Hacking of myoepithelial-to-ductal differentiation programs uncovers inhibitors of retinoid signaling as anti-tumor agents in Adenoid Cystic Carcinoma (ACC).

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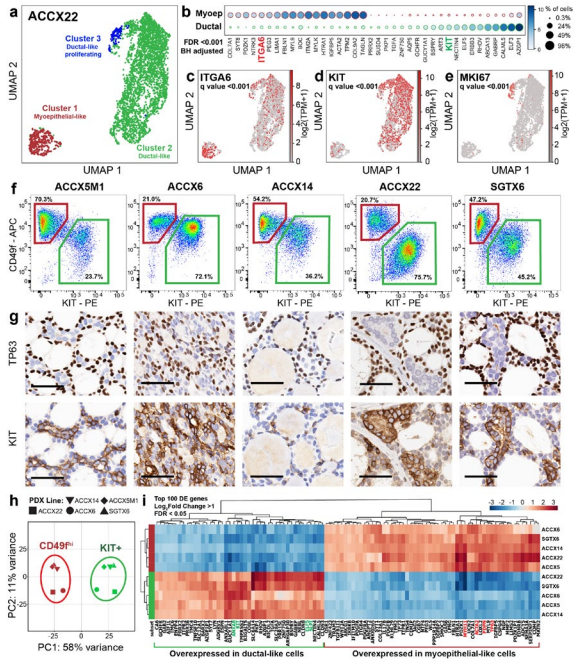
Research summary overview: Adenoid Cystic Carcinoma (ACC) is a rare but highly-metastatic and treatment-refractory form of salivary gland cancer, for which there are no clinically approved therapies[1]. A hallmark feature of ACCs is the co-existence within tumor tissues of two distinct populations of malignant cells, termed myoepithelial-like and ductal-like based on their phenotypic similarities to the myoepithelial and ductal lineages of normal salivary glands. However, the developmental relationship between these two cell populations, as well as their differential sensitivities to anti-tumor treatments are unknown. In this pre-clinical study, we leveraged our expertise in single-cell transcriptomics, fluorescence activated cell sorting (FACS), and three-dimensional organoid cultures to characterize the two distinct ACC cell populations from patient derived xenograft (PDX) models. We examined the role of the two populations in ACC tumor formation, and identified pathways which control their cellular differentiation. Using *in vitro* organoid cultures, we found that pharmacological manipulation of retinoic acid (RA) signaling can affect ACC cell identity. Finally, we demonstrated that inhibition of RA signaling by RA inverse agonist BMS493 led to selective cell death of ACC ductal-like cells and translated to *in vivo* anti-tumor activity against multiple models of human ACCs.

Methods: Utilizing a novel computational approach for single-cell RNA-seq (scRNA-seq) analysis derived from random matrix theory (RMT)[2], we performed transcriptional characterization of a human ACC tumor from a patient derived xenograft model. We identified cell-surface markers (CD49f, KIT) that enable the reproducible purification by FACS of myoepithelial-like ($CD49f^{high}/KIT^{neg}$) and ductal-like ($CD49f^{low}/KIT^{+}$) cells from ACCs. We then used prospective xeno-transplantation experiments in immune-deficient animals to examine the populations' tumorigenic capacity and investigate their developmental relationship. Using three-dimensional organoid cultures[3], we examined signaling pathways that control differentiation and survival of the two ACC cell types. Finally, we treated immune-deficient mice (NSG; NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) engrafted with three distinct ACC PDX lines with BMS493, an inverse agonist of RA signaling, to examine whether inhibition of RA signaling results in anti-tumor activity against human ACCs *in vivo.*

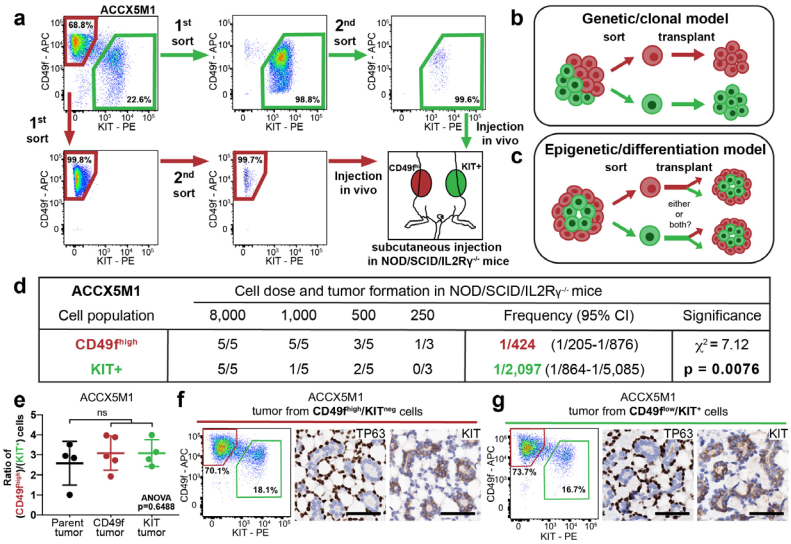
Results:

1. Identification of cell-surface markers for the differential purification of myoepithelial-like and ductal-like cell types from human ACCs. We performed scRNA-seq on a PDX line (ACCX22) established from a patient tumor which displayed two classical morphological features of human ACCs: a well-differentiated "cribriform" histology and a bi-phenotypic cell composition. ACC cells clustered into two main subgroups **(Fig. 1a)** which displayed mutually exclusive expression of known myoepithelial-like (e.g., *ACTA2*, *CNN1*, *TP63*) and ductal-like (*KRT7, KRT18, ELF5*) markers. Among the most differentially-expressed genes, we identified cell-surface markers CD49f (*ITGA6*), and KIT/CD117 (*KIT*), which were preferentially detected in cells associated with myoepithelial-like and ductal-like markers, respectively **(Fig. 1b-d)**. We then tested whether CD49f and KIT proteins could be leveraged to visualize the two subsets of malignant cells using FACS and found that they allowed us to discriminate two clearly distinct cell populations ($CD49f^{high}/KIT^{neg}$ vs. $CD49f^{low}/KIT^{+}$), across 5 independent PDX lines **(Fig. 1f)**. Analysis of the same tumors by immunohistochemistry (IHC) confirmed that KIT expression was

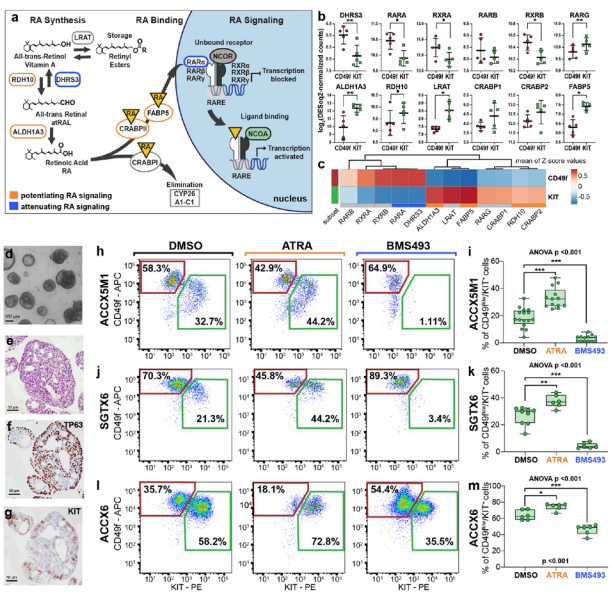


restricted to cells with ductal-like morphology, and mutually exclusive to expression of TP63, a marker of myoepithelial-like cells (**Fig. 1g**) To obtain robust transcriptional profiles of the two ACC cell types, we used FACS to sort autologous pairs of cells from 5 bi-phenotypic PDX lines and analyzed them by bulk RNA-seq. We performed a Principal Component Analysis of the 10 samples in the dataset **(Fig. 1h)**, and found that the samples grouped tightly into two separate and distinct clusters, corresponding exactly to the two sorted cell phenotypes. We then performed a differential expression analysis to identify genes specific to each cell population **(Fig. 1i),** and confirmed that our sorting strategy indeed isolated cells with myoepithelial-like and ductal-like features.

