An organoid platform to study alveolar stem cells in lung generation and cancer

by

Santiago Naranjo

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ABSTRACT

Lung adenocarcinoma (LADC) remains the most common and lethal cancer type worldwide. Although recent breakthroughs using a new class of immune-modulatory therapeutics have improved patient survival in the clinic, the majority still invariably succumb to this disease, highlighting the importance of improving treatment strategies.

A wide variety of models have been developed to study LADC. Cell line- and transplant-based models offer rapid and flexible platforms for discovering and testing novel therapeutics using patient-derived specimens. On the other hand, genetically engineered mouse models (GEMMs) recapitulate key aspects of human LADC including initiation from normal pulmonary epithelial cells and progression into a malignant state. The development of organoid technology has revolutionized the way we model cancer and a vast number of other biological phenomena. Organoids are cultured miniature organs derived from normal adult stem cells that display self-renewal, differentiation capacity and remarkable genetic stability. These features have facilitated the creation of next generation cancer models that combine the best features of their predecessors.

The alveolar type 2 (AT2) cell represents the most prominent cell-of-origin of LADC. These cells serve as stem cells in the adult lung to support tissue turnover during homeostasis and regeneration after injury. They accomplish this by self-renewing and differentiating into alveolar type 1 (AT1) cells. Using organoid technology, we have developed an improved system to cultivate alveolar organoids from normal murine lungs. We demonstrated that these organoids are positive for AT2 and AT1 markers and completely lack expression of basal and club cell makers. Critically, we observed long-term proliferative potential in these organoids. Using this improved culture system, we generated organoid models of LADC, representing three distinct molecular subclasses of this disease. We found that *Kras*-, *Braf*-, and *Alk*-mutant organoids with *Trp53* deficiency displayed mitogen independent growth *in vitro*. Most strikingly, *Kras*-mutant *Trp53*-inactivated organoids orthotopically transplanted into immunocompetent recipient mice formed tumors that displayed histopathological characteristics of human LADC.

Taken together, the work presented here demonstrates the power of organoid technology for building clinically relevant and experimentally flexible cancer models.

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PUBLICATIONS

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2019	Pentinmikko N, Iqbal S, Mana M, Andersson S, Cognetta AB, Suciu RM, Roper J, Luopajärvi K, Markelin E, Gopalakrishnan S, Smolander OP, **Naranjo S**, Saarinen T, Juuti A, Pietiläinen K, Auvinen P, Ristimäki A, Gupta N, Tammela T, Jacks T, Sabatini DM, Cravatt BF, Yilmaz ÖH, and Katajisto P. Notum produced by Paneth cells attenuates regeneration of aged intestinal epithelium, Nature (2019).
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2015	**Naranjo S***, Smith JD*, Artieri CG, Zhang M, Zhou Y, Palmer M, Fraser HB. Dissecting the genetic basis of a complex *cis*-regulatory adaptation, *PLOS Genetics* (2015).

PRESENTATIONS

2019	**Naranjo S**, Liao LZ, Tang A, Jacks T. Modeling Lung Adenocarcinoma using Alveolar Organoids, *Cell Symposium: Engineering Organoids and Organs* (2019). San Diego, California. **Selected for poster presentation**.

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First, I would like to thank Tyler for providing me with the opportunity to be a part of the amazing community he has created and fostered for over 25 years. The culture of the Jacks Lab encourages us to ask big questions and answer them using extraordinary and innovative approaches. I can say without a shadow of a doubt that I have benefitted tremendously from experiencing this environment and that it has shaped the way I now approach science. I fervently believe that the growth I have experienced here would not have occurred anywhere else. The Jacks Lab is a special place and I will miss it dearly. Tyler, you gave me the freedom to pursue my interests from the very beginning. Although most of my ideas were (and still are) crazy and ultimately did not work out, you never pressured or faulted me for trying. I have always taken pride in my creativity. It is thanks to you that I was able to express it freely and allow it flourish. Your deep commitment to your mentees and students has always inspired me. I have always been amazed that despite your incredibly busy schedule, you make always yourself available to us and your students (except that time you went to New York City and missed the 7.45 lecture, but it was okay because Laurens and I got to stand in front of the classroom and pretend we were running the show for a hot minute). I highly appreciate the emphasis you place on developing our careers, and your approach of supporting of our choices instead of pushing us towards a perhaps undesired path. Without hesitation, you used your resources and vast network of contacts to open so many doors for me. I remain eternally grateful to you for that. I owe my future successes to you. It is no surprise to anyone that I endured many lows in my PhD. Your constant support and encouragement at every step of the way kept me going. It means a lot to know that you value my scientific and experimental skills. I will never forget the conversation when you uncharacteristically encouraged me to pursue a career in science. You said that you believe I possess what it takes to excel as a scientist. I know you wouldn't have said this if you didn't truly believe it, so I thank you for your honesty. I am confident that you will continue inspiring many more students and mentees for years to come.

I would like to express my gratitude to my thesis committee members, Jackie Lees and Omer Yilmaz, for the years of support and mentorship. I have learned a great deal from both of you. I would like to acknowledge and thank Carla Kim for serving as the external member for my thesis defense committee. Your work has inspired and informed my own efforts and I look forward to the surely illuminating and engaging discussions we will have about organoids.

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out helps the lab run smoothly and no one would be able to accomplish anything without them. Judy, thank you for being such a great second/adoptive mother. I really appreciate everything you have done and continue to do for me and the lab, especially your famous slow cooker meatball subs. I am sad that we might not get to celebrate the start of the summer to the beat of Summertime by Will Smith. I will continue to carry out that tradition wherever life takes me next.

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ropes when I decided to start culturing organoids. A giant shout-out to my biggest cheerleader, Lindsay. I never felt smarter and more attractive than that time you showered me with compliments during the ski trip. On a more serious note, you have made valuable contributions to my project and I know that your capable hands will make good use of the organoids. I want you to know that I am infinitely grateful for your offer to help me finish my project. I am confident we will make a great team. Grissel, Nemo, Banu, Jason, Leanne, Ryan, Will H, Amanda, Amy, David and Zack, you have all contributed to my experience and success in the lab in one way or another and I can't thank you enough.

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CHAPTER 1

INTRODUCTION

PART 1: TISSUE CULTURE

1.1 The birth of tissue culture

The first successful cultivation of animal tissue occurred in 1882, when Sydney Ringer maintained dissected frog hearts beating for hours at a time using a balanced salt solution (Ringer, 1882, 1883). Subsequent failed attempts to culture tissues and cells using different versions of “balanced salt solutions” made it apparent that additional components were necessary for cells to survive and proliferate outside of the body.

Ross Harrison’s seminal work several years later helped provide insights into what the balanced salt solutions were missing (Harrison et al., 1907). He was originally interested in developing a system that would allow him to observe embryonic nerve fiber growth. To accomplish this, he dissected out pieces of frog embryos and placed them inside a drop of freshly isolated lymph, which was sitting on top of a cover slip. He then inverted the cover slip and placed it over a hollowed-out slide. This technique later became known as the “hanging drop” culture method. Astonishingly, he observed outgrowth of nerve fibers extending from the tissue chunks out into the lymph. He noted that specimens cultured this way could be kept alive for up to four weeks. Building on this work, Alexis Carrel and Montrose Burrows later established that lymph could not support cells from warm-blooded animals such as chickens. Instead, they discovered that plasma from chicken blood could stimulate growth in this context and that this general strategy was also effective for mammalian cells from normal and even tumor tissue (Burrows, 1911; Carrel and Burrows, 1911). Importantly, they also established that routine media changes and sub-culturing could effectively extend the lifespan of

cells in culture (Carrel and Burrows, 1911). Carrel actually went on to later declare that vertebrate cells are immortal. Carrel and his assistants had reportedly been able to continuously cultivate chicken heart fibroblasts for over 30 years (Shay and Wright, 2000; Reviewed in Witkowski, 1980) by routinely sub-culturing them and feeding them fresh chick embryo tissue extract.

Many others had failed to establish immortal cell strains from human and murine tissues and even Carrel's trusty embryonic chick fibroblasts, but the researchers who carried out these experiments attributed their failures to inadequate culture conditions (Reviewed in Witkowski, 1980). Meanwhile, Carrel never reported a similar phenomenon.

1.2 Replicative Senescence

Leonard Hayflick challenged Carrel's long held belief in 1961. His seminal article convincingly demonstrated that normal human fibroblasts from embryonic tissue have a finite lifespan and don't survive more than 50 population doublings (PDs) – this phenomenon would later become known as replicative senescence (Hayflick and Moorhead, 1961). These results were clearly at odds with Carrel's lifework and left many asking for an explanation. Hayflick and others argued that Carrel may have been inadvertently replenishing his cultures with fresh cells every time he fed them with embryo extract (Shay and Wright, 2000; Reviewed in Witkowski, 1980).

Consistenly, reports of immortal cultures indicated that indefinite growth was an acquired and not intrinsic property of mammalian cells. In order to achieve long-term growth, cells needed to undergo fundamental changes in their genomes. As such, immortal cultures resulted from karyotypically abnormal rare clones that grew out in

dying cultures. For example, murine embryonic fibroblasts (MEFs) only become immortalized when put through the 3T3 protocol (Todaro and Green, 1963).

It is now known that one of the main causes of replicative senescence in human cells is a progressive shortening of the telomeres that cap the ends of chromosomes. This is a natural consequence of genomic replication during cell division (Harley et al., 1990). Uncapped telomeres cause havoc upon the genome and lead cells through a two-step process that ultimately ends in death, or immortalization for the rarest few. Mammalian cells do have a mechanism for elongating their telomeres, but Hayflick's experiments suggested that primary cells did not express the necessary components, at least when they were cultured in 2D and using serum-based medium.

As primary human cells divide, their shortening telomeres cause chromosomes to form end-end associations. The resulting DNA damage triggers cell cycle checkpoint genes such as *P16* and *TP53* and ultimately replicative senescence, also known as the Mortality 1 (M1) stage (Alcorta et al., 1996; Shay and Wright, 2005). One of the many consequences of cell cycle checkpoint activation is preventing passage through the cell cycle by maintaining RB in a hypo-phosphorylated state (Shay and Wright, 2005; Stein et al., 1990). DNA tumor viruses such as Simian Virus 40, Human Papilloma Virus 16, and Adenovirus 5 can readily facilitate bypass of M1 through expression of proteins that bind and inactivate TP53 and RB1 (Reviewed in Shay et al., 1991). Alternatively, rare cells can spontaneously inactivate cell cycle checkpoints and survive M1 in some cases (Shay and Wright, 2005).

Continued proliferation following the M1 stage causes further telomere attrition until chromosomes become uncapped. Shortly thereafter, the cells enter into crisis or

the Mortality 2 (M2) stage, in which severe chromosomal damage from breakage-fusion-bridge cycles causes rampant cell death (Shay and Wright, 2005). As more and more chromosomes become uncapped, they start “fusing” and creating dicentric chromosomes. During the anaphase stage of the cell cycle, the miotic spindle can engage both centromeres and pull these aberrant chromosomes in opposite directions, effectively creating “bridges” between its two poles. Eventually these chromosomes are ripped apart at a weak point along the bridge, resulting in two new “broken” chromosomes with uncapped ends ready to go through cycle again. This process continues until the cell is unable to handle the damage and commits suicide. Ectopic expression or spontaneous activation of telomerase can bypass M2 (Shay and Wright, 2005).

Hayflick’s landmark discovery and the work that followed led many to believe that replicative senescence was an intrinsic property of most normal cells. A few even argued that telomere attrition was linked to organismal aging (Lombard et al., 2005). However, there were skeptics who argued that replicative senescence was largely an artifact of culture, specifically due to a process they called culture shock (Sherr and DePinho, 2000). According to the skeptics, a cell is not able to cope with being ripped from its native environment and placed on plastic with a random cocktail of serum/plasma-derived factors. Furthermore, the fact that murine cells with their characteristically ultra-long telomeres (Kipling and Cooke, 1990) and human cells ectopically expressing telomerase (Kiyono et al., 1998) are unable escape senescence clearly indicated that other factors were at play. The culture shock argument implied that long-term culture of primary cells could be achieved under the correct set of conditions.

Indeed, Carrel prophetically quipped that a lack of continuous replication resulted from an ignorance of appropriate culture conditions.

Experiments demonstrating that specific medium components and other culture conditions could induce or delay senescence lent strong support for the culture shock hypothesis. For instance, one group reported that human mammary epithelial cells grown in different medium formulations displayed distinct proliferative life spans–MEG medium induced senescence after 10 PDs, whereas cells in WIT medium could undergo at least 40 PDs without exhibiting signs of senescence (Ince et al., 2007). A more dramatic example involves rat oligodendrocyte precursors. These cells can be expanded indefinitely in a serum-free chemically defined medium, but the addition of serum or thyroid hormone invariably causes senescence (Tang et al., 2001). Relatedly, co-culturing normal human keratinocytes or mammary epithelial cells with a feeder layer of fibroblasts significantly delays the onset of senescence, and ectopic telomerase expression in the co-culture setting prevents senescence all together (Ramirez et al., 2001). Lastly, MEFs can divide continually without senescing when the O2 concentration is decreased from the standard 20% to the more physiological 3% (Parrinello et al., 2003).

1.3 Stem cells

Hayflick's limit was also at odds with the fact that somatic stem cells in proliferative tissues continuously divide throughout the lifetime of their host (Reviewed in Rubin, 2002). At least in the intestine, the number of divisions (~1000 in the mouse) that the resident stem cells undergo far exceeds Hayflick's limit (Reviewed in Rubin, 2002). The two defining characteristics of a stem cell are an unlimited potential for self-

renewal and the ability to differentiate into specialized cell types. Self-renewal is accomplished through symmetric cell division, where one stem cell gives rise to two new stem cells. Differentiation is a result of asymmetric cell division, in which one stem cell produces a new stem cell and one or more differentiated cell types. Adjacent niche cells, whose main job is to direct stem cell behavior, determine the type of division that stem cells execute through the actions of secreted factors.

Given that the sole purpose of a stem cell is to divide and that specific factors control this behavior, it was at least theoretically possible that stem cells in the proper culture environment would be capable of continuous proliferation outside of the body. Early research involving stem cells from embryonic tissues provided strong support for this idea. For example, murine and human embryonic stem cells exhibited long-term proliferative capacity and retained full differentiation potential when cultured on top of a layer of fibroblasts (Evans and Kaufman, 1981; Thomson et al., 1998). Likewise, neurospheres derived from embryonic neuronal stem cells can be passaged at least 40 in the presence of Egf. Differentiation into neurons, astrocytes, and oligodendrocytes is readily observed when serum is added to the culture medium (Reynolds and Weiss, 1996).

1.4 The advent of organoid technology

Although a lot of valuable research involving adult stem cells from the blood and the brain suggested they behaved similarly to their embryonic counterparts, a definitive and compelling confirmation came from work focused on the intestine. These efforts were led by the laboratory of Hans Clevers and they ultimately paved the way for the

development of modern organoid technology, which provides a nearly universal system to create immortal cultures of normal stem cells from both adult and embryonic tissues.

The Clevers Lab was originally interested in learning what molecular programs governed intestinal stem cell behavior. They eventually made the seminal discovery that Wnt signaling is a crucial player in this context (***Figure 1***). The intestinal epithelium is organized into crypt and villus compartments. The crypts contain intestinal stem cells and niche cells, otherwise known as Paneth cells. Differentiated cells such as enterocytes, goblet cells, and enteroendocrine cells make up the villi. One of the first hints that Wnt signaling regulates intestinal stem cell behavior came from the finding that the intestines of mice lacking *Tcf-4*, a key member of this pathway, lack proliferative crypt compartments and are solely composed of differentiated villus cells (Korinek et al., 1998). Subsequent studies employing genetically engineered mice confirmed the earlier findings by showing that the secreted Wnt inhibitor Dkk1 greatly reduces epithelial proliferation while the secreted Wnt activator R-spondin1 has the opposite effect (Kim et al., 2005; Pinto et al., 2003). The discovery that kickstarted the development of organoid technology was made several years later, when an elegant analysis revealed that the R-spondin1 receptor Lgr5 marks intestinal stem cells (Barker et al., 2007). A rather striking observation made in this study was that Lgr5 seemed to mark cells with stem properties in various other tissues including the stomach, mammary glands, and in hair follicles. This strongly implied that Wnt signaling might play a widespread role in mammalian stem cell biology.

Armed with increasing knowledge about the molecular programs that govern intestinal stem cell function, Clevers's group set out to develop a culture system

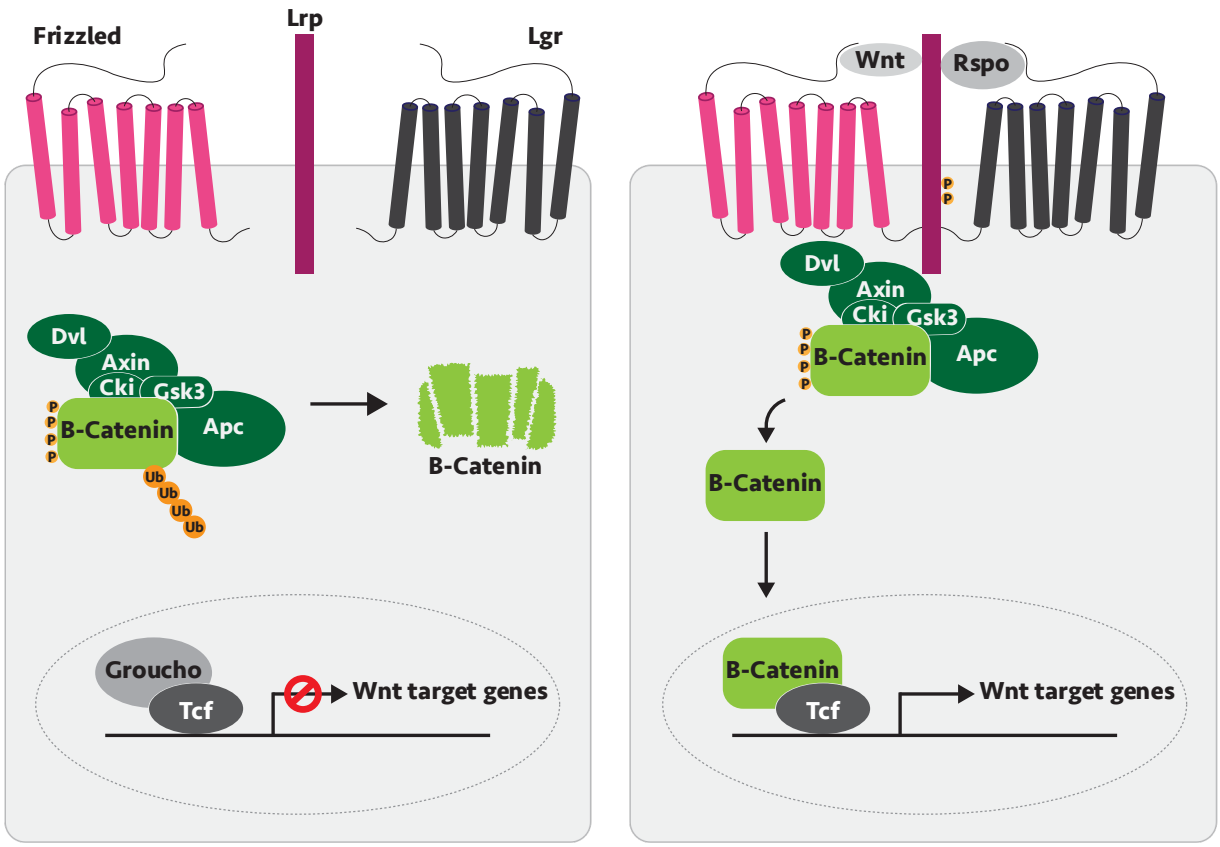

Figure 1. The Wnt pathway.

(Left) In the absence of activating ligands such as Wnt and R-spondin, ß-catenin. is sequestered and targeted for proteasomal degradation by the multi-protein destruction complex through Ck1 and Gsk3 mediated phosphorylation of ß-catenin. (Right) Upon ligand binding, ß-catenin is released from the complex, begins to accumulate, and enters the nucleus, where it associates with Tcf proteins to stimulate transcription of target genes.

for murine intestinal epithelial cells. Previous attempts at building culture systems had been largely unsuccessful as they were unable to maintain continuous proliferation and crypt-villus physiology (Evans et al., 1992; Fukamachi, 1992; Perreault and Beaulieu, 1996), leading many to rely on SV40-immortalized cell lines for their studies (Whitehead et al., 1993). Toshiro Sato and colleagues formulated a culture system that consisted of embedding isolated crypts in matrigel drops and incubating them in a chemically defined medium containing niche inspired ligands. Matrigel is laminin-rich gel isolated from Engelbreth-Holm-Swarm (EHS) mouse sarcomas. In addition to providing a 3D environment, matrigel helps recapitulate the laminin-rich extracellular environment of the crypt base. The importance of the extracellular matrix and a 3D environment for enabling cells to retain their identity in culture had been long established decades before thanks to the pioneering work of Mina Bissell. For example, her earliest efforts revealed that kidney and mammary epithelial cells placed in collagen sandwiches arranged themselves into structures whose morphology resembled their tissues of origin (Reviewed in Simian and Bissell, 2017). Sato and his colleagues found that their physiological media and matrigel scaffold stimulated long-term (on the order of months) expansion of epithelial structures containing crypt and villus domains (Sato et al., 2009). Due to their cellular composition and features, the structures were named organoids, reflecting their resemblance to miniature organs. The specific components of the medium included the mitogen Egf, the Bmp antagonist Noggin, and Wnt agonist R-spondin1 (ENR media). Addition of Wnt3a (WENR media) produced organoids completely composed of undifferentiated stem cells, and made it possible to cultivate colonic organoids (Sato et al., 2011) (***Figure 2***).

