction

The pituitary gland, which integrates multiple central and peripheral signals to masterfully control several endocrine axes, has a high propensity to tumor formation, pituitary tumors being the third most frequently encountered intracranial tumor [1]. Increased morbidity and mortality often result from hormonal hypersecretion [2], particularly in Cushing disease (CD), where an adrenocorticotropin (ACTH)-secreting pituitary tumor, drives excess adrenal-derived cortisol [3]. The molecular pathogenesis of CD is still largely unknown [4–6], although the discovery of somatic deubiquitinase USP8 mutations in ~30% of CD leading to EGFR pathway activation highlights the role

human models of CD could play to test therapeutic targets [7]. Although murine corticotroph tumor AtT20 cells, and transgenic zebrafish expressing POMC promoter-directed green fluorescent protein (GFP) share many transcriptional components with human corticotroph tumors and represent robust *in vitro* and *in vivo* CD models [8–10], they are not human cells. Consequently, extrapolation of findings from these models is not ideal, and we and others have utilized primary cultures of fresh surgically resected human corticotroph tumor tissues to substantiate animal model findings [11–15]. However, these human corticotroph tumor cells typically lose ACTH hormone production after 1–2 weeks [11–15]. Purported reasons for their loss of endocrine secretory function have included altered cell adhesion [11,16], lack of intrinsic hypothalamic factors [17], cellular trans- or de-differentiation [18] and/or deactivation of prohormone convertases [19]. However, no clear changes in specific molecular

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## Research in context

### Evidence before this study

No human pituitary corticotroph tumor cell lines for translational research exist. Surrogate murine or fish corticotroph tumor models are useful *in vitro* and *in vivo* CD models but they cannot fully recapitulate human corticotroph tumors, and therefore extrapolation of findings from these experimental models is not ideal.

### Added value of this study

For the first time, this study describes transcriptional make-up at the single cell level in human corticotroph tumors, characterizes transcriptional shifts in individualized corticotroph tumors as they lose ACTH secretion, and utilizes this combined knowledge to develop a two-step 3D culture methodology that can maintain human corticotroph tumor ACTH secretion and propagate sufficient human corticotroph tumor cells for research use.

### Implications of all the available evidence

Our studies take a critical step in advancing methods to culture human corticotroph tumors. This tool will be an extremely important resource to study the pathogenesis of corticotroph tumors and will provide an important model to identify novel therapeutic options for these disabling and often fatal tumors.

candidates or pathways, that might provide insight into the mechanisms for loss of corticotroph tumor ACTH secretion, have been demonstrated.

In our current studies, we used single cell RNA-sequencing (scRNA-seq) to characterize pituitary corticotroph tumor cellular composition. We also compared microarray transcriptomic profiles of paired individual patient-derived CD primary cultures that were collected at one passage interval in the presence and absence of ACTH secretion and observed striking alterations in cell adhesion signaling and mobility genes. Combined analysis of our scRNA-seq and microarray findings highlighted deficiencies in current 2-dimensional culture methods which appeared to select for non-endocrine cells that exhibited stronger adhesion properties. Based on these findings, we radically modified our culture method to transit to a two-step 3D culture system that can maintain human corticotroph tumor ACTH secretion and propagate sufficient human corticotroph tumor cells for research use.

## 2. Methods

### 2.1. Study ethic approval

This study was approved by University of California, Los Angeles Institutional Review Board (IRB, #20-011275) and informed consent was obtained from all patient in this study.

### 2.2. Human pituitary tumor specimens

Fresh surgically resected human pituitary corticotroph tumor tissues (total n=10; scRNAseq n=4, microarray sample n=1, cell culture n=5) were collected from patients who underwent endoscopic transnasal trans-sphenoidal (TNTS) surgery in accordance with University of California, Los Angeles IRB guidelines. Diagnosis was confirmed in all patients using standard clinical and biochemical criteria and pathological evaluation (Supplementary Table 1). Resected tumor tissues delivered to the laboratory on ice immediately after surgical removal

were washed three times in sterile PBS, then minced with sterile surgical scalpel to 0.5-1 mm<sup>3</sup> fragments in approximately 1mL of PBS.

### 2.3. Conventional 2D primary cultures of human pituitary tumor tissues

Minced surgically resected human pituitary corticotroph tumor tissues were digested with Dulbecco's modified essential medium (DMEM) containing 0.5% bovine serum albumin (BSA), 0.35% collagenase, and 0.1% hyaluronidase at 37°C for 20 min. After centrifugation cell pellets were resuspended in culture medium supplemented with 10% fetal bovine serum (FBS) and cultured in normal culture vessels or extracellular matrix (ECM) coated plates. CytoMatrix screening kit containing individual 96-well plates coated with collagen I, and collagen IV, fibronectin, vitronectin, and laminin (EMD Millipore, Cat# ECM205), was tested for ACHT secretion and proliferation in human corticotroph cultures as described in Figs. 3c & 3d. BioCoat™ TC-treated variety packs containing individual 6-well plates coated with collagen I, collagen IV, fibronectin, and laminin (Corning, Cat# 354431), were used for culture as described in Fig. 3e. The cells were passaged upon confluence. The medium supplements were purchased from Life Technologies, Inc., including DMEM, FBS, trypsin, antibiotics, and glutamine.

### 2.4. 3D human pituitary corticotroph tumoroid cultures

Enzymically dispersed resected human corticotroph tumor tissues (described above) were either plated onto dishes coated with a thin layer of BioCoat matrigel matrix (Corning, Cat# 354601, Thin gel), or cell suspensions were mixed with ice-cold matrigel basement membrane matrix (Corning, Cat# 354234) at 1:1 ratio and then seeded onto plates coated with a thick layer of BioCoat matrigel matrix (Corning, Cat#354433, Thick gel). Once the 50%:50% matrigel/cell suspension mixture solidified at 37C for 15 min, DMEM medium supplemented with 10% FBS or defined medium containing advanced DMEM/F-12 supplemented with B27 (1x), NAC (1.25μM), EGF (5ng/mL), IGF-1 (100ng/mL), Noggin (100ng/mL), FGF-8 (10ng/mL), Y27632 (5μM), T3 (5nM), TRH (12.5nM) and BHE (5μg/mL) was added to plates. ~50% of the medium was changed weekly. In the two-step culture system, cell aggregates were transferred to a 250mL spinner flask (VWR, Cat# 89166-898) following culture in the thick gel. ~50% of the spinner flask medium was refreshed weekly and cells were passaged and cryopreserved when they reached a density of 10<sup>5</sup> cells/mL. The medium supplements were purchased from commercial sources, including advanced DMEM/F-12, and B27 from Life Technologies, Inc; human epidermal growth factor (EGF) from Genzyme; bovine hypothalamus extract (BHE) from ScienCell Research Laboratories, Inc. (Carlsbad, CA, Cat# 0613); N-acetyl-L-cysteine (NAC, Cat# A9165), BMP inhibitor Noggin (Cat# SRP4675), ROCK inhibitor Y-27632 dihydrochloride (Cat# Y0503), 3,3',5-Triiodo-L-thyronine sodium salt (T3, Cat# T6397-100MG) and thyrotropin releasing hormone (TRH, Cat# P1319-50MG) from Sigma; recombinant human insulin-like growth factor-1 (IGF-1, Cat# cyt-2216) from Prospect Protein Specialists; recombinant human fibroblast growth factor-8 (FGF-8, Cat# PHG01844) from ThermoFisher Scientific.

### 2.5. Cell proliferation assay

To measure cell proliferation, aliquots from primary cultures of human pituitary corticotroph ACTH secreting tumors were trypsinized and 2 × 10<sup>3</sup> viable cells were suspended in 100μl DMEM supplemented with 10% FBS. They were then added to 96-well ECM coated plates (EMD Millipore, Cat# ECM205) and cultured for 3 days in the presence or absence of various concentration of FBS supplement. The viability of the cells was determined using CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI) with a luminometer (Wallac 1420 Victor 2 multipliable counter system).

Results are presented as proliferation index (relative luminescence signal to medium control).

## 2.6. Hormone assays

RIAs for human ACTH were performed in triplicate using reagents purchased from Biomerica Inc. (Irvine, CA, Cat# 7023). ACTH level in the supernatant was corrected to supernatant volume, cell number and collection period, and presented as pg/10<sup>4</sup> cells/24 h.

## 2.7. RNA microarray processing and bioinformatic analysis

Total RNA was extracted from consecutive pituitary tumor primary culture passages that either secreted or had lost ACTH secretion using a Qiagen RNeasy Mini kit. RNA quantity and quality were assessed using Agilent TapeStation system and amplified to biotin-labelled cDNA using NuGEN cDNA systems. Illumina HumanHT-12 Expression BeadChip v4 microarray was used for whole-genome gene expression profiling, and data were analyzed by the UCLA Neuroscience Genomics Core (UNGC) using R and Bioconductor. FC<sub>>2</sub> was used to define DEGs for hierarchical cluster analysis. The gene ontology (GO) biological process enrichment analysis of DEGs was performed using a Functional Annotation of NIH DAVID program with Fisher exact t test p value <0.05 [20,21]. Processed data are deposited in NCBI BioSample database with BioSample accessions of SAMN18317203 (ACTH+) and SAMN18317204 (ACTH-).

