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Los Angeles

Multi-omics Transcriptional Profiling of Neuroendocrine Trans-differentiation in Small Cell

Carcinoma

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of

Philosophy in Molecular and Medical Pharmacology

by

Chia-Chun Chen

2024

ABSTRACT OF THE DISSERTATION

Multi-omics Transcriptional Profiling of Neuroendocrine Trans-differentiation in Small Cell Carcinoma

by

Chia-Chun Chen

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2024

Professor Owen N. Witte, Co-Chair

Professor Thomas G. Graeber, Co-Chair

Cellular differentiation is a fundamental process in growth and multicellular development. Trans-differentiation from adenocarcinoma to small cell neuroendocrine carcinoma in lung and prostate cancers can occur *de novo* or induced by targeted therapies. To understand the evolving process of the trans-differentiation, a temporal multi-omics study on a forward transformation *in vitro/in vivo* model of small cell neuroendocrine prostate cancer was performed. By analyzing samples taken from various time points including human basal cells, *in vitro* transformed organoids, early, transitional, and late xenograft tumors, an arc-like transformation trajectory with bifurcated endpoints defined by ASCL1 and ASCL2 is identified. Concurrently, the transcriptional programs shift from stress-response to cellular reprogramming, to neuroendocrine differentiation. With further experimental testing, additional transcription factors such as TFAP4 exhibit dynamic control between ASCL1 and ASCL2 expression. This study provides comprehensive epigenetic landscape of trans-differentiation and potential therapeutic targets for advanced prostate cancer.

The dissertation of Chia-Chun Chen is approved.

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2024

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DEDICATION

This dissertation is dedicated to my angel grandfathers and my parents, for their life-long

devotion to the family and unconditional love and support for me. This work would not be

possible without them.

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I would like to thank my PhD mentors, Dr. Owen Witte and Dr. Thomas Graeber. I am very grateful to have them as my co-mentors during this years-long journey at UCLA. They have taught me not only the scientific principles in research, but also emotional intelligence in life. Owen and Tom have completely different personalities and mentoring styles. They are the Yin and Yang to my scientific practice. I frequently describe our relationship to other people as I am a chef who is making a feast (dissertation) with an air fryer (Owen) and a slow cooker (Tom). The hardest part of this culinary experience has been learning how to incorporate both at the right time with the right intention and attitude.

Owen has a firecracker personality with sharp instincts and right-to-the-point advice. The air conditioner in his office is blowing so strong, yet you are constantly sweating through the conversation with him because you know you are getting the most practical and critical opinions about yourself and your work. There is no filter in front of him. I very much admire his way of approaching life and scientific questions-logically, but with a lot of curiosity. I am very thankful for his patience for the time when I was doubting myself, his honesty when I wasn't improving myself, and his encouragement when I started to feel my feet on the ground. He is a one-of-a-kind mentor with a strict father mentality.

While the air fryer provides a bombing deliciousness without grease, the slow cooker offers a tender and long-lasting tastiness. I would like to thank Tom for his continuous and warm-hearted support during my PhD training and personal growth. He has taught me that the strength of humility carries a long way in one's career and in one's life. His attention to detail and his calmness has guided me to overcome my own recklessness and obstinacy. He is always encouraging about almost everything inside and outside of the lab, and I am grateful for having the freedom and trust when it comes to what I want to do for my PhD. Having two great mentors from different fields could mean an excellent training opportunity, but it also comes with double the amount of work

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PEER REVIEWED PUBLICATIONS

- **Chen, C.-C**., Song, K…, Witte, O. and Graeber, T. *Temporal evolution reveals bifurcated lineages in aggressive neuroendocrine small cell prostate cancer trans-differentiation*. Cancer Cell 41, 2066-2082 e2069. 10.1016/j.ccell.2023.10.009, 2023

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- Kwan, J., …, **Chen, C.-C**., Ratkovic, S., Klezovitch, O., Attisano, L., McNeill, H., Emili, A., Vasioukhin, V. *DLG5 connects cell polarity and Hippo signaling protein networks by linking PAR-1 with MST1/2*. Genes & Development, 30(24), 2696–2709, 2016.

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Chapter 1 Introduction

Chapter 1: Introduction

Treatment resistance remains a serious hurdle in modern medicine. One of the mechanisms adopted by cancer cells to escape from targeted therapies is to take advantage of cellular "plasticity" – an intrinsic potential to change and modify the cell identity in response to outside stimulus and environmental cues. Both genetics (DNA sequence) and epigenetics (how a cell controls gene expression without changing the DNA sequence) contribute to how a cancer cell adapts and responds to the changes. This thesis focuses on the epigenetic regulation in the process of trans-differentiation and highlights the pan tissue parallels between prostate and lung cancers.

The first part of this chapter (1.1, 1.2 and 1.3) takes a deep dive into the basic theories and pivotal experiments of cellular differentiation from developmental and stem cell biology. A pathological example in cancer, trans-differentiation, is an adverse consequence in adenocarcinoma evolving into more aggressive small cell neuroendocrine (SCN) carcinoma. The second part of this chapter (1.3 and 1.4) examines in detail specific transcription factors and chromatin modifiers in SCN trans-differentiation, where the discoveries were made using Next Generation Sequencing with single cell resolution and bioinformatic algorithms.

1.1: Cellular differentiation and lineage plasticity - From One to Infinite Possibilities

1.1.1 Introduction of cellular differentiation

Cellular differentiation is broadly defined as the process of a cell becoming specialized and maturing during development [1,2]. In adults, different types of stem cells (undifferentiated) give rise to various types of specialty cells (differentiated) in the body. For example, hematopoietic stem cells become white and red blood cells, and neural cells give rise to astrocytes and oligodendrocytes in the brain [3]. The concept of cellular differentiation can be dated back to as far

as 1893 in the book, The germ-plasm: A theory of heredity by August Weismann [4], invoking a one-way evolution in the multicellular organism by two cell types: one that passes down genetic material to the next generation (germline cells), and another produces other cell types in the body (somatic cells). This instigated the idea that each somatic cell is destined with an irreversible path in the development of certain cell type[5].

However, the concept was challenged by decades of research in stem cell biology and tissue regeneration, in which new tissue is generated from the pre-existing tissue or terminally differentiated cells[5]. In the early twentieth century, Spemann-Mangold experiment showed that a secondary body axis at the gastrula of an amphibian embryo can be generated by grafting a series of cells taken from the left dorsal lip[6]. This pivotal finding demonstrated that the fate of embryonic cells is not irreversible, and more than one differentiation path is possible.

The concept of "competence" - the ability of cells to react to inducing signals, was first introduced in 1940 by Conrad Waddginton[7]. Subsequently, he introduced "epigenetic landscape" in 1953, one of the most popular metaphors for non-singular cellular differentiation pathways[8]. The landscape was demonstrated as a multi-dimensional surface with a marble rolling down various permitted valleys/pathways ("chreodes"). Each valley signifies a differentiated state. Besides the conceptualization of cellular differentiation, the "Waddington landscape" emphasized how epigenetic (modification to epigenome), rather than the altercation in DNA sequence, affects the plasticity of a cell in lineage differentiation.

1.1.2 Trans-differentiation, dedifferentiation and reprogramming

Trans-differentiation refers to a cell that goes from one cell type to another without going through de-differentiation (or back to stem cell)[9,10], also known as direct reprogramming[11]. The concept of trans-differentiation was first introduced as a statement describing the transformation from the cuticle-producing cells to salt-secreting cells in silk moths by Selman and Kafatos in 1975[12]. This

was subsequently adopted by Okada and Euchi to describe the lens cells generated from pigmented epithelial cells in newt[13,14]. In 1987, the first experimental evidence of rewriting the cell identity was demonstrated by overexpressing MyoD, a mammalian transcription factor expressed in skeletal muscle that directly converts mouse embryonic fibroblasts to myoblasts[15]. This pioneering work opened a new era of discovering critical transcription factors that can induce specific cell fates through reprogramming.

In parallel, reprogramming cellular differentiation from a differentiated to an undifferentiated state *in vitro* was demonstrated by a revolutionary discovery: Yamanaka factors[16,17]. Yamanaka factors are composed of four transcription factors (proteins that control the transcription and expression of certain genes), Sox2, Oct4, Klf4, and c-Myc. When ectopically expressed, they can reprogram adult somatic cells to induced-pluripotent stem cells (iPSC) *in vitro*[16,17]. At the same time, Yu et al also published a different set of transcription factors that can reprogram human somatic cells to iPSC exhibiting embryonic stem cell characteristics[18]. Since then, decades of research in the regeneration field have showcased successful reprogramming both *in vitro* and *in vivo,* as well as their therapeutic application[19-25].

These breakthrough discoveries convey several critical messages. That 1) cellular differentiation is a dynamic process, and 2) master regulators in epigenetics are capable to sufficiently reprogram a cell fate and control lineage differentiation.

1.2: Trans-differentiation: Part I - From Adenocarcinoma to Small Cell Carcinoma

1.2.1 Cancer differentiation

Cancer cells, often described as resulting from an error during the normal cellular development, exploit cellular stress response to differentiate or de-differentiate under various condition and stress[26-28]. For example, hypoxia and metastasis, in which cancer cells promote undifferentiation

or undergo epithelial-mesenchymal transition to disseminate to a new site during the tumor progression[29-31]. Moreover, as a resistance mechanism to targeted therapy or response to different stress conditions, cancer cells can also trans-differentiate or de-differentiate to a different cellular state[32-34], such as the transition from an adenocarcinoma state to a stem-like small cell neuroendocrine state[35,36]. In cancers, the differentiation stage of tumor cells is central for the histopathological classification[27]. Well differentiated tumors tend to be less aggressive and grow slower than their less differentiated counterparts[27]. A poorly differentiated tumor such as small cell neuroendocrine tumor exhibits more aggressive characteristics including uncontrolled cell proliferation and visceral metastasis[37].

<u>1.2.2 Neuroendocrine differentiation and small cell carcinoma of prostate and lung</u>

Small cell carcinoma is a histopathological classification that often occurs with neuroendocrine characteristics. The histological characteristics include small and rounded cells with scant cytoplasm, as well as positive immunohistochemical staining of clinical markers such as SYP, CHGA, NCAM1 and NSE[38-40]. Despite its rarity, small cell carcinoma occurs in multiple cancers including but not limited to prostate, lung, pancreatic, head and neck, and ovarian cancers[37,41-43]. In this thesis, emphasis is placed on small cell lung cancer and neuroendocrine prostate cancer, as they share high degrees of overlap in molecular features and epigenetics[44].

Early-stage prostate cancer including localized disease has mostly an adenocarcinoma phenotype, which is often treatable with surgery (radical prostatectomy) and radiotherapy (i.e. brachytherapy)[45,46]. Androgen deprivation therapy (ADT) is a hormonal therapy that targets advanced prostate cancer systematically through various regime. For instance, surgical castration (orchiectomy), luteinizing hormone-releasing hormone agonists (i.e. leuprolide), and next generation androgen signaling inhibitors (i.e. enzalutamide and abiraterone acetate) that inhibits androgen receptor[47-49]. However, castration resistant prostate cancer (CRPC or CRPC-adeno) tumors no longer respond to ADT therapy[50]. When treatment resistance persists, CRPC trans-

differentiates into an aggressive variant, small cell neuroendocrine prostate cancer (SCNPC or NEPC) or CRPC-NE, a variant of CRPC with neuroendocrine features and mixture of adenocarcinoma[35,51-56]. *De novo* SCNPC is very rare and stemmed from a small population of neuroendocrine cells[39,57], whereas trans-differentiation cases of SCNCP accounts for 15-20% of CRPC and become an increasing challenge in the clinic[40].

In parallel, trans-differentiation from adenocarcinoma to small cell carcinoma is a salient problem in lung cancer. Lung cancer is broadly divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC)[58]. NSCLC includes lung adenocarcinoma (LUAD), large-cell carcinoma and squamous carcinoma based on histopathological classification[58]. SCLC is a malignant variant that is highly associated with tobacco carcinogen and accounts for about 15% of all lung cases[41,42,59]. Besides arising *de novo* from neuroendocrine cells, SCLC has a rising number in clinical cases reported as transformed SCLC from LUAD patients treated with tyrosine kinase inhibitors (TKI). TKI targets epidermal growth factor receptors (EGFR), such as afatinib, erlotinib, gefitinib, dacomitinib and osimertinib[60-63]. LUAD with EGFR mutation and transformation to SCLC are the major resistance mechanisms to the first and second lines of EGFR-TKIs[64]. LUAD with wild-type EFGR also experiences transformation, however at a much lower rate[65].

Small cell neuroendocrine trans-differentiation poses a major setback in the clinic due to the lack of options in the current treatment regime[66]. Both SCNPC and SCLC have poor prognosis with a median survival of 7 months and 2-4 months if left untreated, respectively[57,67,68]. There's no effective standardized treatment for small cell carcinoma. Platinum-based chemotherapy such as cisplatin and etoposide are only effective for a short amount of time until the cancer progresses[69,70]. To develop better therapeutics against small cell neuroendocrine cancers, a systematic molecular profiling study is crucial to understand the underlying biology and vulnerability of this challenging malignancy[35,66,70].

1.2.3 Pre-clinical SCNPC models

To study neuroendocrine trans-differentiation in prostate cancer, there are different *in vitro* and *in vivo* models available, with various degrees of advantages and disadvantages[71]. The Genetically Modified Mouse Model (GEMM) is one of the most common models that may be beneficial for the complex studies of the tumor environment, trans-differentiation, and therapy resistance. Examples include the usage of PBCre4, Nk3.1CreERT or Tmpress2CreERT2 with the combination of double floxed alleles of *Pten*, *Rb1* and/or *Trp53*[72-75]. Overexpression of MYCN has also shown promising in modeling neuroendocrine progression such as the PRN model driven by T2-Cre and Pten loss[76,77]. In addition, co-expression of constitutively active AKT and MYC in basal cells lead to the neuroendocrine transformation *in vivo*[78,79].

Patient Derived Xenograft (PDX) is a useful model to study tumoral heterogeneity and genetic assessment of SCNPC. For example, the LuCAP series encompasses a collection of genomic and phenotypic features in prostate cancer patients[80]. Another group has also established LTL series (Living tumor Laboratory) with comprehensive histopathology and gene expression profiling of donor tumors, with LTL331R being the most widely used model with an induced neuroendocrine phenotype[81]. The MDA PCa series has provided valuable information of SCNPC[82]. In parallel, the patient derived organoid model has gained popularity over the years. Beltran's group developed patient-derived organoids from metastatic biopsies from SCNPC patients[83]. These types of patient-derived organoids present a useful tool for drug screening and testing[83,84].

Despite these models, there is a very limited number of SCNPC cell lines available: NCI-H660 and LASCPC-01[78]. Other groups have utilized a LNCAP cell line, which is derived from a patient with metastatic prostate carcinoma[85], to study trans-differentiation from adenocarcinoma to SCNPC[86-88].

1.3: Trans-differentiation – Part II: From Epigenetics to Key Transcription Factors

1.3.1 Transcriptional convergence of small cell carcinoma

Besides the resemblance of small cell histology and the neuroendocrine marker expression, prostate, bladder, and lung cancers with small cell neuroendocrine phenotypes share similar transcriptomic and gene expression pattern [44,79,89]. In SCNPC and SCLC, the majority of clinical cases have inactivating mutations of P53 and RB1[90-92]. Experimental evidence has also shown that the loss of P53 and RB1 is essential for the advanced transformation and lineage plasticity in various small cell neuroendocrine cancer model in addition to the amplification of oncogenes, such as MYC and AKT[72,73,78,79,93-96]. These factors together contribute to the overall epigenetic landscape, promote neuroendocrine trans-differentiation, treatment resistance and malignant transformation in small cell carcinoma.