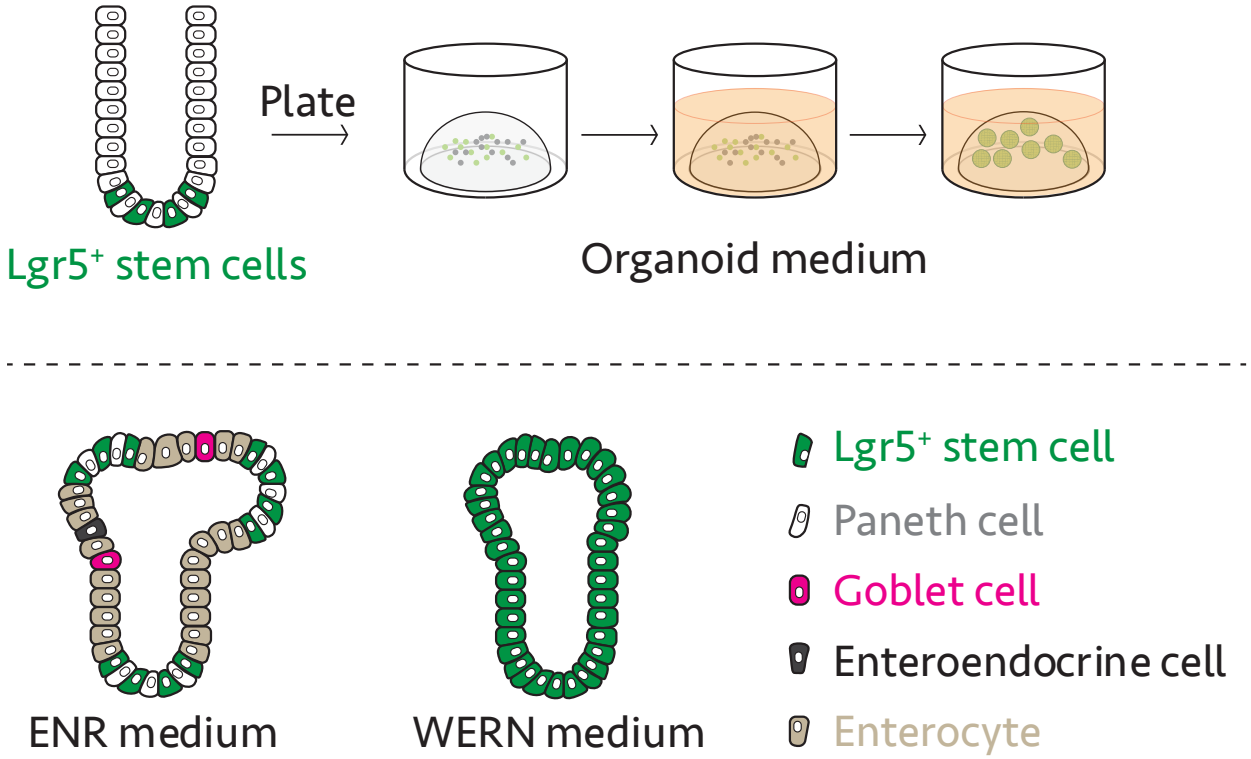

Figure 2. Culturing intestinal organoids.

(Top) The intestinal organoid culture system consists of embedding freshly isolated crypts in matrigel drops and incubating them in medium containing a niche-inspired cocktail of growth factors. (Bottom) The ENR (Egf, Noggin, R-spondin1) formulation stimulates stem cell proliferation and permits differentiation into specialized cell types. Addition of Wnt to the medium (WENR) inhibits differentiation and produces organoids entirely composed of stem cells.

Encouraged by their successes, the same group attempted to apply their updated culture system to grow organoids using human colon, but were unsuccessful. Although organoids did form initially in WENR media, they disintegrated within 7 days. They reasoned that the media required additional factors. Therefore, they screened a small panel of growth factors and vitamins, and found that the combination of nicotinamide and gastrin significantly extended proliferative lifespan to about a month. Interestingly, organoids in the improved media exhibited a two-phase growth arrest consistent with the growth prolife of senescent cells.

Sato and his colleagues once again took a page out of Alexis Carrel's book, and hypothesized that the lack of continuous growth was a result of inadequate culture conditions. Emphatically, a second screen led them to find the missing factors: the Alk4/5/7 inhibitor, A83-01, and the p38-Mapk inhibitor, SB202190. The resulting media formulation, which now included eight factors, supported long-term expansion (at least 6 months) of human colonic organoids that remained genomically stable and retained differentiation capacity (Sato et al., 2011).

Building on this work, similar culture systems for organoids from a wide array of tissues have been reported, including the stomach, prostate, biliary tract, and pancreas (Reviewed in Clevers, 2016). The realization that Wnt signaling broadly drives epithelial stem cell function (Reviewed in Clevers et al., 2014) was instrumental for the development of these culture systems. The vast majority rely on the presence of activating ligands or small molecule agonists of the Wnt pathway. Essentially all described systems generate organoids exhibiting three universal characteristics:

unlimited proliferative lifespan, retention of physiological identity or differentiation capacity, and genomic stability.

1.5 Organoids likely model an injury state

Perhaps it is not surprising that intestinal stem cells are able to persist in culture for extended periods of time given that the entire gastro-intestinal epithelium is programmed to continually turnover throughout the life of the organism. In humans, that can mean tens of decades. What is truly amazing is that it's possible to create organoid cultures from relatively quiescent tissues, such as the pancreas and the liver, which only become proliferative upon injury. Pancreatic organoids arise from duct cells and only require a relatively generic organoid medium not dissimilar from the intestinal equivalent for growth (Huch et al., 2013). In stark contrast, hepatocyte derived organoids rapidly deteriorate in a similar medium, which is extremely surprising given that the liver has a profound capacity for regeneration—in rats, full hepatic restoration is observed in less than a week following surgical resection of two-thirds of its mass (Michalopoulos and DeFrances, 1997). Recognizing the essential role of inflammatory signaling in liver regeneration (Michalopoulos and DeFrances, 1997), the laboratory of Roel Nusse discovered that hepatocytes actually require the inflammatory cytokine Tnf-α to thrive in culture (Peng et al., 2018). An important implication here is that organoids likely model an injured state. Not much is known about this yet, but this phenomenon is likely due to the harsh processing procedures that accompany initial isolation and passaging. Cells living inside an organism only experience similar stresses upon injury.

1.6 Applications of organoid technology

Organoids have emerged as powerful tools for basic and translational biological research. They have been applied in various ways to investigate diverse biological phenomena, ranging from genetic diseases to host-pathogen interactions (Reviewed in Drost and Clevers, 2017). One study leveraged patient-derived organoids to establish an assay for developing personalized treatment strategies against cystic fibrosis (CF). This disease causes a buildup of viscous mucus in the respiratory and gastro-intestinal tracts due to disruptive mutations in the *CTFR* gene. The idea for the assay stemmed from the observation that the small molecule, forskolin, which activates CTFR by increasing intracellular cyclic AMP levels, induces a rapid swelling of organoids from healthy individuals but not CF patients (Dekkers et al., 2013). Therefore, one could easily test the efficacy of CF drugs that stabilize the CTFR receptor by applying the swelling assay in patient-derived organoids. This assay has been successfully employed in the clinic to help predict which patients would benefit from a new class of CF drugs whose efficacy is highly dependent on the specific *CTFR* mutation (Dekkers et al., 2016). A second study used normal intestinal organoids from humans to explore the role of the microbiota in promoting colorectal cancer (CRC). Genotoxic strains of *E.coli* have long been associated with this disease, but it was unclear whether they contributed directly to the occurrence of oncogenic mutations. These genotoxic strains harbor a *pks* operon encoding a group of enzymes that synthesize the genotoxin colibactin, which induces double-stranded breaks in cultured cells and is believed to alkylate adenine residues. To determine whether genotoxic bacterial strains directly induce mutations in intestinal cells, *pks*-proficient and deficient *E.coli* were repeatedly

injected into the lumens of clonal intestinal organoids over a period of 5 months. Whole genome sequencing analysis revealed that organoids exposed to the *pks*-proficient strain accumulated significantly more mutations. Furthermore, they observed that patient tumor genomes harbor *pks* mutational signatures and that *pks* can mutate *APC,* the most commonly altered gene in CRC (Pleguezuelos-Manzano et al., 2020). Altogether these findings provided compelling evidence that the genotoxic bacterial species play a direct role in promoting CRC.

These efforts demonstrate the potential of organoids to help us develop powerful tools for patient care and understand fundamental aspects of disease biology. As will be discussed in **Section 3.4** of this thesis, the unique qualities of genomic stability and retention of physiological identity makes organoids an ideal platform to model cancer and study its progression from a normal state into malignancy.

PART 2: ALVEOLAR TYPE 2 CELLS

2.1 The biology of alveolar type 2 (AT2) cells

Pulmonary alveoli contain two type of epithelial cells: flat alveolar type 1 (AT1) cells and cuboidal AT2 cells. These cells serve three main functions (***Figure 3***). They produce and release pulmonary surfactants to prevent alveolar collapse at the end of expiration by lowering the surface tension at the air-liquid interfaces in the alveoli. Surfactant comprises a combination of four proteins (Sftpa-d) plus a blend of phospholipids, and it is stored inside organelles known as lamellar bodies. AT2 cells also act as facultative stem cells to maintain alveolar spaces in the lung by dividing and differentiating into AT1 cells, which conduct gas-exchange between inhaled air and the blood (Barkauskas et al., 2013; Desai et al., 2014; Evans et al., 1975; Kaplan et al., 1969). Additionally, AT2 cells create an immune-tolerogenic environment in the lung to non-pathogenic antigens. They accomplish this by engulfing and presenting extracellular antigens on MHC Class 2 molecules. Because AT2 cells don't express co-stimulatory molecules such as Cd80, Cd86, and Cd40, which are required for T-cell activation, they suppress and ultimately inactivate any helper T-cells that interact with them (Lo et al., 2008). Lastly, AT2 cells serve as the main cell-of-origin for lung adenocarcinoma. This will be discussed extensively in **Section 4.3**.

2.2 Culturing AT2 cells as cell lines

Initial attempts to culture AT2 cells were largely fruitless. Under standard culture conditions, which include a plastic surface and serum complemented serum, AT2 cells rapidly lose their physiological characteristics, differentiate into AT1 cells and stop dividing (Diglio and Kikkawa, 1977). Specific phenotypic changes include a shift from

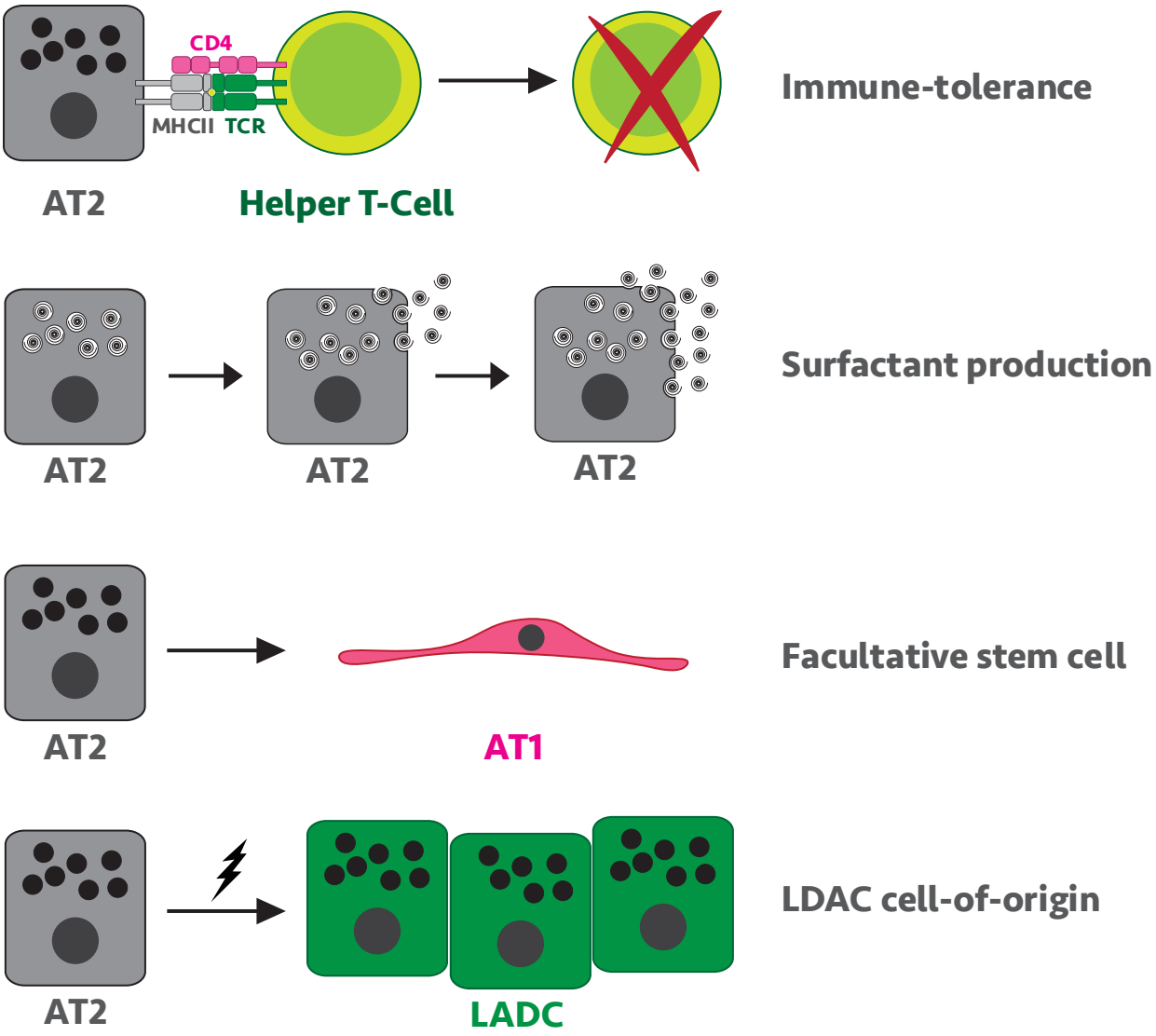

Figure 3. The biological characteristics of alveolar type 2 cells.

AT2 cells have three main functions in the lung: They promote immune-tolerance to non-pathogenic antigens; produce surfactants to prevent alveolar collapse during breathing; and act as facultative stem cells to maintain alveolar spaces in homeostasis and injury. AT2 cells also serve as a cell-of-origin for LADC.

cuboidal to flat morphology, downregulation of surfactant protein expression, and activation of AT1 makers (Reviewed in Dobbs, 1990; Zhao et al., 2013). Having realized that conventional cell culture conditions were insufficient for cultivating AT2 cells, the field focused on finding ways to improve them. Most of the reported methods centered around creating a more physiological environment. One strategy leveraged matrigel-coated dishes or floating collagen gels to better model the substratum of the alveolus (Reviewed in Dobbs Dobbs, 1990). Another strategy involved providing increased or direct exposure to the air to mimic the oxygen-rich environment of the lung. This could be accomplished by either maintaining the culture vessel on top of a rocking platform (Xu et al., 1998) or employing an air-liquid-interface (ALI) system, where cells are seeded on top of a permeable culture insert and medium is only supplied to the basal compartment, leaving the apical side directly exposed to the air (Alcorn et al., 1997; Dobbs et al., 1997).

Surprisingly, less attention was paid to defining the alveolar molecular niche to improve culture medium compositions, but a few studies revealed key roles for Fgfr2 and c-Met mediated signaling. In mice, intratracheal administration of the Fgfr2 ligand, Fgf7, induces alveolar hyperplasia driven via AT2 proliferation (Ulich et al., 1994)–much later, it was found that targeted deletion of Fgfr2 in *Sftpc*-expressing cells has the opposite effect (Dorry et al., 2019). In culture, Fgf7 stimulates proliferation and transcription of surfactant proteins (Sugahara et al., 1995). The c-Met ligand Hgf induces division *in vitro*, but, interestingly, it does not activate AT2 markers (Panos et al., 1993). Consistent with the mitogenic effects observed *in vitro*, c-Met inactivation in *Sftpc*-expressing cells produces mice with malformed alveolar septa (Yamamoto et al.,

2007). An unexpected finding concerns Egfr and its ligand, Egf. Despite Egfr's clear involvement in lung cancer, the ability of its ligand to stimulate mitotic activity in culture (Leslie et al., 1985), and its crucial role in lung development (Miettinen et al., 1995, 1997), targeted deletion of this receptor in AT2 cells is not lethal and does not cause any overt deficiencies or changes in lung structure (Manzo et al., 2012).

One important point to note is that many of these studies assessed the effects of different conditions over a relatively short period of time—on the order of days to weeks. The main reason being that the phenotypic shift from an AT2 to AT1 differentiation state manifests itself shortly after initial isolation. In fact, the majority never even attempted to passage their cultures. As a result, none of these studies addressed how niche-inspired environmental conditions or Fgfr2 and c-Met signaling impacted proliferative lifespan.

2.3 Initial attempts at cultivating AT2 cells as organoids

Shortly after the advent of organoid technology, two independent groups combined matrigel embedding with the ALI system to culture murine AT2 organoids. They reported that AT2 cells cannot form colonies unless they are co-cultured with a supporting feeder cell line (Barkauskas et al., 2013; Lee et al., 2013). Interestingly, Barkauskas's organoids comprised a mixture of AT2 and AT1 cells and could be passaged at least thrice. In contrast, Lee's organoids contained club cells instead of AT1 cells, as well as AT2 cells that could not self-renew. The specific composition of the culture media and the identity of the feeder cells in each case likely accounts for these differences. The former utilized freshly sorted alveolar fibroblasts plus lipo-fibroblasts and MTEC/Plus medium, which contains serum, pituitary extract, and Egf. The latter opted to use an immortalized lung fibroblast cell line known as MLg and medium only

containing serum as a source of growth factors. A subsequent study from Lee et al. later confirmed their original finding that AT2 derived organoids cultured in serum supplemented media lack self-renewal capability even in the presence of primary lung endothelial cells (Lee et al., 2014). Crucially, Barkauskas's culture system is able to establish human alveolar organoids, but it is unclear how long they can persist in culture. Although these observations don't inspire confidence in the idea that alveolar organoid cultures with unlimited proliferative potential can be established, the organoid culture systems discussed above still lacked a physiological culture medium. In fact, essentially all currently available culture systems utilize ill-defined formulations containing serum and it is highly likely that the answer lies in leveraging formulations that better recapitulate the alveolar molecular milieu.

2.4 Defining the alveolar molecular niche

The field has only recently focused more attention on defining the niche factors that drive AT2 self-renewal and differentiation. Perhaps unsurprisingly, AT2 cells rely on the same molecular programs that drive stem cell function in many other tissues. Given that the lung is a relatively quiescent tissue, the vast majority of efforts have employed alveolar injury models in mice to activate and study the stem functions of AT2 cells.

Three independent groups have reported that Wnt signaling plays a key role in supporting the stem properties of AT2 cells *in vivo* (Frank et al., 2016; Nabhan et al., 2018; Zacharias et al., 2018). Under homeostatic conditions, only a small subset make up the alveolar stem cell compartment and they display active Wnt signaling. This population self-renews and differentiates into AT1 cells. However, in the setting of

severe lung injury, the normally quiescent bulk AT2 population acquires stem cell properties that are supported by autocrine Wnts.

Several lines of evidence have suggested that Tgf-ß signaling blocks AT2 cell self-renewal and promotes differentiation. A single cell transcriptomic analysis of lipopolysaccharide treated lungs found that AT2 cells occupy one of three states upon injury: proliferation, cell-cycle arrested, and differentiation. Differential expression analysis between the subsets revealed that Tgf-ß signaling was highly up-regulated in the cell-cycle arrested population and relatively down-regulated in the differentiating population. Consistently, activation of this pathway in cultured AT2 cells imposes cell cycle arrest and its subsequent inactivation permits differentiation (Riemondy et al., 2019). In agreement with these results, two independent analyses of primary AT2 cells demonstrated that they potently and rapidly upregulate Tgf-ß, its receptor, and downstream effectors upon being placed into culture (Bhaskaran et al., 2007; Zhao et al., 2013).

A role for Bmp signaling has also been recently uncovered using a pneumectomy injury model and Barkauskas's organoid system. Bmp signaling was found to be active in the resting state, transiently downregulated shortly after injury to enable proliferation, and restored to promote differentiation (Chung et al., 2018). Consistently, Bmp4 inhibits AT2 proliferation in organoids whereas Bmp inhibitors enhance it, but at the expense of differentiation (Chung et al., 2018).

Echoing the biology of liver regeneration, inflammation likewise regulates alveolar stem cell behavior. Interestingly, different flavors of inflammation impact function and differentiation in unexpected ways. For example, the inflammatory

cytokines Il-1 and Tnf-α promote mouse AT2 organoid proliferation without impacting differentiation (Katsura et al., 2019). Curiously, Il-13 has the same effect on proliferation but it causes transdifferentiation into bronchiolar lineages, as evinced by expression of basal and club cell markers upon treatment with the cytokine (Glisinski et al., 2020).

A few groups have carried out comprehensive inquiries to attain a more complete understanding of the alveolar niche. The laboratory of Edward Morrisey employed a combination of single cell RNA sequencing and fluorescent lineage reporters to characterize the mesenchymal subsets that support alveolar function and repair in mice. They identified two functionally distinct populations they term mesenchymal alveolar niche cells (MANCs) and Axin2+ myofibrogenic progenitor (AMP) cells. Using their transcriptomic data, they defined MANC/AMP secretomes as well as AT2 receptomes to pinpoint paracrine signaling pathways that support and inhibit alveolar growth. Among many factors, their analysis indicated that the supportive MANCs secrete Bmp inhibitors, Fgfr2 ligands, and the inflammatory cytokine Il-6. Whereas, the inhibitory AMPs release Tgf-ß. Interestingly, both niche subsets secrete Bmp ligands (Zepp et al., 2017). Similarly, Kouji Matsushima ‘s group generated epithelial-mesenchymal interactome through transcriptional profiling of sorted cells from embryonic and postnatal murine lungs. Their findings coincided with countless others before them. The interactome suggested that the mesenchyme regulates alveolar epithelial behavior via the following signaling pathways: Bmp, Tgf-ß, Fgfr1-2, Egfr, Igf, Il-6, Lif, Vegf, Wnt, and Notch (Shiraishi et al., 2019).

2.5 Culturing AT2 cells as organoids

Armed with a much more refined understanding of the pathways that govern alveolar stem cell behavior, two alveolar organoid systems employing niche-inspired media have been described. The first system involves cultivating freshly sorted murine lung epithelial cells embedded in matrigel at the air-liquid-interface with MTEC/Plus medium supplemented with Fgf7, Noggin, SB431542 (a small molecule inhibitor of Tgf-ß signaling), CHIR99021 (a small molecule inhibitor of Gsk3-ß, a negative regulator of Wnt signaling), and Jagged1 (an activator of Notch signaling). These conditions are sufficient to stimulate outgrowth of alveolar organoids composed of AT2 and AT1 cells without fibroblast feeders (Shiraishi et al., 2019). A follow up study successfully applied this system to grow organoids from frozen fetal human lung tissue (Shiraishi et al., 2019b). The second system consists of culturing freshly sorted AT2 cells in matrigel drops and overlaying chemically defined medium whose main components include Fgf7, Fgf10, Egf, A83-01 (a small molecule inhibitor of Tgf-ß signaling), R-spondin1, Wnt3a, and Noggin. Organoids expressing AT2 makers developed under these conditions. However, It was not determined whether AT1 cells were present in the cultures (Weiner et al., 2019). The first system permits at least three passages. On the other hand, it is not known whether the second system supports serial passaging. Once again, it is still unknown whether AT2 organoids are capable of continuous proliferation under these optimized conditions.

