Exploration of possible molecular mechanisms of action of clinical effects of opioids in lung adenocarcinoma and triple negative breast cancer through RNA sequencing.

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Abstract

Anesthesia, in particular opioids, may have significant clinical implications in cancer. This effect cannot be generalized across different cancer types, and this likely holds true across cancer subtypes as well. From unpublished retrospective clinical data, lung adenocarcinoma and triple negative breast cancer outcomes were shown to be negatively and positively impacted, respectively. Using retrospective genomic analysis and general knowledge of opioid mechanism of actions, bulk RNA sequencing and single cell RNA sequencing was analyzed to determine not only what cellular pathways are being impacted by opioid exposure, but also where in the tumor microenvironment this mechanism of action. Through this analysis, initial guesses to the cells of action indicate that the tumor in lung adenocarcinoma and immune cells in triple negative breast cancer are the likely site. This work hopes to inform future research at all levels of experiments: clinical, animal models, genomic, and computational.

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1 Introduction

While the 20th century was focused on bringing medical care to a high standard, the 21st century will focus on precision medicine. Increasingly, precision medicine has looked at transcriptome level information.[1,2] Though genomics offers a great amount of detail, the complex regulatory networks and feedback loops that determine cell state are failed to be fully captured by just studying the genome. Incorporating transcriptome-level sequencing allows for consideration of these important elements of biology. Recently, these techniques have been applied to cancer,[3,4] allowing for more specialized treatment of cancer.

Cancer is one of the leading causes of death in the developed world. Though it is generally thought of as a single disease state due to a similar basic clinical presentation, cancer is a highly complex group of diseases that results, generally, from broad disregulation in the human body. Thus, to understand both the pathology and the best course of precise treatment, analysis is needed at a very specific level. In this work, we look a the rapidly evolving field of oncoanesthesia, or the study of how anesthetics and pain management affect cancer. Particularly, we analyze how perioperative techniques may affect patient survival at a detailed molecular biology level, both to corroborate clinical data and to generate unique biochemical hypotheses as to the exact mechanisms that cause these techniques to have their oncological effects. The goal of this research is primarily exploratory; it is supposed to drive future research into this growing field of how our medicine must be increasingly precise to lead to the best outcomes for patients.

2 Background

Cancer is a complex, heterogeneous set of disease. While treatment regularly focuses on the tumor if the cancer is solid tissue, a number of changes must occur in the surrounding environment for the cancer to take root and spread in the human body.

2.1 The Tumor microenvironment

In various cases, inflammatory responses to disease and conditions, such as *H. pylori* infection, can lead to an increased risk of cancer.[5] These responses, while completely normal host responses, cause changes in stromal cells around what will later become the tumor. These changes produce altered cell states, which, when tumorigenesis occurs, become known as the tumor microenvironment or TME. The TME is known to have increased acidity, hypoxia, ischemia, and decreased nutrition as compared to normal tissue.[6] The TME is composed of a diverse group of cells, including the tumor, immune cells, extra-cellular matrix (ECM), and other cells that may be involved in the local area. In particular, the stroma of the TME may be associated with cancer-associated fibroblasts (CAFs), innate and adaptive immune cells, such as B cells, T helper (Th) cells, natural killer (NK)cells, cytotoxic T lymphocytes (CTLs), and regulatory T (Treg) cells,[7] and various cancer-producing stem cells (see fig. 1). These cells produce a high amount of cytokines and other growth factors that increase inflammation and cellular reproduction. CAFs produce most of the ECM and are responsible for autocrine and paracrine secretions in the TME.[8] Though there are vast similarities in the TME, as described by [3], cancer TMEs are different enough to be separable and because of our concern of specific response, we address each separately.

2.2 Lung Adenocarcinoma

Lung cancer is one of the most common forms of cancer. A vast majority are non-small cell lung cancer (NSCLC), which is the primary type to be associated with smoking. Of this form, two primary subtypes exist: adenocarcinoma (LUAD) and squamous cell carcinoma. Because squamous cell carcinoma is associated with non-filtered cigarettes, most modern lung cancer patients have adenocarcinoma, which is associated with smoking filtered cigarettes.[10] Clinically, adenocarcinoma presents itself on the outside of the lung and

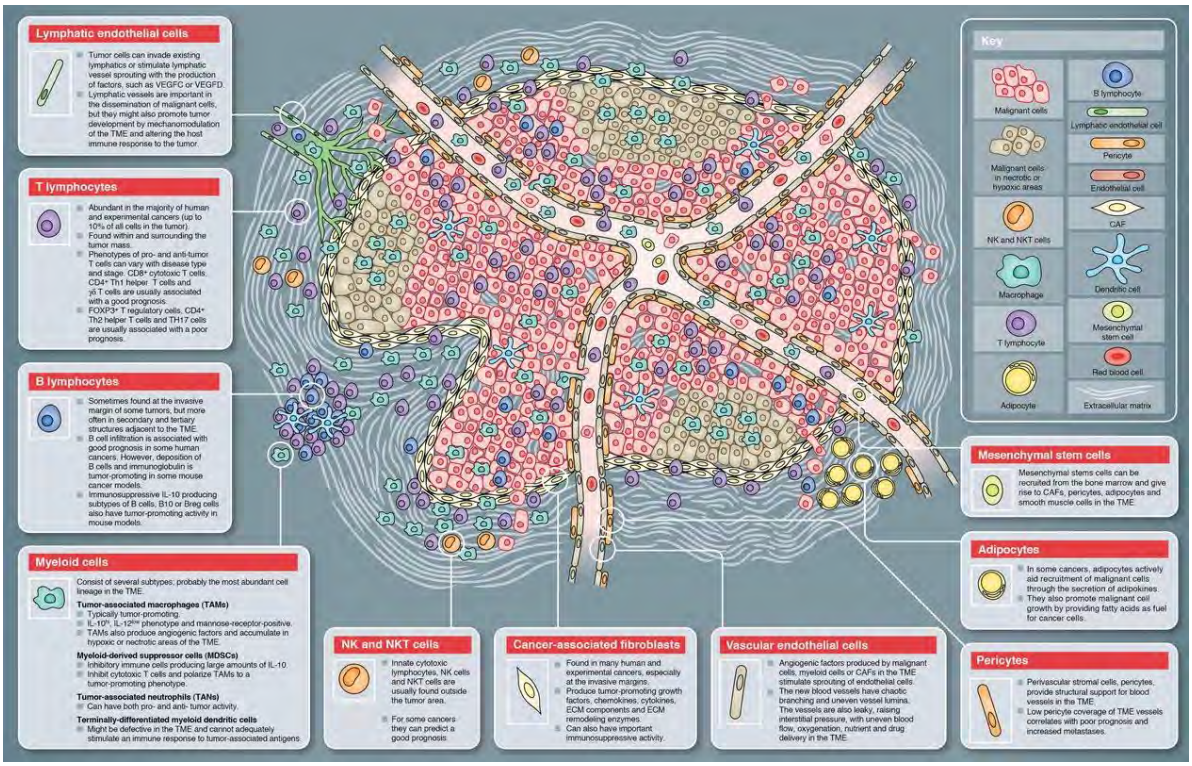


Figure 1: A cartoon representation of the tumor microenvironment. This shows what the environment around a solid state tumor may look like. Note that this also shows adipocytes and pericytes, which are not discussed in this thesis. Reproduced from Balkwill et al.[9]

is thought to originate in Atypical Type II pneumocyte (alveolar) cells, though this is only a hypothesis. Other papers suggest that club cells, which are another form of alveolar cells, though this was only found in mice.[11]

From a molecular standpoint, LUAD presents itself as a complex disease. Weir and colleagues present an early full genome SNP array, showing a large number of copy-number variations and common amplifications and deletions, leading to the hypothesis of NKX2-1 being a major promoter of LUAD tumorigenesis.[12] Similar research at the WGS and exome level level confirmed that in-frame exon mutations of EGFR and SIK2 were likely associated with causing LUAD, as were novel targets like ARID1A, RBM10, and U2AF1.[13] TCGA studied the exome, genome, epistatic regulation, and proteomic analysis of lung adenocarcinoma and was able to corroborate the findings from before.[14] They also found that various

pathways were upregulated in lung adenocarcinoma, including RTK/RAS/RAF pathway activation (76% of cases), PI(3)K-mTOR pathway activation (25%), p53 pathway alteration (63%), alteration of cell cycle regulators (64%), alteration of oxidative stress pathways (22%), and mutation of various chromatin and RNA splicing factors (49%). Because of the heterogeneous nature of mutations, some patients respond well to PD-L1 immunotherapy or checkpoint inhibition, while others fail to benefit from these interventions at all, and other patients respond to cisplatin-based therapies, as well as the use of TK inhibitors to target EGFR-based pathways.[15]

2.2.1 LUAD Tumor microenvironment

The lung TME is similar to other TMEs and can be agitated by inflammatory conditions, like smoking and chronic obstructive pulmonary disease.[16] CAFs secrete a large number

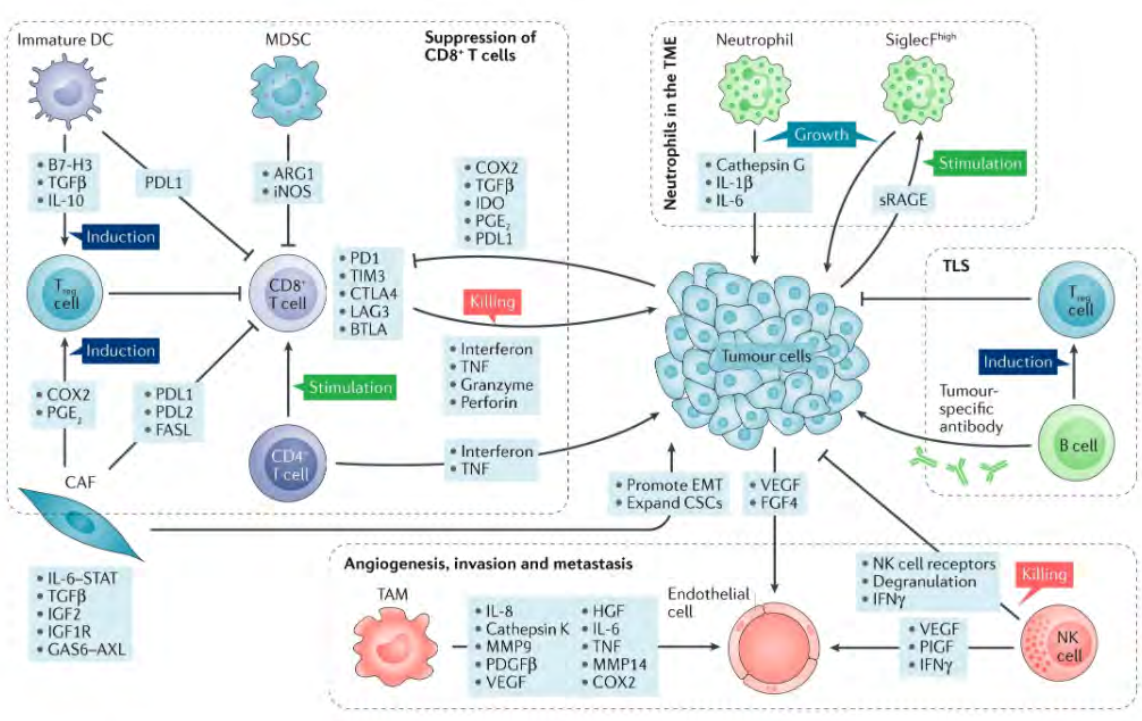


Figure 2: The specific signaling involved in the LUAD tumor microenvironment. This specifically shows the role of immune cells and cancer-associated fibroblasts in great detail. Reproduced from Altorki et al.[16]

of paracrine factors that induce cell metastasis and stem-cell characteristic of cancerous lung cells. CAFs are also known to suppress T cell function, through presentation of PD1 receptors and antigen presentation. CAFs produce the ECM, which promotes a variety of downstream cascades including EGFR production to induce tumorigenesis. Further, ECM density has been shown to prevent T cell infiltration, decreasing the efficacy of immune-based therapies. Immune cells are known to have both tumor-promoting and suppressing activities based on their genetic profile and local signaling. Within myeloid cells, tumors are known to upregulate dendritic cells, so they suppress T cells as opposed to priming them.[17] Furthermore, neutrophils can themselves revert to an immunosuppressive state that is characterized by expression of LOX1. NK cells, which govern targeting of tumor cells, seem to enter dysfunction within the environment, though *in vitro* studies show these cells continue to express tumor suppressing activity.[18] Lymphoid cells also display great variety in responses to TME. Much of the response to immunotherapy is derived from these cells, and a critical balancing act between pro-inflammatory and immunosuppressive activty is necessary for effective response to lung carcinogenesis. Activated B cells are known to be associated with increase T reg activity, which reduces immune function.[19] A summary of these interactions is shown in fig. 2. A closer inspection of the LUAD TME by single-cell RNA sequencing (scRNA-seq) provides a more microscopic view of the vast changes that the TME causes in a broad scheme of cells.[20] It was shown that subtypes of cells outside and inside the TME were vastly more than anticipated, suggesting that many types of biological entities exist within the tumor microenvironment than previously estimated. Importantly, this also showed that most cell subtypes appeared in multiple patients, allowing for generalizations of data to be made from one patient to another.