## 2.8. Single-cell RNA-sequencing and bioinformatic analyses

A single cell suspension of surgically resected human corticotroph tumors was obtained by mechanical and enzymic digestion using a gentleMACS dissociator, and human tumor dissociation kit (Miltenyi Biotec Inc., Germany, Cat# 130-095-929). Library generation was performed on the 10x Genomics Chromium Controller following the manufacturer's protocol for the v3 reagent kit (10x Genomics). In brief, cell suspensions were loaded onto a Chromium Single Cell A Chip, aiming for 10,000 cells per channel for generation of single-cell gel bead-in-emulsions (GEMs), following which reverse transcription was performed and the resulting post-GEM reverse transcription product was cleaned up using DynaBeads MyOne silane beads (Thermo Fisher Scientific, Waltham, MA). The cDNA was amplified, SPRIselect (Beckman Coulter, Brea, CA) cleaned and quantified, and then enzymatically fragmented and size selected prior to library construction. Libraries were quantified by KAPA quantitative PCR for Illumina adapters (Roche, Pleasanton, CA) and size was determined by Agilent TapeStation D1000 tapes. Libraries were sequenced on a NextSeq 500 sequencer (Illumina, San Diego, CA). 10x Genomics Cell Ranger 2.1.1 scRNAseq pipeline was used for data analysis. The pipeline aligned reads to the University of California Santa Cruz (UCSC) human reference (GRCh38) transcriptome using the RNA-seq alignment program STAR. Manually generated clusters, UMAP plots, differential gene expression analyses, and feature plots were produced using Seurat within Rstudio. To select cells for downstream analysis, we removed cells with 1) < 900 unique genes detected. 2) > 7500 unique genes detected. 3) > 20% of their counts mapping to mitochondrial (MT) genes. 4) > 50,000 RNA counts. Genes detected in < 3 cells were also disregarded. Raw data are deposited in NCBI BioSample database with BioSample accessions of SAMN18316925 (CD-1), SAMN18316926 (CD-2, P0), SAMN18316927 (CD-2, P1), SAMN18316928 (CD-3), SAMN18316929 (CD-4). No customized codes were used.

## 2.9. Statistics

All experiments were performed in triplicate and at least 3 times. Results are expressed as mean ± SD. Differences were assessed student *t* test. P values less than 0.05 were considered significant.

## 2.10. Role of the funding sources

The funding source provided support for costs of sequencing and consumables. The funding source played no role in study design, data collection, analyses, data interpretation or manuscript writing.

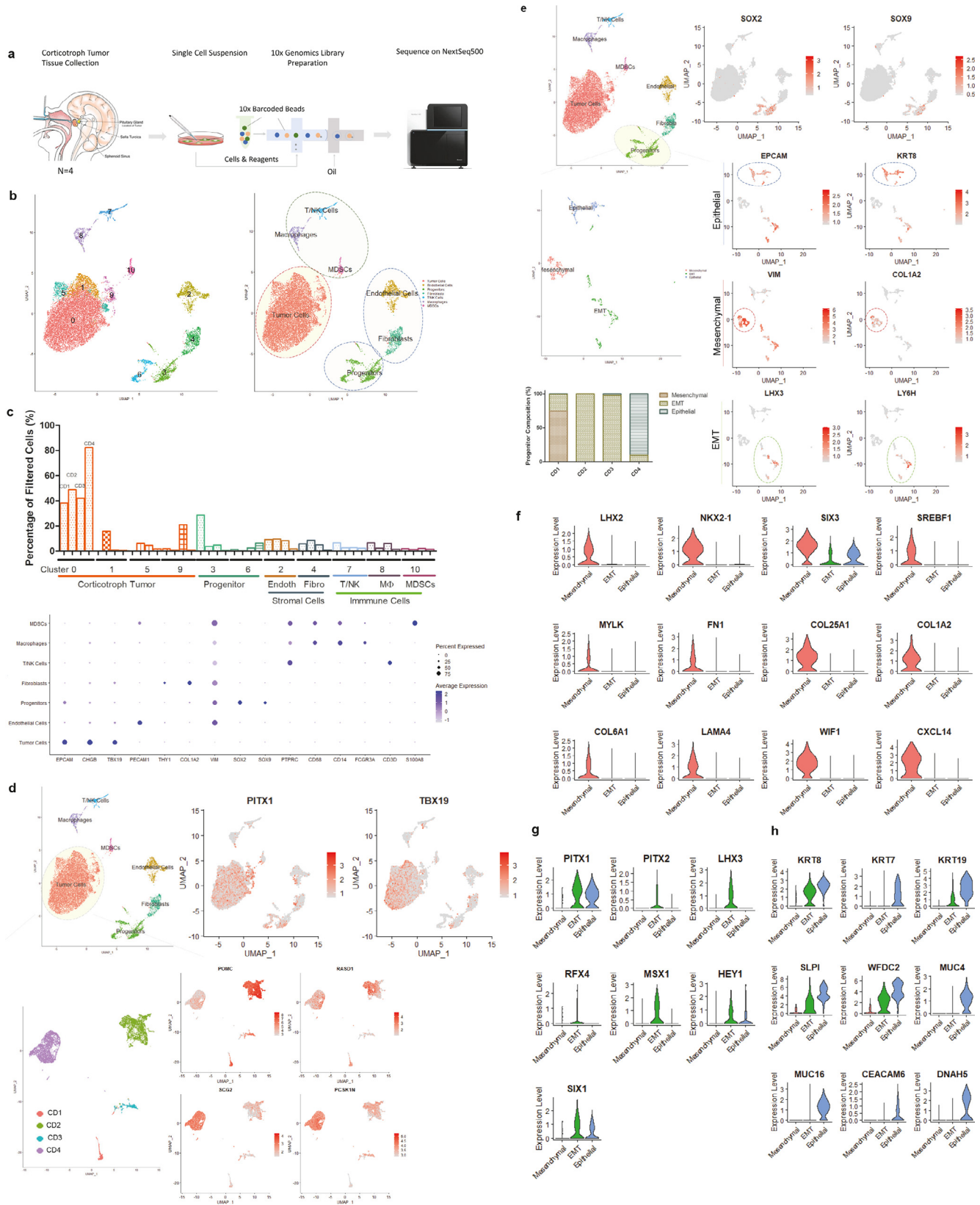
## 3. Results

### 3.1. Classification of cellular composition of human CD tumors based on scRNA-seq analysis

To understand at a deeper level the cellular and transcriptional make-up in human corticotroph tumors, we employed single cell RNA sequencing (scRNA-seq) to analyze 4 individual surgically resected clinically functional corticotroph tumors (CD1-4, Supplementary Table 1) at a single cell resolution using a 10x Genomics platform (v3, Fig. 1a). Using Cell Ranger pipeline for alignment and normalization of scRNA-seq data, we detected a total of 26,126 cells, with an average of 6,532 cells per patient (Supplementary Table 2). An average of 269 million reads, and 24,538 genes per patient were obtained with an average of 1,373 genes expressed and 3,886 unique molecular identifiers (UMIs) detected per cell (Supplementary Table 2). Seurat v3 was then used for read pre-filtering, integration and cluster identification [22], which resulted in 15,623 cells (CD1 1,691 cells, CD2 6,435 cells, CD3 1,186 cells and CD4 6,311 cells) grouped into 10 clusters (Fig. 1b). By profiling differentially expressed genes (DEGs), we identified gene signatures for each of the 10 distinct cell clusters (Fig. 1c, Table 1 and Supplementary Table 3). We assigned the clusters putative identities according to their enriched markers, and annotated them as corticotroph tumor cells (EPCAM+/CHGB+/PITX1+/TBX19+, cluster-0, 1, 5 and 9, containing 11,500 cells, 73.6%, Figs. 1c & 1d), progenitors (SOX2+/SOX9+, cluster-3 and 6, 1,299, 8.3%, Figs. 1c & 1e), stromal cells (including endothelial cells, PECAM1+/VWF+/CLEC14A+/VEGFC+, cluster-2, 982 cells, 6.3%; and fibroblasts, THY1+/COL1A1+/COL1A2+/ACTA2+/PDGFRB+/FN1+, cluster-4, 764 cells, 4.9%, Supplementary Fig. 1), and immune cells (including T/NK cells, PTPRC+/CD2+/CD3D+/CD3E+/CCL5+/NKG7+, cluster-7, 441 cells, 2.8%; macrophages, CD14+/FCGR3A+/CD68+/CD86+/APOE+/C1QA+, cluster-8, 414 cells, 2.6%; and myeloid-derived suppressor cells, CD68+/CD14+/S100A8+/VCAN+/FCN1+, cluster-10, 223 cells, 1.4%, Supplementary Fig. 2). POMC, glucocorticoid responsive gene RASD1 [23], and hormone processing genes such as SCG2 and PCSK1N were highly expressed across all 4 corticotroph tumor cell populations as expected (Fig. 1d).