Related systems that employ induced pluripotent stem cells (iPSCs) to derive and culture AT2 cells leverage conditions that are very similar to what has been used for organoids. For example, Darrell Kotton's group has cultured induced alveolospheres

for long periods of time using media containing CHIR99021 and Fgf7, among other factors (Jacob et al., 2017). However, although these spheres expressed classic AT2 markers and were capable of differentiating into AT1 cells, they did not harbor lamellar bodies and were transcriptionally closer to cultured fetal AT2 cells than to adult AT2 cells. Similarly, Michiaki Mishima's group successfully created AT2 organoid cultures from iPSCs utilizing an air-liquid-interface and medium containing CHIR99021, Fgf7, and SB431542 (a Tgf-ß pathway inhibitor) (Yamamoto et al., 2017). The major limitation of this system is that passaging requires sorting for AT2 cells specifically.

In summary, further work is required to definitively establish whether AT2 cells derived directly from adult lung tissue are capable of continuous proliferation in organoid culture while maintaining their physiological identity and functionality. A major focus of this thesis is to leverage the previously accumulated knowledge regarding AT2 stem cell biology in combination with organoid culture to establish improved conditions to create long-lived cultures of AT2 cells.

PART 3: MODELING CANCER

Cancer models have enabled the research community to gain invaluable insights into the causes and consequences of cancer. A vast variety of cancer models exists, ranging from simple cell line-based models to complex genetically engineered animal models. Each model brings with it a unique set of useful features and important caveats. In this section, the four major types of cancer models that exist today will be discussed: Cancer cell lines, cell line and tumor transplants, genetically engineered mouse models (GEMMs), and organoids. The discussion will mainly highlight advantages and disadvantages associated with each model.

3.1 Cell line models of cancer

Cell line-based models of cancer remain an invaluable resource for the study of cancer. They facilitate rapid interrogation of tumor intrinsic phenotypes for three main reasons. First, cultured cancer cells are easy to manipulate genetically or otherwise. Second, deriving, maintaining, and expanding them is straight-forward, fast, and cost-effective. In fact, an impressive number of cell lines have been derived from almost every imaginable tumor type. Most importantly, they permit the study of cancer in human cells directly.

3.1.1 Cell lines are genetically tractable

The experiments that led to the discovery of cellular oncogenes best illustrate how researchers made use of facile genetic manipulation to understand that genetic changes are at the heart of carcinogenesis. The characterization of chicken and rat tumor viruses in the 1970s provided a simple yet powerful explanation of how cancer arises. Unfortunately, epidemiological studies of human cancer made it clear that the

disease was not contagious and thus did not spread from patient to patient as an infectious disease. Attempts to reconcile the theory of viral carcinogenesis with mounting observations that established a link between carcinogen exposure and human cancer proved to be fruitless. The focus then shifted to understanding how carcinogens triggered cancer formation. The prevailing theory at the time posited that carcinogens introduce mutations into genes that alter their function to promote aberrant cellular growth. To test this theory, researchers first transformed immortalized mouse fibroblasts, which have the ability to persist in culture indefinitely but are not tumorigenic, through carcinogen treatment. They then introduced DNA isolated from the transformed cells back into the original cells from which the donors were derived. The idea was that if the carcinogen-exposed DNA contained mutated cancer-causing genes, the recipient cells should become transformed. The results demonstrated that the DNA from carcinogen exposed cells could indeed endow normal cells with tumorigenic properties, whereas DNA from normal cells could not, strongly implying that carcinogens create mutations in normal genes that program cells to divide uncontrollably.

3.1.2 Cell line biobanks facilitate rapid testing of therapeutics

Many clinically impactful drug candidates have been tested and validated in cell line-based cancer models. In this arena, cancer cell lines have been used as the workhorse, due to the availability of a vast number of samples representing a wide spectrum of tissue and molecular subtypes. In 2005, two research groups independently uncovered that inhibiting PARP1 function represented a powerful strategy to treat *BRCA*-mutant cancers using human cancer cell lines (Bryant et al., 2005; Farmer et al.,

2005). PARP1 is a key member of the base excision repair pathway, and previous evidence demonstrated that its inactivation forces the cell to rely on homologous recombination to mend genetic lesions and preserve genomic integrity. Failure to do so would result in accumulation of DNA damage and ultimately cell death from genotoxic stress. These observations led to the hypothesis that a deficiency in homologous recombination, through mutations in *BRCA* genes, would render cells exquisitely sensitive to PARP1 inhibition. As expected, combination treatment of MCF-7 and MDA-MB231 cell lines with the PARP inhibitor NU1025 and *BRCA2* directed siRNAs resulted in profound cell death. The initial work of these two groups culminated in FDA approval of PARP inhibitors for the treatment of *BRCA*-mutant ovarian and breast cancers. More recently, cancer cell lines were deployed to validate the specificity and efficacy of a novel class of covalent inhibitors targeting $KRAS^{G12C}$, a protein that has proved notoriously difficult to target in the past (Cox et al., 2014). There is much excitement around these inhibitors because KRAS mutations are initiating or “truncal” events in tumorigenesis. Consequently, every descendent of the cell that originally “seeded” the tumor should carry the same KRAS mutation and is likely reliant on the altered gene product for survival. The G12C mutation is highly common in LADC, and found in a number of other cancer types, albeit at lower frequencies (Seton-Rogers, 2020). The inhibitors make a covalent bond with the highly reactive mutant cysteine residue that replaces glycine to endow KRAS with oncogenic properties, locking it in an inactivate state (Seton-Rogers, 2020). Furthermore, the requirement of cystine for binding means that the inhibitors do not disrupt wildtype KRAS function. To test the specificity of the compounds, a panel of human cancer cell lines carrying the G12C mutation (n=12) or

other related but distinct mutations in KRAS (n=10) were treated with the compound. The inhibitor was only able to reduce cell viability in lines carrying G12C mutations (Canon et al., 2019). Excitingly, this inhibitor is showing efficacy in early stage clinical trials (Canon et al., 2019). Collectively, these examples demonstrate that cell line cancer models provide an effective platform to rapidly translate findings from the bench to the clinic. The wide availability of specimens from a variety of disease types and their easy-to-use nature make this possible.

3.1.3 Mega-scale genetic screening is feasible in cell lines

The discovery of PARP-inhibitor therapy perfectly illustrates that cancer cells are not invincible and that they undoubtedly contain many therapeutically-exploitable genetic dependencies. The advent of technologies such as RNAi and CRISPR/Cas9 (briefly discussed in **Section 3.3**) has provided scientists with an efficient way to perform genome-wide genetic screens and identify all such vulnerabilities (and growth promoting genes as a bonus). An example of such an effort is known as the Cancer Dependency Map project, which is being spearheaded by the DepMap team at the Cancer Program of the Broad Institute of Harvard and MIT. The DepMap team aims to conduct genome wide screens in thousands of human cancer cell lines representing a panoply of cancer types. Needless to say, this herculean endeavor requires a system that can support mega-scale expansion of a wide variety experimental samples that are amenable to genetic manipulation. Cell line-based cancer models are perfectly suited for this purpose. The DepMap project, and others like it, would simply not be possible without them.

3.1.4 Complex physiological interactions cannot be modeled in culture

Despite the tremendous progress we have made using cell line cancer models, there are a number of disadvantages associated with them. Naturally occurring tumors develop in the context of a complex and dynamic microenvironment, meaning that tumor cells undergo constant crosstalk with their neighboring cells. For example, one of the most important interactions involves the immune system. It is now widely appreciated that tumor cells employ an array of mechanisms to avoid immune-surveillance and destruction, often leading to immune cell dysfunction in the tumor microenvironment. Groundbreaking work has revealed that reinvigorating dysfunctional immune cells is an effective and potentially universal strategy to cure cancer (briefly discussed in **Section 3.2**). This and other related findings have led to an explosion of interest in the field of tumor immunology in recent years, with the goal of better understanding the causes and consequences of immune cell dysfunction in cancer. Although co-culture strategies can help model how the presence of certain immune cells influence cancer cells, it is nearly impossible for culture models to capture the full extent of the cancer immunity cycle, which involves cancer cells, a variety of T-cells, different types of antigen presenting cells, and innate immune cells acting in concert across different tissues in the body (Chen and Mellman, 2013). In fact, any *in vitro* based cancer system cannot fully recapitulate four out of the ten hallmarks of cancer: avoiding immune destruction, tumor promoting inflammation, inducing angiogenesis, and metastasis (Hanahan and Weinberg, 2011). Again, these processes involve dynamic interactions occurring between the tumor and a variety of normal cells in the body.

3.1.5 Two-dimensional culture induces irrelevant genomic alterations

While the simplistic nature of cell culture makes it an attractive platform to study cancer, it also renders it unable to faithfully model cancer beyond providing an indefinitely persisting culture of cells carrying a hodgepodge of genetic perturbations. Mounting evidence has demonstrated that the choice to culture cells in 2D without proper tissue architecture is at the heart of the problem. A recent elegant study explored the question of how the tissue culture environment impacts genomic integrity in normal cells. Strikingly, this study found that whereas proliferative cell types experience low or no chromosome mis-segregation *in vivo*, freshly dissociated versions of the same cell types are unable to properly segregate their chromosomes in 2D culture (Knouse et al., 2018). Many different reasons can account for the observed difference because tissue and cell culture environments differ in many ways: the cellular and molecular milieu, the atmosphere, and the architecture. The same study, however, went on to show that chromosome segregation improves significantly when dissociated cells are cultured in 3D by embedding them in matrigel droplets (Knouse et al., 2018). The authors propose that disruption of tissue architecture may underlie the rampant genomic instability in cancer.

A deeper implication of this work is that cancer cell lines constantly acquire genomic alterations as they are propagated in 2D culture. However, unlike tumor cells growing inside an organism, the evolutionary trajectory of cultured cells is guided by non-physiological or otherwise artificial selection pressures. Ultimately, this could mean that cell lines derived from the same tissue sample or even different stocks of the same cell lines will progressively diverge away from their source in an incongruent fashion.

Analyses of independent cancer cell line stocks grown in different laboratories have found extensive clonal diversity and significant phenotypic heterogeneity between different “strains,” including differential response to drug treatment (Ben-David et al., 2018). This phenomenon is known as “cancer model evolution,” and it has been argued that it is an important contributing factor to the reproducibility crisis in cancer research (Ben-David et al., 2018).

According to recent reports, only 11-25% of high-profile cancer studies could be replicated in industrial laboratories (Begley and Ellis, 2012; Prinz et al., 2011). A large-scale initiative known as “The Reproducibility Project: Cancer Biology” was initiated in order to address growing concerns about the reproducibility crisis. The results from a substantial number of replication studies have already been published, and while many previous findings still hold true in these studies, a troubling amount of research is not reproducible. There is hope amid this seemingly insurmountable crisis. Han et al. recently performed genome-wide loss-of-function genetic screens using human lung cancer cells that were grown either as monolayers in 2D or aggregates in 3D. Their most striking finding was that inactivation of canonical lung cancer tumor suppressors or oncogenes did not impact cell growth as expected in the monolayers, but did so in the aggregates (Han et al., 2020). This work points to 3D culture as a simple and effective strategy to improve cell line cancer models.

3.2 Transplant models of cancer

Transplant cancer models were originally developed in the early 1900s, when the scientific community was vigorously searching for a platform in which to test cancer therapies. Pioneering work performed by Morau, Jensen, and Clowes at the turn of the

20th century demonstrated that implantation of dissociated tumor material from mice who had spontaneously developed cancer could seed new tumors in healthy recipient mice (Triolo, 1964). A live animal represents the most relevant environment for cancer cell growth, and it helps overcome most of the issues inherent to *in vitro* culture systems, namely a lack of tissue architecture and interaction with stromal cell types. A broad range of transplant models exist today. They are categorized according to the site of injection (subcutaneous/subQ vs orthotopic), source of inoculum (cell lines or tumors), and identity of the recipient (immune-competent vs immune-compromised).

SubQ transplants are generally preferred over orthotopic transplants because they offer a system that is tractable and quantitative—subQ injections are simple to execute and the external location of the resulting tumors facilitates quantitative tracking of growth over time without the need for imaging. On the other hand, orthotopic transplant models offer the distinct advantage of growth in the native microenvironment. Comparative analyses have shown that orthotopic injections produce tumors that better recapitulate the histopathological characteristics, drug response profiles, and nutritional milieu of naturally occurring human tumors compared to their subQ counterparts (Erstad et al., 2018; Sullivan et al., 2019; Wilmanns et al., 1993; Zhang et al., 2018). Human cancer cell lines have traditionally been the inoculum of choice, but the field has recently designated patient derived xenograft (PDX) lines as the gold standard for pre-clinical studies. The defining feature PDX lines is that they are established and propagated as grafts in mice, never experiencing the excessively artificial environment of tissue culture or its associated selection pressures. Due to these features, PDX

models have been presumed to faithfully recapitulate the genomic features of human tumors.

The source of the inoculum and the specific goals of a particular experiment dictate the identity of the transplant host. Immune-compromised mice are required when injecting human cancer cells or when a syngeneic host for a particular murine cancer cell line is not available. Immune-competent mice are compatible with tumor cell lines of the same or a closely similar genetic background. Inbred mice typically serve as the source for syngeneic hosts and tumor cells lines.

3.2.1 Transplant tumor models enable investigation of tumor-stroma crosstalk

Transplant cancer models were instrumental to the discovery of perhaps the most transformative cancer therapy ever, which is now known as immune-checkpoint blockade. The basic idea behind this therapy is that inhibitory signals mediated by immune checkpoint molecules prevents T-cells from mounting productive responses against tumors. Therefore, blocking these signals should theoretically enhance anti-tumor immunity. The first demonstration of this was performed by the laboratory of James Allison. His group observed that blocking the immune checkpoint receptor Ctla-4, which suppresses T-cell activation by binding stimulatory ligands expressed on antigen presenting cells, leads to immune mediated rejection of two independent syngeneic tumor models, 51BLim10 and Sa1N (Leach et al., 1996). Soon after, the laboratories of Tasuku Honjo and Nagahiro Minato reported that inhibiting *Pd1,* whose role is to inhibit T-cell function by antagonizing stimulatory signaling and inducing apoptosis, induces potent anti-tumor immunity against the murine multiple myeloma line J558L (Iwai et al., 2002). The success of checkpoint blockade in pre-clinical transplant

based models paved the way for the eventual approval of checkpoint blockade therapy by the FDA for a variety of disease types (Vaddepally et al., 2020). The most striking feature of the therapy is that it produces long lasting responses in specific subsets of patients, to the point where they can even consider themselves to be cured. Altogether, these results clearly demonstrate that transplant cancer models are a relevant and powerful way to study cancer *in vivo*.

3.2.2 Large-scale genetic screening is feasible in transplant cancer models

As briefly discussed in **Section 3.1**, genetic screening offers a powerful strategy to rapidly and systematically identify growth promoting and inhibitory genes in cancer cells. Transplant models afford us the opportunity to carry out genetic screening in an *in vivo* context. This is feasible because the cells used to generate transplant tumors can be easily manipulated *in vitro* prior to implantation. Many efforts have leveraged genetic screening in transplanted tumors to identify cell-intrinsic mechanisms that directly suppress (Chen et al., 2015; Katigbak et al., 2016) or promote (Bajaj et al., 2020; Kodama et al., 2017) tumor growth. Astoundingly, some of these screens were performed at the genome-wide scale.

Additionally, a number of laboratories have even showed that it is possible to leverage *in vivo* genetic screening to find genes that mediate the interaction between cancer cells and the surrounding microenvironment (Ishizuka et al., 2019; Li et al., 2020; Manguso et al., 2017). An illustrative example involves a screen that tested about 2300 genes for their ability to potentiate or mediate resistance to PD1 checkpoint blockade. This screen identified two promising candidates, *Ptpn2* and *Adar1*, whose inactivation conferred sensitivity to checkpoint therapy. Deletion of the former boosted

the effects of interferon-γ on antigen presentation and growth suppression (Manguso et al., 2017). Loss of the latter increased sensing of endogenous dsRNA species leading to increased tumor inflammation and growth inhibition (Ishizuka et al., 2019). These studies highlight the utility of transplant models for rapidly dissecting the interplay between tumors and stromal cells in an *in vivo* setting using high throughput methodologies.

3.2.3 Transplanted tumors might not accurately recapitulate human cancer biology

Transplant cancer models have clearly helped us make significant strides in the fight against cancer, but no model is perfect. Ironically, the features that have led them to be heavily relied upon for the majority of cancer research also represent their biggest flaws. The rapid growth of transplanted tumors–lasting just a few weeks before the host succumbs to the disease–is not representative of the latencies observed in patients, which are on the order of years and sometimes even decades. The exact consequences of this difference are not yet known, but it could certainly steer the evolutionary trajectory of tumor cells and their neighbors towards an artificial endpoint. Although orthotopic transplants are demonstratively the best transplant modality in terms of their clinical relevance, they are not employed as often as their subQ counterparts because the injections are often prohibitively difficult to conduct. They require lengthy, specialized, and sometimes invasive surgical procedures. Likewise, measuring tumor growth is not always straightforward since many orthotopic tumors develop inside the body and require complicated imaging strategies to visualize and measure.

Most strikingly, transplanted tumors appear to experience model evolution in a fashion similar to cell lines. Evidence for this comes from recent work conducted using a large collection of PDX models and sought to answer two main questions: Do PDX samples acquire genomic changes over the course of continued propagation? If they do, what is the identity and relevance of the observed changes? The researchers leading this work found that PDXs rapidly accumulate copy number alterations that differ from those that are recurrently found in primary tumors of the same cancer type (Ben-David et al., 2017). These results indicate that genetic events that are important for progression in patients are actually dispensable in mice and that PDXs are subject to model evolution just like cell lines.

3.3 GEMMs of cancer

Brinster and Palmiter serendipitously generated one of the first GEMMs of cancer using a microinjection technique (Jaenisch and Mintz, 1974) that allowed introduction of exogenous DNA into the mouse genome. In an effort to generate mice with highly expressed transgenes, they introduced an expression construct carrying the entire early region of the SV40 genome, containing a strong enhancer but also the coding regions of the small and large T-antigens, upstream of the transgene of interest. To their surprise, they found that the construct actually caused mice to develop choroid plexus tumors in the brain, as well as other growth abnormalities in the thymus and liver (Brinster et al., 1984) Upon further examination, they discovered that the large T-antigen portion of SV40 was responsible for this phenotype (Palmiter et al., 1985).

Recognizing the potential of their discovery, Brinster and Palmiter soon adopted a more directed and rational approach to build models for various cancer types by

leveraging transgene constructs that express known oncogenes under the control of tissue specific promoters. Two illustrative examples include the *Eμ-Myc* model of B-cell lymphoma, which relies on *Eμ* driven overexpression of *Myc* in B-cells (Adams et al., 1985), and the *Elastase-Hras*^{G12V} model of pancreatic cancer, which uses the *Elastase* promoter to direct *Hras*^{G12V} expression in acinar cells (Quaife et al., 1987). This approach is still in use today and has produced a wide variety of tumor models.

While the approach employed by Brinster and Palmiter provided a rapid and efficient platform to study the process of tumorigenesis in live animals, there are two main disadvantages associated with this technology. Transgenes are driven by highly active promoters and integrate multiple times into the genome randomly. As a result, transgene expression levels are supraphysiological, can vary considerably across mice, and most importantly, are not regulated by endogenous transcriptional machinery. Furthermore, the capabilities afforded by transgene technology are generally not well suited to study tumor suppressors, which requires gene inactivation.

In the decades that followed, two important technological developments paved the way for the development of improved mouse models of cancer relying on conditional alleles of endogenous genes. The ability to make targeted modifications to the mouse genome overcame the issues associated with transgenes and made it possible to model cancer by introducing germline mutations into endogenous tumor suppressors or oncogenes (Jacks et al., 1992, 1994b, 1994a; Johnson et al., 2001). However, it soon became apparent that creating animals with whole body mutations was not feasible (due to embryonic lethality) or desirable (due to multiple tumor types forming in the same animal) for certain genes.

The incorporation of site-specific recombinase (SSR) technology into GEMMs helped overcome these issues. SSR systems allowed for the creation of conditional alleles whose activation or deletion is dependent on the expression of a recombinase enzyme. SSRs (Cre/Flp) catalyze recombination between two specific sequences of DNA (LoxP, Frt). The placement and relative orientation of the recognition sites dictate the outcome of the recombination reaction: excision of the intervening sequence (two sites in the same DNA molecule facing the same direction) or inversion of the intervening sequence (two inverted sites in the same DNA molecule). For example, an allele can be deleted by introducing LoxP sites around its body (Flanked by LoxP or floxed alleles), or activated by removing a strategically placed LoxP-flanked transcriptional termination cassette (Lox-STOP-Lox or LSL alleles). When employed as a transgene, the timing and location of Cre activity can be controlled by placing its expression under the control of tissue-specific promoters and through modifications which make its activity dependent on binding of small molecules (Cre-ER/Tamoxifen or DD-Cre/Shield). Alternatively, Cre can be delivered to specific sites at defined time points in viral vectors.

Various Cre-LoxP-based mouse models have been developed representing a wide variety of disease types. These models can be simple or very complex, employing anywhere from one to three or more conditional alleles. For instance, a model for colorectal adenocarcinoma is based conditional deletion of *Apc* only (Shibata et al., 1997), whereas a model for high-grade astrocytomas employs floxed alleles of *Nf1*, *Trp53*, and *Pten* (Kwon et al., 2008).

3.3.1 GEMMs recapitulate the full extent of tumor evolution

GEMMs offer two important advantages over all previously discussed models. Unlike transplanted tumors, which are generated using material derived from established tumors, GEMM tumors arise from healthy cells and develop in their native microenvironment. The main consequence is that GEMM tumors closely recapitulate the phenotypic changes that accompany progression into a malignant state in humans. A clear indication of this comes from observations that the histological progression pattern of GEMM tumors is almost indistinguishable from the human equivalent. As an example, the *Kras*$^{LSL\text{-}G12D}$ *Pdx1-Cre* (KC) mouse model of pancreatic ductal adenocarcinoma (PDAC) produces lesions resembling pre-invasive human pancreatic intraepithelial neoplasias that progress to advanced stages, culminating in metastatic spread to stereotypical sites including the liver and the lung (Hingorani et al., 2003). In stark contrast, unpublished experiments from our lab indicate that orthotopically transplanted PDAC cell lines give rise to tumors exhibiting sarcomatoid or mesenchymal histology. Moreover, GEMMs of cancer are not subject to model evolution (discussed in **Section 3.1**) because the normal cells from which GEMM tumors arise are karyotypically normal and do not rapidly accumulate copy number alterations over time, as is the case with cell lines.

Due to the above outlined features, GEMMs have been repeatedly utilized to identify the molecular changes that underlie progression from a healthy to a malignant state, and every step in between. Similar to the KC model of PDAC, activation of oncogenic *Kras* in lung epithelial cells brings about hyperplasias that progress to adenocarcinomas (LADC) (Guerra et al., 2003; Johnson et al., 2001). Using the “K-only”

model of LADC, the laboratory of Mariano Barbacid compared the transcriptional profiles of hyperplastic lesions and adjacent normal cells. This analysis revealed that Ddr1 is highly upregulated in nascent tumor lesions. Consistently, inactivating *Ddr1* in *Kras*-driven tumors severely impairs their growth (Ambrogio et al., 2016). Related approaches have been adopted to dissect the molecular programs dictating progression to a metastatic state (Chiou et al., 2017; Chuang et al., 2017; Denny et al., 2016; Winslow et al., 2011) and have led the field to gain a better appreciation of patient relevant cancer biology.