2.3 Triple Negative Breast Cancer

Breast cancer is the second leading cause of cancer death in women. Breast cancer is heterogeneous group of diseases, with the genetic and immunohistochemical profile of the tumor being important to treatment response and prognosis. These types include HR positive, which is estrogen and progesterone receptor positive, HER-2 positive and triple negative breast cancer, which is negative to all of the other 3 receptors.[21] Triple negative breast cancer, or TNBC, has the poorest long term prognosis of any of the breast cancers. Furthermore, though tumors have been shown to be responsive to various chemotherapy agents, including taxanes and platinum-containing compounds, these do not seem to shown any changes in outcome.[22] Such difficulties complicate treatment, even worsening outcomes after surgery.[23]

Triple negative BC is also noted for a high number of tumor infiltrating lymphocytes (TILs), with extremely high numbers being associated with better outcomes.[24] In particular, this has suggested that TNBC may be a valuable target for immune checkpoint blockade therapy. Furthermore, based on molecular subtype, TNBC can be further classified by gene set analysis of which major pathways are enriched.[25] This molecular subtyping has allowed for some precision in driving possible clinical regimes that are effective in the face of the poor prognosis of the disease. However, because of the diversity of genetic signatures, it has not been possible to find global signals that relate to causal expression in most TNBC subtypes.[26]

2.3.1 TNBC Tumor microenvironment

As mentioned before, one distinguishing feature of TNBC microenvironment is the presence of tumor infiltrating lymphocytes. Many TILs are switched to the Treg state, which blocks immune response and enhances cancer development and progression.[27] A marker of Tregs is gene FOXP3, and the number of T cells expressing FOXP3 is significantly upregulated in TNBC. This implies that TILs may serve as a harmful, as opposed to protective, role

in some cases. Another associated cell type are macrophages, which generally spit into two groups, M1 and M2.[28] M2 macrophages are known to be tumor-promoting as they produce a high level of cytokines leading to enhanced inflammation, growth factors and proteases, angiogenesis, enhance metastasis, and contribute to extracellular matrix remodeling. Cancer associated fibroblasts are known to accelerate tumor progression in TNBC by activating TGF-β, a known oncogene, as well as mediating signaling between different groups of cells in the microenvironment.[29] CAFs also cause a metabolic increase in tumor cells and were also shown by Tchou and colleagues to promote epithelial-mesenchmyal transition, leading to tumor spread and cell migration.[30] Other major features of the the TNBC TME include endothelial cells that are thought to promote angiogensis and vascularization of tumor tissue, the extra-cellular matrix, where much of the signaling to cause the epithelial-mesenchmyal transition are thought to be found, such as loss of E-cadherin and Snail expression,[31] and finally certain genetic expression that can induce different tissue metastasis.[32] It is clear that TNBC tumors can effectively recruit their microenvironment to further alter conditions and promote both tumor growth and metastasis, worsening prognosis and outcomes. Some recent scRNA-seq studies have suggested that as opposed to all-or-nothing type scenarios for various cells that exist in the TME, a continuous spectrum of continuous activation and diffrentation trajectories may exist.[33]

2.4 scRNA-seq

RNA-sequencing is a genomic approach for the detection and quantification of messenger RNA molecules and is useful for studying cellular responses to changes in conditions. However, this is generally done in millions of cells at once and can wash out signal from specific cells. Until recently, it was not possible to probe with better resolution. Some experimenters used forms of cytometry that could detect expression of protein, including mass cytometry that could magnify protein expression in a cell.[34] Still, this fails to grab a full cellular picture

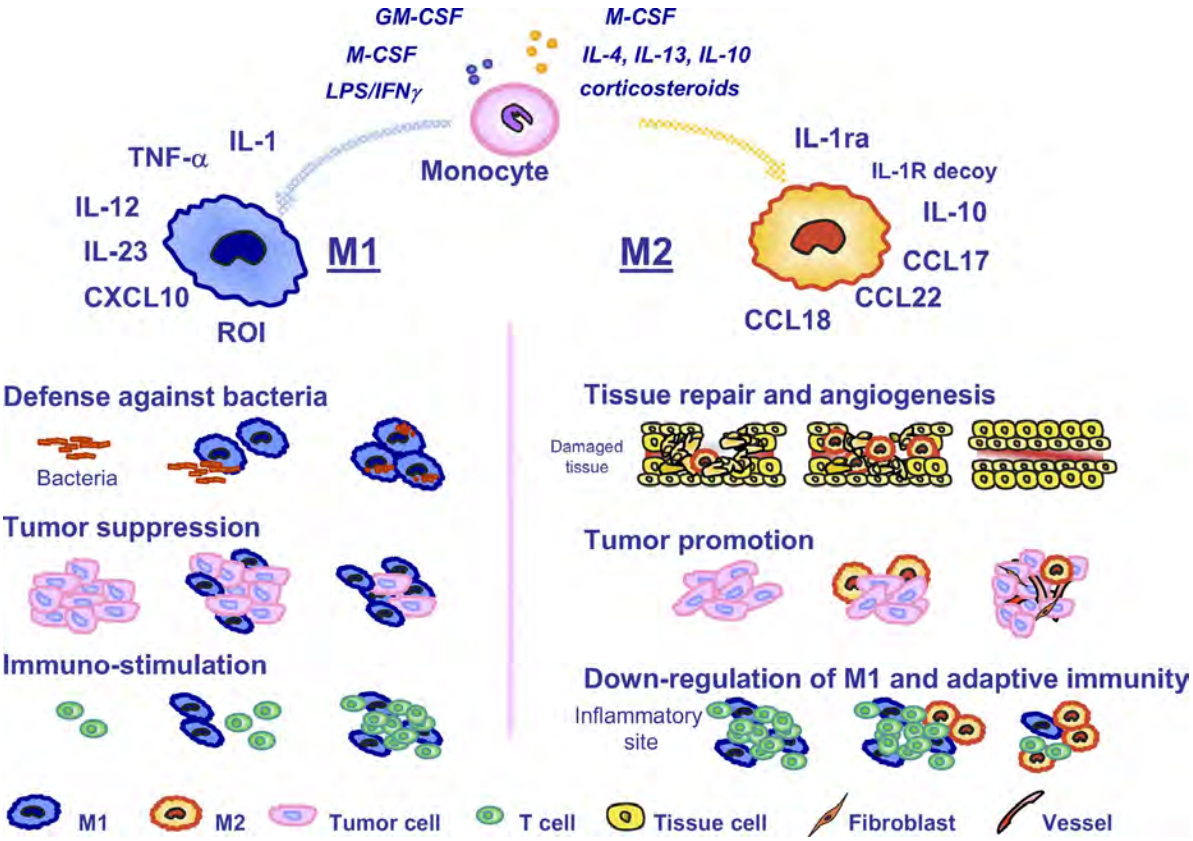


Figure 3: Macrophages exist as either M1 or tumor suppressing, or M2 or tumor promoting. This process is known as macrophage polarization. It has other uses beyond the body from activity in tumors. This has recently been shown to be a continuum by high-resolution sequencing and not a sharp division as previously assumed.[20] Reproduced from Solinas et al.[28]

for the thousands of protein expressed at a given time in a cell. However, within the last decade, the use of single cell-RNA sequencing has taken off as a cellular level map that can help determine exactly where the effect of a change is taking place, while describing most of the cellular machinery.

2.4.1 scRNA-seq Technique

In general, scRNA-seq is composed of four sequential steps: isolation and lysis of target cells, reverse transcription, cDNA amplification, and sequence library preparation, and finally analysis using computational biology methods, as shown in fig. 4. The last step is generally carried out by fragmentation and sequencing with a next generation sequencer.[35] Isolation

of cells is the first and most limiting step in scRNA-seq, as cells must be liberated from ECM and from adhering to themselves. This step causes damage to many cells and an