Using the same methodologic approaches, we defined 3 progenitor sub-groups, namely epithelial progenitors (408 cells, 2.6%) which expressed the epithelial markers (EPCAM/KRT8), mesenchymal progenitors (378 cells, 2.4%) expressing markers of VIM/COL1A2, and mesenchymal-epithelial transitioning cells (EMT, 513 cells, 3.3%), which expressed both EPCAM and VIM as well as pituitary stem cell markers (LHX3 and LY6H, Fig. 1e). The mesenchymal progenitor cells also expressed several posterior pituitary lineage TFs including LHX2, NKX2-1 (aka TTF1) [24], SIX3 and SREBF1 [25], in addition to several genes characteristic of fibroblasts (MYLK, FN1, COL25A1, and COL1A2) and a plethora of distinct secretory genes involved in ECM construction (COL6A1/LAMA4) and cell-cell communication (CXCL14 and WIF1, Fig. 1f and Supplementary Table 4). In contrast, the EMT cell subgroup expressed several well-defined anterior pituitary TFs, including PITX1/2, LHX3, RFX4, HEY1 and MSX1, mimicking features of early embryonic anterior pituitary progenitor cells which dictate endocrine cell commitment [26–29] (Fig. 1g and Supplementary Table 4). Epithelial progenitors comprised the third subgroup and expressed cyokeratin (KRT8/7/19+), serous cell markers SLPI/WFDC2, mucins (MUC16/MUC4) and cilium organization components (CEACAM6/DNAH5), imitating some features of ciliated luminal sub-mucosal epithelial cells within a microcystic fibrosis structure [30]





**Fig. 1.** Single-cell RNA-sequencing (scRNA-seq) analysis of surgically resected human corticotroph tumors demonstrating their heterogeneous tumor cell make-up and microenvironment. (a) Schematic of surgically resected corticotroph tumor isolation (n=4, CD1-4, Supplementary Table 1) for single cell RNA sequencing and analytical workflow. (b) Uniform manifold approximation and projection (UMAP) was used to visualize corticotroph tumor cellular composition and revealed 10 clusters. (c) Each cluster was annotated based on specific marker expression. The number of cells detected in the individual cluster is depicted in the upper panel. (d) Tumor cells were further analyzed by subset, and found to cluster in a patient origin-dependent manner. (e-h) Analysis of the progenitor cell population revealed 3 sub-groups, namely mesenchymal- (f), endocrine- (g) and epithelial-progenitors (h).

**Table 1**

The annotation and number of cells in each cluster.

| Cell Types | Corticotroph Tumor Cells |      |     |     | Progenitors             |                        | Stromal Cells     |             | Immune Cells |          |       | Total |
|------------|--------------------------|------|-----|-----|-------------------------|------------------------|-------------------|-------------|--------------|----------|-------|-------|
|            | Corticotroph Tumor Cells |      |     |     | Mesenchymal Progenitors | Epithelial Progenitors | Endothelial Cells | Fibroblasts | T/NK         | M $\Phi$ | MDSCs |       |
| Cluster    | 0                        | 1    | 5   | 9   | 3                       | 6                      | 2                 | 4           | 7            | 8        | 10    | 10    |
| CD1        | 646                      | 0    | 2   | 32  | 485                     | 21                     | 156               | 96          | 110          | 111      | 32    | 1691  |
| CD2        | 3145                     | 1014 | 403 | 48  | 240                     | 21                     | 620               | 559         | 162          | 145      | 78    | 6435  |
| CD3        | 499                      | 11   | 54  | 249 | 57                      | 29                     | 99                | 58          | 32           | 73       | 25    | 1186  |
| CD4        | 5195                     | 36   | 115 | 51  | 40                      | 406                    | 107               | 51          | 137          | 85       | 88    | 6311  |

(Fig. 1h and Supplementary Table 4). In summary, our scRNAseq analysis of 4 corticotroph tumors revealed 4 major cell types, namely corticotroph tumor cells (73.6%), stromal cells (11.2%), progenitor cells (8.3%), and immune cells (6.8%).

### 3.2. Transcriptional changes across loss of hormone secretion in *in vitro* human CD primary cultures

As summarized schematically (Fig. 2a), surgically removed fresh human ACTH-secreting pituitary tumor tissues were harvested and prepared for primary culture using standard conditions. To characterize transcriptional changes that occurred during corticotroph tumor culture, we extracted mRNA from paired corticotroph tumor primary cultures that were one passage interval in the presence and absence of ACTH hormone secretion for microarray analysis. To identify differentially expressed genes (DEGs) between the consecutive passages, we used a conservative threshold taking a natural logarithm ratio of 1.0 that corresponded to a 2-fold-change (FC) in gene expression. Concordant with loss of ACTH secretion in the human corticotroph tumor primary cultures, we identified 415 DEGs (429 probes), 214 of which were upregulated (227 probes) and 201 of which were downregulated (202 probes, Fig. 2b). Gene Ontology analysis revealed changes primarily in genes that regulated cell migration and motility, extracellular matrix (ECM) protein make-up, and cell adhesion molecules (Fig. 2c). For example, several ECM proteins, including collagen type XI alpha 1 chain (COL11A1), stanniocalcin 1 (STC1), tenascin-C (TNC), and reelin (RELN), as well as the cytoskeleton gene, neurofilament medium (NEFM), were amongst the top 10 most upregulated genes (Fig. 2d), and our scRNAseq analysis also showed their expression in the stromal population (Supplementary Fig. 3). This suggested to us that our existing 2D culture conditions were selecting cells that exhibited strong adhesion to the supporting surface. Interestingly, amongst the top 10 most downregulated DEGs, we noted several tissue specific genes including integrin  $\beta$ 2 (ITGB2) [31,32] and neutrophil cytosolic factor 2 (NCF2) [33,34] which are enriched in leukocytes and granulocytes; elastin (ELN) [35] and myomesin 1 (MYOM1) [36] which are enriched in muscle cells and myofibroblasts; as well as  $\beta$ -tubulin (TUBB2B) which is a neuronal specific tubulin gene [37,38] (Fig. 2d). Reduced expression of these tissue specific genes suggested that certain cell populations such as immune cells, blood vessel/endothelial cells, and neuronal components were being lost from the initial heterogenous tissue of the corticotroph tumor during *in vitro* culture. This hypothesis was further supported by reduced expression of the respiratory regulatory protein, cytoglobin (CYGB, Fig. 2d), which senses oxygen homeostasis and is typically induced in anoxic or hypoxic conditions to sustain cell survival [39,40]. Taken together, the transcriptional pattern we observed in the corticotroph tumor cells as they lost ACTH hormone secretion suggested to us that our current 2D culture system may have been inadvertently generating a selective stress which favored selection of cells with strong adherent properties, such as stromal cells. We further hypothesized that certain types of tumor components, including endocrine hormone secreting tumor cells, were being lost either due to dislodgement during passaging or apoptosis caused by their inability to appropriately adjust to the culture conditions.

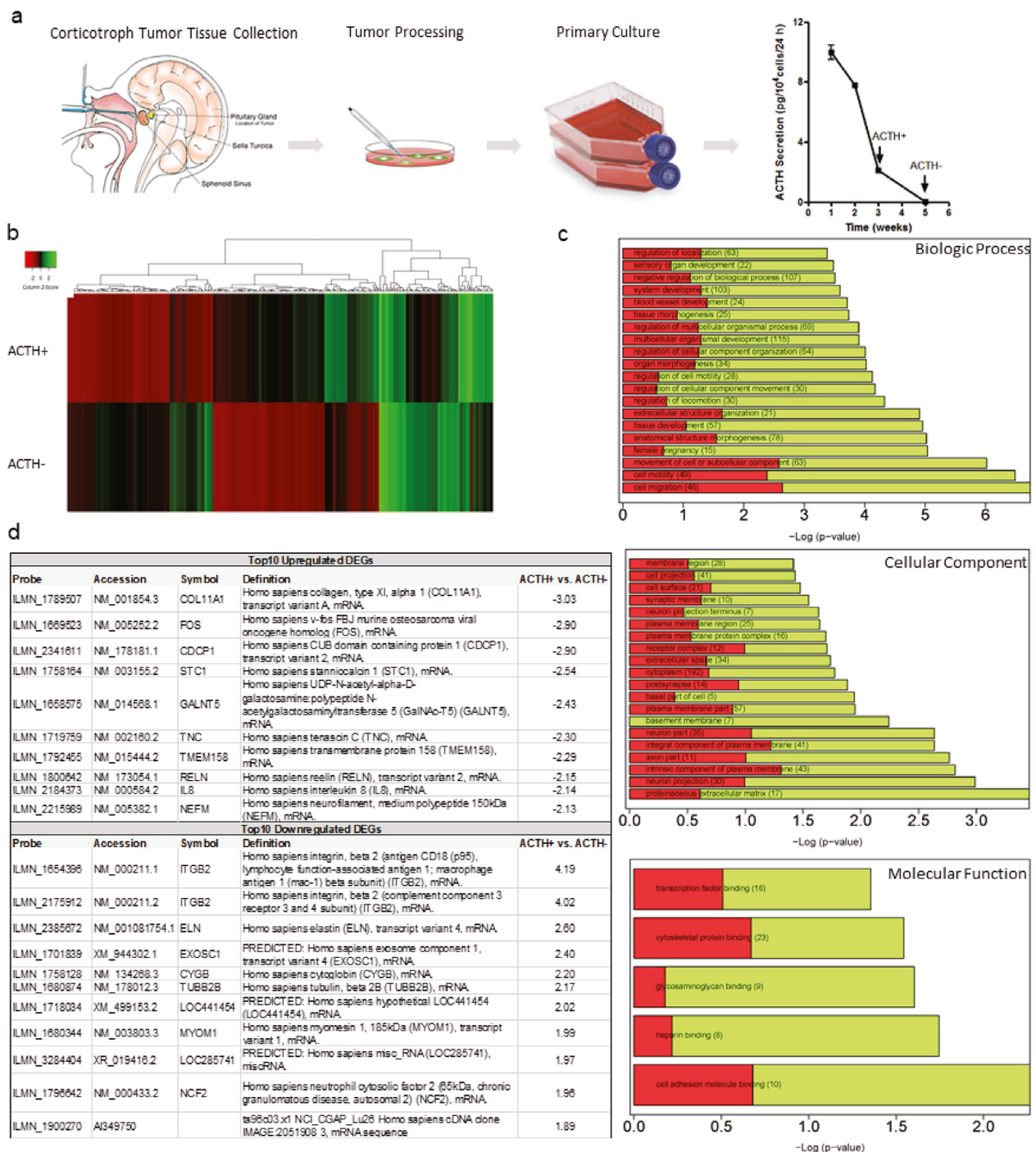
### 3.3. Optimization of the nutrient milieu and ECM coatings for human pituitary tumor cell ACTH secretion