3.3.2 GEMMs facilitate identification of cancer driver events

Recent large-scale tumor sequencing studies have shed light on the mutational landscape of cancer and at the same time uncovered a vast number of previously unappreciated candidate driver genes. Although GEMMs provide a physiologically and biologically relevant platform to assess putative drivers, the process of generating and crossing the relevant conditional alleles into established cancer models is labor intensive and time consuming. The advent of CRISPR/Cas9 genome editing technology has provided a simple but powerful solution to this problem. The CRISPR/Cas9 system makes it possible to mutate any gene of interest in somatic tissues without having to go through the laborious process of engineering conditional alleles beforehand. This system simply requires expression of the Cas9 protein and a small sgRNA molecule that is complementary to the target of interest. Cas9 introduces double stranded breaks that result in indel mutations by virtue of the repair mechanism cells employ to repair the damage. Shortly after its discovery in bacterial cells and implementation to genome editing in mammalian cells (Reviewed in Doudna and Charpentier, 2014), our group and

others quickly devised clever ways to incorporate CRISPR/Cas9 technology into established cancer models (Reviewed in Sánchez-Rivera and Jacks, 2015), including building all-in-one viral vectors expressing Cre to induce tumorigenesis plus Cas9 and an sgRNA to mutate additional genes (Sanchez-Rivera et al., 2014). Others created LSL-Cas9 alleles which could be crossed into cancer models of choice (Chiou et al., 2015; Platt et al., 2014).

CRISPR/Cas9 based somatic genome editing has been used extensively to model cooperating genetic events in established cancer GEMMs (Annunziato et al., 2016; Romero et al., 2017; Walter et al., 2017; Wu et al., 2018). In doing so, we have been able to rapidly sift through the catalogs of putative cancer drivers and validate a decent proportion of them. Furthermore, the combination of CRISPR/Cas9 and GEMMs has helped identify combinations of mutations that mediate tumorigenesis in live animals. As an example, one study used pooled genetic screening in LSL-Cas9 mice to identify genes that when inactivated alongside *Trp53* cause mice to develop gliomas. To achieve this, the authors injected pooled adeno-associated viral vectors expressing sgRNAs against genes found to be significantly mutated across all cancer types, an sgRNA targeting *Trp53,* and Cre under the direction of the GFAP promoter (AAV-mTSG) into the brains of mice. In parallel, they delivered an empty control vector that did not express the sgRNA library. The mice that received the AAV-mTSG vectors developed tumors that closely resemble human glioblastomas. Sequencing analysis revealed that a variety of genes can cooperate with *Trp53* to initiate glial tumors and, more importantly, these same genes were found to be mutated in patient samples (Chow et al., 2017). These and other efforts highlight the power of genetically

engineered mice for understanding cancer genetics and building an arsenal of relevant models.

3.3.3 GEMMs are not suitable for high-throughput screening

Despite the undeniable benefits that GEMMs offer in terms of physiological relevance, they are not well-suited for medium- or large-scale screening even in the pooled setting. The study that searched for genetic events causing gliomas only screened 56 genes using 288 sgRNAs and they required about 56 mice to achieve their desired coverage (number of clones/cells infected per sgRNA). Close to 1000 mice would have been required to perform an arrayed screen with the same parameters, excluding coverage because it doesn't apply in the arrayed setting. Likewise, other pooled genetic screens in autochthonous tumor models have been very small in scale, employing libraries comprising fewer than 20 sgRNAs (Maresch et al., 2016; Rogers et al., 2017, 2018). Needless to say, conducting genome-wide screens in GEMMs would be close to impossible.

3.3.4 GEMMs do not recapitulate the mutational landscape of human cancer

A highly disappointing shortcoming of GEMM models is that they do not recapitulate the mutational landscape of human tumors (McFadden et al., 2016; Westcott et al., 2015). Autochthonous tumors harbor dramatically fewer non-synonymous mutations than their human counterparts, and it appears that their progression is mainly driven by copy-number alterations. While an overall low mutational frequency provides an opportunity to engineer and assess the effect of defined alterations, it also means these tumors do not generate many neoantigens. Given recent observations that the tumor mutational burden is a determining factor for

the efficacy of immune checkpoint blockade (Samstein et al., 2019), GEMMs might not represent an ideal platform for testing novel immune checkpoint therapeutics or exploring tumor-immune interactions. A few groups including our own have ectopically expressed model antigens (DuPage et al., 2011; de Galarreta et al., 2019) or knocked out DNA repair pathways (unpublished) in GEMMs to overcome this issue. However, it is still unclear whether these modified models accurately reflect the biology of human disease.

3.4 Organoid models of cancer

Organoids provide a powerful platform to model and study cancer. They represent a hybrid model that combines the best features of cell line, transplant, and autochthonous cancer models–they are genetically tractable, straightforward to establish and maintain, suitable for large-scale experiments such as genetic screens, transplantable into mice, and generate tumors that maintain a stable and biologically relevant phenotype over time. Two main types of organoid cancer models exist: *in vitro* transformed organoids (IVTO) and tumoroids. IVTOs as their name suggest are derived from healthy organoids that have been transformed in culture through targeted genetic manipulation. Tumoroids are established from tumor tissue. Given that this model type is still in its infancy, not as much is known about them compared to the rest. However, organoid-based cancer models hold significant promise to become the model of choice for cancer research in the future.

3.4.1 In vitro transformed organoids and tumoroids

As discussed at length in **Section 1.2**, organoids are continuously proliferating cultures of normal cells that remain genetically stable and retain their physiological

identity. These features make them an ideal culture-based platform to replicate the events that occur in patients and in genetically engineered mice: oncogenic transformation of a normal cell via mutations in one or more genes. Drost and colleagues were among the first to elegantly demonstrate the feasibility and utility of building IVTOs from normal human organoids (Drost et al., 2015). These researchers leveraged CRISPR/Cas9 technology to introduce different combinations of colorectal cancer associated mutations (*APC*^{KO}*, KRAS*^{G12D}*, TP53*^{KO}*,* and *SMAD4*^{KO}) into normal human intestinal organoids. Strikingly, they found that not only did the different mutations relinquish the need for corresponding niche factors in culture, but triple (*APC, KRAS, TP53*) and quadruple mutant lines (*APC, KRAS, TP53,* and *SMAD4*) were able to form tumors following subcutaneous transplantation into mice. Notably, organoids begin to acquire copy number alterations upon transformation, indicating that they can theoretically experience model evolution. However, it is unclear whether alterations are continuously acquired over time or whether the genome stabilizes after a few alterations accumulate. Nevertheless, IVTOs can be regenerated from their stable wild-type counterparts to circumvent any problems related to artificial genetic drift. Similar IVTOs have been described encompassing cancer types such as the colorectal cancer (Iannagan et al., 2019; Li et al., 2014; Matano et al., 2015; Roper et al., 2017), pancreatic cancer (Boj et al., 2015; Li et al., 2014; Seino et al., 2018), the prostatic cancer (Karthaus et al., 2014), and others. Follow up studies demonstrated that orthotopically implanted IVTOs give rise to tumors displaying clinically relevant histological properties in the context of colorectal cancer (Fumagalli et al., 2017; Roper

et al., 2017), pancreatic cancer (Boj et al., 2015), prostate cancer (Leibold et al., 2020), and ovarian cancer (Zhang et al., 2020).

Tumoroids are derived from tumor samples directly. Mounting evidence excitingly indicates that tumoroids capture and maintain interpatient variation, can be derived with high efficiency and xenotransplanted, and accurately predict drug response of the corresponding patient (Reviewed in Tuveson and Clevers, 2019). Due to these highly desirable characteristics, many groups have begun to establish patient-derived tumoroid biobanks. Although a few studies suggest that tumoroids are susceptible to genetic drift, it appears to be small in magnitude (Fujii et al., 2016; Kopper et al., 2019).

3.4.2 Large-scale genetic screening is feasible in organoids

There is significant interest in using organoids for genetic screening. Recent work has only just started to explore whether organoids are suitable for medium to large scale experimentation. In the case of pooled genetic screening, minimizing the amount of biological noise is essential for being able to robustly identify true hits in a sea of false positives. The optimal parameters have been extensively worked out for cell lines, but it is not known whether these will translate to organoids. Recently, two research groups performed medium and genome-scale pooled genetic screening in organoids and reported promising results. Both groups conducted enrichment screens looking for genes that either mediate resistance to TGF-ß induced killing *in vitro* or enable pre-tumorigenic organoids to form tumors *in vivo*. The first screen was carried out in wildtype and *APC*-mutant human organoids using recombinant TGF-ß to apply selection and tested around 76,000 sgRNAs targeting approximately 19,000 genes. The screen uncovered that inactivating the TGF-ß receptor and a handful of others including

chromatin remodelers, *SMARCA4* and *ARID1A,* allowed cells to evade death (Ringel et al., 2020).

The second screen employed a small library containing 2,600 sgRNAs targeting 85 genes. Although the screen found previously validated colorectal cancer tumor suppressors and novel candidates, there was a significant amount of noise in the data due to clonal drift. The authors propose and validate a method involving unique molecular identifiers to account for clonal drift and subtract noise from their data (Michels et al., 2020). The studies above demonstrate that while large scale genetic screening is feasible in organoids, more work is needed to establish the correct parameters and protocols to produce robust and trustworthy data. This is crucial for dropout screens, which provide drug targets for the clinic, but generate even more noise. Collectively, these two studies highlight the potential for organoids to be used for large-scale experimentation.

PART 4: LUNG ADENOCARCINOMA

Lung cancer remains the most commonly diagnosed malignancy and the leading cause of cancer related deaths in the United States (Siegel et al., 2020). Cigarette smoking causes 80% of lung cancer deaths, making it the top risk factor for lung cancer. Large efforts to raise awareness of this fact has led to significant and steady decreases in incidence and mortality rates over the past several decades. Unfortunately, the disease burden is still high and affects hundreds of thousands of patients every year. It is estimated that 228,820 new cases will be diagnosed and 135,720 individuals will die from lung cancer in 2020 (Siegel et al., 2020). The remaining 20% of lung cancer cases occur in people who have never smoked. The disease burden is so large that if never-smoker cases were assigned a separate category, it would still rank among the top 10 deadliest cancers. An even scarier corollary is that even if we eliminate cigarette smoking from our society, lung cancer would still pose a significant health problem. Therefore, it is critically important that we continue to study and find effective ways to treat lung cancer.

Lung cancers are broadly categorized into two distinct types: small cell lung cancers (SCLCs) and non-small cell lung cancers (NSCLCs). NSCLCs are further sub-categorized into three histological subtypes: squamous cell lung carcinoma (SQCC), large cell lung carcinoma (LCC), and lung adenocarcinoma (LADC). The heterogenous nature of this disease is a reflection of the rich cellular diversity in the mammalian lung, with each subtype primarily arising from distinct lung cell types. Each cell type is susceptible to particular combinations of oncogenic mutations and capable of endowing their resulting tumors with distinctive biological properties. In this section, LADC will be

described in detail, with a specific focus on its histological, molecular, and genetic, characteristics.

4.1 Histological characteristics of LADC

LADC is the most common form of lung cancer, comprising 40% of cases. The histopathological characteristics of LADC tumors can vary considerably. Tumors can arise in either the central (associated with bronchi and terminal bronchioles) or the peripheral (associated with alveoli) lung, and they can adopt one or more of the following five histologic growth patterns: lepidic, acinar, papillary, micropapillary and solid. LADC tumors typically express the lung lineage transcription factor *TTF-1*, also known as *NKX2-1* (Rekhtman et al., 2011).

4.2 LADC Genetics

The LADC subtype is the most genetically diverse form of lung cancer, especially in terms of its driver events. Unlike SCLC and SQCC, about half of LADCs carry mutationally activated oncogenes. The other half are classified as “oncogene-negative” and lack mutations in known driver oncogenes (The Cancer Genome Atlas, 2014). *KRAS* (32.2%) and *BRAF* (7%) are two of the most frequently mutated oncogenes in LADC. These two genes typically harbor single nucleotide substitutions, which render their protein products constitutively active, but don’t directly affect their mRNA expression. The most commonly affected residue in *KRAS* is glycine^{12} (G12) and oncogenic mutations replace it with cysteine (G12C, 39%), valine (G12V, 21%), aspartate (G12D, 17%), or alanine (G12A, 10%). Similar alterations affect G13. *BRAF* acts directly downstream of *KRAS* in the cell, but it is much less frequently mutated. In contrast to *KRAS*-mutant tumors, there is much less variety in the types of activating

BRAF alterations, with the vast majority of *BRAF*-mutant tumors harboring the valine^{600}-glutamine (V600E) substitution.

A separate class of LADC oncogenic mutations involve fusion genes. Such alterations result from chromosomal rearrangements and are considerably less frequent than the mutations listed above. Gene fusions typically bring together highly expressed genes with kinases. The end result is a highly expressed and constitutively active kinase. For example, about 1% of patient tumors harbor the *EML4-ALK* gene fusion, which is formed through a paracentric inversion between *ALK* and *EML4* in chromosome 2 that places the kinase domain of ALK directly downstream of the promoter and N-terminal portion of EML4, leading to increased expression and aberrant activation of *ALK*. Gene fusions involving *RET* and *ROS* kinases are found in similar proportions of patients. A unique aspect of gene fusions is that they are therapeutically targetable due to their dependence on kinase activity to carry out their function. Indeed, clinical studies have demonstrated that the kinase inhibitor crizotinib has potent anti-tumor activity in *ALK* and *ROS*-positive lung cancers (Camidge et al., 2012; Shaw et al., 2014).

The genetic alterations discussed above have been designated as LADC driver mutations based on several lines of evidence. First, these mutations occur much more often than what is expected by chance, strongly implying that they play a functional role in tumor development. Passenger mutations, on the other hand, are expected to occur at much lower frequencies—although recurrence is considered to be the gold-standard metric to nominate candidate driver genes, recent work demonstrated that recurrent yet inconsequential mutations can result from endogenous mutational processes (Buisson

et al., 2019). Second, pre-clinical and clinical studies have demonstrated that LADCs become “addicted” to the signaling mediated by these activated oncogenes, such that their inhibition leads to tumor shrinkage (Arai et al., 2013; Canon et al., 2019; Dankort et al., 2007; Maddalo et al., 2014; Mazieres et al., 2020; Shaw et al., 2014). Third and most important of all, expression of the above mutant oncogenes in the absence of other alterations drives the formation of LADCs in mice (Arai et al., 2013; Dankort et al., 2007; Johnson et al., 2001; Maddalo et al., 2014)

As mentioned above, a large fraction of LADCs lack alterations in established driver oncogenes. Much less is known about this class of tumors, but it is thought that different combinations of tumor suppressor mutations drives them, as is the case in SCLC, where inactivation of *TP53 and RB1* is sufficient for tumorigenesis (Meuwissen et al., 2003). Two pieces of evidence lend support to this idea. A large sequencing study uncovered that mutations in the tumor suppressors *TP53, STK11*, and *KEAP1* are highly enriched in oncogene negative tumors (Jordan et al., 2017). Consistently, simultaneous knockout of *Keap1* and *Pten* in lung epithelial cells leads to LADC formation (Best et al., 2018). Similar efforts, especially those leveraging new generation genome editing tools, will help us identify a more exhaustive list of tumor suppressor combinations that can transform healthy lung epithelial cells in the absence of activated oncogenes.

4.3 Genetically engineered models of LADC

Various genetically mouse models of LADC have been developed. Three models will be discussed here, each of which utilizes mutationally activated versions of proto-oncogenes recurrently mutated in LADC (**Figure 4**). The “*Kras*$^{LSL\text{-}G12D}$” model makes

use of a modified allele of *Kras* containing the oncogenic G12D mutation along with a loxP-flanked transcriptional termination (LSL) cassette upstream to prevent its widespread expression. Cre-mediated removal of the stop cassette and inactivation results in expression of $Kras^{G12D}$ and development of lung tumors (Jackson et al., 2001). The related "$Braf^{LSL\text{-}V637E}$" model leverages a *Braf* allele bearing the V637E mutation (the murine equivalent to V600E) and a loxP-flanked mini-gene that enforces expression of wildtype *Braf* and prevents expression of the oncogenic mutant. The mini-gene is located in the intron preceding the mutant exon and contains the remaining wildtype exons as well as a polyA sequence to terminate transcription. Cre treatment loops out the mini-gene and induces oncogenic *Braf* expression (Dankort et al., 2007). Lastly, the "*Eml4-Alk* or EA" model utilizes CRISPR/Cas9 mediated genome editing to induce an inversion inside chromosome 17, bringing together the promoter and N-terminal portion of *Eml4* and the C-terminal portion of *Alk*. This is accomplished via expression of Cas9 and sgRNAs targeting *Eml4* intron 14 and *Alk* intron 19. The inversion occurs spontaneously at a very low frequency after Cas9 introduces double-stranded breaks at both sites (Maddalo et al., 2014).

All three models initiate LADCs whose histological characteristics closely resemble their human counterparts and can be accelerated by combining them with *Trp53* inactivation. In order to achieve lung specific recombination or editing, the inducing agents are delivered in viral vectors via intratracheal administration.

4.3 The cell-of-origin of LADC

The lung epithelium contains two main compartments. The proximal epithelium lines the conducting bronchi and contains at least nine different cell types: pulmonary

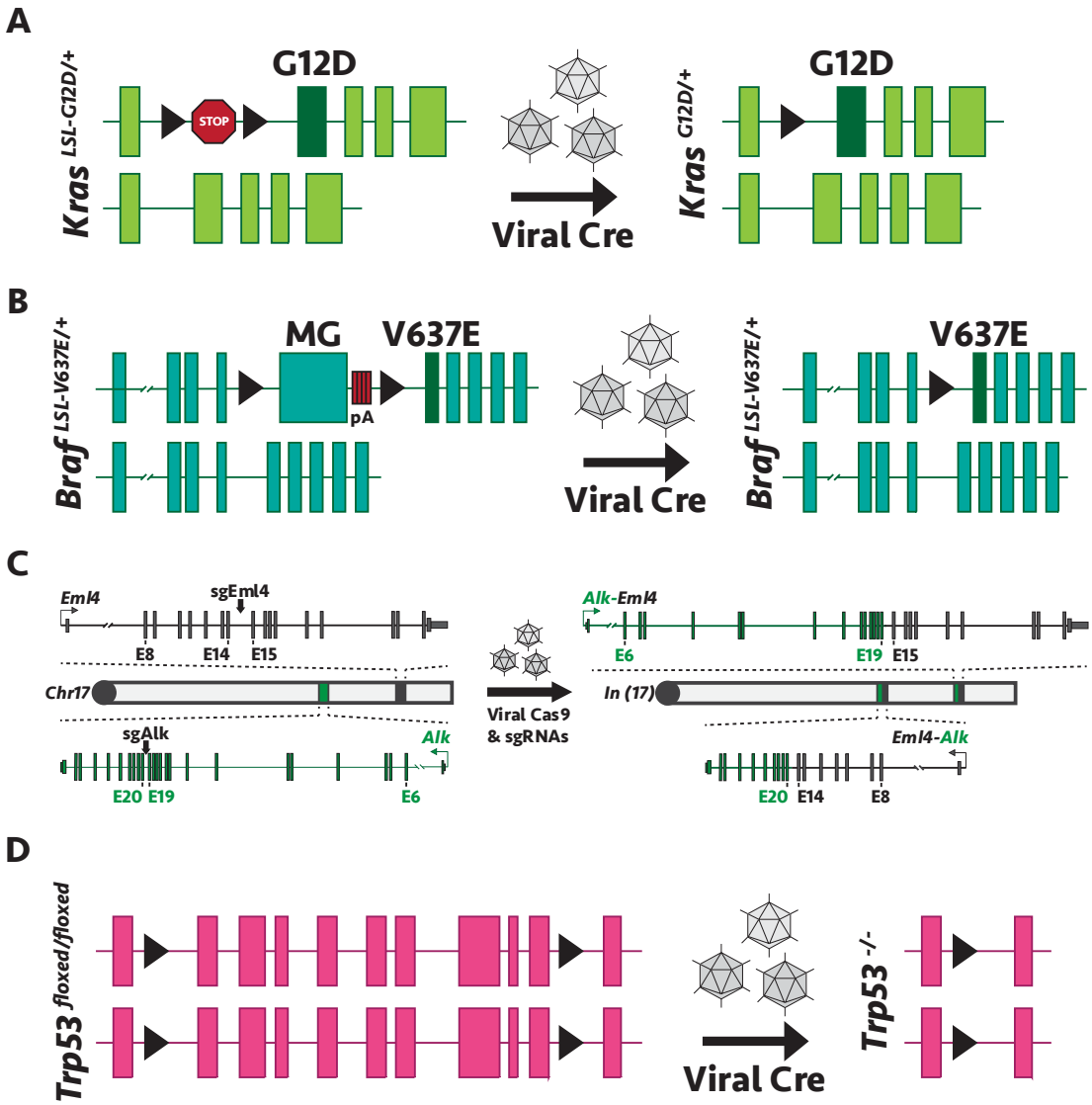

Figure 4. Genetically engineered mouse models of LADC.

A. *Kras*$^{LSL\text{-}G12D}$: encodes the oncogenic *Kras*^{G12D} and remains in a latent state until the stop cassette is removed by Cre.
B. *Braf*$^{LSL\text{-}V637E}$: expresses wildtype *Braf* through the minigene (MG). Cre excises the MG and activates *Braf* ^{V637E}.
C. *Eml4-Alk*: Introduction of double stranded breaks at *Eml4* intron 14 and *Alk* intron 19 using Cas9 and two sgRNAs induces an inversion that fuses the *Eml4* and *Alk* genes, resulting in an aberrantly expressed and constitutively active *Alk* kinase.
D. *Trp53*$^{fl/fl}$: Exons 2-10 are flanked by loxP sites. Cre loops out the flanked region and inactivates *Trp53*. Combining this allele with any of the above models accelerates lung tumor development.

neuroendocrine cells, basal cells, club cells, goblet cells, multi-ciliated cells, mucous cells, serous cells, tuft cells and ionocytes. The distal epithelium mainly covers the alveoli, where gas-exchange between inhaled air and the blood occurs, and contains at least three different cell types: bronchioalveolar stem cells (BASCs), alveolar type 2 (AT2) cells and alveolar type 1 (AT1) cells (**Figure 5**).