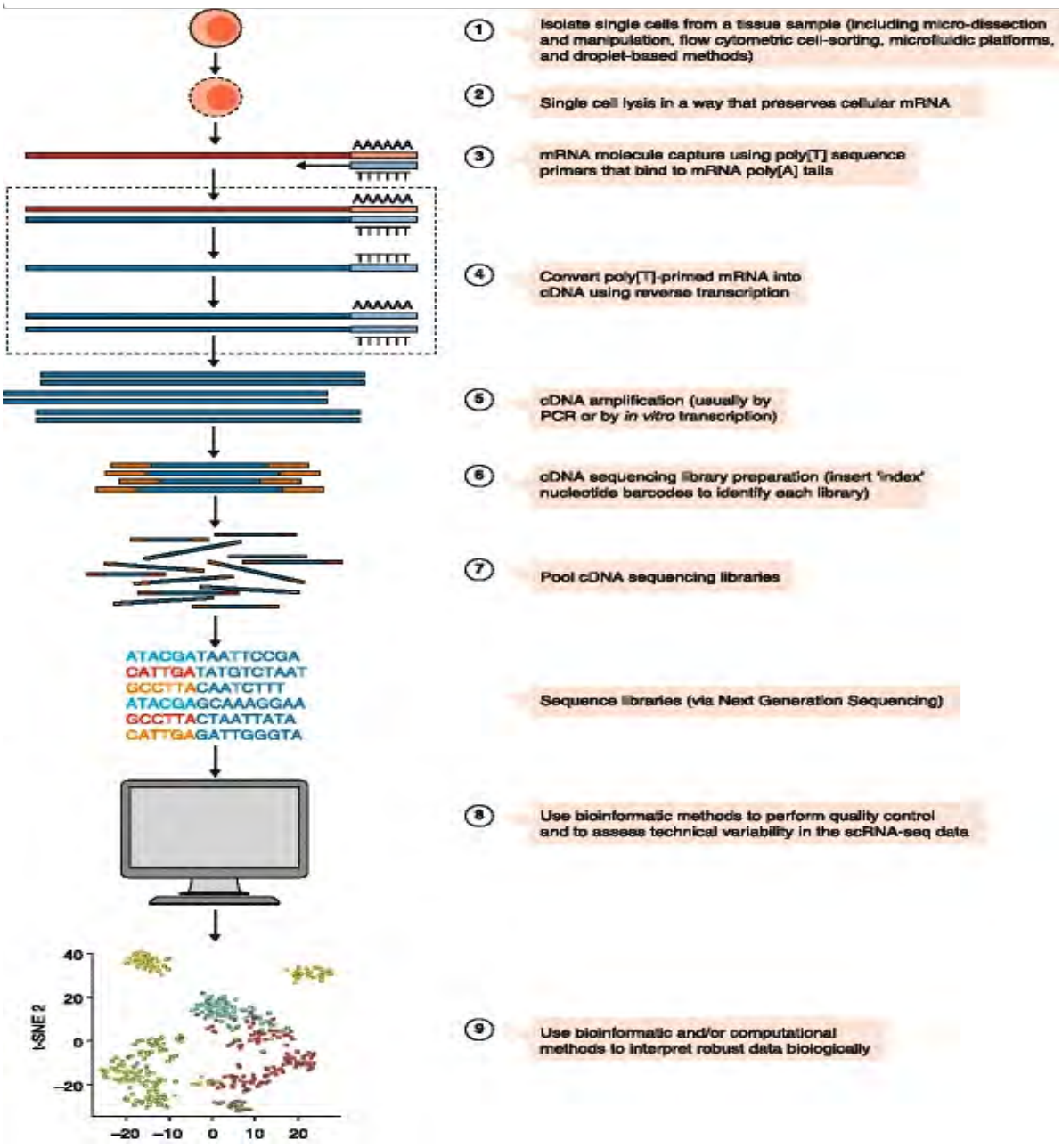
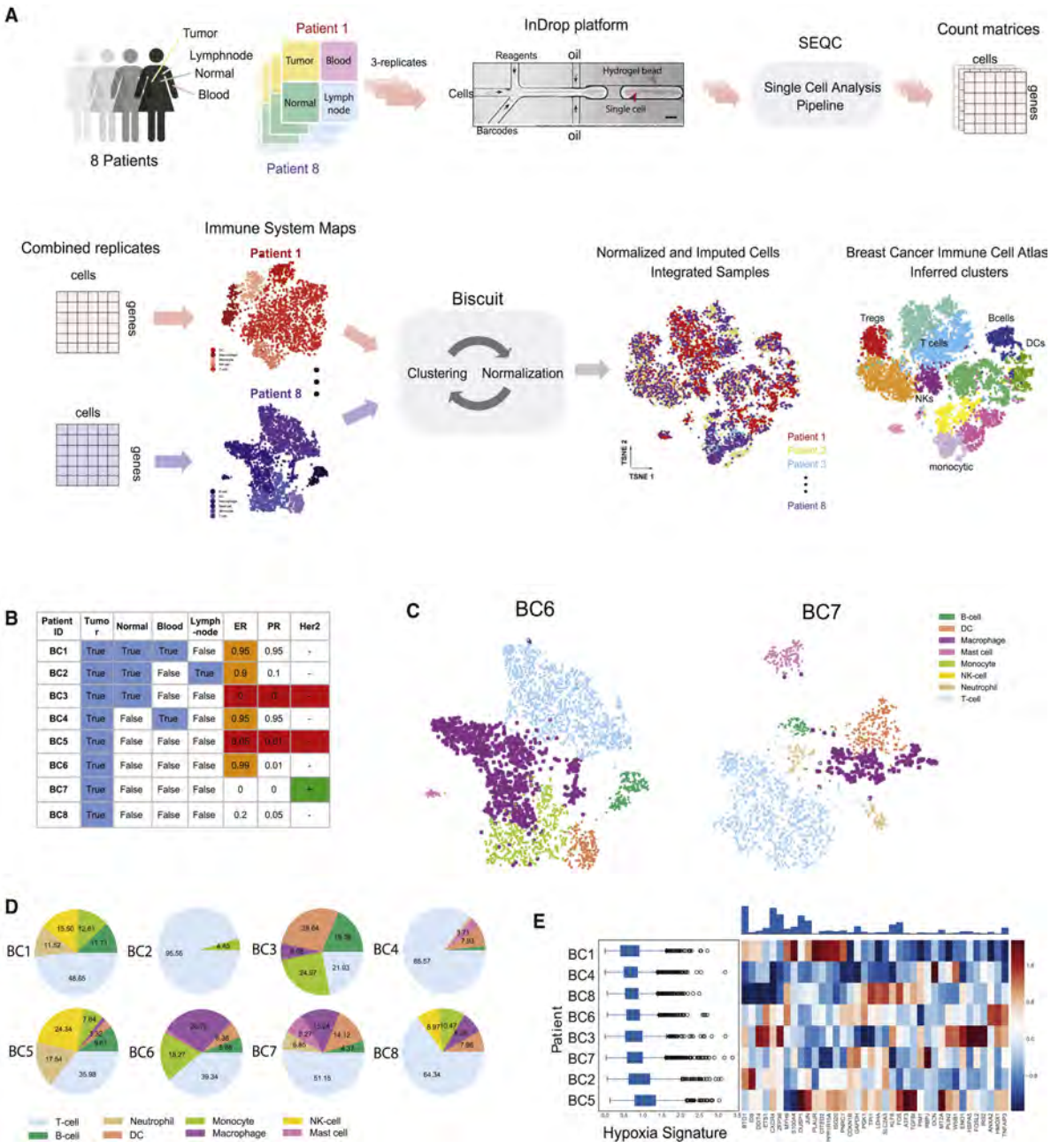


Figure 4: A standard scRNA-seq workflow. The following 9 steps are condensed into 4 steps plus analysis in this paper with step 1 corresponding to image step 1 and 2, step 2 corresponding to image step 3 and 4, step 3 corresponding to image step 5, and step 4 corresponding to image step 6 and 7. Reproduced from Haque et al.[34]

over-representation of circulating cells that are not as stuck as other cells. Many commercial sequencers use microfluidics to accomplish this goal, such as 10X Chromium.[36] Reverse transcription is the next step and generally all protocols must avoid amplifying rRNA, which composes the vast majority of RNA contained in cells. The next step is cDNA amplification, which generally uses adaptations of PCR or a newly described technique called *in vitro* transcription. In general, cells are tagged with certain unique molecular identifiers, a short genetic read, that allow researchers to later determine all the transcripts that came from a cell.[35] Challenges to scRNA-seq are numerous due to the experimental error involved. All scRNA-seq experiments suffer from dropout, where a number of truly expressed transcripts do not reach the level of sensitivity to be detected due to technical and biological noise, and the batch effect, where slight variations in experimental conditions between batches of cells can alter transcriptions reads in a statistically significant way. Thus, downstream statistical analysis of single cell data is a critical part of any successful scRNA-seq experiment.

2.4.2 scRNA-seq Computational and Statistical Analysis

Because of the complexities of scRNA experiments, significant computational and statistical-based analysis of the systems must occur. First and foremost, imputation can be carried out to partially fill in for dropout events. However, though many tools exist to impute gene values such as SAVER, DrImpute, and scone , many analyses do not require strict imputation to give accurate cell type information[37] and imputation is known to give incorrect results in some cases.[38] Batch effects must also be normalized against, as do individual stochastic cell effects such as cell cycle etc.[39] Normalization is generally the single most important part of a scRNA-seq experiment, and poor data normalization can harm down stream performance.[37] State of the art normalization techniques, such as those used in the R package scran, involves computing deconvolutions on clusters of cells, which is similar to other methodologies that use pools of similar cells to compute correction factors,[40] or known values of added genetic



Patient ID	Tumor	Normal	Blood	Lymph-node	ER	PR	Her2
BC1	True	True	True	False	0.95	0.95	+
BC2	True	True	False	True	0.9	0.1	-
BC3	True	True	False	False	0	0	-
BC4	True	False	True	False	0.95	0.95	-
BC5	True	False	False	False	0.01	0.01	-
BC6	True	False	False	False	0.99	0.01	+
BC7	True	False	False	False	0	0	+
BC8	True	False	False	False	0.2	0.05	-

Figure 5: A prototypical analysis involved in an scRNA-seq experiment. **A** corresponds to experimental gathering of cells, library generation, normalization and imputation, and lower dimensional embedding and clustering. **B** shows how patient metadata may be used to segregate patients. **C** Another image showing two low dimensional embeddings for two different patients. **D** Pie charts that show the percentage of identified cells in each patient. **E** Differential expression analysis of hypoxic signatures, a common differentially expressed pathway in cancer. Reproduced from Azizi et al[33]

information called spike-ins to normalize against. Finally, as the number of observed genes in a full scRNA-seq data set is generally on the order of 10000, dimensionality reduction or low-dimension embedding techniques are used to find the most important or varied genes and then genes are clustered into possible clusters. This is generally checked against expert knowledge of known marker genes for different cell lineages to show the clusters are expressing those markers as expected. From these clusters, various forms of differential analysis can be carried out, including on entire pathways of genes. Because of the vast number of genes measured, statistical power of these experiments can only be improved by using multiple hypothesis correction tests. These include false detection rate and family-wise error rate, with the former used more in hypothesis generation, while the latter is used to verify the statistical significance of conclusions. A sample overview of general analysis undertaken in most scRNA-seq experiments is shown in fig. 5

2.5 Anesthesia and Inflammation

As mentioned in section 2.1, inflammation is a major cause of cancer. Anesthetic compounds, especially those used in general anesthesia, are known to reduce immune function. It is thought that this function is protective against certain type of surgical-related complications like ischemia and reperfusion injury, though it may be harmful in stopping cancer from metastasizing. In particular, the volatile anesthetics (sevoflurane, halothane, isoflurane etc.) inhibit the function of neutrophils, increases NO production to surpress inflammation from macrophages, and decreased secretion of IFN affecting both NK cells and lymphocytes.[41] Propofol is shown to have a similar dose-dependent effect on neutrophils and macrophages, though research still needs to be done on T cells and NK cells. Finally, opioids are known to have immunosuppressive effects through activation of the hyptohalmic-pituitary-adrenal axis. This may be due to the fact that most immune cells express at least some of the canonical opioid receptors. However, synthetic agonists like remifentanil and fentanyl do not

have the same attenuating effect as the other anesthetics.

2.5.1 Cancer and Anesthesia

Because of the effects on the immune system by anesthesia, there is a growing body of evidence that suggests that cancer outcomes, especially in the post-operative period, are affected by choice of anesthetic in the perioperative period. Different anesthetic compounds are thought to have different effects, as mentioned in the previous section, but this is also sensitive to cancer type. For instance, the volatile anesthesia halothance was shown to accelerate post-operative metastasis in lung and liver carcinomas in mice models.[42] However, sevolflurane, a similar compound, was shown to suppress lung cancer metastasis and growth of liver cancer cells.[43] Surprisingly, sevoflurane is known to increase proliferation, migration, and invasion of primary breast tumor *in vitro*, showing that more precise solutions may be necessary.[44] Despite these studies indicating association, both causal evidence and biological rationale have been failed to be developed in human population, as most studies have been carried out in either animal models or are retrospective analysis of survivorship without investigation of underlying molecular physiology.

In this work, we focus on the opioids for reasons mentioned in section 3. Opioids have been shown to negatively affect outcome in cancer like LUAD[45] and renal cell carcinoma,[46] while leading to anti-tumor activities in esophageal cancer.[47] Molecular evidence, as mentioned insection 2.5, suggest that opioids are immunomodulators, indicating that the effect may be modulated through the TME. Both cancer and immune cells are know to express a variety of opioid receptors[48] and non-canonical opioid targets that are known to affect cancer outcomes, such as the Toll-like receptors,[49,50] which can activate receptor-dependent cascades and lead to broad effects across the cancer transcriptome.

2.5.2 Lung Adenocarcinoma and Opioids

As mentioned, opioids are shown to negatively impact outcomes in lung adenocarcinoma.[45] The finding was also confirmed in a second retrospective analysis by Oh and colleagues.[51] The assumption of causality comes from the finding that LUAD is an immune-infiltrated form of cancer.[46] Animal clinical models also suggest that the μ-opioid receptor may influence lung cancer progression,[52] as well as *in vitro* models.[53] However, most studies only manage to suggest an associative mechanism. Very few focus on suggesting exactly what actions the drugs take to cause this acceleration of tumor progression.

2.5.3 Triple Negative Breast Cancer and Opioids

The influence of anesthetic intervention on breast cancer is still an open question.[54] Studies indicate that volatile anesthesia and propofol-based techniques do not have significantly different rates of recurrence.[55] However, while work by Sessler and colleagues was a large, randomized experiment, it should be noted that segregation of patients by breast cancer type was not carried out. As mentioned in section 2.3, triple negative breast cancer is known to have a vast number of tumor infiltrating lymphocytes. This suggests that studies that look in breast cancer in bulk may fail to account for the immunogenic responses of the different subtypes, as the most common form of BC is the least affected by immunosuppressive or other effects by anesthetic compounds. Thus, this paper will focus on TNBC that has not been studied in depth. Only a few experimental *in vitro* papers report any results on TNBC, suggesting that it might be protective.[56] However, studies on bulk cancer suggest that no effect is present.[57,58] This suggest the importance of carrying out more fine-grained studies that do not wash out effects from "one size fits all" thinking.