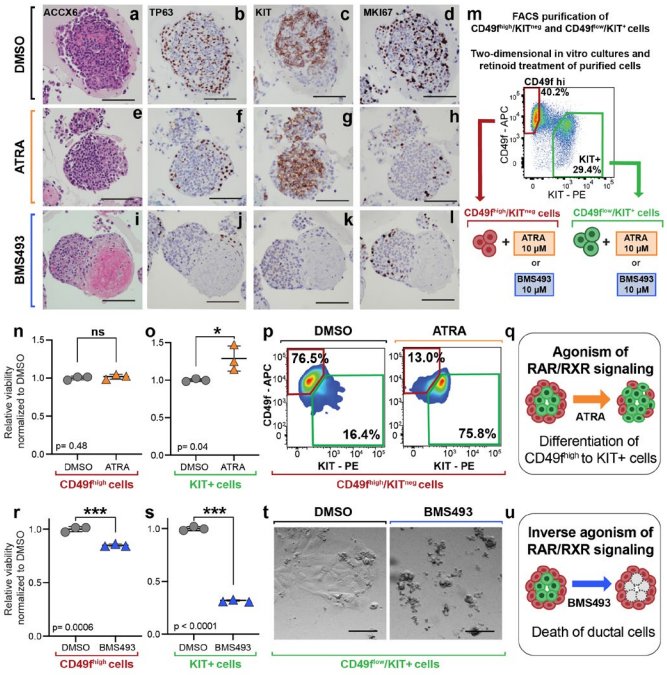
2. CD49f^{high}/KIT^{neg} cells display a higher tumor-initiating capacity as compared to CD49f^{low}/KIT^{+} cells and the two cell identities emerge as a result of epigenetic differentiation. To understand whether the two populations display functional differences, we compared their tumor-initiating capacity upon prospective xeno-transplantations by Extreme Limiting Dilution Analysis (ELDA)[4]. We used FACS to purify autologous pairs of CD49f^{high}/KIT^{neg} and CD49f^{low}/KIT^{+} cells from bi-phenotypic PDX lines and injected them, side-by-side, in NSG mice, at progressively decreasing doses (10,000-250 cells/mouse) (**Fig. 2a,d**). We found that the frequency of tumorigenic cells was substantially higher in CD49f^{high}/KIT^{neg} cells (**Fig. 2d**), indicating that myoepithelial-like cells represent an aggressive component of the malignant tissues, capable of initiating and sustaining tumor growth. To understand whether the two cell populations represented different genetic clones (**Fig. 2b**) or different developmental lineages (**Fig. 2c**), we examined the tumors originated from our ELDA experiments. We found that tumors derived from CD49f^{high}/KIT^{neg} cells recapitulated the histology and bi-phenotypic cell composition of parent tumors, with both cell populations present at identical ratios to those observed in the parent lines **(Fig. 2e-f)**, indicating that CD49f^{high}/KIT^{neg} cells can differentiate into CD49f^{low}/KIT^{+} cells, thus excluding the "genetic/clonal" hypothesis.



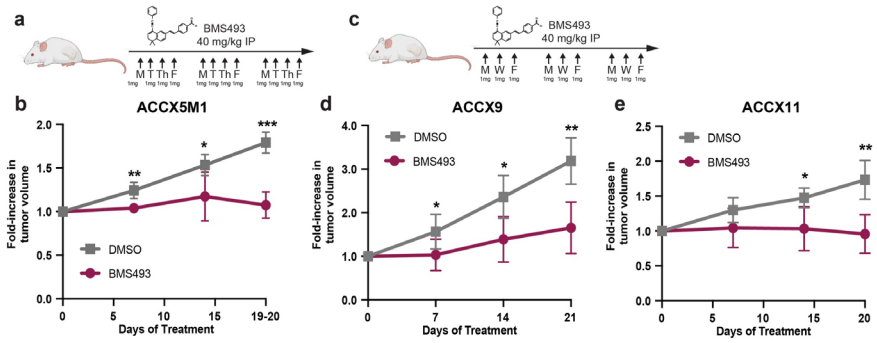
3. CD49f^{high}/KIT^{neg} and CD49f^{low}/KIT^{+} cells are characterized by differential activation of the *retinoic acid* (RA) signaling pathway. To shed light on the molecular mechanisms involved in regulating the differentiation of CD49f^{high}/KIT^{neg} cells into CD49f^{low}/KIT^{+} cells, we decided to investigate the RA signaling pathway. RA signaling is critical for proper morphogenesis and differentiation of salivary gland tissues during development[5-7]. Furthermore, recent studies have demonstrated that stimulation of RA signaling can antagonize oncogenic MYB signaling and slow tumor growth of ACC PDX models[8, 9]. We examined whether genes encoding effectors and modulators of RA signaling, including enzymes necessary for RA biosynthesis, RA binding proteins, and RA receptors, were differentially expressed between CD49f^{high}/KIT^{neg} cells and CD49f^{low}/KIT^{+} cells **(Fig. 3a)**. We found that most of these genes displayed statistically significant differences in expression levels **(Fig. 3b-c)**. We then tested whether activation or inhibition of RA signaling alters cell differentiation in ACCs using 3D organoid cultures **(Fig. 3 d-g)**. We found that activation of RA signaling with agonist ATRA led to an increase in the percentage of CD49f^{low}/KIT^{+} cells, while inhibition of RA signaling by pan-RAR inverse agonist BMS493 caused a dramatic loss of CD49f^{low}/KIT^{+} cells, across three independent, bi-phenotypic PDX models.