As previously described, rapid fibroblast outgrowth is observed during early *in vitro* pituitary tumor culture [41], and replacement of L-valine with D-valine in culture medium has been shown to selectively inhibit fibroblast proliferation [42]. However, we reasoned that the L-valine in fetal bovine serum (FBS) would largely attenuate the value of D-valine selective media [43], and therefore we compared effects of different FBS concentrations on human corticotroph tumor ACTH secretion in 2 CD tissues (CD-6 and CD-7). In short, we observed that changes in FBS concentrations did not alter ACTH secretion (Fig. 3a), although higher FBS concentrations (20%) increased cell proliferation (Relative Proliferation Rate, 10%FBS  $0.91 \pm 0.1$ , 20% FBS  $1.6 \pm 0.2$ ,  $p < 0.05$ ) whereas lower FBS (2%,  $0.5 \pm 0.1$ ,  $p < 0.05$ ) alone or combined with 15% horse serum (HS) reduced cell proliferation ( $0.3 \pm 0.1$ ,  $p < 0.005$ , *t* test, Fig. 3b). Opti-MEM reduced both ACTH secretion and cell proliferation ( $p < 0.05$ , *t* test, Fig. 3b), and therefore 10%FBS was used as routine medium.

As described above, as the corticotroph tumor cells lost ACTH secretion, we had observed a transcriptional pattern that suggested our standard 2D culture system was generating a selective stress favoring cells with strong adhesive capabilities, such as fibroblasts. Therefore, we next investigated ways to provide some form of *in vitro* structural support that might enable maintenance of corticotroph tumor cell-cell and cell-ECM interactions, potentially preserving their longer term ACTH secretory capability. Variation of ECM components, including collagen I (Col-I) & four (Col-IV), fibronectin (FN), laminin (LAM) and vitronectin (VIT) did not alter ACTH secretion (Fig. 3c) or corticotroph tumor cell proliferation (Fig. 3d) in the corticotroph tumor cultures [44,45]. Regardless of which ECM component we used, ACTH secretion decreased after the first passage (P1, ~4 weeks, Fig. 3e). We did observe that the only cells cultured in LAM-coated plates continued to secrete ACTH beyond passage 2 (~6 weeks, Fig. 3e).

### 3.4. Role of matrigel in maintaining long-term human pituitary corticotroph tumor ACTH secretion

Having noticed the effect of LAM coating to prolong ACTH secretion, we investigated matrigel as a scaffold matrix given it contains high LAM concentrations (60%) and to mimic a 3D culture condition. Matrigel is a heterogenous gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, rich in a variety of ECM adhesive proteins including LAM (60%), Col-IV (30%), and entactin (8%) the latter of which acts to bridge between LAM and Col-IV. It also contains heparin sulfate proteoglycans, and a number of growth factors, including transforming growth factor beta (TGF- $\beta$ , 1.7-4.7ng/mL), EGF (0.5-1.3ng/mL), insulin-like growth factor-1 (IGF-1, 11-24ng/mL), bFGF ( $< 0.2$ pg/mL), nerve growth factor (NGF,  $< 0.2$ ng/mL), platelet derived growth factor (PDGF, 5-48pg/mL), and other growth factors which have been reported to be important in pituitary function [46]. As depicted in Fig. 4a, we observed that when corticotroph tumor cells were embedded in 50% matrigel and cultured on top of a thick matrigel coating (200  $\mu$ l/cm<sup>2</sup>), ACTH hormone

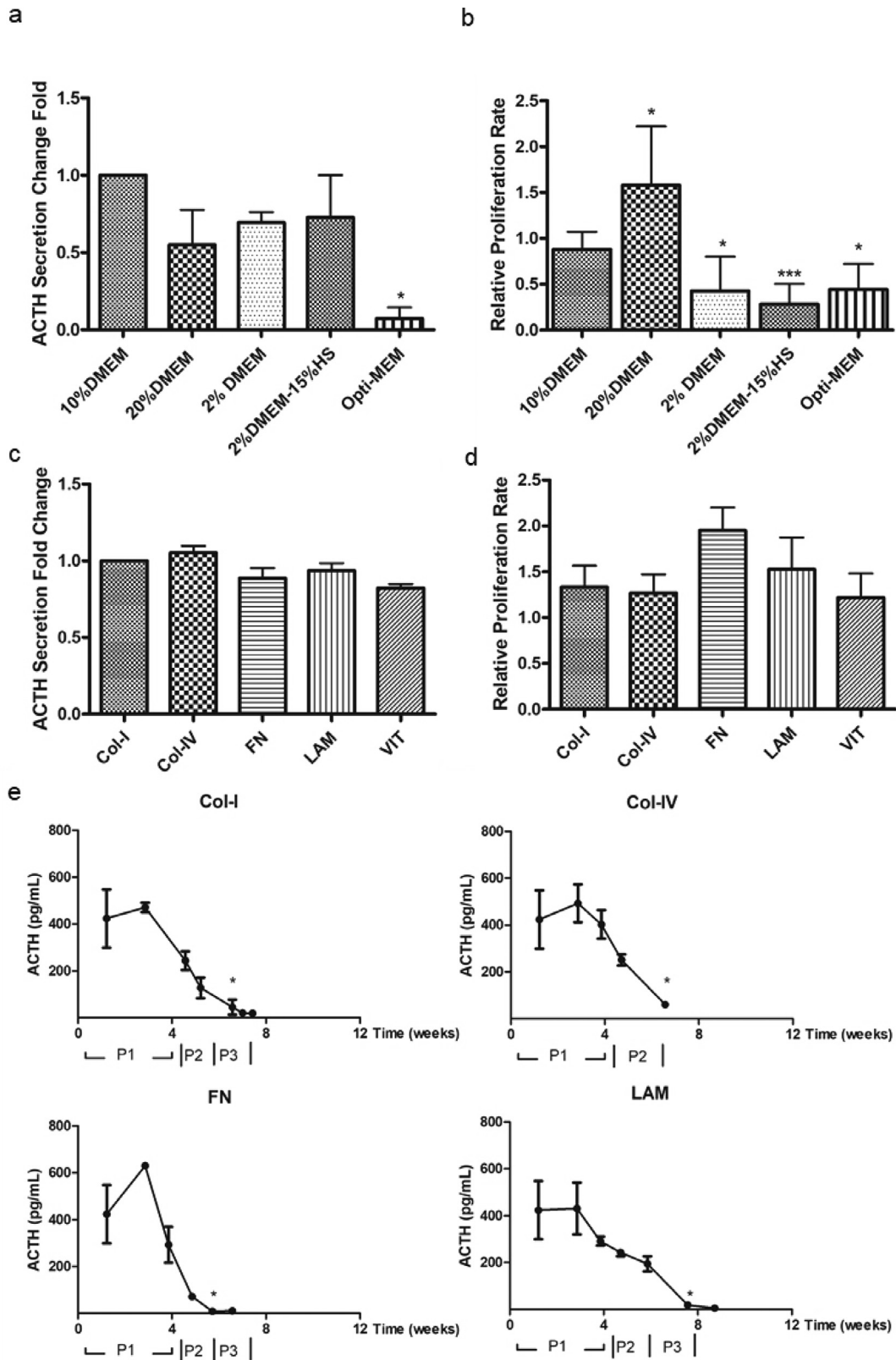


**Fig. 2.** Changes in human corticotroph tumor transcriptome across loss of ACTH secretion. (a) Schematic of surgically resected pituitary corticotroph tumor collection, enzymatic digestion and conventional 2-dimensional culture with quantitation of secreted ACTH levels. mRNA was harvested from paired primary cultures from a patient with CD at one passage interval between active and absent ACTH secretion and subjected to microarray analysis. (b) Heat-map of the 415 genes that exhibited  $\geq 2$ -fold change in expression between paired human CD samples. (c) Gene Ontology analysis of the 415 DEGs depicting their biological processes, cellular component and molecular function. (d) Table depicting the top 10 upregulated and downregulated DEGs respectively from the human corticotroph tumor microarray analysis.

secretion increased and was sustained up to 18 weeks in culture compared to no gel-coating or thin-gel coating ( $50 \mu\text{l}/\text{cm}^2$ ) conditions (Fig. 4b). Additionally, photomicrographs of the cultures demonstrated that those grown on a thick layer of matrigel formed suspended cell aggregates (Fig. 4e), whereas higher fibroblast numbers were observed in primary cultures grown on vessels with either no coating (Fig. 4c) or a thin matrigel coating (Fig. 4d). These encouraging results suggested to us that our matrigel-based 3D culturing method could help sustain corticotroph tumor ACTH secretion.