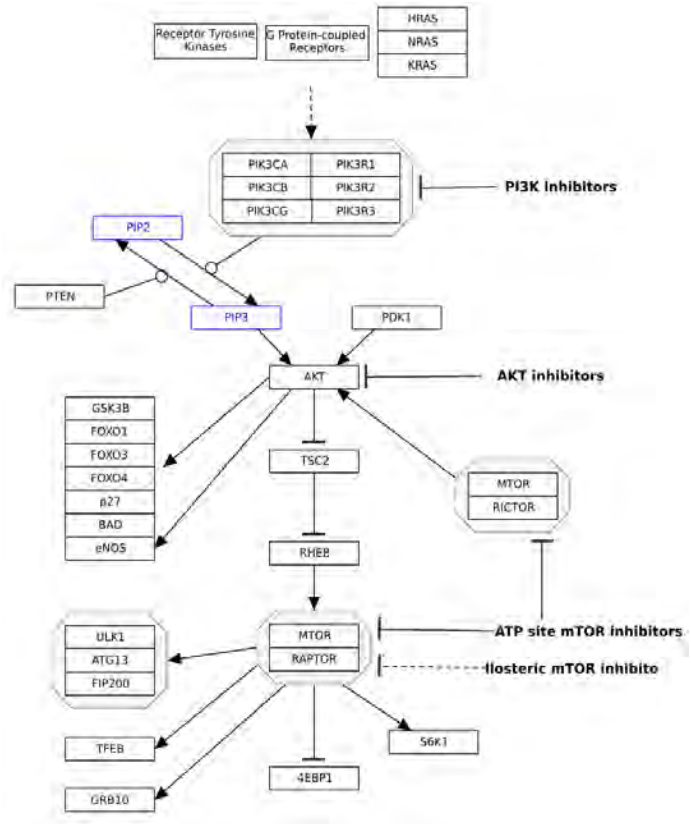
3 Motivation

The motivation of this work is to study the effects of anesthesia, specifically opioids, on lung adenocarcinoma and triple breast cancer. Based on currently unpublished retrospective clinical, genomic, and pathological analysis carried out at Memorial Sloan Kettering Cancer Center in New York, opioids do show a sign of affecting both of these types of cancer. Based on this data and the influence of tumor microenvironments in cancer, published scRNA-seq data[20,59] corresponding to the TMEs of these cancers was analyzed to find which cells were most likely driving the effects of these compounds. By analyzing disregulation from within and outside the TME, hypotheses can be generated to guide future prospective research.

3.1 Lung Adenocarcinoma Motivation

A retrospective study was carried out on a cohort of lung adenocarcinoma patients. These patients also had IMPACT panels, which are genetic panels that check a variety of SNPs in patient. The retrospective study confirmed that opioids had a harmful impact on lung adenocarcinoma outcomes. However, combined with the genetic analysis, putative pathways and genes of interest that could impact patient survival through mediation of the opioid activity were identified. This was the PI3K pathway and the CDKN2A gene. The PI3K pathway is a commonly disregulated pathway in cancer, as it contains the tumor suppressor PTEN and other that acts on PI3K, with downstream effects on the AKT-mTOR canonical pathway.[61] This downstream pathway is known to activate increased protein synthesis due to mTORC1 (a protein complex containing mTOR) activity and cell survival through mTORC2 (a separate complex containing mTOR) (see fig. 6a). A curated gene list was selected from the mSigDB[62] and combined with another list from an internal Memorial Sloan Kettering IMPACT panel-derived list to generate the list found in fig. 6b. A partial representation of this list and reg CDKN2A, or cyclin dependent kinase inhibitor 2a is an oncogene that codes

(a)



(b)

ACACA	ACTR2	ACTR3	ADCY2
AKT1	AKT1S1	AKT2	AKT3
AP2M1	ARF1	ARHGDIA	ARPC3
ATF1	CAB39	CAB39L	CALR
CAMK4	CDK1	CDK2	CDK4
CDKN1A	CDKN1B	CFL1	CLTC
CSNK2B	CXCR4	DAPP1	DDIT3
DEPDC5	DEPTOR	DUSP3	E2F1
ECSIT	EGFR	EIF4E	EIF4EBP1
FASLG	FGF17	FGF22	GNA14
GNGT1	GRB2	GSK3B	HRAS
HSP90B1	IL2RG	IL4	INPP4B
ITPR2	LCK	MAP2K3	MAP2K6
MAP3K7	MAPK1	MAPK10	MAPK8
MAPK9	MAPKAP1	MKNK1	MKNK2
MLST8	MTOR	MYD88	NCK1
NFKBIB	NGF	NOD1	NPRL2
NPRL3	PAK4	PDK1	PFN1
PIK3CA	PIK3CB	PIK3R1	PIK3R2
PIK3R3	PIKFYVE	PIN1	PITX2
PLA2G12A	PLCB1	PLCG1	PPP1CA
PPP2R1A	PPP2R1B	PRKAA2	PRKAG1
PRKAR2A	PRKCB	PTEN	PTPN11
RAC1	RAF1	RALB	RHEB
RICTOR	RIPK1	RIT1	RPS6
RPS6KA1	RPS6KA3	RPS6KB1	RPTOR
SFN	SLA	SLC2A1	SMAD2
SQSTM1	STAT2	STK11	TBK1
THEM4	TIAM1	TNFRSF1A	TRAF2
TRIB3	TSC1	TSC2	UBE2D3
UBE2N	VAV3	YWHAB	

Figure 6: (a) The canonical PI3K/AKT/mTOR pathway. The RAPTOR and RICTOR containing complexes are mTORC1 and mTORC2, respectively. The PI3K complex contains 3 structural elements (CA,CB, and CG), and 3 regulatory elements (R1,R2,R3). Figure from wikipathways and based on work[60] by Edlind and Hsieh. (b) A list of genes identified from MSK IMPACT and mSigDB that are relevant to the PI3K/AKT/mTOR pathway that is used in analysis. Note that this gene list is more comprehensive than the picture of the pathway.

for the cell-cycle inhibitor p16. It prevents cellular proliferation and is part of a broader pathway of cell-cycle control genes that are mainly governed by cyclin dependent kinases; hence, the inhibition by CDKN2A downregulates this pathway.[63] Thus, the goal of this study is to find where in the TME these genes may be upregulated or downregulated.

3.2 Triple Negative Breast Cancer Motivation

Similar to LUAD, a retrospective cohort of triple negative breast cancer was analyzed for response to opioids and other anesthetic compounds. A protective relationship was suggested between opioids and breast cancer. While there was no genetic screen done on this data,hypothesis generation to determine where opioids could be acting would be informative. The aim is similar to LUAD, except with less precise targeting. Instead of a pathway, a list of canonical and non-canonical opioid receptors[64] was collected to be analyzed: OPRM1,OPRL1,OPRD1, TLR4,OGFR,OGFRL1, OPRK1,and TLR2.

4 Methods

The basis of this project arose from the analysis of the two scRNA datasets in section 3. Though there is some merit to rerunning a full analysis pipeline, the normalized data from both experiments was used as the starting point. Because both involved expert analysis of whether clustering and other methods were being accurately applied, renormalizing and clustering may introduce more errors than they fix. Though a thorough examination of the strategies used to normalize in both papers is out of the scope of this thesis, both used well-established methods to normalize data.

4.1 Lung-specific Methods

Lung adenocarcinoma tumor microenvironment data was drawn from a massive scRNA-seq experiment run by Lambrechts and colleagues[20] that had over 50k . In the initial data set, 5 patients were analyzed. However, only 2 of these patients had LUAD, and so the remaining patients were left out from analysis. These 2 patients represented approximately 30k cells. There were 22180 genes present in this data set. The breakdown of the cells is presented in section 4, as clustered by the authors. When referring to cell type, this refers to the overall

Cluster	Total Cells	Percentage Tumor Derived	Annotation from Lambrechts et al. [20]
Alveolar_0	197	18.8%	AT2 Cells
Alveolar_1	221	43.9%	
Alveolar_2	238	10.5%	AT1 Cells
Alveolar_3	47	70.2%	COPD-Injured Cells
Alveolar_4	143	7.0%	Respiratory Epithelial Cells
Alveolar_5	5	60.0%	Club Cells
Alveolar_6	7	100.0%	Basal Cells
Alveolar	858	24.7%	
B_cell_0	758	85.9%	Follicular B Cells
B_cell_1	277	91.3%	Follicular B Cells
B_cell_2	390	97.7%	Plasma B Cells
B_cell_3	252	73.0%	Mast Cells
B_cell_4	272	90.4%	MALT B Cells
B_cell_5	175	96.6%	Plasma B Cells
B_cell_6	230	91.7%	MALT B Cells
B_cell_7	53	66.0%	Plasmacytoid Dendritic Cells
B_cell_8	18	38.9%	Erythroblasts
B_cell	2425	88.1%	
EC_0	320	0.3%	Non-Malignant EC
EC_1	262	37.4%	Lower Quality EC
EC_2	119	94.1%	Tumor EC
EC_3	185	94.1%	Tumor EC
EC_4	237	10.1%	Non-Malignant EC
EC_5	47	36.2%	Lymphatic EC
EC	1170	36.4%	
Epi_0	37	51.4%	
Epi_1	13	61.5%	
Epi	50	54.0%	
Fibro_0	197	99.5%	Tumor Enriched
Fibro_1	191	84.3%	Myofibroblasts
Fibro_2	105	80.0%	Lower Quality Cells
Fibro_3	117	79.5%	
Fibro_4	30	66.7%	
Fibro_5	156	13.5%	Non-Malignant Enriched
Fibro_6	2	0.0%	
Fibro	798	72.1%	
Myeloid_0	243	30.0%	Macrophages
Myeloid_1	1772	97.6%	Tumor-Associated Macrophages
Myeloid_2	583	40.1%	Tumor-Associated Macrophages
Myeloid_3	605	96.2%	Tumor-Associated Macrophages
Myeloid_4	394	86.3%	Langerhans Cells
Myeloid_5	463	6.7%	Lung-Associated Macrophages
Myeloid_6	361	31.0%	Granulocytes
Myeloid_7	392	1.0%	Lung-Associated Macrophages
Myeloid_8	134	43.3%	Lung-Associated Cells
Myeloid_9	213	12.7%	Lung-Associated Macrophages
Myeloid_10	8	0.0%	Lung-Associated Macrophages
Myeloid_11	90	53.3%	Cross-Presenting Dendritic Cells
Myeloid	5258	61.6%	
T_cell_0	2587	89.1%	CD4+ T Cells
T_cell_1	2868	88.0%	CD8+ T Cells
T_cell_2	1951	18.9%	CD4+ T Cells
T_cell_3	3183	99.8%	CD8+ T Cells
T_cell_4	1421	87.5%	CD8+ T Cells
T_cell_5	912	33.8%	Natural Killer Cells
T_cell_6	1040	92.0%	Regulatory T Cells
T_cell_7	784	93.6%	CD8+ T Cells
T_cell_8	481	96.9%	CD4+ T Cells
T_cell	15227	79.3%	
Tumor_0	139	97.1%	Primarily Patient 5 Derived
Tumor_1	3	100.0%	Primarily Patient 5 Derived
Tumor_2	1180	96.6%	Primarily Patient 4 Derived
Tumor_3	750	98.0%	Primarily Patient 3 Derived
Tumor_4	582	100.0%	Primarily Patient 4 Derived
Tumor_5	498	99.8%	Primarily Patient 3 Derived
Tumor_6	494	97.0%	Primarily Patient 4 Derived
Tumor_7	456	99.6%	Primarily Patient 4 Derived
tumor	4102	98.1%	

Figure 7: Cell data that was used in the scRNA LUAD analysis. EC stands for endothelial cell, Epi stands for epithelial cells, and Fibro stands for Fibroblasts. These abbreviations are used in other graphics.

cell type such as T cell or B cell, while cluster or subtype refers to the clusters present in the figure. The data also includes normal tissue that allows for differential analysis to be done

between cells within the TME and cells outside of the TME. Bulk RNA-sequencing data from TCGA was also collected, with 535 primary cancer samples and 59 normal samples, all from patients with LUAD. These were normalized using DESeq2[65] for further downstream analysis, and p-value adjusted using Benjamini-Hochberg (FDR correction).