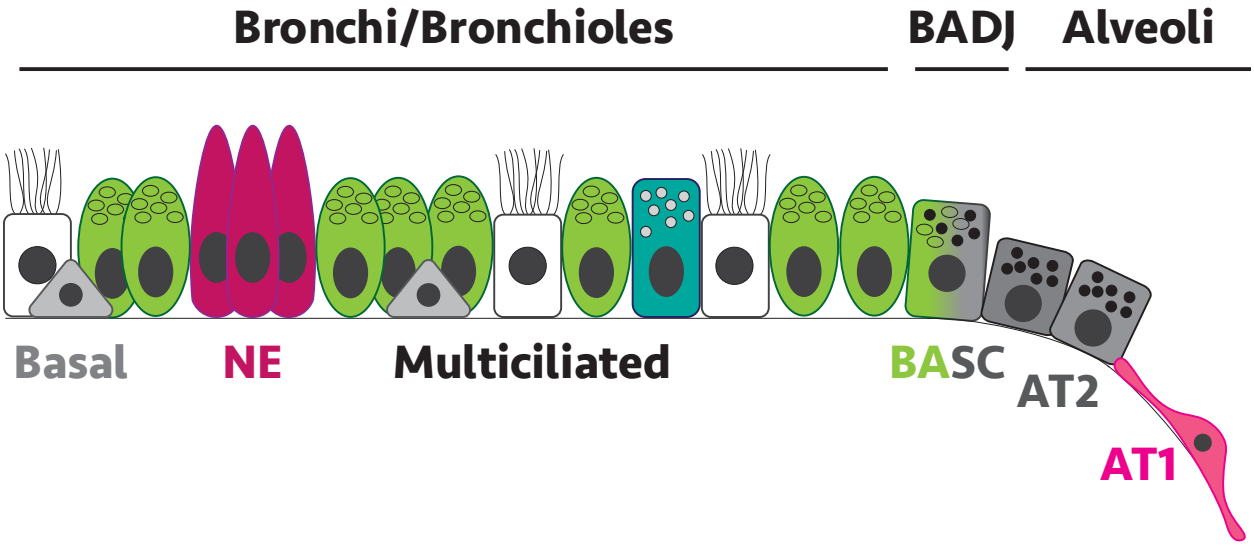

Figure 5. Major cell types in the lung epithelium.
The lung contains two major compartments: the bronchi/bronchioles and the alveoli. The bronchioalveolar duct junction (BADJ) marks the transition point between these two compartments. The major cell types in each compartment is illustrated above. Mucous, serous, tuft cells and ionocytes are not pictured. NE = Neuroendocrine.

Although LADC has several proposed cells-of-origin (club cells, BASCs, and AT2 cells), AT2 cells are considered to be the most predominant. The earliest attempts to identify a cell-of-origin for LADC focused on using marker analysis to pinpoint what, if any, normal cell type tumors resemble. One such effort found that LADC tumors universally express the AT2 marker gene SFTPA, while a subset of late stage tumors express the Club cell maker CC10 (Kitamura et al., 1997). Similarly, murine LADCs

induced by the carcinogen urethane express the full suite of surfactant proteins characteristically found in AT2 cells, but not *Cc10* (Mason et al., 2000). While these guilt-by-association approaches strongly implied that AT2 cells are potentially the predominant cell-of-origin of LADC, they could not exclude the possibility that oncogenic transformation induces lung cells to reprogram their identity to resemble AT2 cells. This idea together with the observation that a fraction of late stage human LADCs express *CC10* suggested that club cells, or perhaps a bi-potent progenitor capable of differentiating into both AT2 and club cells, could also give rise to LADCs.

The $Kras^{LSL\text{-}G12D}$ mouse model of LADC has been especially useful in answering these questions. Detailed analysis of $Kras^{G12D}$-driven murine tumors revealed that many contain “dual positive cells” that express both *Sftpc* and *Cc10*, which supported the notion that progenitor cells might play a significant role in LADC tumorigenesis. Kim et al. later demonstrated that these dual positive cells are present in the normal lung, specifically in the bronchioalveolar duct junction that connects the airway and alveolar epithelia, and presented evidence that they function as stem cells in the context of naphthalene injury (Kim et al., 2005). The latter finding prompted the authors to rename these dual positive cells to BASCs. Strikingly, they found that activation of the oncogenic *Kras* allele in lung epithelial cells stimulated BASC expansion and that naphthalene pre-treatment increased tumor size and number, leading them to argue that BASCs represent a cell-of-origin of LADC.

While these experiments convincingly linked BASC expansion to tumor initiation, the authors did not determine whether BASCs are required for tumorigenesis. Furthermore, their method of non-specifically expressing Cre in lung epithelial cells

made it impossible to tease apart the relative contribution of different cellular compartments to the observed tumor phenotypes. Therefore, it was still very possible that AT2 and/or club cells played a significant role in this context.

Subsequent studies made use of genetic tools which restrict Cre expression, and in turn activation of oncogenic *Kras*, to *Cc10* or *Sftpc* expressing cells to unambiguously assess the tumorigenic capacity of BASCs, AT2, and club cells. For instance, Xu et al. combined *Cc10-CreER* or *Sftpc-CreER* knock-in alleles with the $Kras^{LSL\text{-}G12D}$ model and a fluorescent Cre reporter to explore this question. They demonstrated that Cc10+ and Cc10+Sftpc+ dual positive cells can only form hyperplasias in response to oncogenic stimuli, whereas Sftpc+ cells give rise to frank tumors, all of which remain Sftpc+ (Xu et al., 2012). Two similar studies employing closely related tools reported similar findings (Lin et al., 2012; Sutherland et al., 2014). Furthermore, AT2 cell-initiated LADC is not idiosyncratic to $Kras^{G12D}$ mediated oncogenic signaling. $Braf^{V637E}$ and *Eml4-Alk* driven LADC tumors in mice uniformly display AT2 molecular characteristics such as *Sftpc* expression (Dankort et al., 2007; Maddalo et al., 2014). Altogether, this body of evidence has led to the field to consider *Sftpc*-expressing AT2 cells as the major cell-of-origin of LADC.

While the evidence in support of AT2 cells as being the main site of LADC tumor initiation is compelling, one important consideration is that most of the studies described above assessed tumorigenic potential in the context of a single activated oncogene under homeostatic conditions. LADC is a genetically diverse disease. A great variety of oncogene and tumor suppressor mutants can drive LADC, and patient tumors often harbor complex combinations of driver mutations. It is possible that different lung cell

types have the ability to undergo transformation and progression into LADC only in specific genetic contexts. While there is not much evidence for this yet, one of the studies mentioned above demonstrated that simultaneous *Trp53* inactivation and oncogenic *Kras* activation in club cells enables progression beyond the hyperplastic stage (Sutherland et al., 2014). Additionally, while not discussed here, micro-environmental factors such as inflammation or fibrosis likely play role and should be explored in future studies, especially given the fact that most lung cancers are caused by cigarette smoke which causes a host of changes in the lung microenvironment. A second important consideration is that no study has systematically evaluated the ability of the many other epithelial cell types in the lung to initiate LADC tumors. Although this may seem unlikely at the surface level given the evidence discussed above, recent evidence hints that LADCs might be able to originate from diverse cell types, that undergo cell-state transitions as they progress. One study demonstrated that expression of oncogenic *Kras* in *Hopx* expressing AT1 cells engenders lung tumors showing LADC characteristics, including expression of the AT2 marker *Sftpc* (Jain et al., 2015). Follow up studies using genetic tools that allow cell-type specific introduction of LADC-specific oncogenic insults should help build a comprehensive list of players and shed light on the role cellular plasticity in LADC oncogenesis.

CONCLUSION

The advent of organoid technology is revolutionizing the way we study normal and disease biology. Organoids provide a powerful platform to create improved cancer models that combine the tractability of cell line and transplant models and the physiological and clinical relevance of genetically engineered mouse models. Despite this, a robust alveolar organoid system to build models of lung adenocarcinoma has not yet been developed. This thesis describes an improved system to culture murine alveolar organoids that can be propagated for at least 3 months while maintaining the their cellular identity. Preliminary experiments also suggest that this culture system might be effective for human AT2 cells. Critically, this culture system is effective for modeling diverse molecular subtypes of LADC *in vitro* and *in vivo*. Collectively, these experiments demonstrate the feasibility of creating immortal organoid cultures from alveolar tissue and their versatility for modeling the diverse molecular subtypes of lung cancer.

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CHAPTER 2

An organoid system to culture alveolar type 2 cells and model lung adenocarcinoma

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S.N. and T.J. designed the study and wrote the manuscript with comments from all co-authors; S.N., and L.Z.L. performed all experiments; D.Y. performed all single cell RNA sequencing bioinformatic analyses; A.G. and A.B. performed all bulk RNA sequencing bioinformatic analyses. R.B. provided human tissue samples. All experiments were performed in the laboratory of T.J.

ABSTRACT

Lung adenocarcinoma (LADC) is a very common and deadly cancer type. A variety of models have been developed to study this disease. While genetically engineered mouse models (GEMMs) faithfully recapitulate the biology of human LADC, cancer cell line and transplant-based models are fast and allow for the use of human tissue. The advent of organoid technology has provided a method to create next-generation cancer models that bring together the best features from current cancer models. Here, we describe a novel platform to build organoid based models of LADC. We first develop improved organotypic culture conditions to continuously expand and maintain the identity of primary alveolar type 2 (AT2) cells, a prominent LADC cell-of-origin, from normal murine lungs as organoids. We observe that these organoids strongly express AT2 markers, weakly express a panel of alveolar type 1 cell markers and are completely negative for basal and club cell markers. We also demonstrate that clinically relevant gain-of-function mutations in *Kras*, *Braf*, or *Alk* with concomitant loss of *Trp53* promotes growth in the absence of mitogenic factors. Critically, *Kras*-mutant *Trp53* deficient organoids are capable of establishing lung tumors displaying histopathological characteristics of human LADC. In summary, we present a novel organoid based platform to model LADC. In addition to providing a powerful way to create diverse and physiologically relevant transplant-based models of LADC, we anticipate that this culture system will facilitate the investigation of normal alveolar stem cell biology in lung regeneration and cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide (Siegel et al., 2020). Lung adenocarcinoma (LADC) represents the most common histological subtype of this disease, accounting for 40% of all cases (Siegel et al., 2020). Alveolar type 2 (AT2) cells are a major cell-of-origin for LADC (Lin et al., 2012; Sutherland et al., 2014; Xu et al., 2012). AT2 cells secrete pulmonary surfactant, create an immune-tolerogenic environment to non-pathogenic antigens through unproductive interactions with helper T-cells (Lo et al., 2008), and serve as tissue resident stem cells. Their main function as stem cells is to self-renew and differentiate into alveolar type 1 (AT1) cells during homeostasis and wound repair (Barkauskas et al., 2013; Desai et al., 2014; Evans et al., 1975; Kaplan et al., 1969).

Our lab and others have developed various genetically engineered mouse models (GEMMs) to gain a better understanding of the genetic and molecular events that drive LADC (Dankort et al., 2007; Jackson et al., 2001; Maddalo et al., 2014). In these models, tumors arise from healthy lung epithelial cells and follow a progression pattern that faithfully recapitulates their human counterparts at the molecular and histopathological level. While GEMMs offer a physiologically relevant platform to study cancer initiation and all stages of progression, they lack the tractability of traditional culture and transplant models, and preclude the use of human tissue.

Organoid technology provides a powerful means to create improved murine and human cancer models that are both practical and faithfully recapitulate clinical disease. Two key features of organoids make this possible: (1) they have the ability to proliferate continuously while remaining genomically stable and retaining their physiological

identity (Boj et al., 2015; Sato et al., 2011; Schutgens et al., 2019), and (2) *in vitro* transformed organoids orthotopically transplanted into mice form tumors closely resembling their naturally arising counterparts (Boj et al., 2015; Fumagalli et al., 2017; Leibold et al., 2020; Roper et al., 2017; Zhang et al., 2020). Here, we describe a novel organoid-based platform to model LADC. We first establish an improved organoid culture system that permits long-term feeder-free expansion of AT2 cells for at least 3 months. We subsequently apply our culture system to rapidly model distinct molecular subtypes of LADC *in vitro* and *in vivo*.

RESULTS

Developing an improved alveolar organoid culture system

Established protocols to culture primary AT2 cells as organoids lack flexibility because they require an air-liquid-interface, co-culture with feeder cells, or both (Barkauskas et al., 2013; Lee et al., 2013, 2014; Shiraishi et al., 2019b, 2019a; Weiner et al., 2019). Moreover, the self-renewal capacity of AT2 cells in organoid culture has not yet been fully established. To develop an improved system to expand primary AT2 cells in organoid culture, we first defined a culture medium that would selectively expand adult AT2 cells from bulk lung cell suspensions in the absence of feeder cells or an air-liquid-interface. Previous studies have shown that Fgfr2 (Dorry et al., 2019; Sugahara et al., 1995; Ulich et al., 1994; Zacharias et al., 2018; Zepp et al., 2017), c-Met (Panos et al., 1993; Yamamoto et al., 2007), and Wnt (Frank et al., 2016; Nabhan et al., 2018; Shiraishi et al., 2019a; Zacharias et al., 2018) pathway activity supports adult AT2 stem cell function *in vivo*, whereas Bmp (Chung et al., 2018; Shiraishi et al., 2019a; Zepp et al., 2017) and Tgf-ß (Bhaskaran et al., 2007; Riemondy et al., 2019;

Shiraishi et al., 2019a; Zhao et al., 2013) signaling antagonizes it. Therefore, we hypothesized that growth medium containing a combination of activators and inhibitors of these pathways would support the growth and maintenance of AT2 organoids.

To test this hypothesis, we embedded freshly dissociated bulk lung cell suspensions from 8-20 week-old transgenic *Sftpc-eGFP* mice (Vanderbilt et al., 2015) in matrigel droplets and incubated them in F^7NHCA medium, containing FGF7 (activates FGFR2), NOGGIN (blocks Bmp signaling), HGF (activates c-MET), CHIR99021 (activates Wnt signaling), and A83-01 (blocks Tgf-ß signaling) (***Figure 6A***). Consistent with our expectations, we found that F^7NHCA medium selectively expands eGFP+ organoids from bulk lung cell suspensions, indicating that they express *Sftpc* and likely represent AT2 organoids (***Figure 6B***). As expected based on the crucial role that Wnt signaling plays in supporting AT2 function *in vivo*, these organoids exhibited a dose-dependent growth response to CHIR99021 (**Supplementary Figure 1A**).

We next assessed the self-renewal capacity of *Sftpc-eGFP* organoids through serial passaging and tracking of eGFP expression by flow cytometry. We could reliably expand *Sftpc*-expressing organoids (n = 8) for at least ~3 months (12 passages) (***Figure 6C***). Single cell mRNA sequencing analysis and immunohistochemical staining demonstrated high expression of AT2 marker genes and low expression of AT1 marker genes, whereas club and basal cell markers were undetectable (***Figure 7A*** and ***B***). Interestingly, the AT1 marker gene, *Pdpn*, is expressed at earlier passages but is downregulated over time. Whereas MHC class 2, which has been previously shown to mark *Sftpc* expressing lung cells *in vivo* (Cunningham et al., 1994; Harbeck et al., 1988; Hasegawa et al., 2017; Lo et al., 2008) and mediates their interaction with helper T-cells

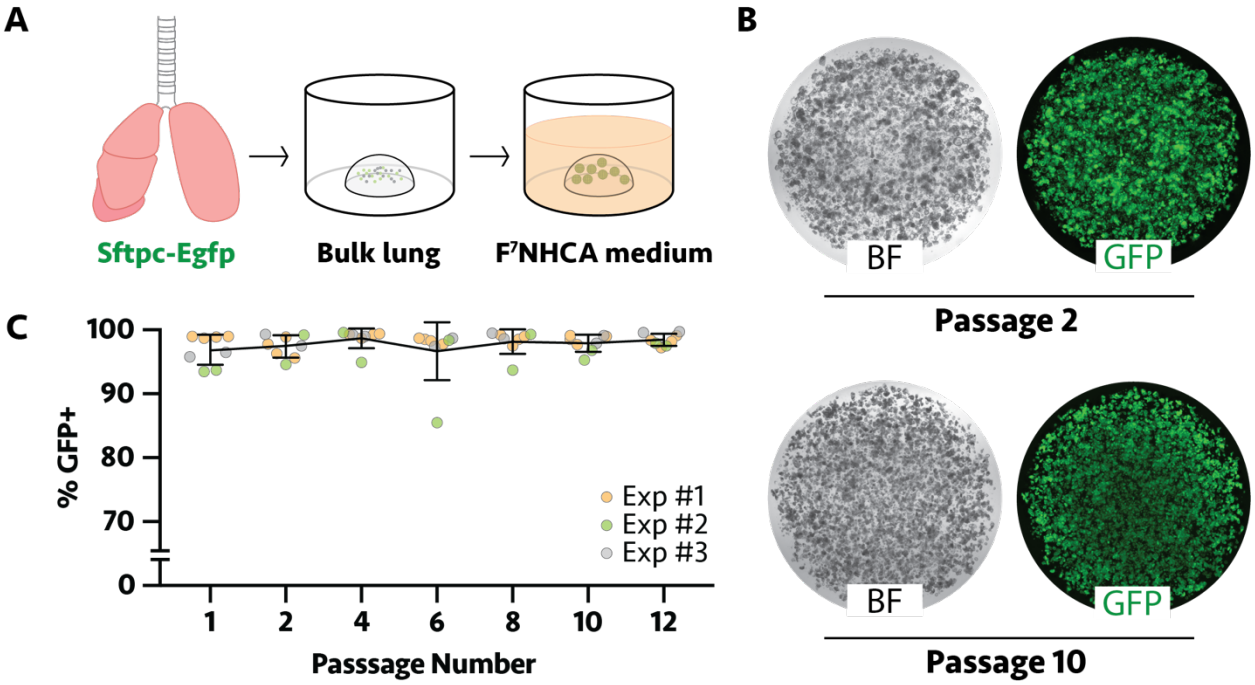


Figure 6. Lung organoids continuously proliferate and maintain *Sftpc* expression in F[7]NHCA medium.

A. Method to culture alveolar organoids from normal lungs.
B. Representative images of *Sftpc-eGFP* organoids at an early (2) and late (10) passage (BF = Bright Field).
C. Flow cytometry based quantification of *Sftpc-eGFP* expressing cells over time. A total of *n* = 8 independent lines were established from *Sftpc-eGFP* mice and tested over three different experiments. Lung organoids cultured in F[7]NHCA media can robustly maintain *Sftpc* expression over the long term.
All data are expressed as mean values ± the standard deviation.

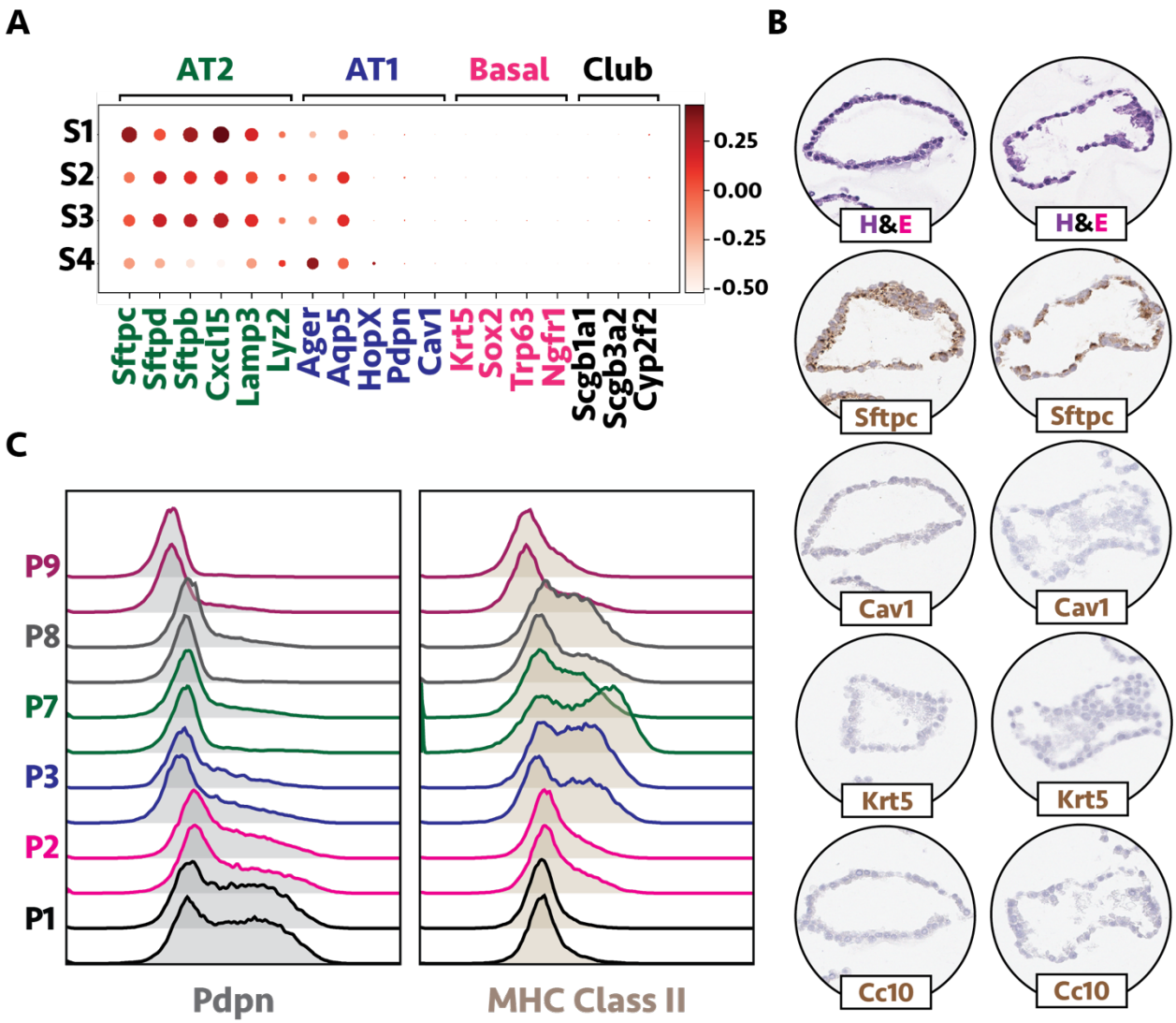


Figure 7. Molecular characterization of organoids.

A. Single-cell transcriptomic analysis of *n* = 4 biologically independent organoid lines demonstrates high expression of alveolar markers (AT2 & AT1) and undetectable expression of airway markers (basal & club).
B. Immunohistochemical staining of *n* = 2 biologically independent organoid lines for lung marker genes (Sftpc - AT2, Cav1 - AT1, Krt5 - basal, Cc10 - club) confirms they strongly express the AT2 marker, Sftpc.
C. Longitudinal tracking of Pdpn (AT1) and MHC Class II (AT2) surface expression in *n* = 2 biologically independent organoid lines. Alveolar organoids gradually downregulate Pdpn and concomitantly upregulate MHC Class II over time.