1.3.2 Epigenetic reprogramming in small cell carcinoma

Trans-differentiation from adenocarcinoma to small cell carcinoma entails epigenetic reprogramming[35,96]. Epigenetic reprogramming includes chromatin remodeling, histone modification and DNA methylation that downstream changes gene expression, driving cancer cells towards plasticity and neuroendocrine differentiation[95,97-99]. There is growing interest in identifying clinically targetable epigenetic regulator in advanced prostate cancer, such as the polycomb group gene family and DNA methyltransferases (DNMT) family[95,98,100-102]. For example, EZH2, a histone methyltransferase and a catalytic subunit of the polycomb repressive complex 2 (PRC2), is responsible for the oncogenic activation in advanced prostate cancer.[77,103,104]. EZH2 is also tightly coupled with DNMTs through recruitment[105]. DNMT1 is also found to be significantly enriched in SCNPC and SCLC compared to non-small cell carcinoma. Ablation of DNMTs in pre-clinical advanced prostate cancer has proven effective attenuation in tumor growth[106]. Other epigenetic modifiers such as SIRT1, a histone deacetylase (HDAC), induces neuroendocrine

differentiation through activation of the AKT pathway[107]. Together, these epigenetic modulators regulate a unique transcriptomic landscape of small cell carcinoma, facilitating a higher level of gene regulation towards neuroendocrine trans-differentiation.

1.3.3 Pioneer factors instigate neuroendocrine differentiation in prostate cancer

Besides epigenetic editing, pioneer transcription factors play an important role in instigating activation of the neuroendocrine transcriptional program in prostate cancer[108,109]. Pioneer factors are capable of binding to closed chromatin and inducing DNA accessibility that leads to cell fate reprogramming[110,111]. Forkhead box protein family genes such as FOXA1 are one of the most studied pioneer factors in prostate cancer[108]. High expression of FOXA1 in prostate cancer is associated with poor prognosis, potentially contributed by its role in activating AR transcriptional program through recruitment and direct interaction with AR[112-114]. Particularly, 25% of SCNPC have FOXA1 mutations that alter the lineage plasticity, including the blockage of luminal differentiation and activation of the neuroendocrine program[115-117]. In parallel, FOXA2 is not only a sensitive marker with specificity in SCNPC, but also responsible for KIT signaling pathway activation and driving the neuroendocrine trans-differentiation[75,118]. Nonetheless, ASCL1, a classical pioneer factor in neuronal and glioblastoma development[119-122], activates neuronal and stem-like programs through chromatin remodeling in SCNPC[123].

1.3.4 Subtypes of small cell carcinoma defined by transcription factors

Small cell carcinoma is a heterogeneous malignancy. SCLC can be divided into two categories: neuroendocrine (NE) and non-neuroendocrine (non-NE). Each category contains two subtypes that are molecularly defined as SCLC-A (ASCL1) and SCLC-N (NEUROD1), as well as SCLC-P (POU2F3) and SCLC-Y (YAP), respectively[124-126]. These four transcription factors represent the subtypes of SCLC based on not only the individual expression pattern, but also comprehensive transcriptomic and histopathological profiling on various tumor tissues and models[124,127]. Distinct

clinical characteristics presented by these four subtypes also implicate therapeutic treatments and outcomes[128,129]. Similarly, there are two clinical subtypes of SCNPC defined by ASCL1 and NEUROD1 through transcriptomic analysis on cell lines, patient-derived xenografts, and clinical samples[130].

Functionally, ASCL1 is a class II basic-helix-loop-helix (bHLH) transcription factor that heterodimerizes with other class I bHLH proteins to activate specific transcriptional programs. It is involved in neurogenesis, neural stem cells, and glioblastoma through various signaling pathways such as Wnt and Notch pathways[119,122,131-136]. Combinatorial expression of ASCL1, BRN2 and MTY1L are sufficient to reprogram fibroblasts into neurons[135,137]. In small cell carcinoma, around 70% of SCLC clinical cases expresses ASCL1 by immunochemical analysis[127,138,139]. ASCL1 is required for pulmonary neuroendocrine differentiation and tumorigenesis, presenting a potential therapeutic target for SCLC[140-144]. In prostate cancer, ASCL1 is further demonstrated to drive and support neural and plastic lineage, as well as contribute to the ferroptosis resistance[123,145,146].

Another neuroendocrine subtype of SCLC and SCNPC is defined by NEUROD1. NEUROD1 is a bHLH factor involved in the development of enteroendocrine cells in the gastrointestinal tract and plays an important role in the central nervous system and neurogenesis[147-149]. NEUROD1 is highly expressed in aggressive SCLC and promotes tumor cell survival and migration, via regulation of the receptor tyrosine kinase tropomyosin-related kinase B (TrkB)[150,151]. The molecular profiling of SCNPC identified that this NEUROD1 subtype, despite sharing similar chromatin accessibility of neuroendocrine transcription factors with the ASCL1 subtype, contains a distinct transcriptional landscape enriched in EBF and LHX transcription factor motifs[130].

In the non-neuroendocrine class of small cell carcinoma, the POU2F3 subtype is identified with higher frequency in SCLC than SCNPC[76,152]. POU2F3, also known as OCT11, is a transcription factor that is important for specification of a rare chemosensory cell type, tuft cells, found in

gastrointestinal and respiratory tracts[153,154]. It is also found to be essential for the tuft cell-like variant of SCLC through binding to OCA-T complex[155,156]. In addition, ASCL2 is identified as one of the unique dependencies in the POU2F3 subtype in SCLC[155], and is co-expressed with POU2F3 in subpopulations of SCNPC tumor cells[76].

The YAP1 subtype is considered the rarest subtype of SCLC due to low expression and lack of exclusivity in clinical cases[127]. Silencing YAP1 by RB1 mutation is found to be important for SCLC cells to metastasize[157]. However, the YAP1 subtype exhibits the worst clinical outcome comparing to other SCLC subtypes and acquires a distinct inflammatory phenotype including high interferon-ɣ response genes, PD-L1 and T-cell functional impairment[158,159]. YAP1 is known to be a transcription co-regulator downstream of the canonical Hippo pathway in controlling organ size and development[160]. Its involvement in tissue regeneration and cancer is extensive[161,162]. Interestingly, YAP1 activity defines pan-cancer into binary classes (YAP on/YAP off) with distinct adhesion phenotypes and vulnerability[163]. The YAP off solid cancer group has neuroendocrine features and loss of RB1, including retinoblastoma, SCLC and SCNPC[163]. YAP1 is also found to drive chemoresistance and change cell fate from neuroendocrine to non-neuroendocrine by induction of REST expression[164].

These four subtypes of small cell carcinoma in prostate and lung defined by these transcription factors are the current foundation in the field to classify clinical cases and identify unique molecular vulnerabilities for targeted therapies. However, with more single cell resolution-based studies, a mixture of these four transcription factors defined cell population have been identified within the same tumor, suggesting a high intra-tumoral heterogeneity as an emerging challenge in targeting small cell carcinoma[130].

1.3.5 Critical transcription factors in SCNPC

Neuroendocrine trans-differentiation requires a rewrite of the transcriptional landscape coordinated by various transcription factors[165]. Several transcription factors have been shown to be responsible for the neuroendocrine trans-differentiation and the survival of small cell cancer cells. For example, BRN2 (also known as POU3F2), is an AR-dependent transcriptional driver for neuroendocrine differentiation in CRPC[86]. Continuous investigation of BRN2 led to a first-in-field orally available inhibitor through the disruption between BRN2 and DNA in SCNPC[166]. Moreover, BRN4 is found to be actively released in prostate cancer extracellular vesicles with BRN2, promoting CRPC to SCNPC transition[167]. In SCLC, ablation of BRN2 significantly decreases neuroendocrine genes such as NCAM1, ASCL1 and CHGA, and vice versa. TTF1 is subsequently identified as a lineage-specific transcription factor through co-expression with BRN2 in SCLC[168].

One of the transcription factors that is BRN2-dependent is SOX2[86]. SOX2 belongs to SRY homolog box protein family. It is well known as one of the reprogramming factors in stem cell biology and has a pivotal role from embryogenesis to adult homeostasis[16,169,170]. In cancers, SOX2 amplification is found in about 27% of the SCLC clinical samples[171,172]. It is identified as an oncogenic driver for neuroendocrine transformation and required for cancer cell proliferation in SCLC[171,173], exhibiting higher sensitivity upon inhibition in the ASCL1 subtype than in the YAP1 subtype[174]. In addition, SOX2 is found to be indispensable for activating lineage plasticity and antiandrogen resistance in P53-RB1 negative prostate cancer[73] alongside the previous report that SOX2 is required for tumor initiation in the RB1 loss retinoblastoma[175]. Despite direct targeting of SOX2 being a challenge in small cell carcinoma, research has shown that the exportin 1 inhibitor, Selinexor, prevents neuroendocrine differentiation through down regulation of SOX2 in SCLC[176].

ONECUT2, also known as OC-2, is associated with a wide range of biological processes including cell proliferation and differentiation in both normal and cancer development[177,178]. In SCNPC, ONECUT2 is identified as a targetable master regulator of the AR signaling network[179,180]. The underlying molecular mechanism is the activation of SMAD3 dependent regulation of hypoxia

signaling in SCNPC, leading to the possibility of targeting hypoxic metastatic CRPC with a hypoxia-activated prodrug such as evofosfamide (TH-302)[180].

1.4: Bioinformatic Deconvolution of Differentiation – From Bulk to Single Cell Resolution

1.4.1 Transcriptional network and chromatin accessibility in small cell carcinoma

With multiple transcription factors identified in regulating neuroendocrine trans-differentiation, more systematic and data-driven approaches have been applied to deconvolute the overall transcriptional network, to investigate the relationship between these transcription factors and associated phenotypes. For example, simulation analysis by Boolean modeling identifies a "hybrid" phenotype that explains the tumoral heterogeneity in SCLC, by using attractor states that are defined by neuroendocrine or mesenchymal signatures[181]. Similarly, the other research group adopts an archetype approach to further characterize the transition of the cellular states and suggests that cell plasticity may be driven by the response to the microenvironmental perturbations and treatments in SCLC[182]. Other analyses rely on more of a structural graph theory concept that recognizes the critical components in a complex network, termed "hubs", which helps generate more leads for functional testing of the targeted pathways in SCLC[183].

Gene expression data from bulk RNA sequencing is crucial for studying the above-mentioned transcriptional network. However, to determination of which and how transcription factors regulate the transcriptional program will rely on chromatin accessibility analysis by the use of Assay of Transposase Accessible Chromatin (ATAC) sequencing. ATAC-sequencing utilizes the hyperactivity of Tn5 transposase with a simple protocol to allow epigenetic profiling at the open chromatin regions[184]. The application of ATAC-sequencing is extensive across fields and downstream analyses such as motif enrichment, nucleosome positioning and transcription factor footprinting are useful tools for studying transcriptional control and epigenetic regulation[185].

1.4.2 Single cell RNA based technology in studying lineage differentiation

Next generation sequencing revolutionized genomic research by providing higher efficiency and throughput capabilities than Sanger sequencing[186]. In cancer studies, bulk RNA sequencing contributes to the most widely available datasets such as TCGA, Cancer Cell Line Encyclopedia, and DepMap[187,188], for its predominant usages in studying general trends, tumor classification, and gene expression patterns in cancers[189]. With the advanced developments in sequencing technologies, single cell-based sequencing offers higher resolution than bulk sequencing, in terms of studying cellular heterogeneity, differentiation status, cell types and the transcriptional landscape of individual cells[188,190,191] .

One of the most common applications of single cell-based technology in studying cellular differentiation is single cell RNA sequencing. It offers a powerful approach in reconstructing differentiation trajectories within a single cell by different algorithm and methods, including inference-based lineage tracing and prediction of differentiation states[192,193]. This type of prediction algorithm, though does not necessarily reflect genetic relationship, overcomes the challenge in experimentally labeling a cell with a heritable mark and tracking its progeny[194]. To name a few, CytoTRACE is a framework that is based on one simple observation: total number of genes expressed in a cell decreases during differentiation. Validation of the platform has been shown in identifying less-differentiated cell population with target genes that contribute to treatment resistance within the luminal progenitor epithelium in breast cancer[195]. RNA velocity, which utilizes the dynamic relationship between unspliced and spliced RNA to predict the differentiation status of a given cell[196]. The extensive application of RNA velocity unravels the transcriptional kinetics of embryonic brain development, pancreatic endocrinogenesis and identification of transient cellular states[196-198]. Another bioinformatic tool such as Monocle2 is based on reverse graph embedding, a type of machine learning, to predict the differentiation state with no requirement of priori genes in the biological process[199].

The other wide application of single cell RNA based technology is *in situ* hybridization (ISH). With the advancement from traditional radiolabel based fluorescence ISH to non-radioisotopic technology, the technique has evolved to allow multiplex detection and spatial arrangement of single cells on tissue sections[200]. One of the popular platforms for researchers is RNAscope. RNAscope utilizes a proprietary double-Z branched DNA probe design strategy to eliminate non-specific hybridization[201]. It allows detection of lower expressed genes and multiple genes in one setting, both of which are the limitation for traditional ISH technique, and has been used to support multiple clinical biomarker discoveries and transcriptional basic science studies since its invention in 2012[201,202].

1.4.3 Studying small cell carcinoma using a multi-omics approach

Small cell carcinoma is a heterogeneous malignancy and challenging to study due to limited models and clinical materials. Efforts have been put into maximizing the usage of the samples and conducting more comprehensive transcriptomic profiling, such as single cell RNA sequencing on needle biopsy samples and small pieces of resected tumors. By comparing over 50,000 single cell transcriptomes of SCLC to lung adenocarcinoma from patient biospecimens, Chan et al identifies a PLCG2-high subpopulation that is responsible for the pro-metastatic phenotype in SCLC[203]. In SCNPC, Dong et al profiles over 20,000 single cell transcriptomes from needle biopsy samples of 6 CRPC patients and determines that neuroendocrine trans-differentiation arises from a luminal-like rather than basal cell compartment[204]. These studies provide valuable insights into tumor heterogeneity and the clinical relevance in studying the pathogenesis of small cell carcinoma.

A multi-omics approach includes usage of a combination of different sequencing and profiling methods to deconvolute a complex biological system[205]. It is a powerful integrative approach to characterize samples and allow more in-depth analysis from various perspectives. In studying neuroendocrine trans-differentiation, Tang et al uses ATAC-sequencing, RNA-sequencing, and

DNA sequencing to profile CRPC organoids, patient-derived xenografts, and tumor derived cell lines. The study identified four subtypes including AR dependent, neuroendocrine, Wnt-dependent and stem cell-like that guide therapeutic decisions[206]. With single cell resolution, Han et al combined both single cell RNA and ATAC-sequencing in a pre-clinical *in vivo* study to investigate the FOXA2-driven lineage plasticity[75]. In SCLC, integration of genomics, transcriptomics, proteomics, and phospho-proteomics of 112 paired tumor and adjacent lung tissues from SCLC patients yields subtype-specific therapeutic strategy[207].

With the advancement of sequencing technology and immense studies on the epigenetic regulation in small cell cancers, we set forth to understand the chronical order of transcriptional changes and to pinpoint each above-mentioned transcription factors during the neuroendocrine trans-differentiation. We performed a comprehensive transcriptomic profiling on an established small cell carcinoma model (termed PARCB)[79] using multi-omics sequencing[208]. The study reveals distinct transcriptional patterns of gene expression and chromatin accessibility at each stage of the SCNPC development, along with single cell based sequencing and histopathological analysis.

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Chapter 2: Temporal evolution reveals bifurcated lineages in aggressive neuroendocrine small cell prostate cancer trans-differentiation

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SUMMARY

Trans-differentiation from an adenocarcinoma to a small cell neuroendocrine state is associated with therapy resistance in multiple cancer types. To gain insight into the underlying molecular events of the trans-differentiation, we perform a multi-omics time course analysis of a pan-small cell neuroendocrine cancer model (termed PARCB), a forward genetic transformation using human prostate basal cells and identify a shared developmental, arc-like, and entropy-high trajectory among all transformation model replicates. Further mapping with single cell resolution reveals two distinct lineages defined by mutually exclusive expression of ASCL1 or ASCL2. Temporal regulation by groups of transcription factors across developmental stages reveals that cellular reprogramming precedes the induction of neuronal programs. TFAP4 and ASCL1/2 feedback are identified as potential regulators of ASCL1 and ASCL2 expression. Our study provides temporal transcriptional patterns and uncovers pan-tissue parallels between prostate and lung cancers, as well as connections to normal neuroendocrine cell states.