4.1.1 Finding "Normal" Cells for Tumor Comparison

One area in which original processing had to be done was finding what the matched normal cells were for comparison with tumor. The tumor cells that existed "outside" of the tumor were likely early stage signs that some tumor cells had drifted away from the original tumor site, not matched normals. Though there is suggestion that alveolar cells are the likely progenitors of tumors in LUAD, it is still not a known fact if this is true or not.[66] To analyze where the tumor cells truly came from, a simple multilayer perceptron or MLP was constructed. An MLP is one of the most basic forms of neural networks is generally used to classify some test set (in this case tumor cells) based on a train set(in this case the other cells). Because an MLP can approximate decision boundaries based on high dimensional space, it is ideal for studying where tumor cells are most likely derived from. The MLP architecture had two hidden layers, with 1024 and 128 nodes respectively. The choice of activation function was RELU for the hidden layers, and a softmax layer was used as an output layer. The model was compiled to use the Adam optimizer[67] and use categorical cross-entropy loss, both of which are common choices in classification tasks. Because of a class misbalance, classes were equilibriated to be much closer to even, with all cell types with over 2000. The MLP converged extremely fast, with a loss on the order of 0.0001 achieved within 5 epochs and accuracy ¿ 99.9%, using a batch size of 100. When applied to the tumor, about 75% of tumor cells were predicted as being alveolar from the output of maximal probability, and another 17% of tumor cells were predicted as being alveolar from the second highest probability. This further suggested that alveolar cells were the likely origin point of tumor cells in lung

adenocarcinoma, and comparisons of differential analysis were done between alveolar and tumor cells. The next highest groups were B cells and fibroblasts. Further analysis of trying to identify which specific subtype using a similar neural network failed to reveal a clear segregation with most tumor cells as being identified as the unannotated due to no specific marker genes and likely undiffrentiated cluster 1, followed by COPD-injured cells (cluster 3). This may fit with the current theory that adenocarcinoma cells are primarily derived from alveolar progenitor cells[68] that have been induced by inflammation (fig. 8c). Because of the poor segregation, all the alveolar cells were treated as tumor normal.

One advantage of using the neural net to train on class labels was the creation of a lower dimension embedding space naturally. The output from the last hidden layer before the softmax was treated as a lower dimension mapping that could be used in data analysis. For instance, t-Stochastic Neighbor Embedding (tSNE)[69] and the newly developed Uniform Manifold Approximation and Projection (UMAP)[70] run significantly faster when run on lower dimensional spaces. This is generally done through principle component analysis, but a neural network is better performing because of its ability to model non-linear spaces (fig. 8).

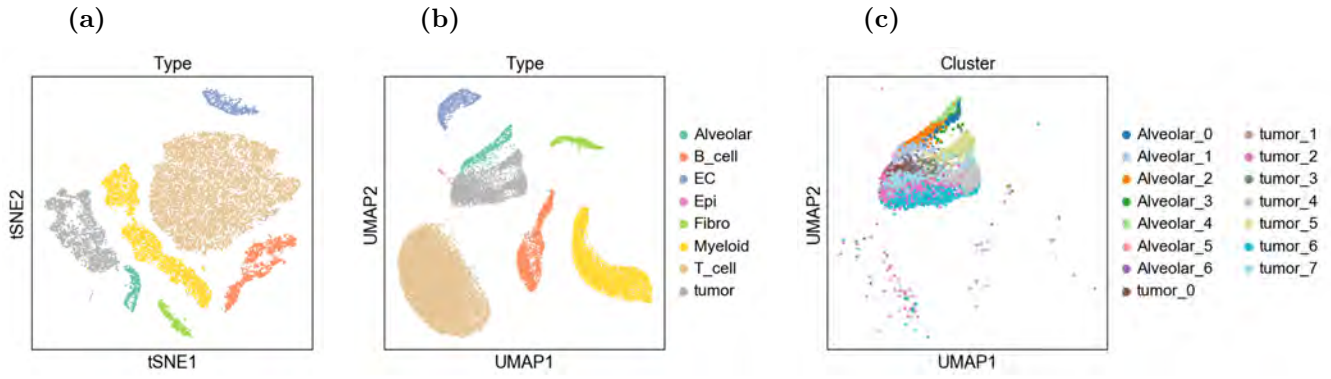


Figure 8: Two low dimensional embeddings of the cells in LUAD, after dimensionality reduction by the neural network to 128 dimensions. (a) The tSNE method to project the 128 dimensions onto 2 dimensions. (b) uses the UMAP method. Note that both show the tumor and alveolar clusters near each other. In particular, UMAP is thought to preserve cell trajectory (i.e. cell lineages) better. This can be seen in (c), a zoomed in UMAP projection where cluster 1 of alveolar is close to the tumor.

4.1.2 Differential Expression in Lung Adenocarcinoma

The differential expression was carried out by comparing intra-type tumor vs non-tumor populations. For each of these, p values were calculated using the T-test. To correct for multiple hypothesis testing, false detection rate was carried out using Benjamini-Hochberg. A gene was labeled as significant if the q-value was lower than 0.05. Log fold change values were also calculated on the differential expression.

4.2 Breast-specific Methods

Triple negative breast cancer scRNA-seq data was drawn from a smaller study than lung.[59] A total of 1189 cells passed quality control measures, of which a further 1112 were assigned clusters by the authors, shown in fig. 9. A total of 13280 genes were reported as being expressed. Because no normal sample was taken, the differential expression was done between cell types, as opposed to outside or inside tumor. For each of these, analysis was done just like in LUAD, in terms of p value calculation, FDR, and fold change.

(a)

Cluster	Cell Count
B_cell	19
EC	14
Epi	868
Macrophage	64
Stroma	94
T_cell	53

(b)

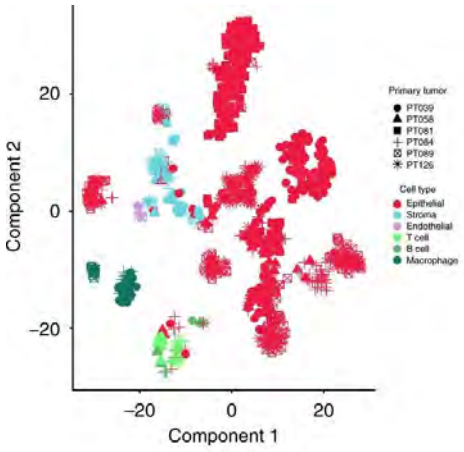


Figure 9: (a) The cell clusters as clustered by the authors.[59] Naming convention is the same as in LUAD.(b) A tSNE projection taken from the original paper. Macrophages (dark green) segregate the most.

4.2.1 Bulk RNA-seq Analysis of TNBC Tissue

Because no normal scRNA-seq data was available, bulk RNA-seq comparison between triple negative breast cancer patients found in TCGA, as reported by Lehmann and colleagues,[25] was used to find differential expression between the tumor and normal. Bulk RNA-seq normal data was analyzed from all breast samples in TCGA, not just the matched normals from the 173 TNBC patients. This accounted for 113 patient samples from solid tissue. This data was normalized using DESeq 2, with the conditions being normal and cancer patients. The assumption for this data was that any genes with differential expression between normal and tumor samples in bulk RNA would map equally to scRNA-seq experiments. Thus, the scRNA-seq data could be used to confirm localization of the increased or decreased expression.

5 Analysis and Results

Analysis of both tumor samples shows the significant differences between cancer types account for the differences seen in exposure to opioids. By analyzing both quantitatively and qualitatively measures of gene expression, putative hypotheses can hopefully be formed.

5.1 Lung Adenocarcinoma Analysis

Because of the retrospective clinical and genetic analysis, the analysis on lung was much more targeted towards the PI3K pathway. It should be noted that the known genes in a pathway are not a stable or singular list. Therefore, the list may not be a full or complete picture of what is occurring in the pathway. The opioid receptors from breast cancer that were selected in section 3.2 were also analyzed. Though differential expression is obviously important, the simple strong signal of a gene can also signify that signals may be integrated through the product of this gene.

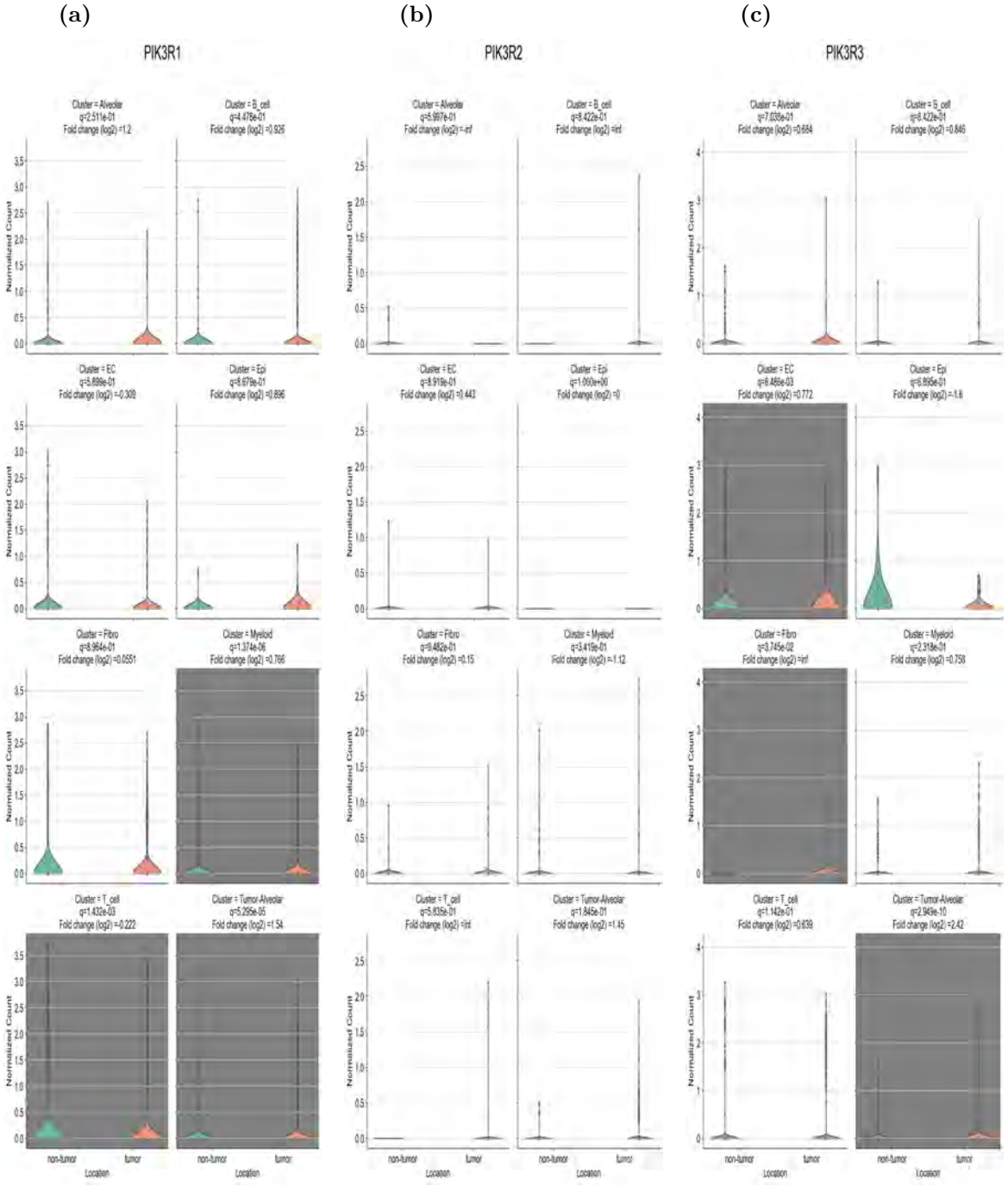


Figure 10: The violin plots show the 3 PI3K regulatory elements. Each plot is annotated with Benjamini-Hochberg adjusted p-values (q-values) and log2 fold changes. Plots highlighted in grey indicate significant data. Though PIK3R2 did not show any statistically significant up regulation (b), all of the genes did shown an increased fold change in tumor tissue.

5.1.1 Tumor Cells

Analysis between normal alveolar cells and tumor cells indicate that most genes in the AKT-PI3K pathway are broadly upregulated. In particular, various downstream products that are positively involved in tumor-suppression in PI3K are upregulated (DEPTOR, EIF4EBP1), suggesting that feedback loops are working. However, the primary regulatory activators of the PI3K pathway were upregulated. These are the PIK3R family of proteins that are shown in fig. 10. A total of 62 of 124 selected proteins were upregulated in tumor cells versus alveolar normal, with 18 being downregulated (fig. 11a). A comparison with the opioid receptors reveals that the major expressed opioid receptor subtype is OGFR, and there is minor expression of OPRK1, OPRD1, and OPRL1 (fig. 11b). Though studies have indicated that OGFR may be a protective mechanism,[71] this may be modulated through the μ opioid receptor (OPRM) initially (see section 6 for a deeper discussion). It should be noted that expression segregated by tumor cluster (fig. 11c). This is likely a side effect of patient populations within the tumor cluster, as patients 3 and 4 were vastly over-represented in cluster 2,3,4,5,6, and 7.