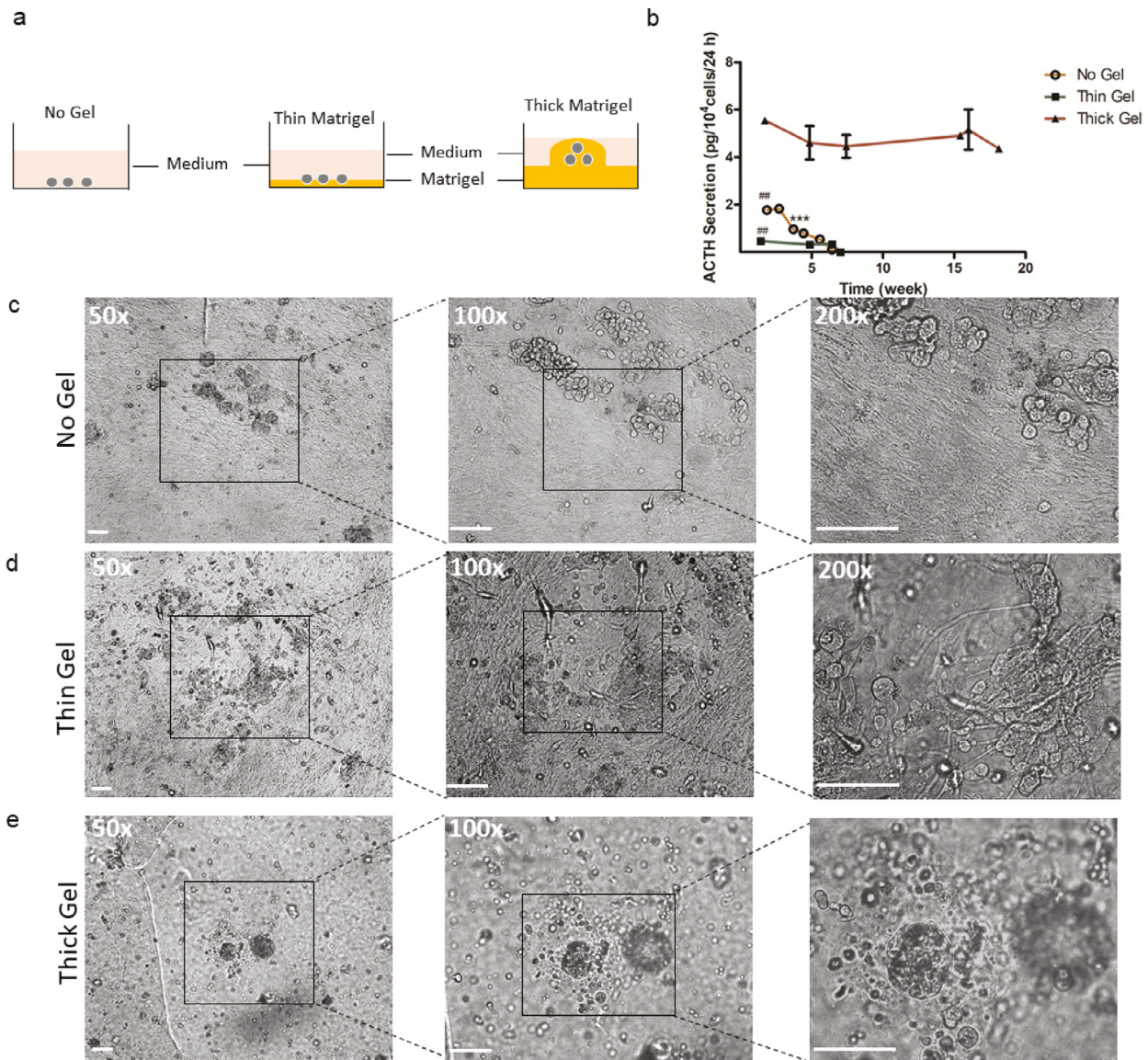
### 3.5. A defined medium for optimal corticotroph pituitary tumoroids in matrigel

Having identified a suitable matrix to sustain the differentiated corticotroph tumor cells, we sought to simultaneously optimize our culture conditions to support the corticotroph progenitor cell population we had noted above (Fig. 1e). Once again, we compared the corticotroph tumoroid culture transcriptome to that of its freshly resected parental corticotroph tumor tissue, this time by scRNA-seq (Fig. 5a) and identified a defined medium (detailed in Materials and



**Fig. 3.** Defining optimal medium supplements and ECM coatings to maintain human pituitary corticotroph tumor cell ACTH secretion. (a & b) Characterization of optimal FBS concentrations on pituitary corticotroph tumor ACTH secretion (a) and cell proliferation after 3-day incubation (b). (c & d) ACTH-secreting human pituitary tumor cells were cultured in various ECM coated 96-well plates for three days, after which ACTH secretion (c) and cell proliferation were measured (d). (e) Effects of the ECM components Col-I, Col-IV, FN, and LAM on longer-term ACTH secretion were tested in corticotroph tumor cells in 6-well plates. Col-I, collagen-I; Col-IV, collagen-IV; FN, fibronectin; LAM, laminin, VIT, vitronectin. \*  $p < 0.05$ , \*\*\*  $p < 0.005$  (t test).





**Fig. 4.** Effect of matrigel on human corticotroph ACTH production in 3D cultures. (a-e) Effect of no-gel, thin or thick matrigel coating (depicted in a) on pituitary corticotroph tumor ACTH secretion over 18 weeks (b) and photomicrographs (50-200x) of corticotroph tumor cell morphology (c-e). Scale bars: 100  $\mu$ m. Reduction in ACTH secretion was seen when the cells were grown on no-gel or thin-gel coating plates compared to thick-gel coating condition, ##  $p < 0.01$ . After the cells were cultured in no-gel plates for 4 weeks, ACTH secretion was reduced, \*\*\*  $p < 0.005$  ( $t$  test).

Methods section) that not only maintained the tumor and the fibroblast cell populations (Baseline: tumor cells 61.65% and fibroblasts 11.91% vs. defined medium for 8 weeks: tumor cells 55.81% and fibroblasts 14.47%), but additionally resulted in expansion of the progenitor cell population (Baseline 4.3% vs. defined medium for 8 weeks 10.3%, Figs. 5b & 5c) with emergence of a new population of “intermediate” cells (19.4%), which expressed PITX1 and TBX19 (Fig. 5d and Supplementary Tables 5 & 6) [47].

### 3.6. A two-step 3D culture platform for sustainable human pituitary corticotroph tumoroids

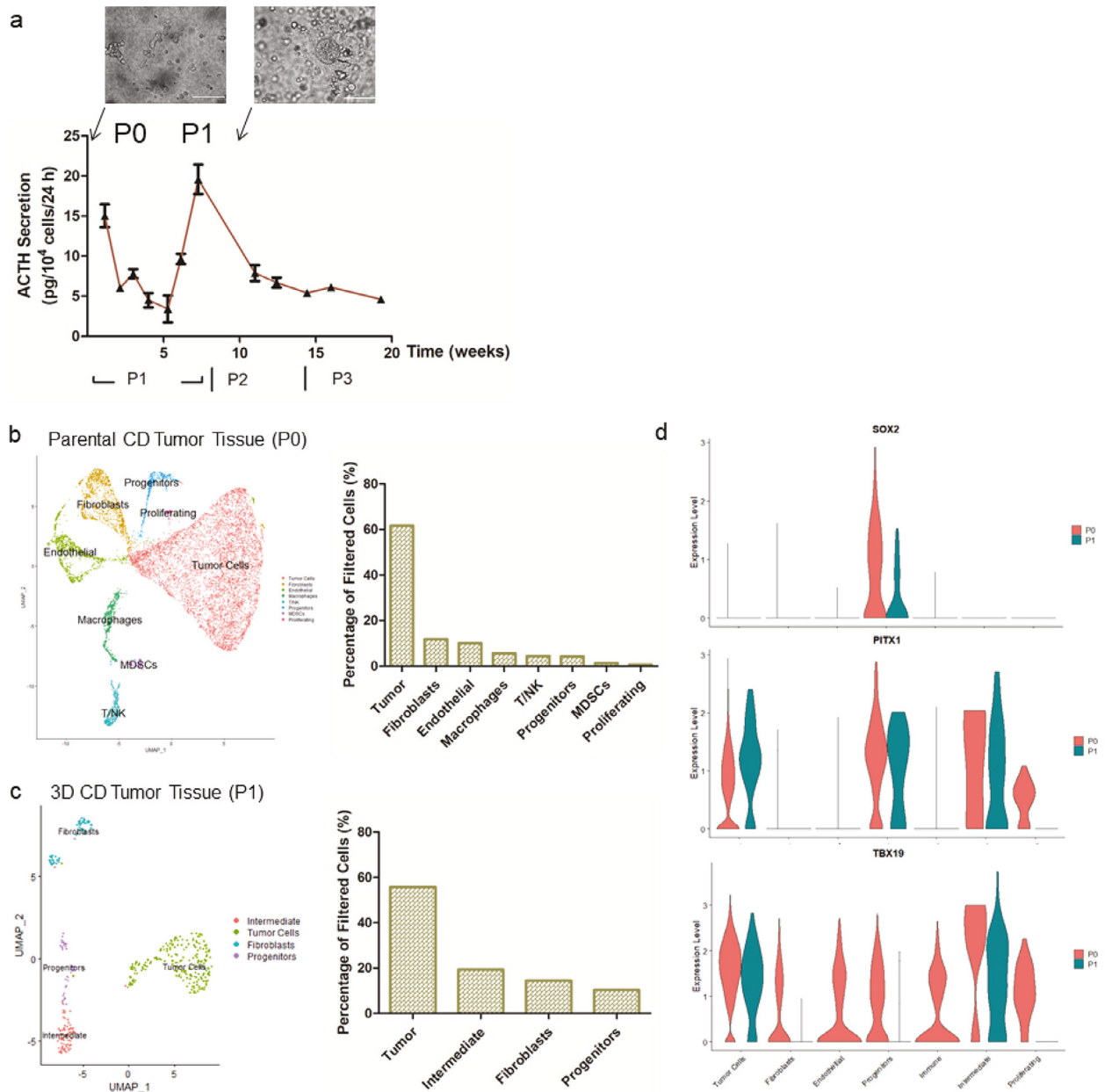
Having defined conditions that supported both the differentiated ACTH-secreting corticotroph tumor and progenitor cell population, we then transferred the established corticotroph tumoroid cultures to spinner flasks to augment corticotroph tumor cell proliferation rate (Fig. 6a) [48]. Using this two-step culture system, whereby established matrigel-embedded corticotroph tumor clusters (steadily propagated for 5 weeks) were then transferred to a spinner flask, we have consistently obtained a high yield of ACTH-secreting human