INTRODUCTION

Small cell neuroendocrine (SCN) cancer is an aggressive variant that arises from multiple tissues such as the lung and prostate [1,2]. SCN is characterized by its histologically defined small cell morphology of densely packed cells with scant cytoplasm, poor differentiation, and aggressive tumor growth, as well as expression of canonical neuroendocrine markers including SYP, CHGA and NCAM1 [3]. In addition to their phenotypic resemblance, SCN cancers across multiple tissues show a striking transcriptional and epigenetic convergence in clinically annotated tumors [4,5]. This molecular signature convergence is recapitulated by our established SCN transformation model that utilizes either normal lung epithelial cells, patient-derived benign prostate epithelial or bladder urothelial cells as the cells of origin [6,7].

Small cell neuroendocrine prostate cancer (SCNPC) occurs either *de novo* (<1% of untreated prostate cancer cases), or through therapy-mediated transversion of castration resistant prostate cancer (CRPC) (~20% of the resistance cases). CRPC is a resistant variant of prostate adenocarcinoma (PRAD), which often responds to androgen deprivation therapy [8,9]. Trans-differentiation from PRAD to the SCNPC state entails complicated epigenetic reprogramming at the chromatin level, resulting in transcriptional changes driven by a number of key master regulators [10,11]. For example, methylation modulated by EZH2 and activation of transcriptional programs by SOX2 are required in *TP53* and *RB1* loss-mediated neuroendocrine differentiation in mouse transgenic models of SCNPC [12,13]. Oncogenic mutation of FOXA1 potentiate pioneering activity and differentiation status of prostate cancer [14,15]. Lastly, knockdown of transcription factors such as ONECUT2 has been shown to inhibit SCN differentiation [16,17]. While the importance of these factors has been demonstrated, the chronological sequence of the associated epigenetic and transcriptional changes remains uncharacterized during the progression to SCNPC. Examination of the temporal evolution of lung cancer revealed a connection between transcription factor defined subtypes and cell plasticity [18,19]. In our study, we sought to answer the following

questions: 1) when do SCN-associated transcription factors emerge during SCNPC progression, 2) how do they coordinate SCN differentiation, and 3) can we identify a transition state defined by transcription factors that can be targeted?

Leveraging our previously developed human pan-small cell neuroendocrine cancer model, the PARCB forward genetics transformation model (driven by knockdown of *RB1*, alongside exogenous expression of dominant negative *TP53*, *cMYC*, *BCL2* and myristoylated *AKT1* via three lentiviral vectors) [6,7], tumor samples were harvested at different time points for multi-omics analyses. The transcriptional and epigenetic status of each time point was determined using integrative bulk RNA sequencing, ATAC sequencing, and single cell RNA sequencing. This longitudinal study provides insight into the temporal evolution of the epigenetic and transcriptional landscape during trans-differentiation and small cell cancer progression. We found consistent transcriptional patterns and differentiation trajectories across samples generated from independent patient tissues, as well as a bifurcation of end-stage neuroendocrine lineages, defined by ASCL1 and ASCL2 and their associated programs.

Achates-scute complex (AS-C) proteins are basic helix-loop-helix (bHLH) transcription factors, first identified in *Drosophila Melanogaster* [20]. They are important in the development of peripheral nervous systems and sensory organs [21]. Mammalian ASCL1 is a well-known neuroendocrine transcription factor in small cell cancers [22-24]. Independently, ASCL2 is involved in embryonic development, colorectal stem cell biology and cancer [25-30]. ASCL2 is largely understudied in SCNPC, mainly shown to be co-expressed with POU2F3 in non-neuroendocrine cell populations [5,31]. Here, our study reveals temporal transcriptional patterns during small cell neuroendocrine differentiation in prostate cancer and associated lineage programs governed by general mutually exclusivity between ASCL1 and ASCL2. Follow-up analysis elucidated a transcriptional network circuity between ASCL1, ASCL2, and the transcription factor TFAP4 which was implicated by the trajectory data.

Temporal gene expression programs of the PARCB transformation model reveal SCNPC trans-differentiation pathways

To determine the timing of SCN differentiation events during prostate cancer development, we utilized the PARCB model system [6]. Independent transformations were performed on basal cells extracted from benign regions of epithelial tissue from 10 prostate adenocarcinoma patients. Basal cells were transformed by the oncogenic lentiviral PARCB cocktail and subsequently cultured in an organoid system *in vitro*[6]. Transformed organoid-expanded cells from each patient tissue sample were subcutaneously implanted into multiple immunocompromised mice to allow for time-course collection of tumors from the matched starting material **(Figure 1A)**. The tumors were collected at approximately two-week intervals until reaching 1 cm^3 in size or occurrence of ulceration, whichever came first. The transformed tumor cells were triply fluorescent due to the lentiviral integration[6], which allowed for cancer cell purification by fluorescence-activated cell sorting (FACS) followed by multi-omics sequencing and analysis **(Figure 1A)**. Each patient series (P1-P10) contains five to six time point samples ranging from basal cells (TP1) to organoids (TP2) to tumors (TP3-TP5/TP6) **(Figure 1A)**. Upon histological examination of the tumor issues by pathologists, we found that the time course tumors transitioned from squamous, to adenocarcinoma, then to mixed and eventually SCN phenotypes **(Figure 1A and Figure S1A-C).** Furthermore, clinically defined neuroendocrine markers, including SYP and NCAM1, emerge during the transition to late stages of the tumor progression **(Figure 1A).** The basal cell marker p63 were only positive in early-stage tumors by immunohistochemistry (IHC) staining **(Figure S1D)**.

We first performed a temporal analysis of gene expression using bulk RNA sequencing to understand the changes in the transcriptional landscape during SCNPC trans-differentiation. By projection of PARCB samples onto principal component analysis (PCA) of clinical lung and

prostate cancer tumor samples[4,10,32-36], we validated that PARCB time course samples follow the transcriptionally defined convergence trajectory from adenocarcinoma to SCN states **(Figure 1B and Figure S1E).** Additional SCNPC associated factors including *ASCL1* and *NEUROD1* were also elevated during the progression **(Figure 1C)**. The mRNA of androgen receptor (AR) was expressed in tumors at the early stage (**Figure 1C**), but the protein level was not detectable by immunostaining **(Figure S1D).** Taken together, the histological and omics data indicate that PARCB time course tumors recapitulate both the phenotypic and transcriptional changes observed in the clinic and provide a model system for studying the temporal evolution of SCNPC development.

To determine the transformation trajectories among the time course series generated from the 10 independent patient samples (P1-P10), we performed clustering and PCA of the transcriptomic data. To account for potential asynchronous development among each patient series and each individual tumor, we defined hierarchical clusters (HCs) of samples by their corresponding differential gene modules and found the resulting 6 clusters (HC1-6) to generally correspond with the time of collection **(Figure 1D and Table S1A).** This provides a clustering-based trans-differentiation reference frame and informs our subsequent multi-omics analyses. Unsupervised PCA demonstrates that the individual transformation paths of each series follow a generally consistent "arc-like" trajectory with a discernable bifurcation in late-stage samples **(Figure 1E, Figure S1E-F and Table S1B).** The late tumors were hence further defined as "Class I" and "Class II" tumors with correspondent HC5 and HC6 gene modules, respectively. HC2 to HC6 had elevated SCNPC signature scores compared to adenocarcinoma signature score **(Figure S1G)**. This last finding supports the existence of two transcriptional programs or end points defining the terminal SCNPC tumor phenotypes.

Gene ontology enrichment analysis of the corresponding 6 differential gene modules identified biological processes enriched uniquely or shared among HCs, including Inflammatory response

(HC1 and HC3, patient derived basal cells and early tumors, respectively), cell proliferation (HC2, *in vitro* organoids), epidermis development (HC3, early tumors), cell activation (HC4, transitional tumors), stem cell differentiation (HC5, Class I late tumors) and neuro-/chemical synapse (HC5 and HC6, both classes of late tumors) **(Figure 1E and Table S1C)**. The transcriptome evolution supports the idea that trans-differentiation from adenocarcinoma to the SCN state is a systematically coordinated process, that involves a transitional stage followed by bifurcated pathways enriched in neuronal/neuroendocrine gene signatures.

Sequential transcription regulators modulate reprogramming and neuroendocrine programs through a highly entropic and accessible chromatin state

Temporal analyses on single transcription factor defined subtypes of small cell lung cancer (SCLC) models have delineated lineage plasticity in the development of lung neuroendocrine tumors [18]. We sought to define the transcriptional evolution in SCNPC through an extensive survey of over 1600 transcription factors [37] by chromatin accessibility analyses using ATAC sequencing [38]. A significant increase in overall accessible chromatin peaks across chromosomes is observed starting at the tumors at transitional stage (HC4) to late stages (HC5 and HC6) **(Figure 2A).** Unsupervised PCA using ATAC-sequencing data showed an arc-like and bifurcated trajectory consistent with the pattern observed using the RNA-sequencing data **(Figure 1D and 2B).** The Shannon entropy has been used to estimate the plasticity potential of a biological sample to change cellular state [39,40]. We found that transitional samples (HC4) have the highest entropy **(Figure 2B),** suggesting there exists a high potential and less well-defined transcriptional state during the trans-differentiation process.

To identify transcription factors that recognize the chromatin accessible regions at each stage of the transformation trajectory, we first looked at the overall accessibility near the transcription start

sites (TSS) **(Figure 2C).** Transitional samples (HC4) have a strong increase in the accessible

peaks as estimated by Shannon entropy calculations **(Figure 2B and 2C)**, consistent with the

gene-expression-based entropy calculations **(Figure S2A).** Next, motif enrichment analysis was

performed on the accessible peaks from each HCs in a "one versus the rest" fashion. Since

transcription factors from the same family share similar motifs and are deposited into a variety of

databases, we used a pipeline that applies an ensemble of existing computational tools and suites

of motifs (*de novo* and known) [41] **(Figure 2D and Table S1D).** Motif enrichment analysis

implicated that 1) representative stress-responsive factors such as NFkB, JUN, ATF and STAT

proteins were active from early to transition stage (HC1-4), 2) reprogramming factors such as

POU/OCT and SOX families were active in Class I (HC5) tumors, and 3) neuronal/neural factors

including ASCL and NEUROD family proteins were found at the later stage in Class II (HC6)

tumors **(Figure 2D).** Due to ASCL1, ASCL2 and other bHLH factors sharing the same E-box motif,

and the stringent "one HC versus the rest" differential accessibility analysis, the motif suite

containing ASCL1 and ASCL2 factors is highly enriched and ranked in HC6 compared to HC5

(Figure 2D). Nonetheless, when HC6 is left out of the analysis, HC5 does demonstrate strong

enrichment for the motif suite containing ASCL1 and ASCL2 factors, compared to HC1-4. **(Figure

S2B)**. The enrichment of stem-like and neuroendocrine programs in HC4-5 and HC6, respectively,

was further confirmed by signature scores derived from the literature [33,42] **(Figure S2C).** This

analysis provides a view of the overall transcriptional shift of the chromatin accessibility during

trans-differentiation.

To determine whether expression of the transcription factors corresponds to their inferred activity

from the motif enrichment analysis, we summarized the top ranked transcription factors (based

on PC1, PC2 and PC3 loadings) across the transformation stages (HC1-HC6) **(Figure 2E and

Figure S2D, Table S1E)** from the perspective of the PCA-based transformation trajectory **(Figure

1E and Figure S1F).** Overall, we observed that 1) *AR* mRNA expression is lost during progression

towards late-stage tumors, 2) *FOXA1*, a known transcription factor of SCNPC [14,15], is shown to emerge at the early-transition stage, and 3) well-known neuroendocrine transcription factors such as *ASCL1*, *NEUROD1*, *ONECUT2*, *SOX2, INSM1* and *FOXA2* were increased towards the late stage **(Figure 2E and Figure S2D)**[43-45]. This analysis also revealed additional candidate stage-specific transcription factors that are largely understudied in SCNPC, such as *LTF*, *ESR1*, *ZIC2* and *TBX10* **(Figure 2E).** *ASCL1* and *ASCL2* expression were elevated in the late tumor stages **(Figure 1C and Figure 2E-F).** Notably, their expression was enriched in separate tumor endpoints (HC5: ASCL2+ and HC6: ASCL1+), supporting their probable contribution to the bifurcated trajectories **(Figure 2E-F and Figure S2D).**

Transcription factor-defined cell populations contribute to lineage divergence and tumor heterogeneity

To determine the degree of heterogeneity within the time course tumors, we performed single cell RNA sequencing on four time-defined serial tumor sets: P2, P5, P7 and P8 (TP3-TP6) **(Figure 3A).** Dimension reduction analysis (Uniform Manifold Approximation and Projection, UMAP) was used to visualize the overall distribution of cell populations at each time point of SCNPC development **(Figure 3A-B and Figure S3A).** Overall, a lineage differentiation from basal (*KRT5*+) to luminal (*KRT18*+) was observed from early to late tumors **(Figure 3B-C)**. *YAP1*, whose expression defines a non-neuroendocrine SCLC subtype [46] and whose high expression is frequently seen in CRPC-PRAD but not SCNPC [47], is enriched in the early tumor cell populations **(Figure 3B-C).**

To understand the association of known SCN transcription factors in contributing to intra-tumoral heterogeneity, we first assigned a SCNPC score [33] to each cell **(Figure S3B).** Despite the high SCNPC scores across populations of single cells, the number of *NEUROD1* and/or *ONECUT2*

positive cells is very low, while deeper single cell sequencing depth would be required to fully investigate this result **(Figure 3C and Figure S3B).** Other well-known neuroendocrine transcription factors such as *ASCL1, INSM1* and *FOXA2* are enriched in the same cell cluster with high SCNPC score **(Figure 3C and Figure S3B).** However, in another cell cluster, *ASCL2, POU2F3* and *SOX9* were co-expressed with a medium level of SCNPC score **(Figure 3C and Figure S3B).** The general mutual exclusivity of *ASCL1* and *ASCL2* in single cells further supports ASCL1 and ASCL2 contributing to the bifurcated endpoint trajectories observed in the bulk tumors **(Figure 3C and Figure S3C-D).**

Single cell datasets available as reference from longitudinal clinical samples in advanced prostate cancer are rare, thus a cell type inference analysis using reference pure cell types was applied to infer the identity of individual cells in PARCB tumors [48]. Five out of a total of 36 reference cell types from the Human Primary Cell Database were highly enriched in the PARCB time course tumor samples **(Figure 3D).** All tumor cells share a similar transcriptome as epithelial cells **(Figure 3D).** Particularly, a majority of tumor cells (other than early-stage cells) exhibit stem-like gene expression patterns reflective of embryonic stem cells and induced pluripotent stem cells, indicative of a de-differentiation shift during SCNPC development and trans-differentiation **(Figure 3D)**. Additionally, later-stage cells expressing either *ASCL1* or *ASCL2* had neuronal-like gene expression profiles, confirming the emergence of SCN differentiation **(Figure 3B-D).**

Single cell analysis supports the overall gene expression and chromatin accessibility patterns observed in bulk tumors. Projection of single cells onto the PCA framework generated from bulk RNA-sequencing samples **(Figure 1E and Figure S1F)** demonstrated that tumors clustering distinctly by bulk RNA-sequencing indeed consist primarily of single cells in the corresponding different transcriptional states, with some degree of heterogeneity **(Figure 3E).** Furthermore, transcription factors with high expression in tumors defined by bulk RNA-sequencing analysis **(Figure 2E**) show heterogenous patterns among single cells **(Figure S3E)**. Tumors at transitional

stage (HC4) and late stage (HC5) have the highest degree of gene fluctuation, further highlighting a potential role for increased intratumoral heterogeneity during the trans-differentiation process **(Figure S3E)**. Importantly, we further validated the mutually exclusive expression pattern of *ASCL1* and *ASCL2* in multiple clinical and GEMM single cell RNA-sequencing datasets[31,49-51]. This analysis confirmed that ASCL2 is generally enriched in non-NE cells/adenocarcinoma and ASCL1 is more abundant in high NE cells/SCNPC clinically **(Figure 3F)**, consistent with the PARCB temporal study **(Figure S3F)**. ASCL1 and ASCL2 double-positive cells are observed at a low frequency, primarily in SCNPC tumors, and may reflect a transitional state between adenocarcinoma and SCN phenotypes **(Figure 3F)**.