5.1.2 Stromal Cells

Stromal cells did not represent a single unified response, and instead had a multitude of responses. Here, we mostly keep the discussion analysis to immune cells, though it should be noted that fibroblasts, endothelial, and epithelial cells do play an important role in the tumor microenvironment. First, it is clear from that B cells are severely unenriched in any genes from the PI3K-AKT-mTOR pathway (fig. 12c. This is further supported by fig. 12a that shows a not a single upregulated gene, and a few downregulated significant genes. Importantly, RPS6, a ribosomal subunit that is activated found downstream of mTORC1, was found to be depleted in B cells. This indicates that all transcriptional activity was decreased and the cells were in an exhausted state and can be seen in fig. 12b.

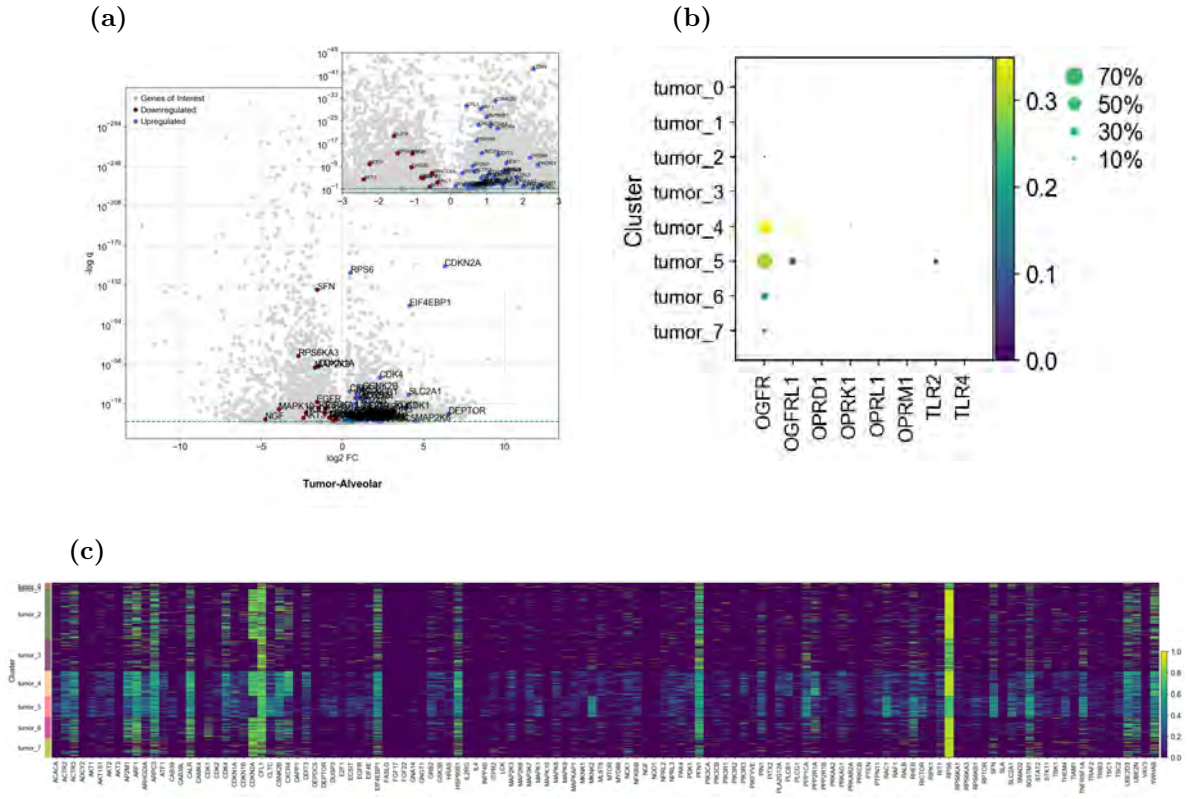


Figure 11: (a)A plot depicting the genes that are upregulated and downregulated in tumor cells. The x-axis is fold change and the y-axis is adjusted p value. The line depicts 0.05.(b) A dot plot showing relative opioid receptor prominence for each tumor cluster. (c) A heatmap showing the the full PI3K pathway as well as CDK2NA expression for the tumor cluster. The data in the plot is normalized N between 0 and 1 by $N = (O - \min(O))/\max(O)$, where O is the data for one gene.

Both myeloid and T cells appear to have a large number of genes represented in the PI3K pathway fig. 12c. However, further analysis shows that the differential expression for myeloid cells is not significant. In fact, more genes in the PI3K pathway are downregulated in myeloid cells (fig. 13a), suggesting that high amount of expression is occurring in the myeloid cells found outside of the tumor. This is corroborated by fig. 13c that shows the primarily non-malignant clusters (0,5,6,7,8,9,11), as having higher expression of the genes. Some of the upregulated genes, such as STAT2 and RIPTOR, form the mTORC2 pathway that is known to regulate cell cycle survival (evidenced by down regulation of TNFRSF1A, a precursor to capsase recruitment), while the downregulated genes RHEB and the upregulated

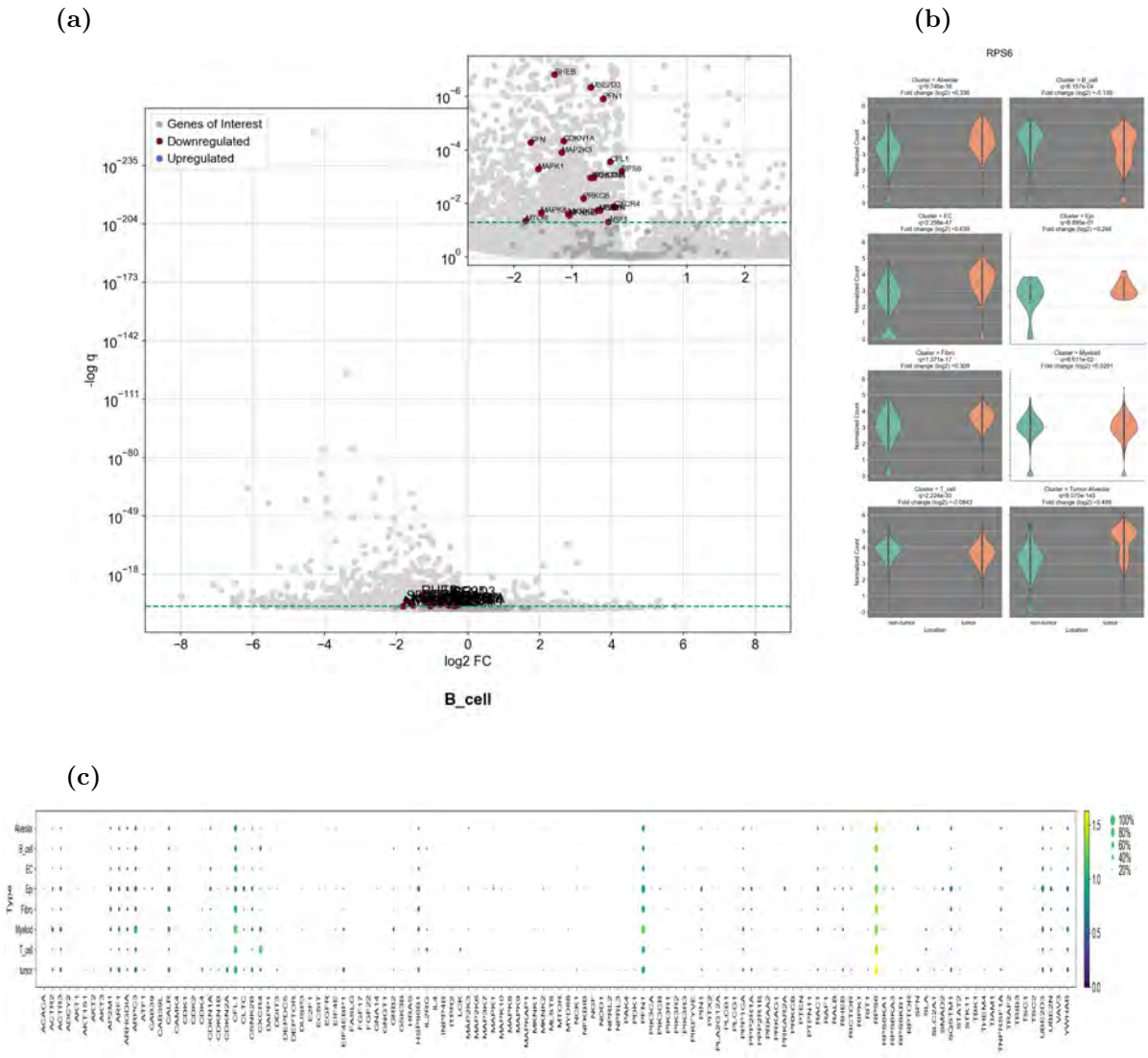


Figure 12: (a)A plot depicting the genes that are upregulated and downregulated in B cells. Note that all genes of interest are downregulated significantly. The line depicts 0.05.(b) RPS6, a ribosomal subunit, is downregulated in B cells. (c) All cell types are shown here, as is the the full PI3K pathway as well as CDK2NA expression. White space indicates no expression.

gene EIF4EBP1 are in the mTORC1 pathway related to protein synthesis. This suggest a reduction in both inflammatory response and cell proliferation, which is consistent with a reduced immune response. T cells also show a similar state, with synthesis inhibitors upregulated and synthesis promoters downregulated (fig. 13b), suggesting a quiescent state, Furthermore, SLA is upregulated; SLA is a T cell response inhibitor that is linked to mTORC

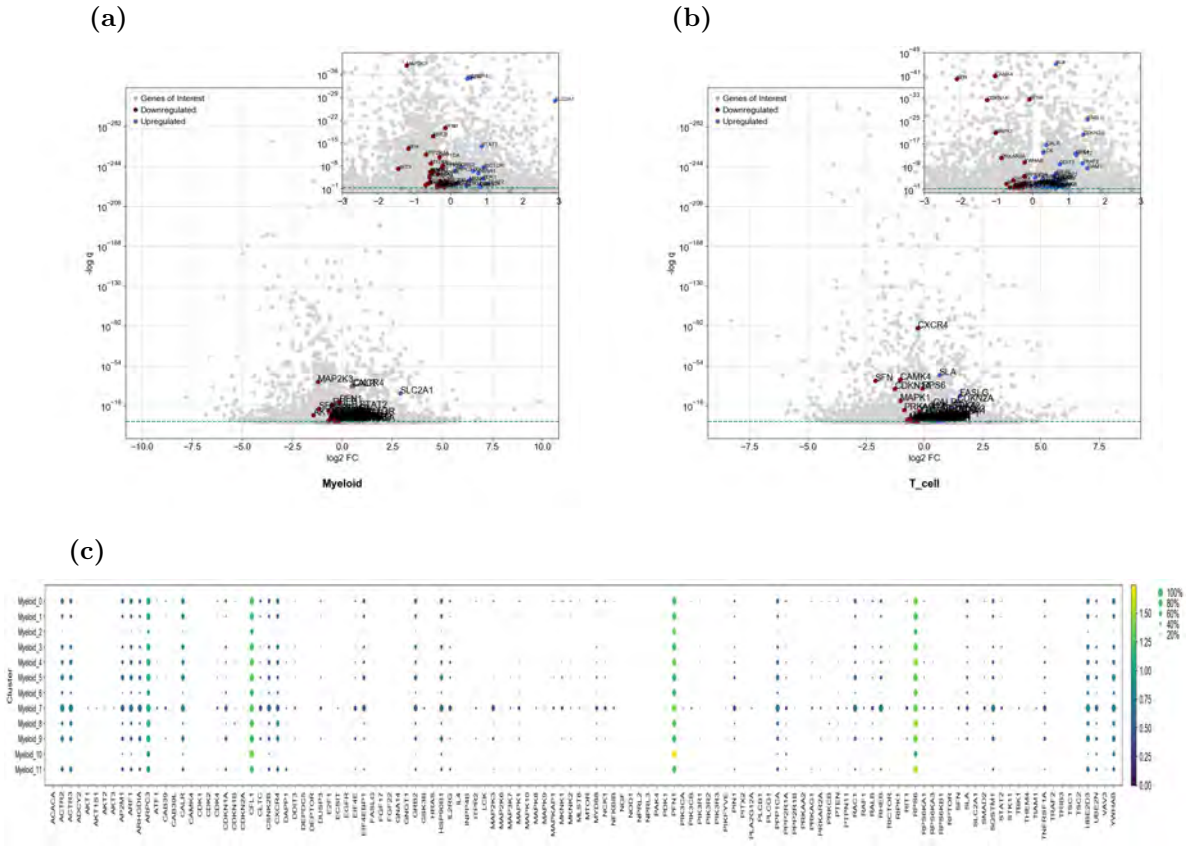


Figure 13: (a)A plot depicting the genes that are upregulated and downregulated in myeloid cells. The line depicts 0.05.(b) The same style of plot as (a), except for T cells (c) All myeloid cell cluster are shown here, as is the the full PI3K pathway as well as CDK2NA expression. White space indicates no expression. White space mainly appears in tumor-derived myeloid cells.

signaling pathways, suggesting that primary activation of T cells is decreased in a mTOR dependent way. There is virtually no opioid receptor presence in T cells, including the TLRs somewhat surprisingly, as TLRs are known to express on T cells.[50] However, OGFR is shown to have a significantly greater presence in the TME in T cells (fig. 14a). The myeloid cells do show some opioid receptor presence (fig. 14b), but primarily in clusters that are found outside of the tumor (fig. 14). This suggests that though some myeloid cells may be responsive to opioids, they are unlikely significantly responsive within the tumor. In fact, TLR4 is found to be negatively expressed in myeloid tumor tissue (q=1.31e-4, log fold change=-0.491).