(Lo et al., 2008), exhibits the opposite expression pattern (***Figure 7C***).

We noticed that *Sftpc* negative cells can progressively take over the culture in some organoid lines (***Supplementary Figure 1C***). We performed bulk RNA sequencing on sorted eGFP-positive and eGFP-negative cells to determine the identity of the eGFP-negative population. This analysis revealed that basal cell markers were among the top differentially expressed genes in the eGFP-negative group (***Supplementary Figure 1D***). Consistently, gene set enrichment analysis (GSEA; Mootha et al., 2003; Subramanian et al., 2005) using the top differentially expressed genes in both groups as gene sets confirmed that the eGFP-negative gene set is significantly enriched in a basal cell signature (FDR p-value = 0), whereas the eGFP-positive equivalent significantly enriches for an AT2 signature (FDR p-value = 0; ***Supplementary Figure 1E***). Interestingly, the cell-surface gene *Egfr* was upregulated 16-fold in the eGFP-negative population (***Supplementary Figure 1E***), suggesting a potential strategy to rapidly identify unstable lines via flow cytometry.

Building on our encouraging results with murine tissue, we attempted to cultivate human alveolar organoids. We used bulk lung cell suspensions from the morphologically normal portions of freshly resected tumor specimens as starting material and incubated these samples in F^7NHCA media. To track the percentage of AT2 cells present in the population over time, we performed surface staining for HT2-280, an established marker of AT2 cells in the human lung (Gonzalez et al., 2010). In cultures derived from two independent donors, we saw outgrowth of organoids (***Supplementary Figure 2A***). 30% and 60% of the cells in these cultures expressed HT2-280 and passaging did not alter these percentages (***Supplementary Figure 2B***).

In vitro transformed *Kras$^{G12D/+}$;Trp53^{KO}* alveolar organoids give rise to LADCs

We next applied our optimized murine AT2 culture system to model LADC. KRAS and *TP53* are mutated in about 35% and 50% of patient lung adenocarcinomas, respectively (The Cancer Genome Atlas, 2014). Consistently, previous studies have shown that expression of oncogenic *Kras* with concomitant deletion of *Trp53* initiates LADC in mice (Lin et al., 2012; Sutherland et al., 2014; Winters et al., 2017; Xu et al., 2012). To test whether the same genetic insults can transform wildtype AT2 organoids, we first established *Kras$^{LSL\text{-}G12D/+}$;Trp53$^{fl/fl}$;R26$^{LSL\text{-}TdTomato/+}$* (n = 7), *Trp53$^{fl/fl}$;R26$^{LSL\text{-}TdTomato/+}$* (n = 2), and *R26$^{LSL\text{-}TdTomato/+}$* (n = 2) organoids. We then infected these three sets of organoids with an adenovirus expressing Cre (*Ad5-Cre*) to generate *Kras*-mutant, *Trp53*-deficient, TdTomato-expressing (*KPT)* organoids as well as control *PT* and *T* organoids (***Figure 8A***). We next performed *in vitro* and *in vivo* tumorigenesis assays (***Figure 8A***). Our *in vitro* assay tested the ability of these organoids to grow in selection medium lacking mitogenic factors. This medium only contained NOGGIN, CHIR99021, and A83-01 (NCA medium). Our *in vivo* assay assessed the ability of organoids to form lung tumors following orthotopic transplantation into immunocompetent syngeneic recipient mice.

We found that *KPT* organoids exhibited growth factor independence *in vitro* (***Figure 8B***). Consistently, six out of seven *KPT* lines gave rise to lung tumors *in vivo* (***Figure 8C*** and ***E***). Control *T* and *PT* lines did not exhibit oncogenic properties in either setting (***Figure 8B* and *E***). As expected, all analyzed tumors express the canonical LADC marker Nkx2-1. Organoid tumors displayed histological features of hyperplasias,

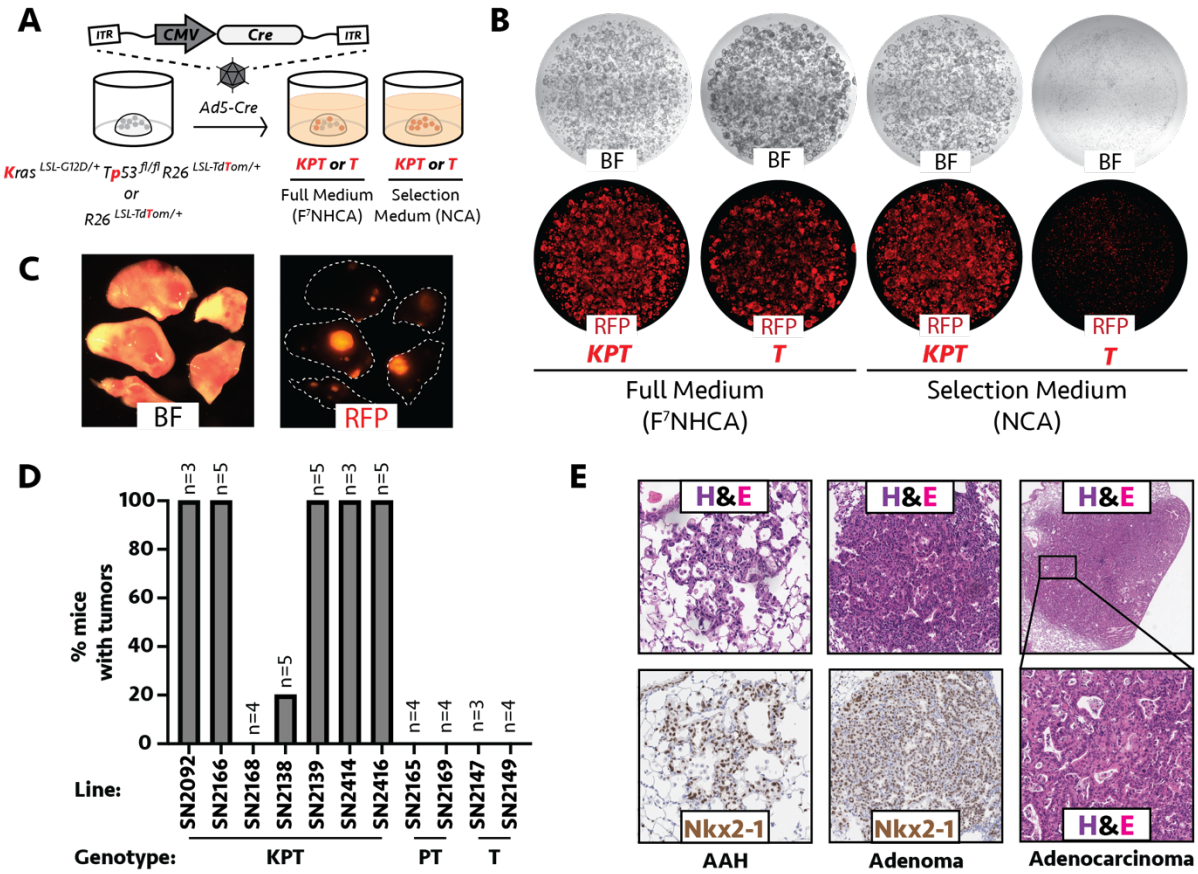


Figure 8. Activation of *Kras*^{G12D} and simultaneous *Trp53* inactivation endow alveolar organoids with oncogenic properties *in vitro* and *in vivo*.

A. Strategy to introduce oncogenic *Kras* and *Trp53* mutations *in vitro* and test their effect on the growth of normal organoids in vitro. The letters in red denote recombined versions of their corresponding alleles. *K* = *Kras*$^{G12D/+}$, *P* = *Trp53*^{KO}, T = *R26*$^{TdTom/+}$.
B. Representative images of transformed *KPT* (*n* = 2) and normal *T* (*n* = 1) organoids in complete or selection medium lacking growth factors. The widespread expression of red fluorescence indicates efficient infection and Cre-mediated recombination. *KPT* organoids can proliferate in the absence of mitogens. BF = Bright Field.
C. Representative gross images of organoid derived tumors 5 months post transplantation.
D. Tumorigenicity of KPT (*n* = 7), PT (*n* = 2), and T (*n* = 2) organoid lines. The numbers above each bar indicate the number of recipient animals used for each transplant experiment.
E. Illustrative Hematoxylin & Eosin (H&E) and Nkx2.1 stained tumor sections. Organoid derived tumors exhibit adenomatous alveolar hyperplasia (AAH - left), adenoma (middle), and adenocarcinoma (right) histology. They also express the canonical LADC marker *Nkx2-1.*

adenomas and adenocarcinomas (***Figure 8D***). Given that all mice were sacrificed and analyzed at 5-7 months post-transplantation, these results suggest that tumors progress from benign to aggressive states at different rates.

Building a *Braf*-mutant organoid model of *LADC*

About 10% of LADC patient tumors are driven by $BRAF^{V600E}$ (The Cancer Genome Atlas, 2014). Experiments using genetically engineered mice have indicated that although expression of oncogenic *Braf* is sufficient for lung tumor formation, these tumors do not progress beyond the adenoma stage. A second hit in *Trp53* is required for progression into adenocarcinoma (Dankort et al., 2007). Therefore, we chose to model *Braf*-driven LADC by introducing $Braf^{V637E}$ as well as inactivating *Trp53* mutations into wildtype organoids.

To that end, we derived and infected $Braf^{LSL\text{-}V637E}$;$R26^{LSL\text{-}Cas9\text{-}eGFP/+}$ (n = 2) and $R26^{LSL\text{-}Cas9\text{-}eGFP/+}$ (n = 1) organoids with *Ad5-Cre* to create $Braf^{V637E}$ mutant, Cas9 expressing organoids (*BC9*) and control organoids only expressing Cas9 (*C9*). We subsequently transduced these lines with lentiviruses expressing an sgRNA targeting *Trp53* under the direction of a U6 promoter as well as eGFP driven by the EFS promoter (Lenti-U6-sgTrp53-EFS-eGFP or Lv-USEG-sgTrp53) to mutate *Trp53* and generate *BPC9* and *PC9* organoids (***Figure 9A***). PCR analysis demonstrated efficient infection and recombination of the $Braf^{LSL\text{-}V637E}$ allele (***Figure 9B***). To confirm that *Trp53* had been successfully inactivated, we cultured *BC9*, *C9*, *BPC9*, and *PC9* organoids in media supplemented with the *Trp53* stabilizing agent, Nutlin-3a. As expected, only the lines that were treated with Lv-USEG-sgTrp53 did not exhibit growth defects in the

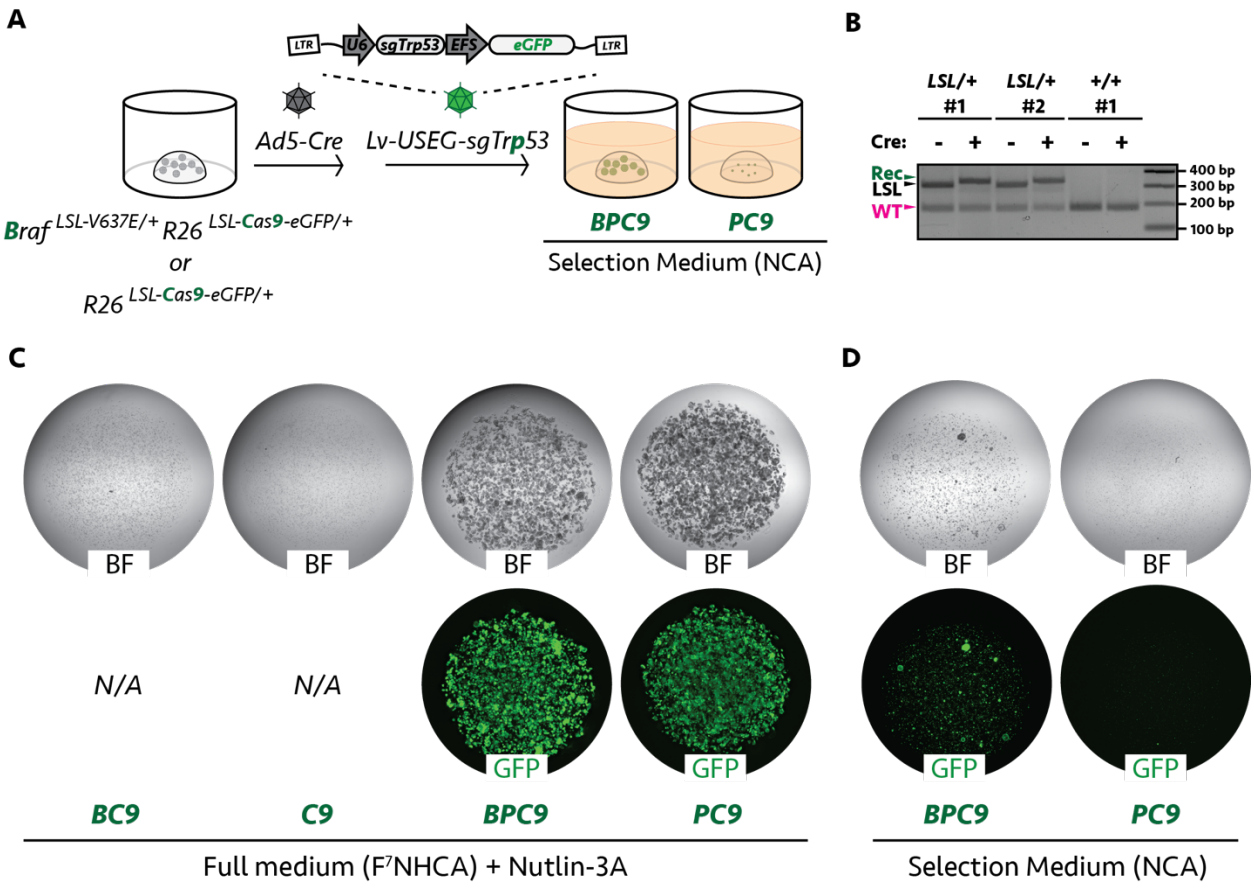


Figure 9. Concomitant *Braf*^{V637E} activation and *Trp53* deletion endow alveolar organoids with oncogenic properties *in vitro*.

A. Strategy to introduce *Braf* and *Trp53* mutations in vitro and test their effect on the growth of normal organoids *in vitro*. The letters in green denote recombined or CRISPR/Cas9 edited versions of their corresponding alleles. *B* = *Braf*^{V637E}, *P* = *Trp53*^{mut}, *C9* = *R26*$^{Cas9\text{-}eGFP}$.

B. PCR-based detection of *Braf*^{V637E} activation. PCRs were carried out on genomic DNA isolated from *Braf*$^{LSL\text{-}V637E}$;*R26*$^{LSL\text{-}Cas9\text{-}eGFP/+}$ and *Braf*$^{+/+}$;*R26*$^{LSL\text{-}Cas9\text{-}eGFP/+}$ organoids before and after Cre treatment using a previously published primer pair (Dankort et al., 2007). The expected product sizes are 185bp (wildtype, pink arrow), 308 (unrecombined, black arrow), and 335 bp (recombined, green arrow).

C. Representative images of *Trp53* proficient (*BC9* and *C9*) and *sgTrp53* targeted (*BPC9* and *PC9*) organoids cultured in the presence of 5μM Nutlin-3A. Outgrowth of *BPC9* and *PC9* organoids confirms successful CRISPR-Cas9 mediated inactivation of *Trp53*. BF = Bright Field.

D. Representative images of *BPC9* (*n* = 2) and *PC9* (*n* = 1) organoids cultured in the presence of selection medium lacking growth factors. Only *BPC9* organoids can proliferate without mitogen support.

presence of Nutlin-3a, strongly implying *Trp53* had been successfully knocked out in a subset of treated cells (***Figure 9C***). Lastly, we conducted the *in vitro* oncogenesis assay described above with *BPC9* and *PC9* organoids (***Figure 9A***). The results indicate that *BPC9* organoids display weak but discernable mitotic activity in NCA medium whereas control *PC9* were not able to divide at all in these conditions (***Figure 9D***).

LADC-associated chromosomal rearrangements transform alveolar organoids

A distinct class of LADCs lack identifiable activated oncogenes, and instead harbor oncogenic fusion genes that result from chromosomal rearrangement events. A prominent example of this class involves an intrachromosomal inversion that generates the *EML4-ALK* fusion gene and is found in approximately 1.3% of LADC patients (The Cancer Genome Atlas, 2014). We employed a previously published strategy to develop an *Eml4-Alk*-driven organoid model of LADC that leverages CRISPR/Cas9 to introduce double stranded breaks into translocation breakpoints to induce the desired fusion event (Maddalo et al., 2014). To that end, we infected *PT* organoids with a previously published adenovirus expressing Cas9 and a pair of sgRNAs targeting the breakpoints of the *Eml4-Alk* rearrangement (*Ad5-EA*) (Maddalo et al., 2014) or a control virus expressing Cas9 alone (*Ad5-Cas9*). We subsequently cultured these organoids in NCA medium to assess their *in vitro* oncogenic capacity (***Figure 10A***). A small subset of cells infected with the *Ad5-EA* virus (*PT-EA* organoids) were able to divide without mitogenic factors and retained their proliferative capacity after passaging (***Figure 10B***). In contrast, control *PT* organoids did display mitotic activity in selection medium. PCR analysis of *PT-EA* organoid genomic DNA and Sanger sequencing of the products

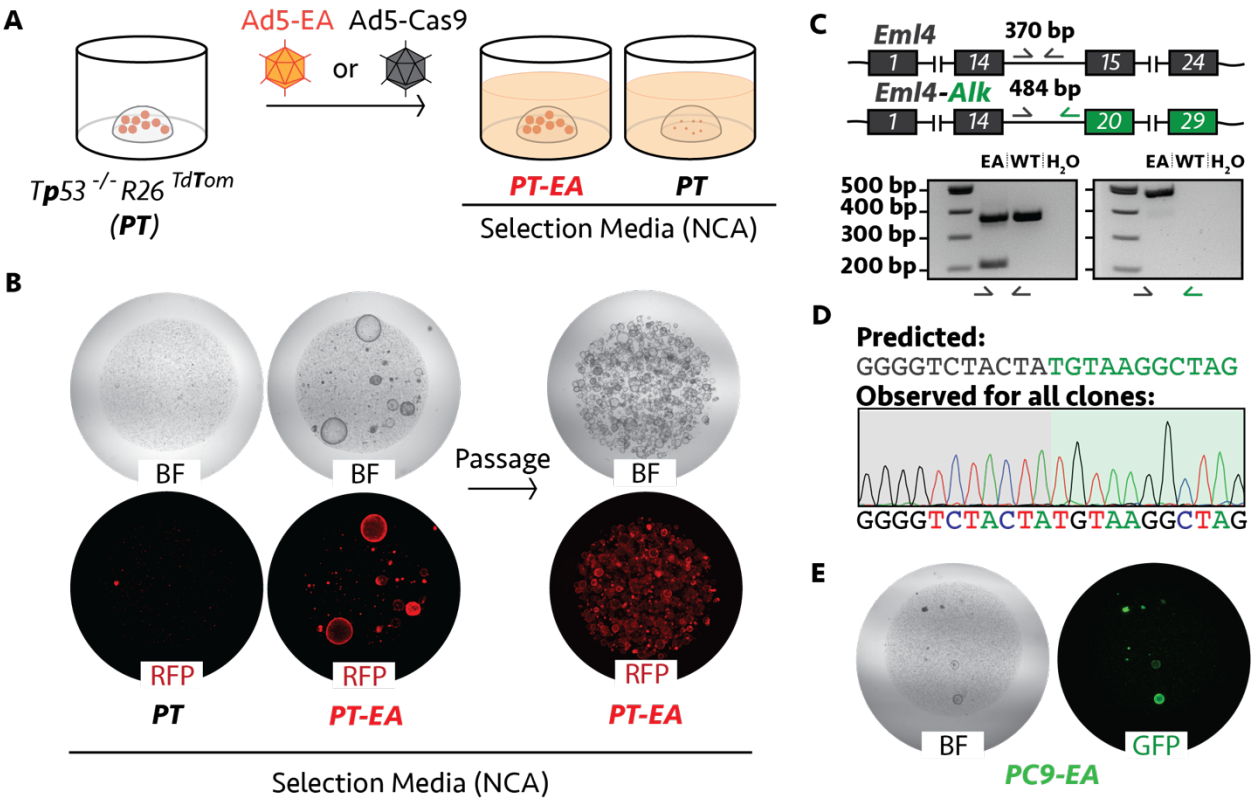


Figure 10. Engineering LADC associated chromosomal rearrangements using CRISPR/Cas9.

A. Strategy to introduce the *Eml4-Alk* inversion and test its effect on the growth of *Trp53* deficient organoids *in vitro*.
B. Representative images of Ad5-EA (n = 1) or Ad5-Cas9 (n = 1) treated organoids in selection media lacking growth factors. PT-EA organoids can proliferate in the absence of mitogens.
C. PCR-based detection of the *Eml4-Alk* inversion. The diagram illustrates the *Eml4* locus before and after the inversion. PCR reactions for the wildtype and inverted *Eml4* locus were carried out on genomic DNA isolated from PT-EA and wildtype organoids using the indicated primer pairs. The lower band in the wildtype specific reaction indicates the presence of large deletions. The single product generated by the inversion specific primer pair demonstrates successful induction of the *Eml4-Alk* fusion.
D. Sanger sequencing analysis of the inversion specific PCR product. The PCR band was subcloned and a total of 6 clones were sequenced. The predicted sequence and a representative chromatogram are shown. All the clones perfectly matched the predicted sequence.
E. The results in Panel B were replicated using an independent organoid line (n = 1) and an orthogonal infection strategy (see main text).

confirmed the presence of the *Eml4-Alk* fusion gene (***Figure 10C*** and ***D***). In parallel, we utilized an orthogonal infection strategy to engineer the rearrangement in an independent organoid line. We generated and infected *Trp53*^{KO}*;R26*$^{Cas9\text{-}eGFP/+}$ (PC9) organoids with Lv-USEG-sgEA (encoding the same sgRNAs in the adenoviral vector described above) and cultured them in NCA media. As expected, we observed outgrowth of colonies, albeit at a lower frequency than with the adenoviral strategy (***Figure 10E***).

DISCUSSION

Organoids provide a powerful means to create improved cancer models owing to their near limitless proliferative potential, long-lasting genomic stability, and ability to durably retain their physiological identity. In this study, we have established a new and improved method to build diverse AT2-derived organoid models of LADC. Building upon previous attempts to culture primary AT2 cells as organoids and efforts that defined the molecular niche of the alveolus, we defined a growth factor cocktail that supports sustained growth of murine organoids that express *Sftpc*, a canonical marker of AT2 cells. We also present evidence that these conditions can expand human AT2 cells as organoids. Finally, using organoids derived from genetically engineered mice and CRISPR/Cas9 genome editing, we demonstrate that three classes of clinically relevant mutations endow these organoids with oncogenic properties *in vitro* and, at least in one case, *in vivo*.