ASCL1 and ASCL2 specify independent transcriptional programs and sub-lineages in SCNPC

Given that *ASCL1* and *ASCL2* expression levels are mutually exclusive in single cells, we asked whether ASCL1 and ASCL2 represent separate cellular sub-lineages by inferred clonal tracing analyses[52]. With KRT5 (basal marker) set as the beginning of the tracing, the inferred tracing results in three primary lineage branches/states **(Figure 4A).** As hypothesized, single cells expressing either *ASCL1* or *ASCL2* are enriched in different lineage branches **(Figure 4A-B)**. This result is further supported by a different analytic tool (RNA velocity) that measures the temporal ratio of un-spliced to spliced mRNAs to infer lineage trajectory [53] **(Figure S4A).** The inferred clonal tracing results complemented the real-time-based analysis visualized as the total composition of ASCL1- or ASCL2- positive, double positive and negative populations over time **(Figure 4C),** supporting that ASCL1 and ASCL2 are associated with independent sub-lineages. Double-positive cells are very infrequent in the PARCB temporal tumors. The double-positive population observed in the P2-TP5 tumor may capture the cells undergoing the transitional state

(Figure 4C), and the overall low double-positive frequency is consistent with the clinical results above **(Figure 3F)**.

To further characterize the transcriptional difference between cells expressing a high level of *ASCL1* or *ASCL2*, we analyzed their differential gene expression profiles **(Figure 4D and Table S1F)**. Genes that are involved in synaptic and neuroendocrine regulation such as *DDC*, *CACNA1A* and *INSM1* are enriched in *ASCL1+* cells. *ASCL2+* cells express genes with stem-like characteristics such as *SOX9* and *POU2F3* **(Figure 4D)**. SOX9 is directly regulated by ASCL2 in intestinal stem cells [29], suggesting a possible contribution to stem-like properties in SCNPC trans-differentiation. Upon further investigation, we observed that genes implicated in the intestinal stem cell program such as *EPBH3* and *TNFRSF9* [29] are positively correlated with *ASCL2*, but not *ASCL1* **(Figure S4B)**. In contrast, a well-known intestinal stem cell marker, *LGR5* [54], has no correlation with either *ASCL1* or *ASCL2*, consistent with it having a more tissue specific intestinal role **(Figure S4B)**.

To identify the transcriptional programs that are associated with either ASCL1 or ASCL2 in prostate cancer, we constructed an inferred network [55] using multiple bulk RNA sequencing prostate cancer and model datasets including The Cancer Genome Atlas (TCGA), additional patient tumor (Beltran), and SCNPC model (Park) datasets [6,33]. The analysis identified 336 and 352 genes regulated independently by ASCL1 or ASCL2 **(Figure 4E and Table S1G)**. Strikingly, there are only 5 genes from the inference analysis that are regulated by both factors: *TMEM74*, *RGS16*, *LHFPL4*, *CDCA7L* and *SOX2* **(Figure 4E)**. This result is consistent with the demonstrated role of SOX2 in regulating neuroendocrine differentiation in null *TP53* and *RB1* backgrounds [13], hence showing that SOX2 is involved in both ASCL1 and ASCL2 associated neuroendocrine sub-lineages. Genes that are regulated by ASCL1 are enriched in neuroendocrine differentiation markers and factors such as *SYP*, *CHGA*, *NCAM1*, and *NEUROD1* **(Figure 4E)**. ASCL2 is associated with genes including *PTGS1/COX1*, *POU2F3*, *ANXA1* which

are generally immune and stress responsive, and stem-like **(Figure 4D-E).** We further confirmed

that PARCB tumor-derived cell lines from different tissues of origin (prostate, bladder, and lung)

all have only one or the other gene expression patterns associated with either ASCL1 or ASCL2

expression **(Figure 4F and Figure S4C).** We next validated the predicted transcriptional

programs of ASCL1 and ASCL2 by exogenously expressing either ASCL1 or ASCL2 in ASCL2+

or ASCL1+ cell lines, respectively. ASCL1 exogenous expression in ASCL2+ cells, increased the

ASCL1 transcriptional program as indicated by increased signature score (**Figure S4D**). However,

ASCL2 exogenous expression in ASCL1+ cells, did not have notable effect, suggesting that the

ASCL1 endpoint state has higher stability (**Figure S4D**).

In situ hybridization of *ASCL1* and *ASCL2* mRNA in the transitional PARCB tumor samples further

confirmed the mutually exclusive expression pattern **(Figure 4G).** The staining patterns

demonstrated both *ASCL1* and *ASCL2* mixed populations **(left, Figure 4G)**, as well as patch

regions potentially resulting from local clonal expansion **(right, Figure 4G)** of *ASCL1+* or *ASCL2+*

cells. Our combined results support that ASCL1 and ASCL2 define independent cellular sub-

lineages and transcriptional programs with stem-like and neuroendocrine enrichment in SCNPC.

ASCL1 and ASCL2 as pan-cancer classifiers

Clinical subtypes are fairly well-defined in SCLC [46,56], but molecular subtyping remains an evolving

challenge in SCNPC [8]. By performing projection analysis of our samples onto a gene expression

or chromatin accessibility PCA framework defined by the Tang et al. dataset of patient metastatic

CRPC phenotypes[57], we found that PARCB temporal samples share similar transcriptome and

epigenome signatures, including a shared stem-cell like (SCL) group and a shared NEPC group

(Figure 5A).

Given the high degrees of similarity in transcriptional profiles of SCLC and SCNPC [4], we compared our HC classification of the PARCB time course samples to the SCLC clinical subtypes: ASCL1 (A), NEUROD1 (N), POU2F3 (P) and YAP1 (Y) **(Figure 5B)** [32,46]**.** The class I/ASCL2+ (HC5) tumor group shares transcriptome similarity with SCLC-P **(Figure 5B),** which is consistent with the co-expression pattern of ASCL2 and POU2F3 observed in multiple analyses **(Figure 3C and 4D)**. Likewise, and concordant, the Class II/ASCL1+ (HC6) tumor group is transcriptionally aligned to SCLC-A **(Figure 5B).**

To investigate whether the ASCL1 and ASCL2 sub-classes from PARCB temporal study recapitulate patterns observed in clinical samples of prostate cancer, we compared *ASCL1* and *ASCL2* expression in PARCB temporal samples versus numerous clinical profiling datasets[10,33-36]. The results demonstrate that the expression levels of *ASCL1* and *ASCL2* are comparable between the PARCB model and clinical samples, and the transcriptional patterns of HC1 to HC6 generally corresponded with the transition from PRAD/CRPC-PRAD to SCNPC **(Figure 5C)**. We further confirmed the general mutual exclusivity and low double positivity of *ASCL1/2* expression using an RNA *in situ* hybridization assay on both CRPC-PRAD and SCNPC clinical samples and CRPC PDX models **(Figure 5D and Figure S5A-B)**.

By comparing the expression levels of *ASCL1* and *ASCL2* across a broad panel of pan-cancer cell lines, we found that almost all cancers, apart from lung cancers, can be divided into three categories (i) demonstrating expression of *ASCL1* (neuroblastoma), (ii) of *ASCL2* (colorectal and breast cancers), and (iii) double negative (other cancers) **(Figure 5E).** Only SCLC and other lung cancer cell lines have mixed levels of *ASCL1* and *ASCL2*. Combined with our results, this suggests a potential role for ASCL2 and POU2F3 in specifying intermediate, and/or heterogenous states in (small cell) lung cancer **(Figure 5E).** Protein expression analysis in lung squamous carcinoma (NCI-H1385), SCLC-A (NCI-H1385, NCI-H146 and DMS79), SCLC-P (NCI-H526 and COR-L311) and SCNPC (NCI-H660) cell lines further highlighted a mutually exclusive pattern of

ASCL1 and ASCL2 **(Figure S5C).** SCLC-N (NCI-H1694) is double negative for ASCL1 and ASCL2 and positive for NEUROD1 as expected **(Figure S5C).** Last but not the least, in patient tumor pan-cancer data, the exclusive expression of either *ASCL1* or *ASCL2* is again observed, highlighting that binary distinctions defined by ASCL1 and ASCL2 occur across multiple tissue types **(Figure 5F).** In sum, an inverse and generally mutually exclusive relationship between ASCL1 and ASCL2 is observed in multiple and pan-cancer contexts, and mutual exclusivity is strongly observed at the single cell level.

Alternating ASCL1 and ASCL2 expression through reciprocal interaction and TFAP4 epigenetic regulation

With the evidence that *ASCL1* or *ASCL2* expression levels are mutually exclusive in single cells during SCNPC trans-differentiation, we explored two hypotheses: 1) These two factors mutually regulate each other's expression, or 2) they share a common upstream transcription factor that alternates their transcription through regulated differential binding to respective gene regulatory elements. To test the first hypothesis, we expressed V5-tagged ASCL2 in multiple PARCB tumor derived cell lines (lung and prostate) and observed that ASCL1 protein expression was significantly suppressed in these cells **(Figure 6A).** In contrast, expression of V5-tagged ASCL1 increased ASCL2 expressions both at protein and mRNA levels **(Figure 6A and Figure S6A).** Thus in our model cells, ASCL1 and ASCL2 mutually regulate each other at the protein level, but each in the opposite manner.

To test the second hypothesis of a common regulator, known promoter and enhancer regions of *ASCL1* and *ASCL2* were first annotated in the PARCB time course ATAC-sequencing data **(Figure 6B).** An opposing pattern of open and closed chromatin formation is found on both the *ASCL1* promoter and the *ASCL2* enhancer regions **(Figure 6B).** A rank list of transcription factors

that have matching motifs in the regions was generated to determine potential shared regulators [58] **(Figure 6C and Table S1H).** An extensive literature search of all the factors whose motifs were found in both *ASCL1* and *ASCL2* regulatory regions revealed that TFAP4 was reported to form different transcription complex to either activate or repress target genes, including facilitating epithelial-to-mesenchymal transition in colorectal cancer and repressing neuronal programs in non-neuronal cells [59,60]. The TFAP4 motif was shared in both the *ASCL1* promoter (ranked 2nd) and the *ASCL2* enhancer region (ranked 6th) in the top 8 list of shared transcription factor motifs **(Figure 6C)**, and is expressed across all the SCLC, SCNPC and PARCB tumor derived cell lines tested **(Figure S5C and S6B)**. Interestingly, NCI-H1385, a lung squamous carcinoma (non-small cell) cell line, has lower TFAP4 expression compared to other small cell neuroendocrine cell lines **(Figure S5C).**

The direct differential binding of TFAP4 to the *ASCL1* promoter and the *ASCL2* enhancer region was confirmed by the CUT&RUN technique [61], a chromatin immunoprecipitation experiment using TFAP4 antibody in both ASCL1+ and ASCL2+ PARCB tumor derived cell lines. Despite cell lines having various degrees of TFAP4 binding signals due to potential mixed cell clones within the cell lines, TFAP4 was found to have higher binding affinity near the *ASCL1* promoter in ASCL1+ cell lines (P7-TP6) than ASCL2+ cell lines (P2-TP6 and T3-TP5) **(Figure S6C).** In contrast, TFAP4 consistently bound to *ASCL2* enhancer regions in ASCL2+ cell lines compared to ASCL1+ cell line **(Figure S6C).** This result supports that TFAP4 potentially regulates transcription of *ASCL1* and *ASCL2* in a context-specific manner.

To determine whether TFAP4 directly regulates the expression of *ASCL1* and *ASCL2*, we introduced a doxycycline-inducible CRISPR sgRNA targeting *TFAP4* in multiple ASCL1+ and ASCL2+ cell lines, including PARCB tumor-derived cell lines and the patient-derived cell line NCI-H660. Both ASCL1 and ASCL2 expression decreased, with various strength, after TFAP4 knockout was induced by the addition of doxycycline in the respective cell lines **(Figure 6F and**

Figure S6D). However, other lineage associated proteins did not change **(Figure 6F and Figure S6D).** Cell growth assays showed a mild decrease in P7-TP6 (ASCL1+) cell growth, and in contrast a drastic increase in P3-TP5 (ASCL2+) growth upon the knockout of TFAP4 **(Figure 6E).** To explore the clinical relevance of TFAP4 in cancer and SCNPC, we surveyed the expression of *TFAP4* across subtypes of cancers compared to normal tissue. There is a substantial increase in *TFAP4* expression in small cell cancers compared to adenocarcinoma, and compared to normal tissue, in both prostate and lung cancer indications **(Figure 6F),** as well as in pan cancer tumors (TCGA) vs. normal tissue (GTEx) **(Figure S6E).**

Thus in our transcriptional regulatory circuit studies, we found a reciprocal, non-symmetric regulatory relationship between ASCL1 and ASCL2; and that within this circuit, ASCL1 and ASCL2 have a shared positive regulatory factor, TFAP4. In the sum of our studies, the PARCB model provided a blueprint of SCNPC trans-differentiation as specified by temporal transcription factors **(Figure 6G).** In particular, ASCL1 and ASCL2 define distinct bifurcated sub-lineage trans-differentiation trajectories in small cell cancers, and binary transcriptional profiles in a pan-cancer context.

DISCUSSION

SCNPC has a rare *de novo* presentation, however, trans-differentiation from prostate adenocarcinoma to SCN cancer is a frequent adverse consequence of cancer cells acquiring resistance to therapeutics repressing AR signaling [8,9]. In a pan-cancer context, therapy-induced trans-differentiation from adenocarcinoma to SCN cancer is a growing clinical challenge in lung cancer with the expansion of effective targeted therapies, such as EGFR, ALK, BRAF, KRAS inhibitors [62]. Genetically engineered mouse models of SCNPC and SCLC have been generated to provide insight into the tumorigenesis of SCN cancers [12,18,31,43,63,64], with some models demonstrating evidence of the adenocarcinoma to SCN cancer transition [13,31,65,66]. Patient tumor-derived organoids and circulating tumor cells have also provided models for monitoring differentiation state transitions [50,67], including reversion to non-SCN states via specific signaling inhibition [50]. Our PARCB froward genetics *in vivo* temporal transformation model further adds to these resources by being human cell-based, recapitulating the adenocarcinoma to SCN phenotype trans-differentiation at both the histological and molecular signature levels, and providing the temporal resolution to reveal an arc-like plasticity trajectory and associated stem cell-like (reprogrammed) intermediate states. A limitation of the PARCB model is that inhibition of the AR axis is not an initiating component of the trans-differentiation process.

Such an arc-like trajectory is commonly observed in unbiased profiling of development and differentiation processes, including in cancer contexts [39,68-74]. The pattern is reminiscent of temporal regulation in development, with the differentiation transition stage promoted by temporally regulated epigenetic and transcriptomic plasticity programs [75-77].

The transcription profiles of the transition stage from adenocarcinoma to SCNPC provide evidence for an initial de-differentiation or reprogramming step when cells enter the trans-differentiation process, with enrichment of stem cell and iPSC programs. Furthermore, we find samples in the transitional state have a higher degree of entropy at both the epigenetic and gene

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expression level. Our findings are in concordance with a recent study in an adenocarcinoma lung cancer mouse model where a highly plastic intermediate state was seen as cells transitioned from lung hyperplasia to adenocarcinoma [19], and with past observations of increased entropy proceeding differentiation processes [39]. Together these findings support the idea that de-differentiation, and epigenetic loosening and/or cellular heterogeneity are prerequisites for further lineage trans-differentiation during cancer evolution.