Both fibroblasts and endothelial cells show similar patterns of upregulation and downregulation, with a large number of relevant genes upregulated (fig. 16). However, a detailed analysis of these phenotypes is outside the scope of this thesis, due, in part, to the lack of in-depth study of these cell types in the tumor microenvironment. Suffice to say, pro proliferation genes are present and upregulated, which may drive pro tumor signaling within the TME. Similar to B cells, fibroblasts show almost no expression of opioid related channels fig. 14b. Endothelial cells do have receptors present in cells likely present in the tumor microenvironment fig. 14b. However, none are significantly expressed and may just be normal expression of receptors that are suggested respond to opioids in normal tissue. Because of the low or non-differential expression of opioid related receptors in stromal cells, it appears

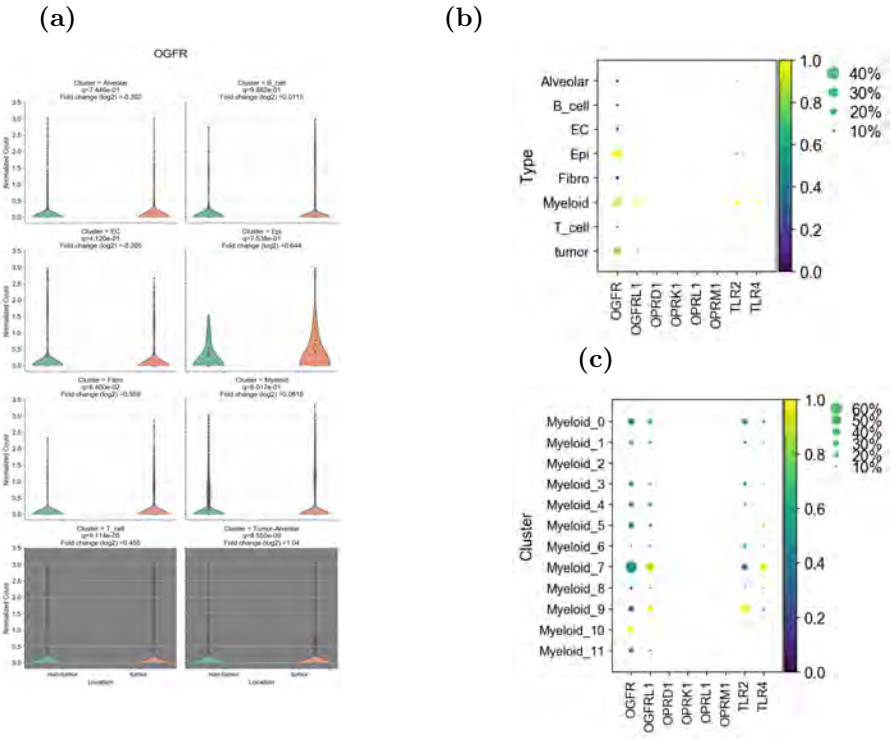


Figure 14: (a) OGFR is differentially expressed only in T cells and tumor cells, even though it shows expression in most cell types. (b) Weak expression for all opioid receptors, except OGFR. Minimal expression is shown for all other cell types. These are normalized using the same strategy in fig. 11c. (c) Myeloid cells show segregation by cluster when the opioid receptor expression is measured. These are normalized using the same strategy in fig. 11c

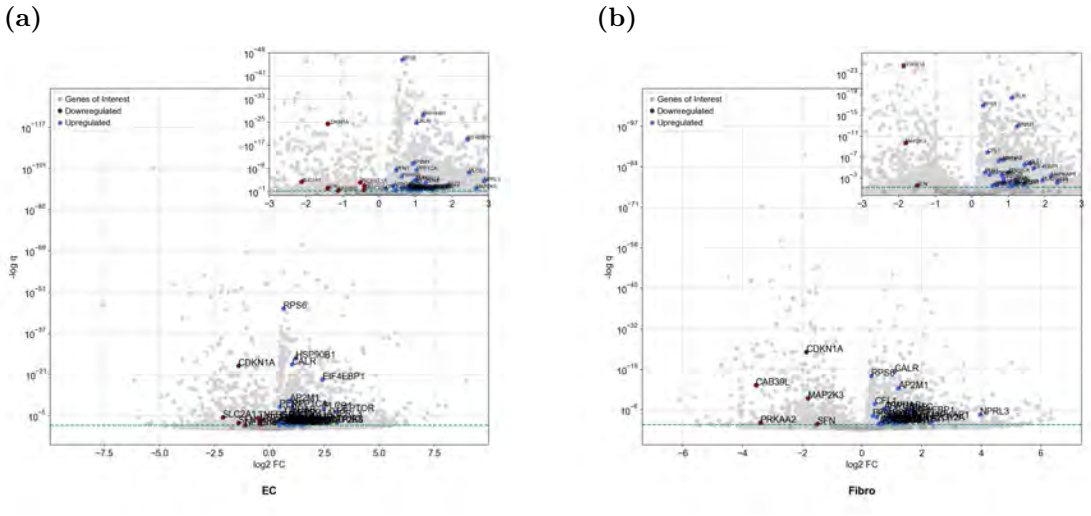


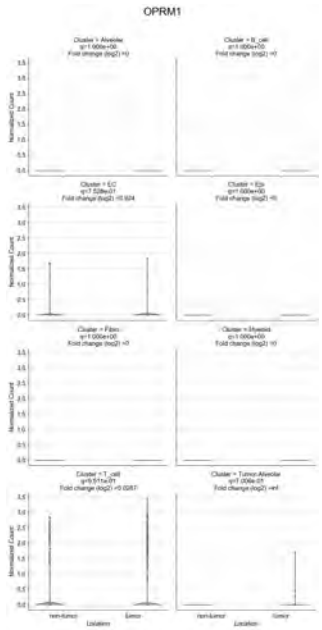
Figure 15: (a) Expression of relevant genes in endothelial cells. (b) Expression of relevant genes in fibroblast cells. Both cell types predominantly show upregulation of relevant genes, though some are downregulated, including a mediator of tumor suppression, CDKN1A.

that the likely target of opioids in lung cancer is the tumor itself. Though μ opioid receptors were not found to be significantly expressed, this may be due to just lack of prominent expression. TCGA data from bulk shows that μ opioid receptors are expressed at very low levels, about 1000x less prominent than OGFR fig. 16b. However, it is still differentially expressed (q=8.877e-5,log Fold Change=2.073) in the bulk data, which may explain some of the results mentioned in section 2.5.2, and a trace amount of OPRM1 is detected in tumor cells (fig. 16a).

5.2 Triple Negative Breast Cancer Analysis

Triple negative breast cancer analysis was less targeted. Because of this, results were more hypothetical and used the bulk sequencing data to greater extent. This was also necessary because of the relatively small number of cells that the original data set had. Because a vast majority of the cells were epithelial, or putative tumor cells, power was significantly reduced to make any type of conclusion.

(a)

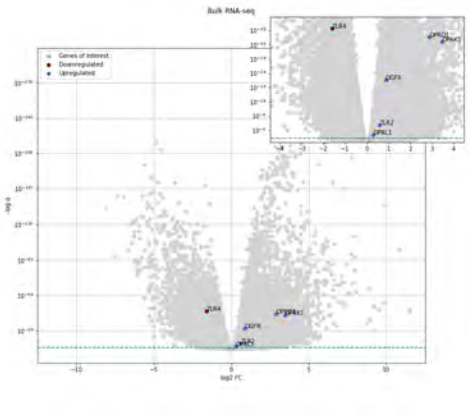


(b)

Gene	Mean	log Fold Change	q Value
OGFR	2542.7	-0.085	0.397
OGFRL1	2372.5	-0.361	0.00382
OPRD1	93.0	2.284	1.19e-17
OPRK1	60.4	2.050	5.88e-07
OPRL1	204.8	0.134	0.351
OPRM1	1.3	2.073	8.88e-05
TLR2	2427.2	-0.531	0.000867
TLR4	1124.7	-1.573	3.47e-27

Figure 16: (a) Plots of OPRM1 expression by cell type in LUAD. Note that only T-cells and tumor cells have any form of expression. This may fit in with the immunosuppressive nature of opioids. (b) Opioid receptor data from TCGA bulk RNA sequencing. Though the OPRs, except L, all showed differential expression in bulk, this signal is not picked up in scRNA likely because of the small amount of expression as shown by the mean.

(a)



(b)

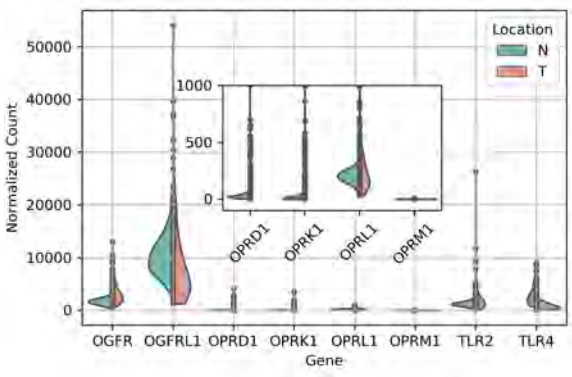


Figure 17: (a) Analysis of triple negative breast cancer TCGA data shows that most opioid receptors are upregulated. Importantly, TLR4 is downregulated and μ opioid receptor is not differentially expressed. (a) TCGA data shows the normalized expression levels of each of the target receptors. Importantly, the OPR receptors all have small mean expression levels, similar to LUAD, as compared to the endogenous opioid receptors and the TLRs.

5.2.1 Triple Negative Breast Cancer TCGA Data

TCGA RNA-seq data on TNBC patients was combined with normal breast tissue RNA-seq data. The panel of opioids used in lung was also used here. fig. 17a shows that 6 of the eight opioids are differentially expressed between bulk tumor and normal tissues. One surprising non-significant gene is the μ opioid receptor. However, similar to lung tissue, the receptor is expressed in very small quantities, approximately a 1000x less prominent than OGFR again (fig. 17b). Thus, it is particularly difficult to measure these values in scRNA-seq, as there just is not enough RNA to amplify.