corticotroph tumor cells from 5 consecutive harvested corticotroph tumoroids thus far (representative tumor, Fig. 6a). This approach has not only sustained corticotroph tumor ACTH secretion but actually resulted in increased corticotroph tumor ACTH secretion in some (ACTH (pg/10<sup>4</sup> cells/24h): week-1; 14.7  $\pm$  0.07 vs. week-6; 93  $\pm$  2.9,  $p < 0.05$ , Fig. 6b) but not all corticotroph tumoroids studied. We have also been able to cryopreserve and retrieve some corticotroph tumor cultures for later translational research studies and demonstrated that these retrieved clones secreted similar ACTH levels to their original parental tumor cells for up to two further weeks after thaw recovery (Fig. 6c). These extensive set of studies indicate that our 2-step culture system can be employed to simultaneously maintain differentiated ACTH-secreting corticotroph tumor cells as well as support corticotroph tumor progenitor cells.

## 4. Discussion

The availability of human tumor-derived *in vitro* cell cultures provides a unique and irreplaceable resource to gain insights into a tumor’s biological features and is critical to the identification of





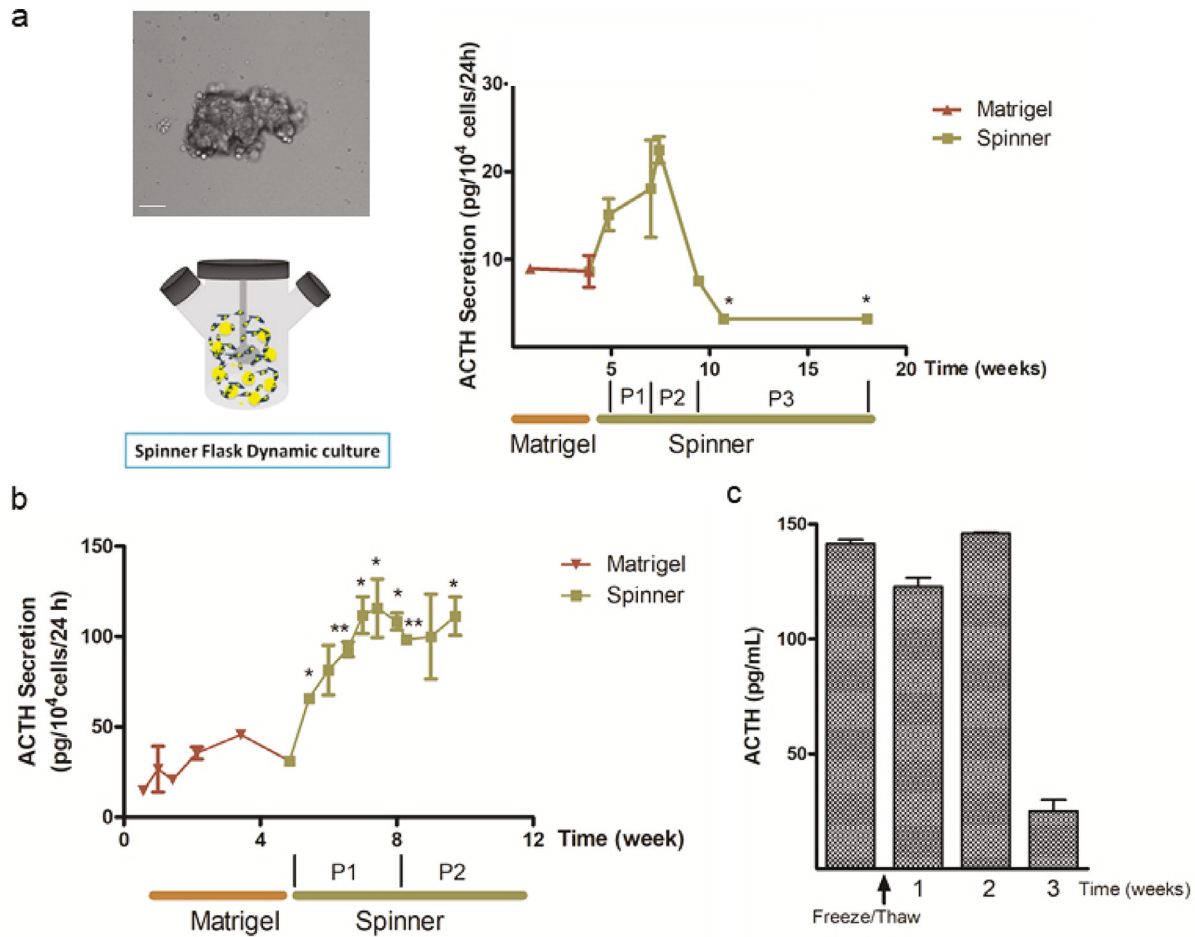
**Fig. 5.** A defined medium for optimal corticotroph pituitary tumoroids in matrigel.

(a) ACTH secretion was monitored to determine the effect of a defined medium on matrigel-based 3D culture. (b-d) scRNA-seq analysis of a corticotroph tumor parental tissue (P0) and its derived passage-1 corticotroph tumor culture (P1), comparing changes in cellular composition after 2-month culture. Scale bars: 100  $\mu$ m.

potential novel therapeutic molecular targets and testing potential therapeutics [49]. Whereas many epithelial cancers can be grown either as patient derived xenograft (PDX) *in vivo* models or used to generate immortalized cell lines for *in vitro* studies, neuroendocrine tumors, including those of the pituitary gland, have not thus far been amenable to *in vitro* culture for any longer than 1-2 weeks and they do not form tumors *in vivo*. Unquestionably, *bona fide* human pituitary tumor cell lines that faithfully recapitulate the highly differentiated features of these tumors including specific hormone secretion would represent a major advance in the field. Such a tool would greatly facilitate advances in evaluating the detailed mechanism(s) of action of various therapeutic agents at the cellular and subcellular level and allow improved understanding of the molecular pathogenesis of pituitary tumors.

Several methods have previously been exploited to generate long-term pituitary tumor cultures, including modifying the extracellular

matrix [11], adjusting culture media components or use of various culture devices [50]. However, no comprehensive molecular analysis of the factors underlying the loss of pituitary corticotroph tumor ACTH secretion in culture that might guide optimization of culture conditions has ever been undertaken. Furthermore, an effective experimental protocol that promulgates long-term human pituitary corticotroph tumor culture has not been established. Cushing disease, a rare orphan disease due to a pituitary corticotroph tumor, is particularly challenging to study as even large pituitary centers only perform 10-15 corticotroph tumor surgeries annually. Compounding this, corticotroph tumors are often small (3-5mm), and sufficient tissue is not always available for research use. Our studies here outline a 2-step culture system that incorporates a 3D matrigel culture model and a defined medium to maintain human ACTH-secreting pituitary corticotroph tumor cells with expansion in a spinner flask to promote their proliferation. They advance our ability to study human



**Fig. 6.** Development of a two-step culture system to preserve pituitary tumor-derived hormone production in *in vitro* cultures. (a) ACTH secretion was maintained in human corticotroph tumoroid culture following culture on thick on matrigel and transfer to a spinner flask for expansion in differentiation medium. (b) Defined medium further augmented ACTH secretion using the 2-step culture approach. (c) Cryopreserved human pituitary corticotroph tumor primary cultures were recovered from frozen cell aliquots, and exhibited ACTH secretion for two weeks after retrieval. Scale bars: 100  $\mu$ m. \*  $p < 0.05$ , \*\*  $p < 0.001$  (t test).

corticotroph tumors and we and others now need to further test these methods to build a living biobank of human pituitary corticotroph tumors.