At the end-stages, the trans-differentiation trajectory demonstrates a bifurcation, resulting in two neuroendocrine states, one characterized by ASCL2 and POU2F3 expression (Class I tumors), the other by ASCL1 expression (class II tumors). Throughout the trans-differentiation trajectory, individual cells demonstrate mutually exclusive expression of either ASCL1 or ASCL2, with emergence of ASCL2 generally earlier and more prominent than ASCL1. Thus, the ASCL2 state and double positive state may reflect a semi-stable and transitional state. The molecular switch from ASCL2 to ASCL1 demonstrates the dynamic transcriptional control in SCNPC. An analogous temporal shift from FOXA1 to FOXA2 orchestrated transcriptional programs was observed in an independent SCNPC temporal GEMM model [43], and the FOXA1 to FOXA2 transition is reflected in the PARCB model (Figure S2D).

SCLC tumor subtypes are canonically defined by the predominant expression of one of four master regulators (ASCL1, NEUROD1, POU2F3, and YAP1) [46], and tumors expressing ASCL1 have been reported in therapy induced SCNPC [5,51]. Nevertheless, single cell data from mouse models of SCNPC have identified both a distinct cell subpopulation with co-expression and open chromatin accessibility of *ASCL2* and *POU2F3* motifs [31], and a *POU2F3* expression-dominant cell subpopulation [50]. Upon close examination of clinical prostate cancer expression datasets [31,49-51], and upon performing RNA hybridization studies of prostate tumor histology sections, we found that ASCL2+ cells are common in castration-resistant and therapy-exposed prostate cancers. Thus, the previous reports combined with our findings support a potential cancer physiology role

for the ASCL2/POU2F3 subtype in prostate cancer trans-differentiation. In parallel, NEUROD1, a marker of a previously defined prostate (and SCLC) cancer subtype [5,46] has relatively low expression in the PARCB temporal study. However, a *NEUROD1*-expressing cell cluster is situated between the *ASCL1* and *ASCL2* cell clusters in the lineage analysis, suggesting a potential facilitating role in lineage bifurcation and trans-differentiation.

Prior links between master regulators POU2F3 and ASCL2 have previously been reported, such as a unique dependency on ASCL2 in the POU2F3 subtype of SCLC cell lines [78]. Whether ASCL2 and POU2F3 regulate highly overlapping transcriptional targets remains to be determined. One potential mechanism is through the co-activation of E-box and octamer DNA binding by ASCL2 and POU2F3, respectively. This interaction mechanism was observed previously between ASCL1 and POU3F2 (BRN2) in neurogenesis [79]. Further work will help answer if ASCL2 facilitates the transitional stage and/or is a more default program during the de-differentiated transition stage.

Despite sharing similar nomenclature and pro-neuronal properties in the literature [20], ASCL1 and ASCL2 are known to play different roles in stem cell, lineage, and cancer biology [22,29]. ASCL1 is a prominent driver for neuroendocrine differentiation in normal cells [22]. However, recent cancer studies have shown that ASCL1 contributes to high lineage plasticity, resulting in subtype changes via remodeling of the global epigenetic state [18,24]. The role of ASCL2 requires further investigation to determine its balance of compensatory and competitive characteristics in regard to ASCL1 function in small cell neuroendocrine cancers. In our mechanistic studies, we found that increased ASCL1 leads to increased ASCL2 expression, whereas ASCL2 suppresses ASCL1 expression using PARCB tumor-derived cell lines from multiple tissues of origin (prostate and lung). This leads to a future testable hypothesis on whether existence or absence of ASCL2 is required to arrive at an ASCL1-positive neuroendocrine state via trans-differentiation.

A dynamic lineage plasticity among subtypes of SCLC has been reported [18]. However, the triggers and mechanisms underlying cancer cells switching to different lineages remain elusive. In SCNPC,

beyond our discovery of the reciprocal regulation between ASCL1 and ASCL2, our results identified TFAP4 as an additional candidate member of this transcriptional circuitry. In particular, TFAP4 can alternate the expression of *ASCL1* and *ASCL2* by differential binding to *cis* regulatory elements on both genes. TFAP4 has been shown to have both activating and repressing properties in gene regulation through interactions with distinct transcription factors [59,60]. TFAP4 demonstrates substantial increased expression in small cell vs. non-small cell cancers and is elevated in cancers compared to normal tissue. Future mechanistic and functional studies on TFAP4 will help clarify its master regulator role in lineage trans-differentiation in SCNPC and SCLC.

In clinical therapy, different forms of tumor plasticity define the battle grounds for acquired resistance. In the primary prostate cancer setting, the vast majority of prostate cancers are adenocarcinomas while all other histologic types are rare. In the castration-resistant setting, especially with the clinical introduction of next-generation anti-AR therapies, many different variant histology has been observed, including rare cases of squamous carcinoma[80]. In this combat, trans-differentiation to a small cell neuroendocrine state in response to otherwise effective molecular therapies is an emerging challenge across multiple cancer types. The temporal profiling of SCNPC development in the human cell based PARCB model revealed that trans-differentiation from an adenocarcinoma to neuroendocrine state is a temporally complicated, yet systematically coordinated process. The combination of bulk and single cell profiling approaches allowed for the identification of an arc-like trajectory and a transitory period characterized by epigenetic loosening, which are shared in general by other differentiation and development processes. Consistent with genetically engineered mouse SCNPC models, and with the multiple subtypes of SCLC, we find a role for both ASCL1 and ASCL2/POU2F3 in trans-differentiation to SCNPC. The results from our model have provided insight into the regulatory crosstalk between different neuroendocrine master regulators and provide a resource for

identifying candidate approaches for blocking this clinically challenging case of trans-differentiation.

DECLARATION OF INTERESTS

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community.

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AUTHOR CONTRIBUTION

Conceptualization, C.C., J.W.P., T.G.G., O.N.W.; Methodology, C.C., L.W., J.W.P., T.G.G., O.N.W. Investigation, C.C., T.S., L.W., L.T., G.V., N.J.B., Z.M., K.S., W.T., M.B.O., K.M.S., D.C., A.H., R.X., Y.Z., M.N.; Resources, C.M., E.C., P.S.N., Y.Z., J.H.; Writing, C.C., T.G.G., O.N.W.; Data Curation, W.T., Y.Z., T.G.G.; Supervision, T.G.G. and O.N.W.

Figure 1. Temporal gene expression programs of the PARCB transformation model reveal SCNPC trans-differentiation pathways. (**A**) Schematic summary of PARCB time course study and representative Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) staining of neuroendocrine markers (SYP and NCAM1) on sequential tumors from the tissue microarray. Time point (TP1-6) samples were sequenced using bulk RNA sequencing (green circle), bulk ATAC-sequencing (red circle) and/or single cell RNA sequencing (blue circle, tumors only). (**B**) Projection of the PARCB time course samples onto the PCA framework defined by pan-cancer clinical tumor datasets [4,10,32-36]. LUAD: Lung adenocarcinoma. LUAD norm: lung adenocarcinoma adjacent normal tissue. SCLC: small cell lung cancer. PRAD: prostate adenocarcinoma. PRAD norm: prostate adenocarcinoma adjacent normal tissue. CRPC: castration resistant prostate cancer. SCNPC: small cell neuroendocrine prostate cancer. (**C**) Average gene expression of selected SCNPC-associated proteins and markers. (**D**) Heatmap of hierarchical clusters (HC) of samples (columns) and corresponding differentially upregulated gene modules (rows). Differential expression defined by one HC vs all other HCs). (**E**) PCA of the PARCB time course samples and trans-differentiation trajectories including primary arc and secondary bifurcation. A 3-dimensional rotatable version of this figure is available on the PARCB Multi-omics Explorer website. [For review, a 3D rotatable version is included as Data S1.] (**F**) Selected enriched GO terms across HC. See also Figure S1.

Figure 2. Sequential transcription regulators modulate reprogramming and neuroendocrine programs through a highly entropic and accessible chromatin state. (**A**) Overall differential chromatin accessibility across HC. (**B**) PCA of chromatin accessibility of PARCB time course samples with entropy analysis using ATAC sequencing. (**C**) Overall mean accessible peaks near TSS of each HC in PARCB time course study. (**D**) Enriched motifs from

suites of transcription factors in each HC using ATAC-sequencing. Top 5 motif suites for each comparison are shown, with additional analysis in Figure S2B, and full results in Table S1D. (**E**) Top ranked transcription factors and known neuroendocrine transcription factors across PARCB time course using bulk RNA sequencing. HOXC TFs avg: Average expression of HOXC4, HOXC5, HOXC6, HOXC8, HOXC9, HOXC10, HOXC11, HOXC12 and HOXC13. (**F**) Expression of ASCL1, ASCL2, NEURDO1 and POU2F3 in each HC. See also Figure S2.

Figure 3. Transcription factor-defined cell populations contribute to lineage divergence and tumor heterogeneity. (**A**) Dimension reduction UMAP analysis of four patient series (P2, P5, P6 and P7) over time (TP3-6) using single cell RNA sequencing. (**B**) Temporal UMAP analysis of all the samples. (**C**) Expression of selected markers and transcription factors. KRT5 marks basal cells. KRT15 marks luminal cells. The expression is presented in log normalized counts. (**D**) Top enriched inferred cell types from the Human Cell Type Database using SingleR [48]. (**E**) Projection of single cell RNA-sequencing samples on PCA framework by bulk RNA-sequencing samples (top panel) and the expression of selected markers and transcription factors (bottom panel). Each data point is a single cell colored by their corresponding HC. (**F**) Expression of ASCL1 (top) and ASCL2 (middle) and percentage of ASCL1/2 positive cells (cells with expression value >0) (bottom) in human biopsy and GEMM model tumors from five single cell RNA-sequencing datasets [31,49-51]. Other: prostatic intraepithelial neoplasia. NMYC_RB_M: *Pten^f/f; Rb1^f/f;MYCN* + (PRN) and RB_M: *Pten^f/f; Rb1^f/f* (PR) mouse model in Brady et al[31]. See also Figure S3.

Figure 4. ASCL1 and ASCL2 specify independent transcriptional programs and sub-lineages in SCNPC. (**A**) Inferred clonal tracing analysis of the PARCB time course samples using

Monocle 2 [52]. (**B**) Relative expression of KRT5, ASCL1 and ASCL2 in the inferred clonal tracing

analysis (pseudo-time). (**C**) Percentages of ASCL1 or ASCL2 positive, double positive and double

negative cell populations over time. (**D**) Volcano plot of differential gene expression in high

ASCL1+ vs high ASCL2+ cell populations. (**E**) Representative genes from the predicted

transcriptional programs of ASCL1 and ASCL2 trained on data from patient and model prostate

cancer tumors ([6,10,33] including TCGA), as determined by the ARACNE algorithm [81]. (**F**) Western

blot of panel of genes in the PARCB tumor derived cell lines from different tissue of origin (prostate,

bladder and lung) [6,7] . (**G**) Representative images of *in situ* hybridization of ASCL1 and ASCL2

mRNA analysis on transitional tumors (P7-TP5 and P9-TP4). See also Figure S4.

Figure 5. ASCL1 and ASCL2 as pan-cancer classifiers. See also Figure S5. (**A**) Projection of

the PARCB time course samples on the PCA framework defined by the CRPC subtypes using

RNA sequencing (left) and ATAC-sequencing (right) [57]. SCL: stem-cell like. NEPC:

Neuroendocrine prostate cancer. 3-dimensional rotatable versions of these figures are available

on the PARCB Multi-omics Explorer website. [For review, 3D rotatable versions are included as

Data S2 and Data S3.] (**B**) Projection of the PARCB time course samples on the PCA framework

defined by the SCLC subtypes [32,46]. (**C**) mRNA expression of ASCL1 and ASCL2 in the PARCB

time course samples and multiple sets of clinical CRPC-PRAD and SCNPC samples including

TCGA and different research groups [10,33-36]. (**D**) Representative images of *in situ* RNA

hybridization of ASCL1 and ASCL2 in clinical SCNPC tissues. (**E**) mRNA expression of ASCL1

and ASCL2 in pan cancer cell lines (CCLE). (**F**) mRNA expression of ASCL1 and ASCL2 in pan

cancer tumors from TCGA.

Figure 6. Alternating ASCL1 and ASCL2 expression through reciprocal interaction and TFAP4 epigenetic regulation. See also Figure S6. (**A**) Western blot analysis of exogenously expressing either V5 tagged ASCL2 in ASCL1+ cell lines [6] (left) or V5-tagged ASCL1 in ASCL2+ cell lines (right). (**B**) Schematic of putative cis regulatory elements (CREs) of ASCL1 and ASCL2 (top) and the heatmap of open chromatin accessibility across CREs of ASCL1 and ASCL2 using the PARCB time course ATAC-sequencing (bottom). Red box: CREs containing predicted TFAP4 binding sites by HOMER motif enrichment analysis [58]. (**C**) Top 8 ranked transcription factor motifs in ASCL1 promoter and ASCL2 enhancer regions, ranked by p-values. (**D**) Western blot analysis of doxycycline-inducible knockout of TFAP4 and proteins of interest in P7-TP6 (ASCL1+) and P3-TP5 (ASCL2+) cell lines. DOX: doxycycline. (**E**) Cell proliferation analysis of P7-TP6 (ASCL1+) and P3-TP5 (ASCL2+) cell lines with doxycycline-inducible knockout of TFAP4. Ctrl: no addition of doxycycline. TFAP4: with addition of doxycycline. (**G**) Schematic summary of the PARCB time course study.

<u>MATERIAL AND METHOD DETAIL</u>

PARCB transformation temporal model

Prostate tissues from donors were provided in a de-identified manner and therefore exempt from Institutional Review Board (IRB) approval. Processing of human tissue, isolation of basal cells, organoid transformation, and xenograft assay were described in detail previously [6]. 20,000 cells FACS-sorted cells per organoid were plated in 18-20ul of growth factor-reduced Matrigel (Cat# 356234, Corning) with PARCB lentiviruses (MOI=50/lentivirus). Organoids were cultured in the prostate organoid media[82] for about 10-14 days. Transduced organoids were harvested by dissociation of Matrigel with 1mg/mL Dispase (Cat# 17105041, Thermo Fisher Scientific). The organoids were washed three times with 1xPBS to remove Dispase and re-suspended in 10µl of growth factor reduced Matrigel and 10ul Matrigel with High Concentration (Cat# 354248, Corning). The organoid-Matrigel mixture was implanted subcutaneously in immunodeficient NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice [83].Tumors were extracted in every two-week window, with the last tumor collection of the time course series determined by either reaching around 1cm in diameter in tumor size or ulceration, whichever came first. NSG mice had been transferred from the Jackson Laboratories and housed and bred under the care of the Division of Laboratory Animal Medicine at the University of California, Los Angeles (UCLA). All animal handling and subcutaneous injections were performed following the protocols approved by UCLA's Animal Research Committee.

Cell lines

NCI-H1385 (Cat# CRL-5867), NCI-H1930 (Cat# CRL-5906), NCI-H1694 (Cat# CRL-5888), NCI-H146 (Cat# HTB-173), DMS79 (Cat# CRL-2049), NCI-H526 (Cat# CRL-5811), and NCI-H660 (Cat# CRL-5813) were purchased from American Type Culture Collection (ATCC). COR-L311 was obtained from Sigma Aldrich (Cat# 96020721). All commercially available cell lines were cultured and maintained based on the instruction from the vendors. PARCB tumor derived cell

lines were generated using the previous method [6]. All the cell lines in the study are free of Mycoplasma using a MycoAlert™ PLUS Mycoplasma Detection Kit (Cat# LT07-703, Lonza).

Lentiviral vectors and lentiviruses

The myristoylated AKT1 vector (FU-myrAKT1-CGW), exogenous expression of cMYC and BCL2 (FU-cMYC-P2A-BCL2-CRW), dominant TP53 mutant (R175H) and shRNA targeting RB1 vector (FU-shRB1-TP53DN-CYW) have been described previously [6]. Exogenous expression of V5 tagged ASCL1 (pLENTI6.3-V5-ASCL1) is obtained from DNASU (Cat#: HsCD00852286) [84]. For making exogenous expression of ASCL2 containing vector (pLENTI6.3-V5-ASCL2), Gateway cloning (Cat# 11791020, Thermo Fisher) was performed using pLenti6/V5-DEST Gateway Vector (Cat# V49610, Thermo Fisher) and the entry plasmid (pDONR221-ASCL2) was obtained from DNASU (Cat# HsCD00829357) [84]. For making doxycycline- inducible sgTFAP4 (TLCv2-Cas9-BFP-sgTFAP4), TLCv2 (Cat# 87360, Addgene) was first digested with BamHI-HF (Cat# R3136, New England Biolabs) and NheI-HF (Cat# 3131, New England Biolabs) at 37℃ for 1 hour and inserted with a synthesized fragment containing T2A-Hpal-BFP sequence (gBlock service provided by IDT) using Gibson Assembly (Cat# E5510, New England Biolabs). sgTFAP4 sequence was cloned into the previously described TLCv2-BFP vector using an established protocol [85]. sgTFAP4-primers are listed in the key resources table. Lentiviruses were produced and purified by a previously established method [86].