Our system to culture AT2 organoids offers two main advantages over existing culture systems. First, it does not require co-culture with a feeder cell line or an air-liquid-interface. While both of these requirements better recapitulate the lung microenvironment, they make it exceedingly difficult to perform large-scale studies such as drug or genetic screens. In contrast, our minimal system is better suited for such applications. Second, and most importantly, the culture conditions we present here enable continuous expansion and durable phenotypic stability in most cases (***Figure 6C***). To our knowledge, no other study has reported similar findings.

The main functions of AT2 cells include surfactant production, tolerizing the immune system to non-pathogenic antigens, and acting as tissue resident stem cells.

They also serve as a site of origin for LADC. Our data strongly indicate that F^7NHCA medium expanded AT2 organoids recapitulate many aspects of these characteristics. Specifically, the strong and consistent expression of the full suite of surfactant proteins (***Figure 7***) implies that these organoids can produce and secrete pulmonary surfactant. Additional studies leveraging electron microscopy are ongoing to search for lamellar bodies—which serve as a storage place or secretory vehicle for pulmonary surfactant—within these organoids. Furthermore, these organoids express MHC Class 2 on their surface, clearly indicating that they can present antigens to helper T-cells (***Figure 7***) as previously described (Lo et al., 2008). A cardinal feature of AT2 cells is their ability to differentiate into AT1 cells. While the alveolar organoids described here weakly express AT1 markers (**Figure 7**), they do not seem to differentiate completely under the conditions we optimized for continuous expansion. We believe that they can be coaxed to differentiate by shutting down Wnt signaling (Nabhan et al., 2018; Zacharias et al., 2018) and activating the Bmp pathway (Chung et al., 2018). This can easily be achieved by removing CHIR99021 and Noggin as well as adding Bmp4 to the medium. Critically, it remains to be established whether these organoids can engraft into injured lungs and participate in the repair process via division and differentiation into AT1 cells. We also demonstrate that these organoids retain oncogenic potential of their native counterparts. The experiments herein indicate that patient-inspired gain-of-function mutations in *Kras*, *Braf*, and *Alk* render normal alveolar organoids growth factor independent (***Figure 8***). Consistently, *Kras* mutant organoids can form lung tumors in mice (***Figure 8***).

The observation that some organoid lines are not phenotypically stable warrants further investigation (**Supplementary Figure 1**). We believe that the cells are either

differentiating into or getting outcompeted by contaminating basal cells. Our current experiments do not allow us to differentiate between these two possibilities. Future studies that compare organoid cultures initiated with bulk lung cell suspensions versus sorted AT2 cells should help shed light on what causes this phenotypic shift. Interestingly, one group reported that AT2 organoids expanded for a week post isolation and transplanted into influenza injured mice differentiate into AT1 cells as expected, but a fraction seem to adopt a basal cell phenotype. However, freshly sorted AT2 cells only differentiate into the AT1 lineage after transplantation (Weiner et al., 2019). These results imply that the unstable phenotype we observed here might be an artifact of culture.

We also present evidence that F^7NHCA medium stimulates growth of human AT2 cells in organoid culture. Surface staining for HT2-280 indicates that the human lung organoids described herein only partially comprise AT2 cells, but it appears that this population is stable (**Supplementary Figure 2**). Experiments are currently underway to measure the proliferative lifespan of these organoids and identify the HT2-280 negative population. It is possible that this latter population might still represent AT2 cells as it has been reported that not every *SFTPC* expressing cell is HT2-280 positive *in vivo* and that this marker is downregulated in culture (Evans and Lee, 2020).

Our organoid system provides a rapid and powerful platform for modeling LADC. Several tumor genome sequencing studies have revealed a rich genetic diversity within and across patients. Tumor driving events can be simple such as the G12D substitution in *KRAS* or as complex as the large chromosomal inversion that aberrantly activates *ALK*. Our efforts demonstrate that alveolar organoids are a suitable platform for

modeling any known or candidate driver mutations. We provide strong evidence that three independent oncogenic mutations (*Kras*^{G12D}*, Braf*^{V600E}*, Eml4-Alk*) are able to transform these organoids and that orthotopic tumors derived from *Kras*-mutant organoids exhibit typical LADC histology and marker expression (***Figure 8***-***Figure 10***). Ongoing work will address whether the other mutations endow similar phenotypes and establish how closely orthotopic tumors resemble autochthonous tumors from mice and humans.

Collectively, our data demonstrate F^{7}NHCA medium expands AT2 organoids from murine lungs that retain the functional properties of their native counterparts. We envision that this organoid system will be useful for studying normal alveolar stem cell biology and uncovering the mechanisms governing their evolution throughout cancer progression.

MATERIALS AND METHODS

Tissue processing

8-17 week old mice were sacrificed and their lungs were inflated with digestion buffer containing Advanced DMEM/F-12, Penicillin/Streptomycin, Amphotericin B, 1 mg/mL Collagenase (Sigma, C9407-500MG), 40 U/mL DNase I (Roche, 10104159001) 5μM HEPES, and 0.36 mM $CaCl_2$. Distal lung tissue was extracted, minced and incubated in 3-5mL digestion buffer at 37ºC for 30-45 min with agitation. The resulting suspension was washed two times with PBS, filtered through 70μm mesh to remove chunks, and incubated in ACK Lysis Buffer (Thermo, A1049201) for 3-5 minutes at room temperature to lyse red blood cells. The suspension was washed two more times and resuspended in PBS. We typically plated 20,000 – 100,000 cells per drop. Human samples were processed identically except without an initial inflation step on the same day as surgery. Normal human lung tissues were acquired from patients undergoing lung tumor resections at Brigham and Women's Hospital. This study was reviewed and approved by an Institutional Review Board. Patients gave their informed prospective consent to have their discarded, de-identified tissue obtained from standard of care treatment used for scientific research.

Organoid culture

Cells were mixed with Growth Factor Reduced Matrigel (Corning) at a ratio of 1:9 and seeded onto multi-well plates as 20μL drops. The drops were incubated at 37ºC for 15 minutes – 3 hours to allow them solidify, then overlaid with F^7NHCA medium supplemented with Y-27632 (see below). The cultures were maintained in a humidified 37ºC / 5% CO_2 incubator at ambient O_2 pressure. Media was replenished every 3-4

days using F[7]NHCA medium (see below) and organoids were passaged 6-12 days after plating. For passaging, matrigel drops were dissolved in TrypLE Express (Sigma, 12604-013) and incubated at 37ºC for 7-15 minutes. The organoid suspensions were then dissociated into single cells by vigorous pipetting, washed twice, resuspended in PBS, and plated as described above. Recommended split ratios are 1:2 - 1:4 for earlier passages and 1:5 - 1:10 for later passages. We typically plated 20,000 – 100,000 cells per drop.

F[7]NHCA medium recipe

Component	*Concentration*	*Vendor, Catalog Number*
Advanced DMEM/F-12	n/a	Thermo, 12634010
HEPES	10 mM	Thermo, 15630080
Nicotinamide	10 mM	Sigma, N0636-100G
N-Acetyl-L-cysteine	1 mM	Sigma, A9165-5G
L-Glutamine	2 mM	Thermo, 25030149
Amphotericin B	250 ng/mL	Thermo, 15290018
Penicillin/Streptomycin	1x	VWR, 45000-652
SB202190	10 µM	Cayman, 10010399
A83-0*	1 µM	Cayman, 9001799
CHIR99021*	5 µM	Cayman, 13122
Y-27632**	10 µM	Sigma, 1005583
rh-FGF7***	80 ng/mL	Peprotech, 100-19-50UG
rm-Hgf***/rh-Hgf #	80 ng/mL	Peprotech, 315-23-50UG / 100-39H-25UG
rm-Noggin	80 ng/mL	Peprotech, 250-38-50UG

*Stock solutions were stored at -80ºC and spiked-in immediately prior to use.
**Added only when plating.
***Removed from media for growth factor independence assays.
#Replaces rm-Hgf in media for culturing human organoids.

Organoid Viral transduction

Viral transduction of organoids was carried out using the previously described “mix-and-seed” method (Wang et al., 2014). Briefly, organoids were processed for passaging, resuspended in concentrated virus, mixed with matrigel, and plated. We do not recommend using spinfection-based protocols described in the literature because the

cells tend to attach to most surfaces, even those that have not been treated for tissue culture. Adenoviral transductions were performed at an MOI of 250-500. Lentiviral transductions were performed using non-tittered viruses. The Ad5-sgEA-Cbh-Cas9 virus was purchased from ViraQuest Inc. The Ad5-CMV-Cas9 and Ad5-CMV-Cre viruses were purchased from the Viral Vector Core at University of Iowa Carver College of Medicine.

Animal studies

Mice were housed at the animal facility at the Koch Institute for Integrative Cancer Research at MIT. All animal studies described in this study were approved by the MIT Institutional Animal Care and Use Committee. *Kras*$^{LSL\text{-}G12D/+}$ (Jackson et al., 2001), *Trp53*$^{fl/fl}$ (Marino et al., 2000), *Rosa26*$^{LSL\text{-}Cas9\text{-}eGFP}$ (Platt et al., 2014), *Rosa26*$^{LSL\text{-}TdTom}$ (Madisen et al., 2010), *Sftpc-eGFP* (Vanderbilt et al., 2015) mice have been previously described and were maintained on a pure C57BL/6 background. For all transplant experiments, approximately 2-6x10^5 dissociated C57BL/6 organoids were transplanted orthotopically into syngeneic C57BL/6 recipient mice via intratracheal delivery as previously described (DuPage et al., 2009). An approximately even distribution of male and female transplant recipients at 8-20 weeks of age were used for all experiments.

Tumor scoring analysis

Mice were sacrificed 5-7 months post organoid inoculation. Lungs were immediately scrutinized for red fluorescent nodules using a dissecting microscope. Tissues were then processed for histology and H&E-stained tissue slides were imaged using the Aperio AT2 slide scanner (Leica Biosystems), visualized using QuPath

(https://qupath.github.io/), and scrutinized for tumors. A positive score was assigned to mice bearing at least one lesion in both analyses.

Flow cytometric analysis

For longitudinal tracking of Sftpc expression, the percentage of cells expressing eGFP was determined via flow cytometry at each passage. For staining cell-type specific surface markers on murine organoids, cells were incubated with either anti-mouse MHC Class II APC-eFluor 780 (1:1000, Thermo, 47-5321-82) or anti-mouse Podoplanin APC/Cy7 (1:300, BioLegend, 127417) antibodies on ice for 20 minutes in 96-well U-bottom plates. For staining human organoids, cells were sequentially incubated with an anti-human HT2-280 (1:100, Terrace Biotech, TB-27AHT2-280) antibody followed by anti-mouse IgM PE/Cy7 antibody for 20 minutes each on ice. Flow cytometric analysis was performed on a Guava EasyCyte flow cytometer. Data analysis was performed using FlowJo software.

Histology

Whole organoids were harvested 7-12 days after plating by incubating matrigel drops in dispase solution (Corning, 354235) for 20-30 minutes at 37ºC. Organoids were then fixed in 4% paraformaldehyde overnight at 4ºC and immobilized in Histogel (Thermo, HG-4000-012) for paraffin embedding. Tissues were fixed in zinc formalin (Polysciences, 21516) overnight at room temperature and maintained in 70% before being processed for paraffin embedding.

Immunohistochemistry (IHC)

Sectioned organoids or tissues were stained with hematoxylin and eosin (H&E) or IHC stained with the following antibodies: *organoids*: rabbit anti-SFTPC (1:10,000, Millipore,

ABC99), rabbit anti-Caveolin-1 (1:10,000, Thermo, C3237), rabbit anti-CCSP (1:10,000, Millipore, 07-623), rabbit anti-Keratin 5 (1:10,000, Biolegend, 905501); *tissues*: rabbit anti-TTF1 (1:5000, Abcam, ab76013). For IHC staining, antigen retrieval was performed in citrate buffer (10 mM, pH 6.0) at 125 °C for 5 min. Endogenous peroxidases and alkaline phosphatases were blocked at room temperature for 30 minutes using the Dako Dual Endogenous Enzyme Blocking Reagent (Dako, S2003). Slides were incubated in primary antibody overnight at 4ºC and subsequently in HRP-polymer-conjugated secondary antibodies (ImmPRESS HRP Anti-Rabbit IgG [Peroxidase] Polymer Detection Kit, Vector Laboratories, MP-7401). The slides were developed with DAB (1 minute development time for all antibodies, Vector Laboratories SK-4100), and counterstained with hematoxylin.

Bulk transcriptome analysis

Total RNA was isolated from sorted cells using the TRIzol Plus RNA Purification Kit (Invitrogen, 12183555). Briefly, cells were sorted directly into TRIzol. Following lysis and phase separation, total RNA was purified from the aqueous phase using the PureLink RNA Mini Kit (included in the TRIzol Plus RNA Purification Kit) according to the manufacturer's specifications. RNAseq libraries were prepared from 300ng of total RNA using the Kapa Hyperprep kit (Roche) with 14 cycles of PCR. RNAseq libraries were quality controlled using Fragment Analysis (Agilent) and qPCR and pooled for sequencing. The libraries were sequenced with single-end 75 base pair reads on an Illumina NextSeq Instrument. Sequence reads were trimmed to eliminate 3' adapter traces using cutadapt (v1.16) (Martin, 2011). Trimmed reads were aligned to the mouse genome (mm9 build, UCSC annotation, genome.ucsc.edu) with STAR (v2.5.3a) (Dobin

et al., 2013). Reads per feature were quantified using the featureCounts utility in the Subread package (v1.6.2) (Liao et al., 2014). Differential analyses were performed using DESeq2 (v1.28.1) (Love et al., 2014) on raw counts. Enrichment analyses were performed using GSEA (v4.0.3) (Subramanian et al., 2005) in the “pre-ranked” mode using DESeq2 reported log2 fold-change values as the ranking metric. The c2 (curated) collection from MSigDB (www.gsea-msigdb.org/gsea/msigdb) was used with the customized addition of two gene-sets (Alveolar type 2 marker genes from (Treutlein et al., 2014); Basal cell associated genes from (Dvorak et al., 2011)).

Single-cell transcriptome analysis

Single cell RNA-sequencing was performed using the 10x Genomics platform following manufacturers guidelines with a few modifications.

MULTI-seq

The MULTI-seq method (McGinnis et al., 2019) was used to multiplex individual samples into the same 10x lane. MULTI-seq libraries were prepared as previously described (McGinnis et al., 2019) using a custom protocol based on the 10x Genomics Single Cell V2 and CITE-seq workflows. Briefly, the 10x workflow was followed up until complementary DNA amplification, where 1µl of 2.5µM MULTI-seq additive primer (oDYT039) was added to the cDNA amplification master mix. After amplification, MULTIseq barcode and endogenous cDNA fractions were separated using a 0.6X solid phase reversible immobilization (SPRI) size selection. To further purify the MULTI-seq barcode, we increased the final SPRI ratio in the barcode fraction to 3.2X reaction volumes and added 1.8X reaction volumes of 100% isopropanol. Eluted barcode cDNA was then quantified using a QuBit before library preparation PCR using primers

TruSeq_RPIX and TruSeq_P5 (95 °C, 5′; 98 °C, 15′; 60 °C, 30′; 72 °C, 30′; eight cycles; 72 °C, 1′; 4 °C hold).

TruSeq_RPIX:

5′-CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA-3′

TruSeq_P5:

5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′

Following library preparation PCR, the library was size-selected by a 1.6X SPRI clean-up prior to sequencing.

10x scRNA-seq library

Single cell RNA-seq libraries were prepared using the Chromium Single Cell 3' Reagent Kit v2 according to the user guide, except for the following modification. A MULTIseq primer was added into the cDNA amplification step for amplifying MULTIseq barcodes. After cDNA amplification, the cDNA pool was purified following MULTIseq library preparation. Purified cDNA pool was then made into scRNA-seq library as directed in the 10x user guide.

Sequencing

Sequencing libraries from each sample were pooled to yield approximately equal coverage per cell per sample. scRNA gene expression libraries and MULTI-seq amplicon libraries were pooled in an approximately 10:1 molar ratio. Libraries were further pooled with approximately 0.5-1% PhiX genomic DNA library added for quality-control. The libraries were sequenced using the on Illumina NovaSeq instrument.

Data analysis

scRNA-seq data was aligned and processed by cell ranger v2.0. Raw data was preprocessed (normalized, filtered and logarithmized) by SCANPY (Wolf et al., 2018).

Dot plot was generated by SCANPY. MULTI-seq data was processed following the published standard MULTI-seq analysis pipeline to call the multiplexing barcode sequence for each single cell. Corresponding sample ID were then assigned to each cell based on the MULTI-seq barcodes (McGinnis et al., 2019).

Cell lines and cell culture

HEK293T cells were maintained in DMEM (Corning, 10-013-CV) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Gibco, 25030), and Penicillin-Streptomycin (VWR,45000-652).

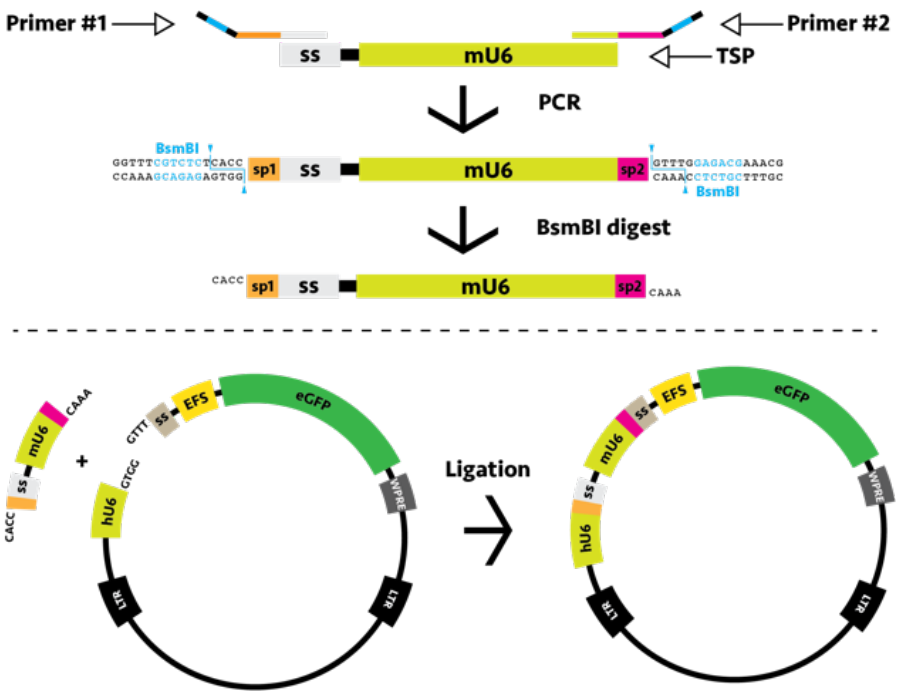
Lentivirus production

Lentiviral vectors were produced by co-transfecting HEK293T cells with lentiviral and packaging plasmids (psPAX2 and pMD2.G). Viral supernatant was harvested 48 and 72 hours after transfection, concentrated by centrifugation at 25,000 rpm for 2 hours at 4ºC, resuspended in OptiMEM (Gibco, 31985-062), and frozen at -80^{o}C.

Vector cloning

The Lenti-U6-Trp53-EFS-eGFP vector was generated in two steps. First, a Lenti-U6-filler-EFS-eGFP vector was generated using the Gibson Assembly-based Modular Assembly Platform (GMAP) (Akama-Garren et al., 2016). In brief, a GMAP compatible lentiviral backbone (derived from LentiCRISPRv2, Addgene #52961) was fused with DNA fragments corresponding to S1-hU6-filler-S3 (PCR amplified from pSECC, Addgene #60820), EFS-pB, and eGFP-gB (PCR amplified from LSL-Cas9-Rosa26TV, Addgene #61408) using Gibson Assembly. The Lenti-U6-filler-EFS-eGFP was subsequently digested with BsmBI (NEB, discontinued) and sgTrp53 was cloned into the cut vector as previously described (Ran et al., 2013).