High-throughput scRNA-seq has provided unprecedented potential to assess and dissect the heterogeneous composition of the tumor microenvironment and generate transcriptional profiles from thousands of cells simultaneously. Our scRNA-seq studies of human corticotroph tumors identified at least 4 major cell populations, namely corticotroph tumor cells, stromal cells, progenitors, and immune cells. We also identified 3 types of progenitor cells that were SOX2 and SOX9 positive. Intriguingly, the mesenchymal progenitor cells also expressed several posterior pituitary lineage TFs. Although we cannot discount some contamination of the corticotroph tumor sample with posterior pituitary tissue, prior studies have reported corticotroph tumors completely located in the posterior pituitary [51], and corticotroph (basophil) invasion of the pars nervosa in the human pituitary is not uncommonly seen at autopsy [52,53]. EMT endocrine progenitor cluster (513 cells, 3.3%) expressed several embryonic pituitary stem cell markers, including HEY1, and LHX3, suggesting that this cluster could potentially be a forerunner of differentiated pituitary cell sub-types under appropriate specific direction. This hypothesis is supported by animal models which have demonstrated that SOX2 lineage progenitor pituitary cells can give rise to new hormone-producing cells throughout life [54]. The epithelial progenitor cluster (408 cells, 2.6%) expressed the epithelial markers, KRT8/18/19 and several markers of serous gland secreting cells, as well as dynein components (DNAHs) of cilium organization

and TF CEACAM6 found in pancreatic ductal carcinoma [55]. In some ways, this population appears to emulate some features of Rathke's cleft cysts [56]. Prior studies using *in vitro* cultures of mouse pituitary derived ciliated cystic organoids which were later engrafted in mice resulted in a limited number of hormone-expressing cells *in vivo* [57]. Therefore, we speculate that the cystic organoids [57] resemble the *in vitro* derivative of our defined epithelium progenitors, as they are mucin-producing cells rather than endocrine progenitors. We speculate that our findings also raise the intriguing possibility that isolation and propagation of these EMT endocrine progenitor cells could offer a novel approach to direct their development to generate functional differentiated pituitary cell lineages. Supporting this idea, a prior study described expression of the stem cell marker SOX2 in short-term (7-10 days) suspension cultures of a human clinically non-functioning and 2 growth hormone secreting pituitary tumors [58].

By careful monitoring of ACTH secretion in serial passages of individual primary human corticotroph tumor cultures, we have been able to characterize changes in gene expression that occur within one passage interval between the presence and absence of active pituitary corticotroph tumor ACTH secretion. These studies demonstrated dramatic changes in expression of transcripts related to cell motility, ECM and receptor binding as these corticotroph tumor cultures lost the ability to secrete ACTH. These findings underpin the important role of the ECM [11] and use of suspension culture [50] to optimize pituitary tumor culture conditions and highlight the potential unintended deleterious effects of current commonly used 2D

culture approaches for these vulnerable cells. Following a systematic analysis of effects of various physical architectural factors in the maintenance of human corticotroph tumor *in vitro* ACTH secretion, we created a two-step culture system, whereby corticotroph tumoroids were first established in a matrigel plug overlaid onto thick matrigel and subsequently transferred into a spinner flask. We did observe a drop in ACTH concentration in the increased spinner flask media volume which we believe is due to reduced cell density and we did note some variability in ACTH secretion between individual patients indicating that optimal culture conditions may vary slightly from patient to patient (Fig. 6). However, we believe these issues can be rectified by further optimization of factors in spinning culture, such as mixing speed; seeding density and cell harvest time to attain optimal sustained ACTH hormone secretion in harvested corticotroph tumors and this is a current focus. This is the first report of a method that maintains the highly differentiated phenotype of pituitary corticotroph tumor cultures for periods greater than 2-3 weeks, with sustained high levels of ACTH secretion for up to 4 months. Furthermore, we have also been able to cryopreserve our corticotroph tumoroids and retrieve these expandable primary cultures, thus permitting a consistent and convenient supply of human corticotroph tumor cells for drug sensitivity screens and other studies.

Altogether, our studies take a critical step in advancing methods to culture human corticotroph tumors and provide an extremely important resource for the study of the corticotroph tumor pathogenesis and provide a critical tool to identify and/or validate novel therapeutic options for these disabling and often fatal corticotroph tumors.

Contributors: Study design: D.Z, A.P.H; Conducting experiments and Acquiring Data: all authors; Analyzing data: D.Z., W.H, A.P.H; Verifying the underlying data: D.Z, W.H, A.P.H; Writing the manuscript: D.Z, A.P.H; Reviewing the manuscript and approval of the final version: all authors.

### Declaration of Competing Interest

The authors declare no competing interests.

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### Data sharing

Raw data are deposited in Gene Expression Omnibus (submission #: SUB9121105).

### Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.ebiom.2021.103294](https://doi.org/10.1016/j.ebiom.2021.103294).