Tissue section, histology, and immunohistochemistry (IHC)

PARCB model tumor tissues were fixed in 10% buffered formaldehyde overnight at 4℃ and followed by 70% ethanol solution. Tissue microarray construction and hematoxylin and eosin (H&E) staining were performed by Translation Pathology Core Laboratory (TPCL) in UCLA using standard protocol. TPCL is a CAP/CLIA certified research facility in the UCLA Department

of Pathology and Laboratory Medicine and a UCLA Jonsson Comprehensive Cancer Center

Shared Facility. For immunohistochemistry staining, formalin-fixed, paraffin-embedded (FFPE)

sections were deparaffinized and rehydrated with a washing sequence of xylene and different

concentration of ethanol. Citrate buffer (pH6.0) was used to retrieve antigens. The sections

were incubated in citrate buffer and heated in a pressure cooker. 3% of H2O2 in methanol was

used to block endogenous peroxidase activity for 10 mins at room temperature. The sections

were blocked then incubated with primary antibodies overnight at 4°C. Anti-mouse/rabbit

secondary antibodies were used to detect proteins of interest and DAB EqV substrate was used

to visualize the staining. All components were included in the ImmPRESS Kit (MP-7801-15 and

MP-7802-15, Vector Laboratories) The slides were then dehydrated and mounted with Xylene-

based drying medium (Cat# 22-050-262, Fisher Scientific).

Western blot

Cells were lysed on ice using UREA lysis buffer (8M UREA, 4% CHAPS, 2x protease inhibitor

cocktail (Cat# 11697498001, Millipore Sigma)). Genomic DNA was removed by ultracentrifuge

(Beckman Optima MAX-XP, rotor TLA-120.1, 48,000 rpm for 90 min). Protein concentrations

were measured using the Pierce BCA Protein Assay Kit (Cat#: 23227, Thermo Scientific).

Samples were electrophoresed on polyacrylamide gels (Cat# NW04120BOX, Thermo Fisher),

transferred to nitrocellulose membranes (Cat# 88018, Thermo Fisher). Western blots were

visualized using iBright CL1500 Imaging system (Cat#44114, Thermo Fisher).

RT-qPCR

Total RNA was isolated from cells using miRNeasy Mini Kit (Cat# 217004, Qiagen). cDNA was

synthesized from 2 ug of total RNA using the SuperScript IV First-Strand Synthesis System

(Cat# 18091050, Thermo Fisher). RT-qPCR was performed using SYBR Green PCR Master

Mix (Cat# 4309155, Thermo Fisher). Amplification was carried out using the StepOne Real-

Time PCR System (Cat# 4376357, Thermo Fisher) and analysis was performed using the

StepOne Software v2.3. with the following primers were used at a concentration of 250 uM:

Relative quantification was determined using the Delta-Delta Ct Method. Primer sequences are listed in the key resources table.

In situ RNA hybridization assay and image analysis

The RNAscope Multiplex Fluorescent V2 kit was used to perform in situ hybridization on FFPE tissue microarray slides following the manufacturer's protocol (Cat# 323270, ACDBio). The Institutional Review Board of the University of Washington approved this study (protocol no. 2341). All rapid autopsy tissues were collected from patients who signed written informed consent under the aegis of the Prostate Cancer Donor Program at the University of Washington. The establishment of the patient-derived xenografts was approved by the University of Washington Institutional Animal Care and Use Committee (protocol no. 3202-01). For multiplex hybridization, the Double Z probes targeting ASCL1 (Cat# 459721-C2, ACDBio) and ASCL2 (Cat# 323100, ACDBio) were hybridized to the samples for 2 hours at 40°C. ASCL1 signal was visualized using Opal dye 520 (Cat# FP1487001KT, Akoya Biosciences) and ASCL2 signal was visualized using Opal dye 570 (Cat# FP1488001KT, Akoya Biosciences). DAPI (Cat# D3571, Thermo Fisher) was used to visualize nuclei. Confocal fluorescence images were acquired using an inverted Zeiss LSM 880 confocal microscope. All images were processed using Fiji (https://imagej.net/software/fiji/).

Cell proliferation assay

3000 cells per cell line in five replicates were seeded on 96-well plates on Day 0. Cell viability was measured on Day 1, 3, 4, 5 and 6. using Cell Titer-Glo Luminescent Cell Viability Assay (Cat# G7570, Promega). Luminescence was measured at an integration time of 0.5 second per well.

Bulk RNA sequencing and dataset collection

Tumors were dissociated into single cells, followed by cell sorting of triple colors (RFP, GFP and YFP) by flow cytometry. Total RNA was extracted from the cell lysates using miRNeasy mini kit (Cat# 217084, Qiagen). Libraries for RNA-sequencing of PARCB time course samples were prepared with KAPA Stranded mRNA-Seq Kit (Cat# KK8420, Roche). The workflow consists of mRNA enrichment and fragmentation. Sequencing was performed on Illumina Hiseq 3000 or NovaSeq 6000 for PE 2x150 run. Data quality check was done on Illumina SAV. Demultiplexing was performed with Illumina Bcl2fastq v2.19.1.403 software. Raw sequencing reads were processed through the UCSC TOIL RNA Sequencing pipeline1 for quality control, adapter trimming, sequence alignment, and expression quantification. Briefly, sequence adapters were trimmed using CutAdapt v1.9, sequences were then aligned to human reference genome GRCh38 using STAR v2.4.2a and gene expression quantification was performed using RSEM v1.2.25 with transcript annotations from GENCODE v23 [87].

The FASTQ files of the Park dataset [6], Beltran dataset [33], George dataset [32] and Tang dataset [57] were all processed through the TOIL pipeline with the same parameters to get RSEM expected counts. The TOIL-RSEM expected counts of TCGA pan cancer samples were obtained directly from UCSC Xena browser (https://xenabrowser.net/datapages) and gene expression (log2(TPM + 1)) of pan-cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE) were downloaded from DepMap Portal (DepMap Public 22Q1) (https://depmap.org/portal/download/all/). The RSEM counts of all combined datasets were upper quartile normalized, log2(x+1) transformed (referred to as log2(UQN+1) counts) and filtered down to HUGO protein coding genes (http://www.genenames.org/) for the downstream analyses. SCLC subtypes [46] and CRPC subtypes [57] were previously defined. The details of the bulk RNA-sequencing of PARCB time course are described in Table S1I.

Differential gene expression analysis and hierarchical clustering

PARCB time course samples were grouped into 6 hierarchical clusters (HC) by performing Ward's

hierarchical clustering (k=6) on log2(UQN + 1) counts using the hclust function from the base R

package, Stats (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html).

Differential gene expression analysis was then performed on each HC in a "one vs. rest" fashion,

i.e., between one cluster vs. the remaining five clusters, using DESeq2 with the following

parameters: independentFiltering=F, cooksCutoff=FALSE, alpha=0.1 [88]. For each HC vs. rest

comparison, genes with a log2FC > 2 and p-adjusted value < 0.05 were considered upregulated

for that HC gene module. However, four genes (IL1RL1, KRT36, PIK3CG, NPY) were upregulated

among multiple HC vs. rest comparisons. As a result, these genes were assigned to the HC gene

module with the smaller p-adjusted value for that gene. Z-scores for upregulated genes in each

cluster were then plotted in a heatmap using pheatmap function. PARCB time course samples

were subsequently categorized by this HC definition in downstream analyses.

GO enrichment analysis

Enrichment analysis was performed using the "GO_Biological_Process_2021" database and the

enrichr function from the R package, enrichR, using upregulated genes for each HC [89]. Pathways

were selected based on their adjusted p-value for each HC. The results were plotted using ggplot().

Bulk ATAC sequencing and dataset collection

Tumors were dissociated into single cells, followed by cell sorting of triple colors (RFP, GFP and

YFP) by flow cytometry. ATAC-sequencing samples were prepared following the previously

published protocol[38]. Bulk ATAC sequencing was performed in the Technology Center for

Genomics & Bioinformatics Core in UCLA. Sequencing was performed on Illumina NovaSeq 6000

for PE 2x50 run. Data quality check was done on Illumina SAV. Demultiplexing was performed

with Illumina Bcl2fastq v2.19.1.403 software. The raw FASTQ files were processed using the published ENCODE ATAC-Seq Pipeline (https://github.com/ENCODE-DCC/atac-seq-pipeline). The reads were trimmed and aligned to hg38 using bowtie2. Picard was used to de-duplicate reads, which were then filtered for high-quality paired reads using SAMtools. All peak calling was performed using MACS2. The optimal irreproducible discovery rate (IDR) thresholded peak output was used for all downstream analyses, with a threshold P value of 0.05. Other ENCODE3 parameters were enforced with the flag-encode3. Reads that mapped to mitochondrial genes or blacklisted regions, as defined by the ENCODE pipeline, were removed. The peak files were merged using bedtools merge to create a consensus set of peaks across all samples, and the number of reads in each peak was determined using bedtools multicov [90]. A variance stabilizing transformation was performed on peak counts using DESeq2 [88] and batch effects were removed using removeBatchEffect() from limma [91]. All downstream ATAC-sequencing analysis was performed using this matrix (referred to as VST peak counts), unless otherwise specified. P1-TP1 was not collected for ATAC-sequencing due to insufficient cell number for sequencing. P7-TP2 was not included for the processing due to low read counts (total of 1536). P1-TP5, P2-TP6 and P10-TP2 were not included in PCA due to reaching within 95th percentile of calculated Shannon entropy for all ATAC-sequencing samples. The details of the bulk ATAC-sequencing processing of PARCB temporal samples are described in Table S1J

Raw FASTQ files of Tang ATAC-sequencing dataset were downloaded from GSE193917 [57]. The raw FASTQ files were processed using the same ENCODE pipeline described above with the same parameters.

Differential chromatin accessibility and Transcription start site (TSS) analysis
Differential peak analysis was performed on each HC in a one vs. rest fashion, as described above in the bulk RNA-sequencing analysis. Peaks were called hyper- or hypo- accessible if the

log2 fold change was greater than 2 or less than 2, respectively, and had an adjusted p-value of less than 0.05. The z-scores of the union of all differentially accessible peaks were used to plot the heatmap using VST peak counts, with the rows ordered by chromosomal location.

For mapping peaks near TSS sites, the bigwig files containing ATAC-sequencing readings were first converted into wig files. Wig files from samples within the same HC were then merged by calculating the mean across peak regions using wiggleTools [92]. The TSS analysis was performed using deepTools and computeMatrix in reference-point mode with parameters referencePoint=TSS, a=2000, b=2000 to compute the scores from merged bigwigs in regions 2 kbp flanking the region of interest. plotHeatmap was used with parameters zMin=0, zMax=5, binSize=10 was to plot the TSS figure from the score matrix [93].

PCA and projection analyses

Unsupervised PCA of the PARCB time course samples using log2(UQN +1) counts was performed using the prcomp function from the stats package available on R (described above). PC2 and PC3 sample scores were then multiplied by a 30-degree clockwise rotation matrix. Ellipses were drawn around samples with 95% confidence based on real time labels using stat_ellipse() from ggplot2. The PCA projection of PARCB time course samples onto the framework using pan small cell cancer combined gene expression datasets have been discussed previously [4]. In brief, the input matrix for this PCA was centered but not scaled. PARCB time course samples were then projected by multiplying the data matrix by the PCA loadings. For projection of PARCB time course samples onto the framework using gene expression data of CRPC subtypes [57] or SCLC subtypes [46], the same methodology was applied.

For projection of PARCB time course samples onto the framework using ATAC-sequencing data of CRPC subtypes [57], peak coverage of the Tang dataset was determined using the consensus set of peaks from the PARCB time course data with function bedtools multicov [90]. Tang dataset peak read counts were then variance stabilized transformed using DESeq2 [88]. PCA was

performed on VST peak read counts of the Tang dataset using the prcomp function with the parameters center = T, scale = F. PARCB time course samples were then projected onto the framework by multiplying PARCB time course VST peak read counts by PCA loadings.

For projection of PARCB time course single cells onto the framework defined by the bulk RNA-sequencing data, the single cell data after integration by batch was down-sampled for 1000 cells within each patient series or cluster. The single cell and bulk RNA-sequencing data were centered separately prior to projection. The projection was carried out by multiplying the single cell data matrix by PCA loadings of PARCB bulk samples.

Transcription factor analysis

Top ranked transcription factors (TF) were selected using the gene loading scores derived from the unsupervised PCA of gene expression described above. PC2 and PC3 loading scores were rotated 30 degrees clockwise by multiplying a 30-degree clockwise rotation matrix to the gene loading scores (resulting components called PC2' and PC3', respectively). The loading scores were then filtered to include only transcription factors [37]. The center of the TF loading scores was determined by taking the average of PC1, PC2', and PC3'. The Euclidean distance from the center was calculated for each TF, and the top 60 TFs furthest from center were selected. Hierarchical clustering (k = 5) was performed on the log2(UQN +1) counts of the top 60 TFs. The z-scores for each TF were plotted using pheatmap. Average z-score of HOXC genes was calculated from HOXC 4-13 (except for HOXC7) in each PARCB time course sample.

Shannon Entropy analysis

Shannon entropy for each PARCB time course sample was calculated on variance stabilized transformed (VST) ATAC-sequencing peak counts using the Entropy() function from the R

package DescTools (https://cran.r-project.org/web/packages/DescTools/index.html). PARCB

samples falling within the 95th percentile of calculated Shannon entropy scores were included in

the following PCA. PCA was performed on VST peak counts and was plotted using ggplot2 with

samples colored by their Entropy scores and ellipses with 95% confidence were drawn around

each time point group using stat_ellipse().

Prostate cancer gene regulatory network analysis

The RNA-sequencing data of PARCB time course study, Park dataset [6], Beltran dataset [33], and

TCGA PRAD/PRAD-norm dataset were included in this analysis. TCGA PRAD/PRAD-norm data

was down sampled to match the sample size of other cohorts. Gene network was built on the

combined datasets using ARACNe-AP [81].

Signature scores (adult stem cell, adenocarcinoma and SCNPC)

SCNPC signature was derived using Beltran dataset [33], following the methods described

previously [6]. The adult stem cell (ASC) signature in our analysis is defined in literature [42]. For

prostate adenocarcinoma signature, differential gene expression analysis was performed on

TCGA PRAD samples vs CRPC-PRAD and SCNPC samples from the Beltran dataset [10,33]

using DESeq2. The adenocarcinoma signature was defined by all the upregulated genes

(log2FoldChange >2 and padj < 0.05) from the differential gene expression analysis.

Adenocarcinoma and SCNPC signature scores of our PARCB time course samples were

calculated using gsva with method="ssgsea".

Motif analysis

Hyper-accessible peaks in each HC from the differential peak analysis described previously were

used for motif enrichment analysis using GimmeMotifs 41,90. Differential motif analysis was

performed on hyper-accessible peaks for each HC against a hg38 whole-genome background

using the maelstrom function with default parameters. The top 5 enriched motifs and their aggregated z-scores for each HC are shown in the heatmap (each individual HC vs all others). Additionally, we performed differential peak analysis on HC5 vs HC1-HC4 and HC6 vs HC1-HC4 with the same parameters as described previously using DESeq2. Likewise, hyper-accessible peaks for HC5 and HC6 in these comparisons were defined by a threshold of log2FoldChange > 2 and padj < 0.05. Differential motif analysis was performed on the set of hyper-accessible peaks from HC5 vs HC1-4 and HC6 vs HC1-4 using the maelstrom function as described above. Note that in the GimmeMotif enrichment analysis, transcription factors are culled to minimize redundancy, and this step is impacted by the exact input data and sample group comparison indicated. Thus, each motif suite may contain slightly different enriched transcription factors. However, the transcription factor sets remain highly consistent between each case.