5.2.2 Triple Negative Breast Cancer scRNA-seq Data

Differential expression was carried out between cell types within the tumor microenvironment. Within breast cancer, it appears that a majority of the differential expression happens within immune cells. For OGFR, TLR2, and TLR4, all show significant upregulation in at least one type of immune call (fig. 18). OPRD and OPRM were both not sufficiently expressed at the single cell level to be included in this analysis. As opposed to lung, B-cells seemed to overexpress OGFR as compared to the other cell types, being significantly upregulated against both epithelial (tumor) and stroma cells. Macrophages and T-cells also had increased OGFR expression over tumor cells. Macrophages contained the highest expression of OGFRL1, as well as TLR2, even amongst the immune cells. T cells also slightly expressed TLR2 more significantly than B cells. Finally, TLR4 showed significant expression in endothelial tissue over epithelial tissue and T cells ,and once again, very strong expressions in macrophages. However, based on the data from the TCGA indicating that TLR4 is downregulated, it may be less expressed. OPRK does not show significant expression quantitatively; however, qualitatively it only seems to be expressed strongly in epithelial or tumor cells fig. 18d. This may be in line with protective effects seen in melanoma.[72] This seems to agree with the conclusion from the retrospective data that opioids are protective,

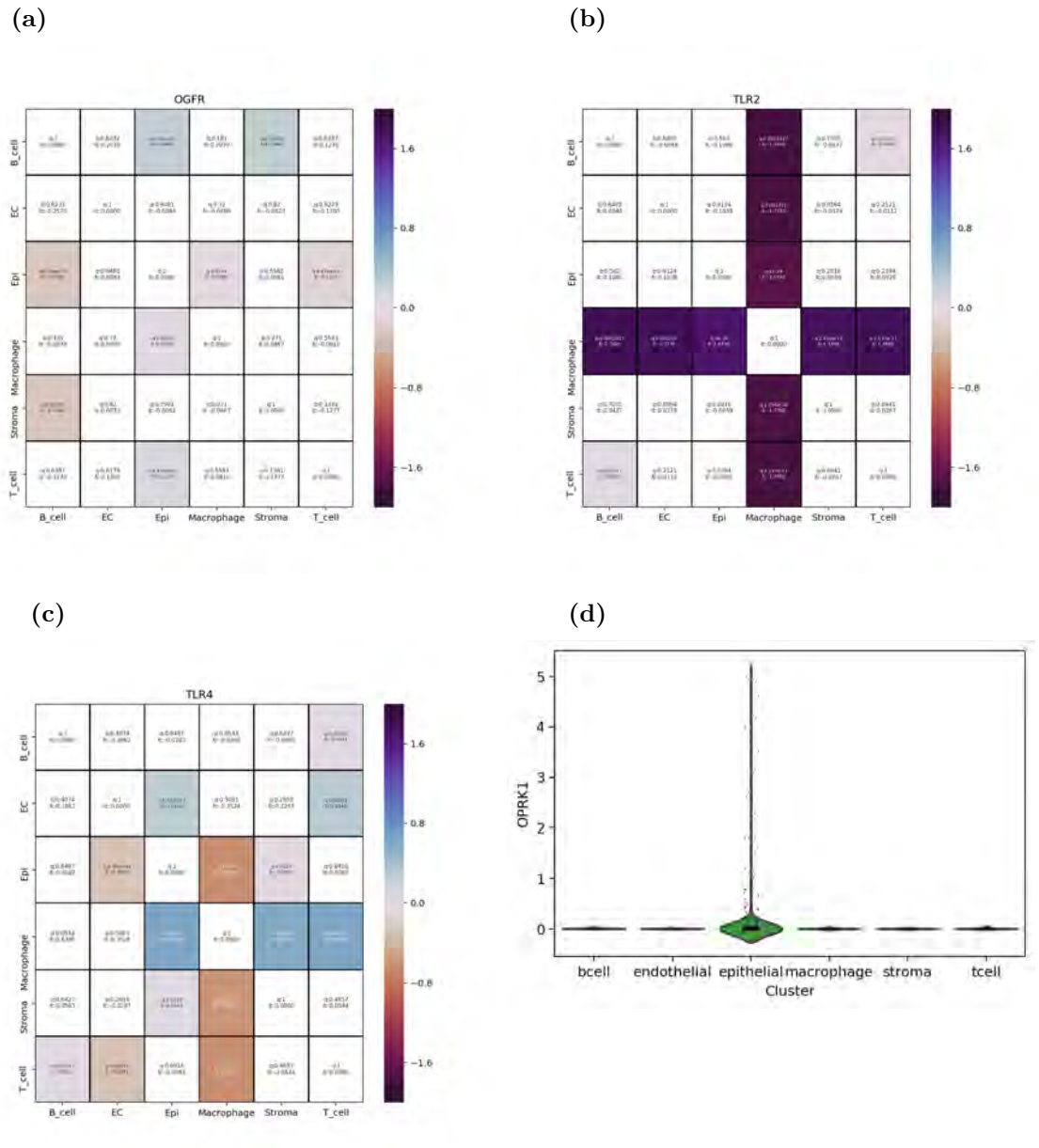


Figure 18: (a)(b)(c) These plots depict the q values and log2 fold changes in each square. The fold change is calculated as y/x. Cells that are colored are significantly expressed, with blue representing up regulation and red representing down regulation when read from left to right.(a) OPRK is expressed highly in epithelial cells, though not significantly.

as TLR4 is supposed to be significantly pro-inflammatory. The evidence in breast seems to suggest the opposite as in lung; the main driver of sensitivity to opioids is mediated through immune cells, as opposed to direct interaction with the tumor.

6 Discussion and Conclusion

Trends can be pulled from this genomic data to establish the effects of opioids in these cancers. Here, we generate some hypotheses as to the biochemical nature, based on the retrospective clinical analysis and this thesis.

6.1 Lung Adenocarcinoma Hypotheses

Based on the IMPACT panel that was used to analyze the clinical cohort, the effect of opioids given intraoperatively is being modulated by mutations in the PI3K pathway. On further analysis, tumor cells were noticed to differentially express many genes in this pathway. Though downstream inhibitors were upregulated, the drivers behind the signaling cascade, the PIK3R regulatory genes, were mostly upregulated as well. This suggest a signaling cascade where negative feedback loops are still working, but not enough to prevent runaway cell proliferation and survival through both mTORC pathways. Further, other cells in the tumor microenvironment, particularly B cells and T cells (aka tumor infiltrating lymphocytes), show significant upregulation of suppressing elements or downregulation of promoting elements in the PI3K pathway. This suggests that an opioid's primary effect is exerted on the tumor directly.

Though scRNA-seq data only picks up OGFR, this is thought to be protective and anti-tumor. However, when expanding to bulk sequencing, it becomes clear that OPRM1, the primary pro-tumor receptor, is upregulated. A minor trace of this signal can be picked up on T cells and tumor cells. This seems to suggest that both the immunosuppressive elements of opioids, as well as the pro-tumor elements can be triggered through this receptor still.[53] This is in line with the analysis from the clinical cohort that opioids are harmful to long-term survival.

6.2 Triple Negative Breast Cancer Hypotheses

Retrospective clinical data showed that in a large cohort of surgical triple negative breast cancer cases, opioids had the opposite effect by being protective. Exploratory analysis into the TCGA suggests that triple negative breast cancer patients do not differentially express OPRM1. Thus, it is likely other pathways that are making an effect. In particular, analysis of the single cell data suggests that any protective pathways are likely to be found in immune cells, as they strongly express the receptors. One exception to this may be the OPRK1 receptor that is thought to be protective and is found on putative tumor cells.

The first gene to stand out is OGFR. This gene is thought to have a suppressive effect on the immune system. However, such suppression may not be negative. An overactive immune system in cancer can lead to a muted immune response, through regulatory compensation. More research needs to be done on the exact role of OGFR in mediating the effect the immune system in diverse cancer phenotypes. Further, both TLR2 and TLR4 show upregulation. TLR2 is somewhat interesting; though it is known to possibly cause a shift from the M2 pro-tumor macrophage type to M1 anti-tumor,[73] the exact role of TLR2, as to whether it is a receptor or a downstream target of opioids, is still unclear. However, it is interesting that either way opioids could be inducing a shift towards the M1 phenotype. Finally, TLR4 appears broadly in immune cells. However, it is quite greatly downregulated in bulk tumor sampling, suggesting that its inflammatory role at worsening tumors may be reduced. These developments suggest many new and exciting avenues that can be opened up from a clinical and basic science view.

6.3 Basic Heuristic Oncoanesthetic Model

Applying the above hypotheses to the broader field of oncoanesthesia may allow creation of a generalized model. While this model may not be generalizable between cancers, it allows

for the development of a parametric model that can combine the two relatively disparate areas of pathway analysis and receptor analysis. Basically, the change in survivability S can be given by

$$S = \sum_{c} \sum_{r} D_c E_{r|c} I_r \tag{1}$$

where r and c are the full receptors and cell types respectively. D represents the downstream pathways and effects for a given cell type, E represents the expressivity of receptors given a single type and I represents whether the impact of the receptor is positive or negative. While this model may be simplistic, it gives a roadmap for future expansion and each term may be expanded upon to give a better model in the future.

6.4 Future Directions

There are many future directions suggested by work in this thesis. Here, a few are described.

6.4.1 Oncoanesthesia Future Experiments

The analysis here suggests that more in-depth study needs to be done on particular pathways, genes, and receptors to discover how they fit into a broader story. This should include both clinical and genomic testing. By analyzing transcriptomes and relating to survival outcomes, the precision in precision medicine really comes into play. Furthermore, more scRNA sequencing should be carried out in triple negative breast cancer and lung adenocarcinoma patients, with focusing on matching normal tissue to tumor tissue. This would enhance the power of any differential expression study. One interesting experiment that may be hard to do at least in tumor could be a time-based RNA sequencing of a patient to discover what the changes that anesthesia induces. This may be more easy to work into an animal model.

Finally, investigations at a cellular level should take place. These can include down-

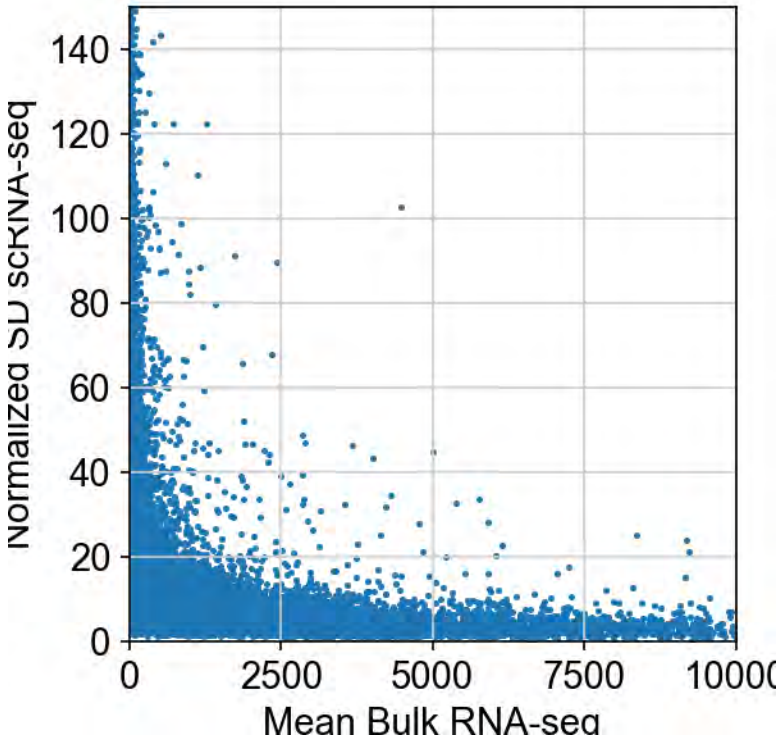


Figure 19: Though scRNA-seq is able to pick up weakly expressed genes, the variance of such measurement is extremely high. The y axis describes mean normalized standard deviation, while the x axis plots the mean of bulk RNA expression. As it can be seen, the variance due to dropout is exceptionally high. This suggests that cells are truly not expressing weak signals at appreciable amounts, and alternate experiments may be needed to probe weakly exhibited gene such as OPRM1. (Kendall's Tau -0.623).

stream analysis of the entire PI3K pathway to binding assay experiments to elucidate where exactly opioids bind and what occurs in cells. In general, experiments contextualized to *in vitro* tumor proliferation may fail to take into account the full impact of the tumor microenvironment. In this regard, animal models may be useful in studying *in vivo* effects do caution should be taken as these animals may not mirror humans. These non-genomic experiments may be useful because signaling cascades do not require vast expression at the primary stages. This can be seen in OPRM1, which is not expressed at any appreciable amount but has vast downstream effects. This is particularly true in the context of scRNA experiments (fig. 19).

6.4.2 Machine Learning Validation of Clustering

One initial area of exploration is the use of machine learning tools to validate possible clustering. By withholding a set of cells of known or hypothesized origin and training a model on the possible clustering, the cells of known origin can be used a validation set. Further, this can confirm whether a clustering strategy was accurate by seeing if decision boundaries are truly replicapable. This is an ongoing field of research and should be studied with detail.

6.5 Conclusion

RNA sequencing offers an in-depth look into cellular processes. Here, it is used to explore the space studying the interplay between oncology and anesthesia. By analyzing at a highly granular level, the specific types of cancer can be interrogated in great detail. The work here suggests that molecular hypotheses can be developed from publicly available data to corroborate restrospective clinical results. Further work in establishing the true nature of these effects lead by these results can provide interesting results.

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