### References

- [1] Perez-Castro C, Renner U, Haedo MR, Stalla GK, Arzt E. Cellular and molecular specificity of pituitary gland physiology. *Physiol Rev* 2012;92(1):1–38.
- [2] Gittleman H, Ostrom QT, Farah PD, Ondracek A, Chen Y, Wolinsky Y, et al. Descriptive epidemiology of pituitary tumors in the United States, 2004–2009. *Journal of Neurosurg* 2014;121(3):527.
- [3] Praw SS, Heaney AP. Medical treatment of Cushing's disease: overview and recent findings. *Int J General Med* 2009;2:209–17.
- [4] Reincke M, Sbiara S, Hayakawa A, Theodoropoulou M, Osswald A, Beuschlein F, et al. Mutations in the deubiquitinase gene USP8 cause Cushing's disease. *Nat Genet* 2015;47.
- [5] Hayashi K, Inoshita N, Kawaguchi K, Ibrahim Ardisasmitha A, Suzuki H, Fukuhara N, et al. The USP8 mutational status may predict drug susceptibility in corticotroph adenomas of Cushing's disease. *Eur J Endocrinol* 2016;174.
- [6] Ma Z-Y, Song Z-J, Chen J-H, Wang Y-F, Li S-Q, Zhou L-F, et al. Recurrent gain-of-function USP8 mutations in Cushing's disease. *Cell Res* 2015;25(3):306–17.
- [7] Kaiser UB. Cushing's disease: towards precision medicine. *Cell Res* 2015;25(6):649–50.
- [8] Ooi GT, Tawadros N, Escalona RM. Pituitary cell lines and their endocrine applications. *Mol Cellular Endocrinol* 2004;228(1–2):1–21.
- [9] Bilodeau S, Vallette-Kasic S, Gauthier Y, Figarella-Branger D, Brue T, Berthelet F, et al. Role of Brg1 and HDAC2 in GR trans-repression of the pituitary POMC gene and misexpression in Cushing disease. *Genes Dev* 2006;20(20):2871–86.
- [10] Liu N-A, Huang H, Yang Z, Herzog W, Hammerschmidt M, Lin S, et al. Pituitary corticotroph ontogeny and regulation in transgenic zebrafish. *Mol Endocrinol* 2003;17(5):959–66.
- [11] Westphal M, Jaquet P, Wilson CB. Long-term culture of human corticotropin-secreting adenomas on extracellular matrix and evaluation of serum-free conditions. *Acta Neuropathol (Berl)* 1986;71(1):111–8.
- [12] Oliva D, Nicosia S, Spada A, Giannattasio G. VIP stimulates ACTH release and adenylate cyclase in human ACTH-secreting pituitary adenomas. *Eur J Pharmacol* 1982;83(1):101–5.
- [13] Sharp B, Melmed S, Goldberg R, Carlson HE, Refetoff S, Hershman JM. Radioimmunoassay detection of endorphins from long-term culture of human pituitary tumour cells. *Acta Endocrinol (Copenh)* 1982;99(2):174–8.
- [14] Zhang D, Bergsneider M, Wang MB, Heaney AP. Targeting the ERK pathway for the treatment of Cushing's disease. *Oncotarget* 2016;7(43).
- [15] Du L, Bergsneider M, Mirsadraei L, Young SH, Jonker JW, Downes M, et al. Evidence for orphan nuclear receptor TR4 in the etiology of Cushing disease. *Proc Natl Acad Sci* 2013;110(21):8555–60.
- [16] Atkin SL, Hipkin L, Jeffreys RV, Foy PM, White MC. Human anterior pituitary adenoma cell attachment *in vitro*. *Vitro Cellular Devel Biol - Animal* 1997;33(3):158–60.
- [17] Knappe UJ, Lüdecke DK, Puchner MJA, Saeger W, Herrmann H-D. *In vivo* labeling with125I-CRH of human ACTH-producing pituitary adenomas heterotransplanted to nude mice. *Endocr Pathol* 1991;2(4):200–9.
- [18] Hofland LJ, Lamberts SWJ. Pituitary gland tumors. In: Masters JRW, Palsson B, editors. *Human Cell Culture: Cancer Cell Lines Part 1*. Dordrecht: Springer Netherlands; 1999. p. 149–59.
- [19] Seidah NG, Chrétien M. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides1. *Brain Res* 1999;848(1–2):45–62.
- [20] Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 2009;37(1):1–13.
- [21] Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2008;4:44.
- [22] Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM. Comprehensive integration of single-cell data. *Cell* 2019;177(7):1888–902 e21.
- [23] Kemppainen RJ, Behrend EN. Dexamethasone rapidly induces a novel ras superfamily member-related gene in AtT-20 cells. *J Biol Chem* 1998;273(6):3129–31.
- [24] Shibuya M. Welcoming the new WHO classification of pituitary tumors 2017: revolution in TTF-1-positive posterior pituitary tumors. *Brain Tumor Pathol* 2018;35(2):62–70.
- [25] Chen Q, Leshkowitz D, Blechman J, Levkowitz G. Single-cell molecular and cellular architecture of the mouse neurohypophysis. *Eneuro* 2020;7(1) ENEURO.0345-19.2019.
- [26] Chen J, Gremeaux L, Fu Q, Liekens D, Van Laere S, Vankelecom H. Pituitary progenitor cells tracked down by side population dissection. *Stem Cells* 2009;27(5):1182–95.
- [27] Kioussi C, O'Connell S, St-Onge L, Treier M, Gleiberman AS, Gruss P, et al. Pax6 is essential for establishing ventral-dorsal cell boundaries in pituitary gland development. *Proc Natl Acad Sci U S A*. 1999;96(25):14378–82.
- [28] Fletcher PA, Smiljanic K, Maso Prévède R, Iben JR, Li T, Rokic MB, et al. Cell type- and sex-dependent transcriptome profiles of rat anterior pituitary cells. *Front Endocrinol (Lausanne)* 2019;10:623–.
- [29] Xie H, Cherrington BD, Meadows JD, Witham EA, Mellon PL. Msx1 homeodomain protein represses the  $\alpha$ GSU and GnRH receptor genes during gonadotrope development. *Mol Endocrinol* 2013;27(3):422–36.
- [30] Stromatt SC. Secretory leukocyte protease inhibitor in cystic fibrosis. *Agents Actions Suppl* 1993;42:103–10.
- [31] Jerke U, Rolle S, Dittmar G, Bayat B, Santoso S, Sporbert A, et al. Complement receptor Mac-1 is an adaptor for NB1 (CD177)-mediated PR3-ANCA neutrophil activation. *J Biol Chem* 2011;286(9):7070–81.
- [32] Bai M, Grieshaber-Bouyer R, Wang J, Schmider AB, Wilson ZS, Zeng L, et al. CD177 modulates human neutrophil migration through activation-mediated integrin and chemoreceptor regulation. *Blood* 2017;130(19):2092–100.
- [33] Jacob CO, Eisenstein M, Dinauer MC, Ming W, Liu Q, John S, et al. Lupus-associated causal mutation in neutrophil cytosolic factor 2 (NCF2) brings unique insights to the structure and function of NADPH oxidase. *Proc Natl Acad Sci* 2012;109(2):E59–67.
- [34] Sancho-Shimizu V, Malo D. Sequencing, expression, and functional analyses support the candidacy of  $\langle \text{em} \rangle \text{Ncf2} \langle / \text{em} \rangle$  in susceptibility to  $\langle \text{em} \rangle \text{Salmonella} \langle / \text{em} \rangle$  typhimurium infection in wild-derived mice. *J Immunol* 2006;176(11):6954–61.
- [35] Mithieux SM, Weiss AS. Elastin. *Adv Protein Chem* 2005;70:437–61.
- [36] Wang L, Geist Hauserman J, Grogan A, Hu LY, Kontrogrianni-Konstantopoulos A. Thick filament protein network, functions, and disease association. *Comprehens Physiol* 2018;8:631–709.
- [37] Jaglin XH, Poirier K, Saillour Y, Buhler E, Tian G, Bahi-Buisson N, et al. Mutations in the  $\beta$ -tubulin gene TUBB2B result in asymmetrical polymicrogyria. *Nat Genet* 2009;41(6):746–52.



- [38] Geiger JT, Schindler AB, Blauwendraat C, Singer HS, Scholz SW. TUBB2B mutation in an adult patient with myoclonus-dystonia. *Case Reports Neurol* 2017;9(2):216–21.
- [39] Bholah TC, Neergheen-Bhujun VS, Hodges NJ, Dyll SD, Bahorun T. Cytoglobin as a biomarker in cancer: potential perspective for diagnosis and management. *Biomed Res Int* 2015;2015:824514.
- [40] Schmidt M, Gerlach F, Avivi A, Laufs T, Wystub S, Simpson JC, et al. Cytoglobin Is a respiratory protein in connective tissue and neurons, which is up-regulated by hypoxia. *J Biol Chem* 2004;279(9):8063–9.
- [41] Peculis R, Mandrika I, Petrovska R, Dortane R, Megnis K, Nazarovs J, et al. Pituitary adenomas contain genetic variants characteristic to pituitary adenoma tumor tissue. *Front Endocrinol* 2020;11(313).
- [42] Hongpaisan J. Inhibition of proliferation of contaminating fibroblasts by D-valine in cultures of smooth muscle cells from human myometrium. *Cell Biol Int* 2000;24(1):1–7.
- [43] Lazzaro V, Walker R, Duggin G, Phippard A, Horvath J, Tiller D. Inhibition of fibroblast proliferation in L-valine reduced selective media. *Res Commun Chem Pathol Pharmacol* 1992;75(1):39–48.
- [44] Walker JL, Assoian RK. Integrin-dependent signal transduction regulating cyclin D1 expression and G1 phase cell cycle progression. *Cancer Metastasis Rev* 2005;24(3):383–93.
- [45] Manabe R-i, Oh-e N, Sekiguchi K. Alternatively spliced EDA segment regulates fibronectin-dependent cell cycle progression and mitogenic signal transduction. *J Biol Chem* 1999;274(9):5919–24.
- [46] Ray D, Melmed S. Pituitary cytokine and growth factor expression and action. *Endocr Rev* 1997;18(2):206–28.
- [47] Kawasaki K, Toshimitsu K, Matano M, Fujita M, Fujii M, Togasaki K, et al. An organoid biobank of neuroendocrine neoplasms enables genotype-phenotype mapping. *Cell* 2020;183(5):1420–35 e21.
- [48] Mather JP. Laboratory scaleup of cell cultures (0.5–50 liters). *Methods Cell Biol* 1998;57:219–27.
- [49] Grozinsky-Glasberg S, Shimon I, Rubinfeld H. The role of cell lines in the study of neuroendocrine tumors. *Neuroendocrinology* 2012;96(3):173–87.
- [50] Thodou E, Ramyar L, Cohen A, W S, Asa S. A serum-free system for primary cultures of human pituitary adenomas. *Endocr Pathol* 1995;6(4):289–99.
- [51] Iglesias P, Guerrero-Pérez F, Villabona C, Díez JJ. Adenohypophyseal hyperfunction syndromes and posterior pituitary tumors: prevalence, clinical characteristics, and pathophysiological mechanisms. *Endocrine* 2020;70(1):15–23.
- [52] Lloyd RV, D'Amato CJ, Thiny MT, Jin L, Hicks SP, Chandler WF. Corticotroph (Basophil) invasion of the pars nervosa in the human pituitary: Localization of proopiomelanocortin peptides, galanin and peptidylglycine  $\alpha$ -amidating monooxygenase-like immunoreactivities. *Endocr Pathol* 1993;4(2):86–94.
- [53] Fan X, Olson SJ, Johnson MD. Immunohistochemical localization and comparison of carboxypeptidases D, E, and Z,  $\alpha$ -MSH, ACTH, and MIB-1 between human anterior and corticotroph cell "basophil invasion" of the posterior pituitary. *J Histochem Cytochem* 2001;49(6):783–90.
- [54] Andoniadou Cynthia L, Matsushima D, Mousavy Gharavy Seyedeh N, Signore M, Mackintosh Albert I, Schaeffer M, et al. Sox2+ stem/progenitor cells in the adult mouse pituitary support organ homeostasis and have tumor-inducing potential. *Cell Stem Cell* 2013;13(4):433–45.
- [55] Bernard V, Semaan A, Huang J, San Lucas FA, Mulu FC, Stephens BM, et al. Single-cell transcriptomics of pancreatic cancer precursors demonstrates epithelial and microenvironmental heterogeneity as an early event in neoplastic progression. *Clin Cancer Res* 2019;25(7):2194–205.
- [56] Shanklin WM. The incidence and distribution of cilia in the human pituitary with a description of microfollicular cysts derived from Rathke's cleft. *Acta Anat (Basel)* 1951;11(2–3):361–82.
- [57] Cox B, Laporte E, Vennekens A, Kobayashi H, Nys C, Van Zundert I, et al. Organoids from pituitary as a novel research model toward pituitary stem cell exploration. *J Endocrinol* 2019;240(2):287–308.
- [58] Mertens F, Gremeaux L, Chen J, Fu Q, Willems C, Roose H, et al. Pituitary tumors contain a side population with tumor stem cell-associated characteristics. *Endocr Relat Cancer* 2015;22(4):481–504.