For identifying transcription factors that recognize ASCL1 and ASCL2 regulatory sequences, ASCL1 and ASCL2 promoter and enhancer regions were mapped using UCSC Genome Browser Gateway (https://genome.ucsc.edu/cgi-bin/hgGateway). Motif analysis was then performed on each ASCL1 and ASCL2 promoter and enhancer region using the findMotifGenome function from HOMER with the parameters -size 200 and -mask [58]. Resulting motifs were then ranked by their p-value. Additionally, ASCL1 and ASCL2 enhancer and promoter regions were mapped to accessible peaks from ATAC-sequencing data of the PARCB time course to identify chromatin changes of ASCL1 and ASCL2 cis-regulatory sequences. Peak regions from the PARCB consensus peak set overlapping with the ASCL1 and ASCL2 enhancer and promoter regions were then plotted in a heatmap using VST peak counts and scaled per sample.

Single-cell RNA sequencing

PARCB time course samples were sequenced in two batches: P2/P5 and P6/P7 series. Single cell gene expression libraries were created using Chromium Next GEM Single Cell 3' (v3.1

Chemistry) (Cat# PN1000123, 10x Genomics), Chromium Next GEM Chip G Single Cell Kit (Cat# PN1000120, 10x Genomics), and Single Index Kit T Set A (Cat# PN1000213, 10x Genomics) according to the manufacturer's instructions. Briefly, cells were loaded to target 10,000 cells to form GEMs and barcode individual cells. GEMs were then cleaned cDNA and libraries were also created according to manufacturer's instructions. Library quality was assessed using 4200 TapeStation System (Cat# G2991BA, Agilent) and D1000 ScreenTape (Cat# 5067-5582, Agilent) and Qubit 2.0 (Cat# Q32866, Invitrogen) for concentration and size distribution. Samples were sequenced using Novaseq 6000 sequencer (Catl# A00454, Illumina) using 100 cycles (28+8+91). The illumina base calling files were converted to FASTQ using the mkfastq function in Cell Ranger suite (https://support.10xgenomics.com/single-cell-gene-expression/). The reads were then aligned to GRCh38 for UMI counting with cellranger count function. The details of the single cell-seq of PARCB time course are described in Table S1K.

UMAP analysis

The downstream quality control, statistics and visualization of PARCB single cell RNAseq data were performed mainly using the Seurat (v3.2.3) R package [94]. Briefly, the data from all four patient series was first filtered for cells with total number of unique features above 500 and below 10000 as well as mitochondria feature counts below 10%. The mitochondrial genes and ribosomal genes were then removed from the count matrix for the downstream analysis. To overcome batch effect, we performed Seurat integration between batch 1 (Series P2 and P5) and 2 (Series P6 and P7). Briefly, for each batch, the two corresponding matrices were combined first, and log transformation and library size normalization were performed with NormalizeData function. Then the 2500 most variable genes were selected as anchor features to integrate for all coding genes. After integration, the top 30 principal components were used to perform UMAP analysis.

Processed single cell RNA-sequencing data of advanced prostate cancers were downloaded from the Single Cell Portal hosted by Broad Institute (https://singlecell.broadinstitute.org/single_cell/study/SCP1244/transcriptional-mediators-of-treatment-resistance-in-lethal-prostate-cancer)[49]. For this dataset, UMAP analysis was performed on TPM values of prostate cancer cells only as defined in the paper using the umap function in base R. For UMAP visualization of this dataset, TPM values were log2 transformed with a pseudo count of +1. Single cell RNA-sequencing data of N-myc GEMM tumors [31], and human biopsy and GEMM tumors [50] were downloaded from the Gene Expression Omnibus (GEO) database with the accession numbers GSE151426 and GSE21035, respectively, and processed with cellranger count. In the Brady et al paper, single-cell data were first filtered for cells with total number of unique features > 200 and < 10000 as well as mitochondrial feature counts < 10%. We then performed Seurat SCTransform integration on each sample. Briefly, for each sample, the matrices were first combined and normalized using SCTransform function. Then the top 3000 most variable genes were selected as anchor features to integrate all genes. After integration, the top 15 principal components were used to perform UMAP analysis. In the Chan et al paper, GEMM single-cell data were filtered with the following thresholds nFeature_RNA >200 & nFeature_RNA < 8000 & percent.mt < 5 and human biopsy tissues single-cell data were filtered with nFeature_RNA > 200 & nFeature_RNA < 10000 & percent.mt < 5. Seurat integration of filtered cells for both datasets were then performed as described above. After integration, the top 50 principal components were used to perform UMAP analysis.

In the Dong et al analysis, the human biopsy scRNA-sequencing data was downloaded from GSE137829. We used the filtration parameters of the manuscript, total number of unique features > 500 and <7000, and mitochondrial feature counts < 10%. We filtered cells to only include epithelial (cancer) cells, as described by the CellType column in the annotation. Seurat

NormalizeData was used with the LogNormalize method and a scale factor of 10000. The top 30 principal components were used to perform UMAP analysis.

Inferred cell type and cellular lineages analysis

The cell type inferences of PARCB single cells were implemented using the singleR R package [48]. For scoring each cell for each general cell type, the Human Primary Cell Atlas data from LTLA/celldex package that contains normalized expression values was used as the reference.

Single cell trajectory analysis of PARCB samples was performed using two different methods, expression-based method Monocle2 [52] and RNA Velocity based method scVelo [53]. For Monocle2, the integrated Seurat object was used as the input for the program. DDRtree was used as the reduction method. Cells were ordered by the most variable 3000 genes in Seurat. For calculating pseudotime, the KRT5 population was selected as the root state. For RNA velocity, the spliced and unspliced counts were quantified by velocyto accounting for repeat masking. The spliced counts were then normalized using Seurat sctransform method followed by integration by batch. The integrated data was used for UMAP visualization. In scVelo, the data was filtered for genes with a minimum of 5 shared counts. The top 3000 highly variable genes were extracted based on the dispersion. Velocities were estimated by dynamical model and then projected onto the UMAP embedding.

Differential gene expression analysis in single cells

FindMarkers function in Seurat R package (described above) was used to identify differential expressed genes between ASCL1+ and ASCL2+ single cell populations. Patient series was regressed out by including it as the covariate. ASCL1+ cells and ASCL2+ cells are defined as cells with log normalized expression counts > 0 for ASCL1 or ASCL2, respectively. Genes that

are differentially expressed in ASCL1+ population were defined by the difference of gene expression in ASCL1+ cells minus the one in ASCL2 expression (log and library size normalized) above 3. Genes that are differentially expressed in ASCL2+ cells were defined by such a difference below -1.

CUT&RUN sequencing

The CUT&RUN experiment was performed using previously established method [61] (Skene et al., 2018) and the manufacturer's protocol (Cat# 86652, Cell Signaling). 100k live cells were used per reaction. 50pg of Spike-In DNA (Cat# 12931, Cell Signaling) was added per reaction for downstream normalization. DNA was purified using MinElute PCR Purification Kit (Cat# 28004, Qiagen), followed by fragmentation by using sonicator (Cat# M202, Covaris). Dual size selection was applied using KAPA Pure beads (Cat# KR1245, Roche). DNA Libraries were prepared with the KAPA DNA HyperPrep kit (Cat# KK8504, Roche).

Sequencing was performed on Illumina HiSeq3000 for a SE 1x50 run. Data quality check was done on Illumina SAV. Demultiplexing was performed with Illumina Bcl2fastq v2.19.1.403 software. Raw FASTQ files were processed using the published ENCODE-TF CHIP Seq pipeline. Batch 1 samples (P3-TP5 and P7-TP6) were processed with the parameter "chip.paired_end" : false while Batch 2 sample (P2-TP6) were processed with the parameter "chip.paired_end" : true. (https://github.com/ENCODE-DCC/chip-seq-pipeline2). For all samples, the reads were trimmed and aligned to hg38 (target) and S. cerevisiae strain S288C (spike-in) reference genomes using bowtie2. After alignment, Picard was used to remove PCR duplicates reads and SAMtools was used to further filter high-quality paired reads (i.e., remove reads that were unmapped, not primary alignment, reads failing platform, and/or multi-mapped). Peak calling was performed using MACS2. Peaks overlapping with blacklisted regions were removed

(). Lastly, spike-in normalization factors were calculated following established protocol [95]. The details of the CUT&RUN sequencing of PARCB time course are described in Table S1L.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were analyzed and processed using R v4.1.2, Python v3.11.5 and Excel. Error bars show mean ± SD unless otherwise specified. Significance was determined by Student's two-tailed unpaired t tests or Wald test with 95% confidence intervals. P values <0.05 is considered statistically significant. P values were adjusted based on various methods dependent on the analysis including Benjamin-Hochberg method (Figure 1D and Figure 1F) and Bonferroni correction (Figure 4D). No statistical methods were used to predetermine sample sizes. Other details such as sequencing processing can be found in Table S1. All statistical methods for the bioinformatic analyses are described in detail in the method section.

ADDITIONAL RESOURCES

PARCB Multi-omics Explorer provides an interactive platform for visualization of gene expression using bulk RNA-sequencing and single cell RNA-sequencing of this time course study (https://systems.crump.ucla.edu/transdiff/).

Table S1. Supplemental information of bioinformatic analyses, related to Figure 1, 2, 4 and 6. A) Upregulated genes in each hierarchical clusters (HC); **B)** PCA scores of PARCB time course samples with HC and timepoint annotations; **C)** Enrichr GO Biological Processes 2021 results of upregulated genes per HC; **D)** Motif enrichment analysis per HC; **E)** List of weighted transcription factors for PCA loadings of PARCB time course data; **F)** Differential genes in ASCL1 vs ASCL2 cell populations; **G)** List of predicted gene network of ASCL1 and ASCL2; **H)** Ranked transcription factors motifs in ASCL1 and ASCL2 cis regulatory sequences; **I)** RNA-seq sequencing statistics; **J)** ATAC-seq sequencing statistics; **K)** Single Cell RNA sequencing statistics of PARCB time course sample; **L)** CUT&RUN sequencing statistics.

Data S1. PCA of PARCB time course as a 3D rotatable object, related to Figure 1E.

Data S2. Projection of RNA-seq as a 3D rotatable object, related to Figure 5A.

Data S3. Projection of ATAC-seq as a 3D rotatable object, related to Figure 5A

Figure 1

Figure 2

A — Overall differential chromatin accessibility across HCs

B — Entropy analysis by accessible peaks

Primary path (1° arc)

Secondary path (2° bifurcation)

C — Overall accessible peak near TSS across HC

D — Top 5 ranked enriched motifs in suites of transcription factors

E — Expression of top ranked transcription factors across HCs

F

Figure 3

Figure 4

A Inferred clonal tracing analysis

B Relative mRNA expression in lineage differentiation

C Composition of ASCL1/ASCL2 positive cell population over time

D Differential gene expression high ASCL1+ vs. high ASCL2+ cells

E Predicted transcriptional programs of ASCL1 and ASCL2

F

G *In situ* hybridization of ASCL1 and ASCL2 mRNA on transitional tumors

Figure 5

A

CRPC subtypes
(*RNA-seq*, Tang et al 2021)

CRPC subtypes
(*ATAC-seq*, Tang et al 2021)

B

SCLC subtypes
(*RNA-seq*, George et al 2015 and CCLE)

C

D

CRPC-PRAD

E

SCNPC

F

mRNA expression of ASCL1 vs ASCL2 in pan cancer tumors (TCGA)

96

Figure 6

A

Overexpression of ASCL1 or ASCL2 in respective cell lines

C

Ranked Homer motifs in ASCL1 and ASCL2 regulatory sequences

Rank	ASCL1 promoter E1638562	ASCL2 enhancer E1515225
1	FOXK1	THRB
2	AP4	NR2E1
3	NFIA	HINFP
4	ZNF189	ZNF341
5	MZF1	OSR1
6	GATA3	AP4
7	VEZF1	MED1
8	TCFL5	BACH1

B

Accessibility of ASCL1 and ASCL2 *cis* regulatory sequences in time course (ATAC-seq)

D

E

F

G

Figure S1

A

squamous cell carcinoma

adenocarcinoma

small cell carcinoma

B

mix squamous/ adeno	adeno	mix small cell/adeno
P2-TP3	P2-TP4	P3-TP4
P4-TP3	P7-TP4	P5-TP4
P5-TP3	P9-TP4	P8-TP4
P6-TP3	P1-TP5	P10-TP4
P7-TP3	P2-TP5	P5-TP5
P8-TP3	P7-TP5	P6-TP5
P9-TP3	P3-TP5	P1-TP5
P10-TP3	P2-TP6	P8-TP6
P4-TP4	P5-TP6	
P6-TP4	P6-TP6	
P8-TP5	P7-TP6	
P4-TP5	P9-TP5	
	P10-TP5	
12/33	**13/33**	**8/33**

C

4 weeks (Early) → 8 weeks (Transition)

Transition (6-8 weeks)

D

benign prostate   early tumors   transitional tumors   late tumors

HLA

P63

AR

E

PC2; 30 degree rotation (clockwise)

PC1

Patient: P1, P2, P3, P4, P5, P6, P7, P8, P9, P10

HC: HC1, HC2, HC3, HC4, HC5, HC6

F

Primary path (1° arc)

Secondary path (2° bifurcation)

-PC3; 30 degree rotation (clockwise)

PC2; 30 degree rotation (clockwise)

PC1

HC: HC1, HC2, HC3, HC4, HC5, HC6

Timepoint: Basal, Organoid, Early, Transition, Endpoint

G

ssGSEA Score

HC1, HC2, HC3, HC4, HC5, HC6

Gene signatures: ADENO, SCNPC

Figure S2

A

Entropy analysis by gene expression

Primary path (1° arc)

Secondary path (2° bifurcation)

Timepoint
- Basal (HC1)
- Organoid (HC2)
- Early (HC3)
- Transition (HC4)
- Endpoint (HC5/HC6)

Entropy Score
13.90
13.85
13.80
13.75

B

(a) HC5 vs HC1-4

GM.5.0.bZIP.0003 (FOS, JUN...)
GM.5.0.bZIP.0082 (JUN)
GM.5.0.p53.0007 (TP53, TP63...)
GM.5.0.bZIP.0053 (MAFF, MAFG...)
GM.5.0.IRF.0019 (JUN, IRF4...)
GM.5.0.p53.0008 (TP53, TP63...)
GM.5.0.Nuclear_receptor.0133 (HNF4A, HDAC2...)
GM.5.0.C2H2_ZF.0068 (GFI1...)
GM.5.0.bZIP.0102 (PAX9, PAX5...)
GM.5.0.Mixed.0029 (PAX5, PAX3...)
GM.5.0.Mixed.0101 (POU3F2, NANOG...)
GM.5.0.Homeodomain_POU.0010 (POU2F1, POU2F2...)
GM.5.0.Homeodomain_POU.0007 (SOX1, SOX2...)
GM.5.0.bHLH.0073 (ASCL1, TFAP4...)
GM.5.0.Unknown.0039 (RUNX2, RUNX1...)
GM.5.0.Mixed.0079 (CTCF, RAD21...)
GM.5.0.Unknown.0109 (unknown)
GM.5.0.bHLH.0061 (ASCL2, SNAI2...)
GM.5.0.Sox.0023 (LEF1, NANOG...)
GM.5.0.C2H2_ZF.0132 (GZF1)