4. Activation of the RA signaling induces differentiation of CD49f^{high}/KIT^{neg} cells into CD49f^{low}/KIT^{+} cells, while inhibition of the RA signaling pathway causes selective death of CD49f^{low}/KIT^{+} cells. To understand whether ATRA or BMS493 induced selective proliferation of one of the two cell populations, we analyzed serial sections of 3D organoids by IHC. We found no increases in the number of MKI67^{+} (proliferating) cells in either their myoepithelial-like (TP63^{+}) or ductal-like (KIT^{+}) compartments **(Fig. 4a-l).** However, we observed an increase in KIT^{+} cells in organoids treated with ATRA **(Fig. 4g)** and a dramatic loss of KIT^{+} cells and a striking change in organoid morphology following treatment with BMS493 **(Fig. 4 i-l)**. To further dissect the effects of activators and suppressors of RA signaling on the two cell populations, we purified CD49f^{high}/KIT^{neg} and CD49f^{low}/KIT^{+} cells by FACS and then treated them individually with either ATRA (10 μM) or BMS493 (10 μM) using *in vitro* monolayer cultures[10] **(Fig. 4m)**. This experiment revealed that agonism of RA signaling with ATRA did not alter cell viability but induced a dramatic change in phenotype in CD49f^{high}/KIT^{neg} cells, which became almost completely CD49f^{low}/KIT^{+} **(Fig. 4p),** indicating that the effects observed in organoid cultures resulted from differentiation of CD49f^{high}/KIT^{neg} cells into CD49f^{low}/KIT^{+} cells **(Fig. 4q)**. Experiments with BMS493, an inhibitor of RA signaling, resulted in fragmentation and death of the majority of CD49f^{low}/KIT^{+} cells **(Fig. 4s-t)**, indicating that the effects observed in organoid cultures following treatment with RA inverse agonists were caused by selective cell death of CD49f^{low}/KIT^{+} cells **(Fig. 4u)**.



5. Inverse agonists of RA signaling can be leveraged as anti-tumor agents against human ACCs. Finally, we tested whether an inverse agonist of RA signaling (BMS493) could be leveraged for the *in vivo* treatment of human ACC. We engrafted three PDX lines in NSG mice and treated tumor-bearing mice with BMS493 (40 mg/kg, i.p.) using two dosing regimens **(Fig. 5 a, c)**. Out of 18 BMS493-treated animals, 33% (n=6/18) experienced reductions in tumor volume. Four animals (4/18 22%) had to be prematurely euthanized due to a deterioration in their health. Nevertheless, treatment with BMS493 was associated with a statistically significant reduction in the growth kinetics of engrafted tumors across all 3 tested models, even after removal of the four mice undergoing premature euthanasia **(Fig. 5 b, d, e)**.



Conclusions: The current study has revealed important differences in transcriptional identities and functional properties (i.e. tumorigenic capacity) of ACC cell populations. We found that myoepithelial-like cells were endowed with higher tumor-initiating capacity than ductal-like cells in ACC tumors, and that myoepithelial-like cells acted as progenitors of ductal-like cells. We demonstrated the importance of RA signaling for ACC cell-fate specification. Agonism of the RA signaling pathway led to differentiation of myoepithelial-like to ductal-like cells, while inhibition with inverse agonists induced lineage-specific toxicity of ductal-like cells. We showed that BMS493 has robust anti-tumor activity against human ACCs, both *in vitro* and *in vivo*, indicating that inverse agonists of RA are deserving of further study as a new class of anti-tumor agents for the clinical treatment of human ACCs. Overall, our study demonstrated that understanding developmental programs involved in the control of multi-lineage differentiation can be "hacked" in rare malignancies to uncover novel pharmacological manipulations with selective toxicity against specific cell types.

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