The Lenti-U6-sgEA-EFS-eGFP vector was cloned using the following strategy. A DNA fragment containing an improved sgRNA scaffold (Chen et al., 2013) and the mU6 promoter was amplified from pDonor_mU6 (Addgene #69350) using two successive PCR reactions and a trio of primers designed to add the improved scaffold sequence to the 5' end of the product. We termed this fragment the PST (paired sgRNA template). A paired sgRNA cassette containing the spacer sequence for sgAlk, the improved sgRNA scaffold, the mU6 promoter, the spacer sequence for sgEml4, and BsmBI restriction sites on either end was then amplified using the PST and a customized primer pair (shown below). This cassette was digested with BsmBI and ligated into the BsmBI digested Lenti-U6-filler-EFS-eGFP vector. This process is illustrated in below:



TSP sequence:

GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAA
GTGGCACCGAGTCGGTGCTTTTTTCGGATTACGGTGTTTACTCCGTACTGCAGTATCTAGAGATCCG
ACGCCGCCATCTCTAGGCCCGCGCCGGCCCCCTCGCACAGACTTGTGGGAGAAGCTCGGCTACTC
CCCTGCCCCGGTTAATTTGCATATAATATTTCCTAGTAACTATAGAGGCTTAATGTGCGATAAAAGAC
AGATAATCTGTTCTTTTTAATACTAGCTACATTTTACATGATAGGCTTGGATTTCTATAAGAGATACAAA
TACTAAATTATTATTTTAAAAAACAGCACAAAAGGAAACTCACCCTAACTGTAAAGTAATTGTGTGTTT
TGAGACTATAAATATGCATGCGAGAAAAGCCTTGTTTG

Key: Improved sgRNA scaffold; Spacer (random sequence); mU6

Primers to generate paired sgEA cassette:

Primer #1:

5'-GGTTTCGTCTCTCACCGTCCTGGCATGTCTATCTGTAGTTTAAGAGCTATGCTGG-3'

Primer #2:

5'-CGTTTCGTCTCCAAACTCCTAGTAGACCCCGACAAACCAAACAAGGCTTTTCTCGC-3'

Key: Leader sequence; BsmBI recognition site; BsmBI overhang; sgRNA spacer; complementarity to improved sgRNA scaffold; complementarity to mU6

Primer and sgRNA sequences

ID	Sequence	Reference
Braf fwd	TGAGTATTTTTGTGGCAACTGC	(Dankort et al., 2007)
Braf rev	CTCTGCTGGGAAAGCGGC	(Dankort et al., 2007)
Eml4 fwd	GGTCAGCTACGGCTGAAGAC	This report
Alk rev	CTTCACCATGGGAAACCAGT	This report
Eml4 rev	GGCAGTTTGGGCTACACAGT	This report
TSP fwd 1	CGAGTCGGTGCTTTTTTCGGATTACGGTGTTTA CTCCGTACTGCAGTATCTAGAGATCCGACGCC	This report
TSP fwd 2	GTTTAAGAGCTATGCTGGAAACAGCATAGCAAG TTTAAATAAGGCTAGTCCGTTATCAACTTGAAAA AGTGGCACCGAGTCGGTGCTTTTTTCGG	This report
TSP rev	CAAACAAGGCTTTTCTCG	This report
sgTrp53	GACACTCGGAGGGCTTCACT	(Maresch et al., 2016)
sgAlk	GTCCTGGCATGTCTATCTGTA	(Maddalo et al., 2014)
sgEml4	GTTTGTCGGGGTCTACTAGGA	(Maddalo et al., 2014)

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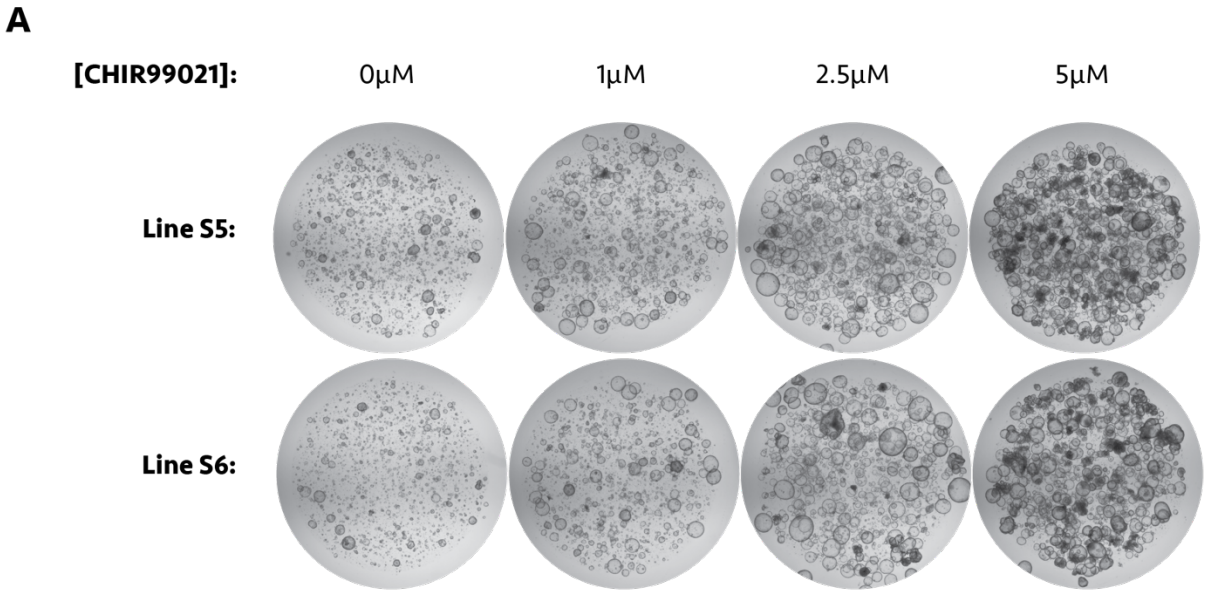
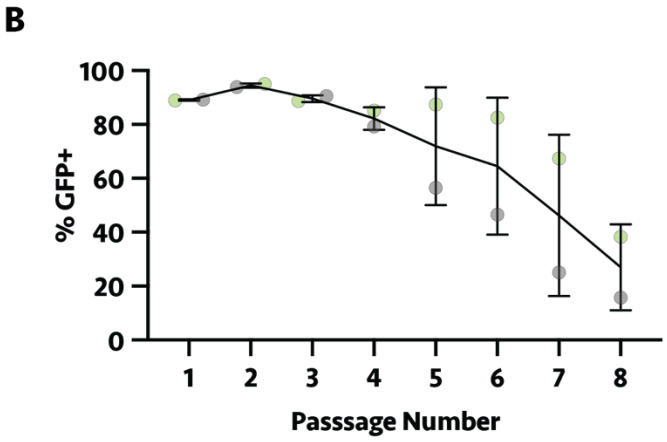
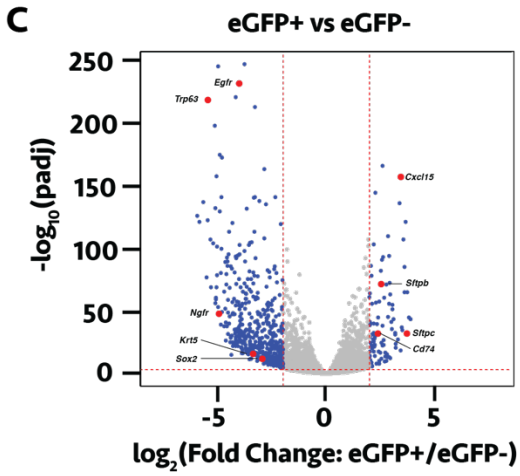
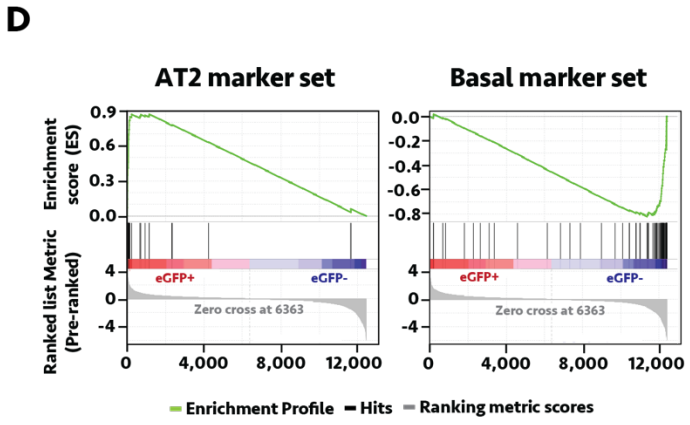
T.J. is a Howard Hughes Medical Institute Investigator, David H. Koch Professor of Biology, Daniel K. Ludwig Scholar; SAB member for Thermo Fisher, Lustgarten Foundation for Pancreatic Cancer Research, Amgen Inc. and Skyhawk Therapeutics; he is also a consultant and founder for T2 Biosystems Inc. and Dragonfly Therapeutics.

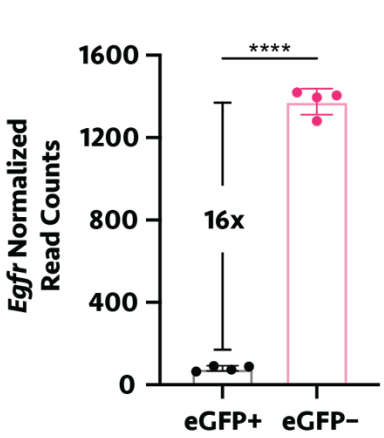
SUPPLEMENTARY FIGURES







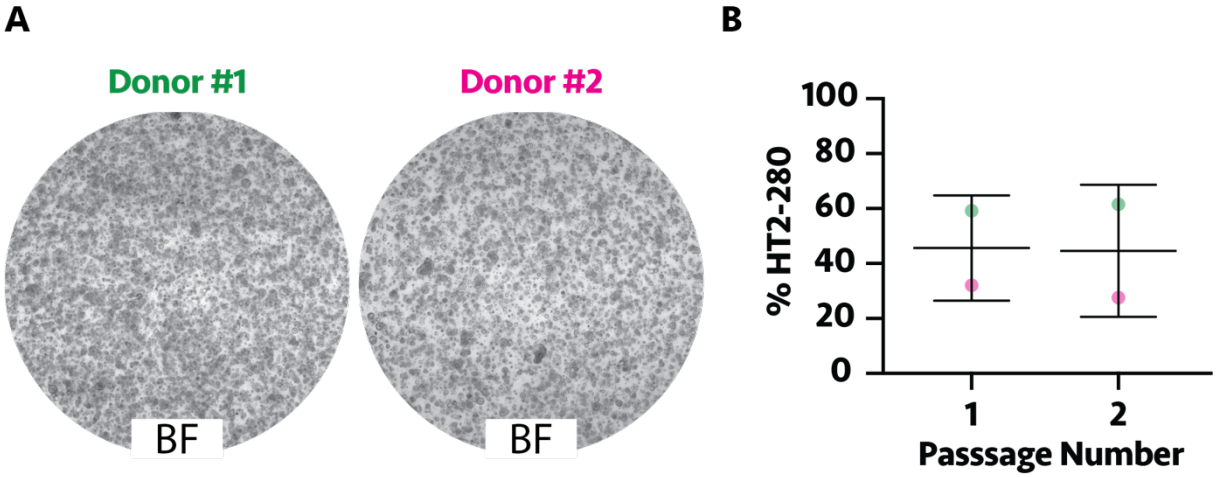




Supplementary Figure 1. Cells expressing basal cell makers occasionally take over F[7]NHCA medium expanded lung organoid cultures.

A. Growth of alveolar organoids in increasing amounts of the Wnt small molecule agonist CHIR99021. Alveolar organoids display a dose-dependent growth response to CHIR99021.
B. Flow cytometry based longitudinal quantification of *Sftpc-eGFP* expressing cells in *n* = 2 independent lines. Certain lines cannot maintain *Sftpc-eGFP* expression.
C. Volcano plot showing differentially expressed genes between the eGFP-positive and eGFP-negative populations. Canonical basal cell markers (highlighted in the plot) are amongst the most highly upregulated genes in the eGFP-negative population.
D. GSEA enrichment plots of AT2 (FDR = 0) and basal (FDR = 0) cell signatures.
E. Egfr expression in the eGFP+ and eGFP– populations.

All data are expressed as mean values ± the standard deviation. Statistical analysis in **D** was performed using a two-tailed students t-test. **** denotes a P-value < 0.0001.



Supplementary Figure 2. F7NHCA can expand and maintain HT2-280 expressing lung organoids.

A. Representative images of passage 2 human lung organoids. BF = Bright Field.
B. Flow cytometry based longitudinal quantification of HT2-280 expressing cells in *n* = 2 independent lines.

All data are expressed as mean values ± standard deviation.

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CHAPTER 3

DISCUSSION AND FUTURE DIRECTIONS

In this thesis, I have used organoid technology to establish an improved culture system for alveolar type 2 (AT2) cells and build next generation models of lung adenocarcinoma (LADC). The culture conditions described herein allow prolonged cultivation of primary AT2 cells that retain their cellular identity without mesenchymal or air-liquid interface support. Due to its minimal nature, this culture system serves as a genetically tractable and scalable model for researchers to rapidly study the various facets of AT2 cell function in homeostasis and how these cells change in the context disease.

Organoid LADC models facilitate the investigation of tumorigenesis, tumor evolution, and tumor maintenance

In this thesis, I primarily focused on applying the AT2 organoid culture system to model distinct molecular subtypes of LADC. Due to the unique advantages that culture-based cancer models offer, I believe the organoid-based models developed in this thesis will help advance our understanding of the molecular mechanisms governing LADC initiation, progression, and maintenance.

LADC is a genetically heterogenous disease. Classic analyses and recent large-scale sequencing studies (The Cancer Genome Atlas, 2014) have identified a wide variety of molecular subtypes within this disease and at the same time generated a long list of candidate driver mutations. Approaches leveraging genetically engineered mouse models (GEMMs) in conjunction with CRISPR-Cas9 technology have helped rapidly functionally validate many of these mutations as drivers and initiators of disease (Annunziato et al., 2016; Chow et al., 2017; Romero et al., 2017; Sanchez-Rivera et al., 2014; Walter et al., 2017; Wu et al., 2018). However, due to the mode of action of Cas9

and the inflexibility of GEMMs, these approaches are mostly useful for interrogating one or two loss-of-function events within a single tumor. Organoids offer a genetically tractable and flexible platform to study the effects of complex combinations of a wide variety of genetic alterations.

One type of mutations that have been relatively unexplored due to the limitations of somatic genome editing in live animals are oncogenic gene fusions. The majority of fusions involve *ALK*, *ROS*, or *RET* (The Cancer Genome Atlas, 2014; Kohno et al., 2015). Lower frequency fusion events encompass diverse kinases such as *NTRK1*, *ERBB4*, *KIAA1967*, *CIT*, or *TACC3*, among others (Kohno et al., 2015). So far, only *EML4-ALK* has been functionally validated as a driver of tumorigenesis in mice (Maddalo et al., 2014). Organoids are particularly suited for this purpose because these events are rare, especially those involving interchromosomal translocations. This model system offers the ability to easily generate large pools of cells, in which several sets of sgRNA pairs can be tested for their ability to induce the desired fusion in parallel. At the same time, it is possible to assess whether the fusion endows the cells with oncogenic properties using growth factor independence and transplantation assays. Such studies would help inform therapeutic strategies for patients with fusion positive tumors, as clinical data has demonstrated that targeting the kinases within these fusion genes elicits potent anti-tumor effects (Camidge et al., 2012; Shaw et al., 2014).

A second set of mutations whose role is relatively unknown are those that are enriched in oncogene negative tumors. Any tumor that does not harbor mutations in well-known driver oncogenes fall into this category (The Cancer Genome Atlas, 2014). Mutations in this tumor subclass typically occur within tumor suppressor genes (Jordan

et al., 2017). It is currently unknown which combinations of tumor suppressor mutations initiate LADC in the absence of activated oncogenes. Experiments leveraging CRISPR-Cas9 to induce rationally selected combinations of mutations in organoids can help to us build a tool kit for studying the oncogene negative tumor subtype, which is the least studied LADC subclass due to the lack of models.

In addition to profound intertumoral genetic heterogeneity, LADCs display a considerable degree of intratumoral heterogeneity at different levels. Previous studies employing our established *Kras^{G12D};Trp53^{KO}* (KP)-driven model of LADC have revealed that tumor subpopulations adopt unique cellular states throughout progression. For instance, the transition from adenoma into adenocarcinoma is accompanied by the appearance of Wnt-producing and Wnt-responsive compartments (Tammela et al., 2017). Similarly, metastatic subclones characteristically lose expression of *Nkx2-1* and gain expression of *Hmga2* (Winslow et al., 2011). Ongoing efforts in our lab applying single cell profiling technologies have further revealed that the previous two examples are only just the tip of the iceberg. In fact, murine LADC tumor cells can occupy a variety of cellular states throughout tumor progression, each of which is characterized by previously unappreciated transcriptional programs (unpublished results).

A big challenge for us now is devising strategies to validate these observations. In principle, our autochthonous KP tumor model is the ideal platform for validation experiments. However, genetically engineered mouse models are generally not amenable to precise genetic manipulation. There are three main ways to achieve this currently. The first method is to create novel conditional knock-out or knock-in alleles using gene targeting technology in embryonic stem cells (ESCs). Although this is a

relatively simple feat, it can take months or even years before a mouse strain can be derived from the newly modified ESCs. The second method is to use somatic genome editing with CRISPR-Cas9 to make the relevant modifications. Although this strategy circumvents the need to generate new mouse strains, there are still significant challenges associated with the technology. CRISPR-Cas9 can efficiently induce loss-of-function mutations in the KP model (Sanchez-Rivera et al., 2014; Tammela et al., 2017), but engineering precise mutations is exceedingly difficult. One study demonstrated that the efficiency of CRISPR-Cas9 mediated homology directed repair in lung epithelial cells is between 0.02-0.1% (Winters et al., 2017). Therefore, it would be close to impossible to build lineage reporters or other more complex tools using somatic genome editing. A third way is to use lentiviral vectors encoding reporters or constructs of interest. Although this method has shown promise (DuPage et al., 2011; Tammela et al., 2017), careful analysis performed in our lab suggest tumor cells downregulate lentiviral construct expression. This happens due to active epigenetic repression, position effect variegation, or a form pseudo-transduction, where viral particles only contain protein from the packaging cells but not the viral genome, resulting in tumor initiation but not continued expression of the viral construct.

In chapter 2, I demonstrated that KP-mutant organoids give rise to lung tumors that exhibit histopathological characteristics reminiscent of the KP mouse model and human disease. Ongoing studies will establish whether organoid derived and autochthonous tumors also exhibit similar epigenetic and transcriptomic profiles. Organoids offer a convenient and relevant platform for swiftly and cleanly generating a diverse set of tools ranging from lineage reporters to knock-out/in lines to answer the

numerous outstanding questions from our profiling studies. A powerful advantage of organoids is that they are amenable to virtually any form of manipulation because they are a culture based system. They have been successfully subcloned (Pleguezuelos-Manzano et al., 2020), genetically perturbed in simple ways such as inducing loss-of-function mutations (Iannagan et al., 2019; Roper et al., 2017) and in complex manners such as knocking-in fluorescent reporters into endogenous loci (Artegiani et al., 2020).

As discussed in Chapter 1, a prominent interest in the field is to leverage high-throughput genetic screening technologies to discover cancer specific dependencies. Most of the efforts have relied on utilizing cancer cell lines due to massive numbers of cells required for carrying out such endeavors. Given the numerous documented issues regarding cell lines, most notably their genomic instability and inability to mimic human tumor biology (Reviewed in Ben-David et al., 2019), a promising alternative is to use organoids. Not only do organoids offer a genetically clean and physiologically relevant platform for conducting genetic screens, the ability to cultivate and use normal organoids as a point-of-comparison enable us to discover true cancer specific vulnerabilities. A number of studies have demonstrated that it is possible to conduct large scale genetic screens using organoids *in vitro* and *in vivo* (Michels et al., 2020; Ringel et al., 2020). Similar studies harnessing the power of organoids can be applied to uncover cancer specific vulnerabilities in the various molecular subtypes of LADC.

Most important of all, organoids offer us the possibility of creating human cancer models that exhibit unprecedented characteristics. An astounding number of efforts focused on building biobanks of tumor derived organoids (tumoroids) have surfaced in the last couple of years. While these specimens will surely provide valuable resources

for studying cancer, another exciting modality has emerged: genetically manipulating normal organoids to generate cancer organoids. The Clevers and Sato groups have demonstrated that this is possible for colorectal and pancreatic tissues, respectively (Fumagalli et al., 2017; Seino et al., 2018). To my knowledge, this has not been done with lung organoids. This is the first time we can assess the immediate consequences of oncogenic mutations in normal human epithelial cells. Furthermore, through transplantation into mice, tumor progression and its influence on the neighboring microenvironment can be evaluated in detail. In Chapter 2, I demonstrated that the conditions enabling outgrowth of murine alveolar organoids support cultivation of their human counterparts. An important next step is to use this platform to build human organoid models of LADC.

Alveolar organoids provide a relevant model for diverse lung diseases

Going beyond cancer, AT2 cells are affected in wide variety of viral diseases, including coronavirus disease 19 (COVID-19). This disease has quickly become a global pandemic in the past few months and is threatening to cause long-lasting negative effects on society. COVID-19 mainly affects the respiratory tract and can cause respiratory distress, or even respiratory failure (Chen et al., 2020). Recent evidence suggests AT2 cells are one of main pulmonary cell types targeted by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, the agent responsible for causing COVID-19 (Smith and Sheltzer, 2020; Ziegler et al., 2020). SARS-CoV-2 cell entry depends on binding of the viral spike protein to ACE2 and subsequent proteolytic priming of the same spike protein by the cellular protease TMPRSS2 (Hoffmann et al., 2020). A cross-species examination of *ACE2* and

TMPRSS2 expression in murine and human pulmonary cell types revealed that these two genes are primarily expressed in AT2 and ciliated cells (Smith and Sheltzer, 2020; Ziegler et al., 2020). Disappointingly, mice are ineffective for studying COVID-19 pathogenesis or testing therapeutics because SARS-CoV-2 cannot bid the murine ACE2 receptor (Cohen, 2020; Li et al., 2004). Two groups generated transgenic mice expressing the human *ACE2* under the direction of either the human *K18* promoter (McCray et al., 2007) or the human *ACE2* promoter (Bao et al., 2020). Although SARS causing coronaviruses can infect these lung cells in mice to different extents, they suffer from one significant shortcoming. Given that *ACE2* is expressed as a transgene, the expression levels are supraphysiological and, at least in the case of the *K18* promoter-driven mice, they likely do not exhibit a relevant expression pattern across tissues. Efforts are underway to engineer mice in which the human *ACE2* replaces its murine counterpart, such that the expression of the gene is under the control of endogenous transcriptional machinery (Cohen, 2020).

Alveolar organoids provide an alternative and complementary model to investigate the interaction between SARS-CoV-2 and pulmonary host cells. Preliminary data (not shown) suggests the murine alveolar organoids developed in this thesis express *Ace2* and *Tmprss2*, suggesting that *Ace2* knockout organoids engineered to express the human ACE2 receptor would serve as a relevant model in this context. More importantly, although we do not know whether the human organoids described in this thesis express viral entry proteins, these would represent one of the best platforms for COVID-19 research. No effective treatment has been developed for COVID-19 yet. Leveraging organoid-based models could be highly effective for identifying its effects on

AT2 cells and potential strategies to slow down or even reverse them. Critically, organoids can be used for high throughput drug or genetic screening to find or test strategies preventing viral entry or disrupting the viral life cycle.

Alveolar organoids offer a system to dissect AT2 biology

One of the most important functions of AT2 cells is to repair damaged alveoli via self-renewal and differentiation into alveolar type 1 (AT1) cells. Even though we have known about this function for decades (Evans et al., 1975; Kaplan et al., 1969), we still have an incomplete understanding about the molecular pathways underlying AT2-to-AT1 differentiation. Three studies employing organoids have recently found roles for Yap (Sun et al., 2019), Bmp (Chung et al., 2018), and senescence related programs (Kobayashi et al., 2019) in this process.

The first two studies took a hypothesis based approach, whereas the third one used transcriptional profiling to arrive at their findings. A particularly exciting and effective way to dissect AT2 differentiation in organoids would be using a technique known as Perturb-Seq (Dixit et al., 2016), which combines pooled genetic screening with single cell mRNA sequencing to identify the effects of gene disruption on the transcriptome. However, the organoid models employed in all three of the above studies require co-culture with fibroblasts, and it is not clear whether these organoids can be serially passaged and expanded. Therefore, they are not suited for large-scale experimentation. In Chapter 2, we presented marker expression data suggesting that the AT2 organoids developed herein have the capacity to differentiate into AT1 cells. We have not proven that this is definitely the case, but ongoing studies leveraging rationally designed differentiation media formulations will answer this question

definitively. Nevertheless, combining this scalable AT2 organoid culture system, which can support high throughput screening in principle, with techniques such as Perturb-Seq, and a closely related cousin Perturb-ATAC (Rubin et al., 2019), would facilitate rapid and comprehensive identification of candidate AT2-to-AT1 differentiation regulators.

Final perspective

The development of organoids technology by the laboratory of Hans Clevers heralded in a new era in biology and has also changed the way we think about cell culture. In the end, the much maligned Alexis Carrel, who claimed that all cells were immortal and accordingly could be cultured outside the body indefinitely under the right conditions, was partially proven correct. Thanks to the unprecedented ability to cultivate living mini-organs in dishes, we have gained a valuable way to study normal and disease biology. This thesis adds to the growing number of organoid systems developed by various labs to continuously culture diverse cell types and provides further evidence that these systems can form the basis of powerful new disease models.

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