HC1thru4   HC5

Z score
4
2
0
-2

(b) HC6 vs HC1-4

GM.5.0.bZIP.0013 (FOS, JUNB...)
GM.5.0.bZIP.0082 (JUN)
GM.5.0.Forkhead.0043 (FOXC1, FOXA1...)
GM.5.0.IRF.0021 (IRF2, STAT1...)
GM.5.0.p53.0007 (TP53, TP63...)
GM.5.0.IRF.0019 (IRF4, BATF)
GM.5.0.TEA.0006 (TEAD1, TEAD3...)
GM.5.0.bZIP.0078 (JUN, MEF2C...)
GM.5.0.TEA.0009 (TEAD2, TEAD4...)
GM.5.0.Nuclear_receptor.0009 (RORA, NR1D1...)
GM.5.0.C2H2_ZF.0002 (ASCL2, SNAI2...)
GM.5.0.bHLH.0061 (ATOH8, TCF3...)
GM.5.0.bHLH.0073 (ASCL1, NEUROD1...)
GM.5.0.bHLH.0032 (ASCL1, ASCL2, TFAP4...)
GM.5.0.bHLH.0072 (MYF6, PRDM9...)
GM.5.0.Unknown.0114 (FOXA2, TCF12...)
GM.5.0.T-box.0019 (ZEB1, EOMES)
GM.5.0.C2H2_ZF.0132 (DMRT1, GZF1...)
GM.5.0.Forkhead.0033 (FOXM1, FOXA2...)
GM.5.0.Mixed.0080 (ZEB1, PAX2)

HC1thru4   HC6

C

HC
- HC1 (Early)
- HC2 (Organoids)
- HC3 (Early Tumors)
- HC4 (Transitory Tumors)
- HC5 (Class I/ASCL2+)
- HC6 (Class II/ASCL1+)

Adult Stem Cell (Smith et al 2018)

Neuroendrocrine (Beltran et al 2016)

Sum Z-Score

D

Top ranked transcription factors (PCA loading)

Type
- Top scored
- NEPC known

ASCL1
HNF4A
NKX2-2
HNF1A
FOXA3   ZIC2   GPR   INSM1
HEY2
NR0B1   TBX10   SOX1
SNAI2   ZIC5   PAX5
FOXA2
ESR1   HES6
AR   KLF8   PNOX1
GLI3   ST18
FOXQ1   SNAI   HOXC12   ZNF804A
PEG3   BNC1   FOXN1
TP63
POU4F1   ZFP42
HOXC8   OLIG1   LIN28B
ZIC1   SIPS   HOXC5
NKX11   HOXC9   CLX3
DMRT81   prl1B   TFAP2B
NFATC1   HMX3   HOXM1
HOXC13   HMX2   HOXC10
HES1   POU2F3 HOXC11
ASCL2   SOX17   OLIG2

PC1

Time
HC1 (Basal)   HC2 (Organoids)   HC3 (Early)   HC4 (Transitory)

HC6 (Class II Late tumors)
HC5 (Class I Late tumors)

Figure S3

A HC defined cell population

B SCNPC score (derived from Beltran 2016)

C Density analysis of ASCL1 vs ASCL2 expression in single cells

D

PCA analysis of single cell RNA-seq

Expression of selected marker and transcription factors

E

Heterogenous expression of transcription factors in HC tumors by single cells
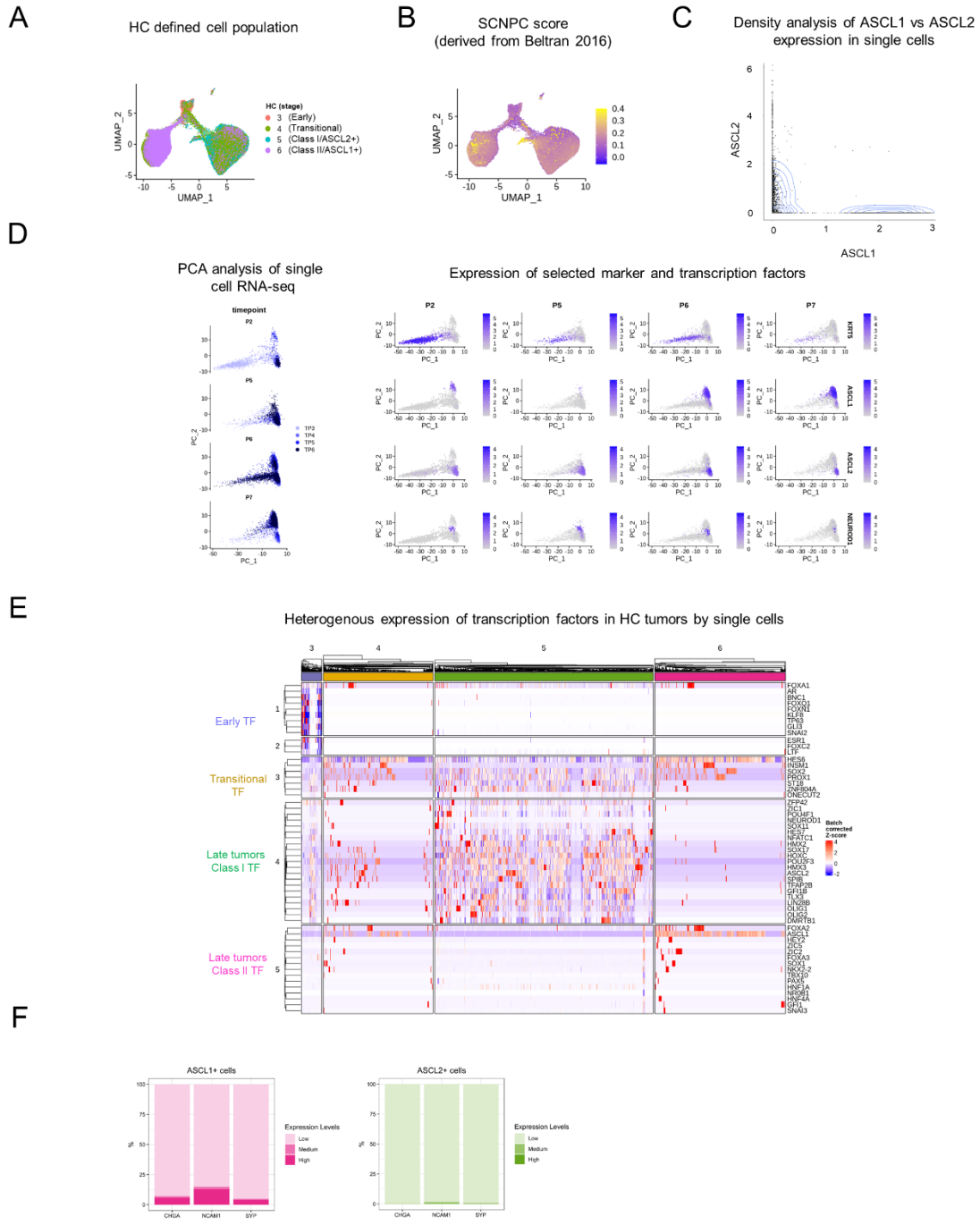

F

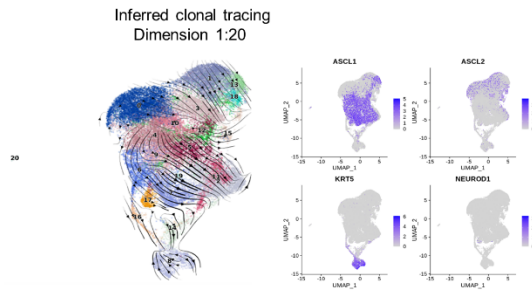# Figure S4

A

Inferred clonal tracing
Dimension 1:20

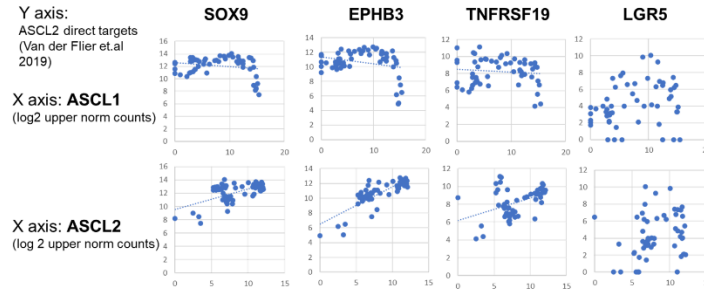## B

Expression of ASCL2 direct targets (Van der Flier et al 2019) vs ASCL1 or ASCL2 in PARCB time course study

C

RT-qPCR of ASCL2 in PARCB tumor derived cell lines

D

Signature score of gene sets from Figure 1D

Signature scores of gene sets from Figure 4E

Figure S5

A

CRPC-PRAD

0014   0016   0002

SCNPC

12021-J1   16071-H3   17001-I1

B

CRPC PDX model

7547B-lung (FHPCX_20-01A)   7394B-liver (FHPCX_20-01B)

C

Figure S6

A RT-qPCR of ASCL1 and ASCL2 in ASCL1 overexpression cell lines

B

C Differential binding of TFAP4 (CUT&RUN)

ASCL1 promoter — ASCL2 enhancers

D

E
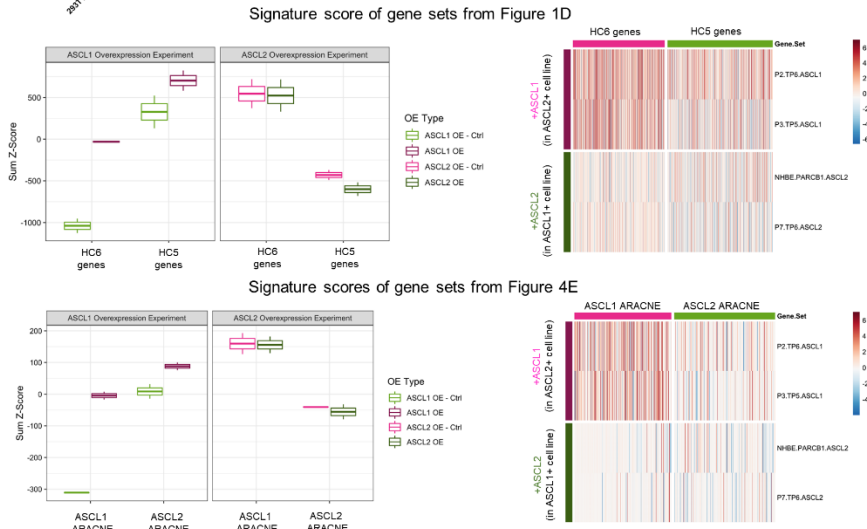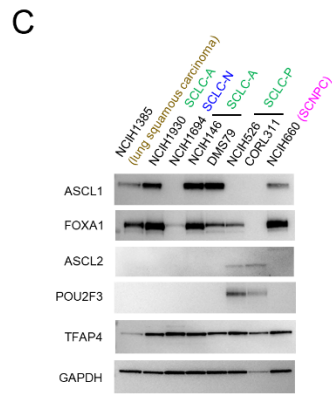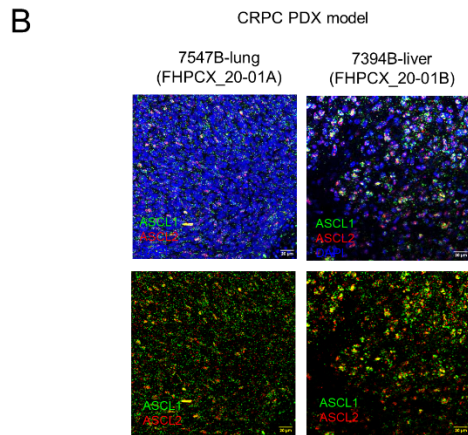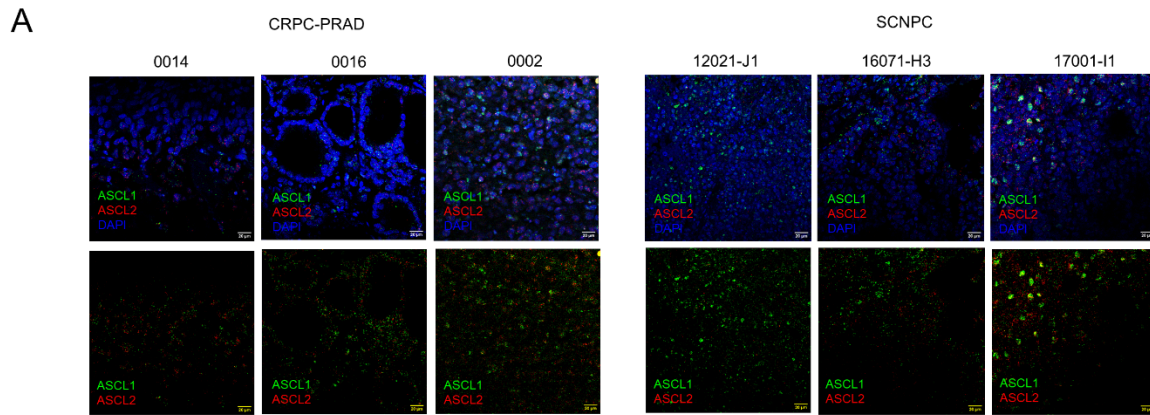
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Chapter 3: Discussion and Closing Remarks

Most trans-differentiation cases from adenocarcinoma to small cell neuroendocrine carcinoma result from acquired resistance against prior targeted treatments in both prostate and lung cancers. It becomes evident that though the underlying biology of trans-differentiation is complicated, it is systematically coordinated by distinct stages of transcriptional changes and master regulators.

This study provides a blueprint for the development of small cell neuroendocrine prostate cancer. With a multi-omics approach integrated with time, the study shows that there exists a bifurcation from a common neuroendocrine differentiation trajectory towards the end stage of the disease progression[96]. Continued work in identifying subtype-specific therapeutic vulnerability presents a potential avenue for precision medicine. Emerging evidence has also suggested that cross-functional profiling such as proteogenomics and spatial transcriptomic analysis could add more dimensions to deepening our understanding of this seemly complex disease[97].

Besides targeting the advanced small cell neuroendocrine stage of cancers, a prevention or re-direction of the differentiation pathway during the disease course may suggest an alternative treatment option for poorly differentiated tumors. Our research opens such potentiality by delineating temporal transcriptional events and pinpointing the emergence of various transcription factors during SCNPC development. Differentiation therapy for solid tumors has been explored but not yet matured in clinical development. Several mechanisms adopted by differentiation therapy include reprogramming, elimination of cell renewal and induction of antitumor immune responses to achieve therapeutic outcomes[98]. Despite the technical challenges in the research and design of clinical trials, which may be difficult for differentiation therapy, identifying more targetable candidates and understanding their mechanisms will accelerate such developments.

Much appreciation and emphasis have been put on identifying transcriptional master regulators in neuroendocrine trans-differentiation. Although some transcription factors may not be directly druggable, targeting the transcriptional axis, such as trans-activator, may provide an alternative

approach. For example, the Chen group identified a BET inhibitor that disrupts the physical interaction between BET bromodomain proteins and NEUROD1, reducing SCLC-N subtype growth *in vitro* and *in vivo*[99]. Besides the extensive study on inhibitors of chromatin modifier EZH2, the other group has explored the possibility of targeting the nucleosome remodeling SWI/SNF complex with a proteolysis-targeting chimera degrader in CRPC[100]. Prospective development of such therapeutic approaches is anticipated in the next generation as more pre-clinical models in prostate cancer are established and our knowledge of pathogenesis is strengthened.

An increasing number of transcriptional parallels and similarities between SCNPC and SCLC have been identified. Outside of tissue-specific molecular events, SCNPC and SCLC share distinct transcriptional patterns from their adenocarcinoma counterparts. In this study, we have validated transcriptional events such as the mutual exclusivity and feedback loop between ASCL1 and ASCL2 expression and their associated transcriptional programs. More work in applying this knowledge to study the molecular mechanism and explore therapeutic opportunity is needed.

This dissertation unravels a timely coordination of transcription factors, suggesting that a combinatorial approach of targeting factors may be warranted. Decades of research has shown that a simple combination of lineage-determining transcription factors are essentially responsible for cell type differentiation[58]. The elimination of passenger events and identification of driver transcription factors is an important attribute for the combinatorial strategy. Our collaborators have made significant progress in determining the minimal number of critical factors that sufficiently transform prostate cancer *in vitro*, which will provide a powerful tool for future studies.

The main objective of the dissertation is to study transcriptional development of SCNPC through multi-omics profiling, however, we anticipate a broader application to pan small cell cancers including lung, bladder and ovarian. Future work is needed to identify the shared molecular mechanism and vulnerability.