Molecular Mechanisms of Airway Epithelial Progenitor Cell Maintenance and Repair.

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

The lungs are vital organs whose airways are lined with a continuous layer of epithelial cells. Epithelial cells in the distal most part of the lung, the alveolar space, are specialized to facilitate gas exchange. Proximal to the alveoli is the airway epithelium, which provides an essential barrier and is the first line of defense against inhaled toxicants, pollutants, and pathogens. Although the postnatal lung is a quiescent organ, it has an inherent ability to regenerate in response to injury. Proper balance between maintaining quiescence and undergoing repair is crucial, with imbalances in these processes leading to fibrosis or tumor development. Stem and progenitor cells are central to maintaining balance, given that they proliferate and renew both themselves and the various differentiated cells of the lung. However, the precise mechanisms regulating quiescence and repair in the lungs are largely unknown. In this dissertation, ionizing radiation is used as a physiologically relevant injury model to better understand the repair process of the airway epithelium. We use in vitro and in vivo mouse models to study the response of a secretory progenitor, the club cell, to various doses and qualities of ionizing radiation. Exposure to radiation found in space environments and in some types of radiotherapy caused clonal expansion of club cells specifically in the most distal branches of the airway epithelium, indicating that the progenitors residing in the terminal bronchioles are radiosensitive. This clonal
expansion is due to an increase in p53-dependent apoptosis, senescence, and mitotic defects. Through the course of this work, we discovered that p53 is not only involved in radiation response, but is also a novel regulator of airway epithelial homeostasis. p53 acts in a gene dose-dependent manner to regulate the composition of airway epithelium by maintaining quiescence and regulating differentiation of club progenitor cells in the steady-state lung. The work presented in this dissertation represents an advance in our understanding of the molecular mechanisms underlying maintenance of airway epithelial progenitor cells as well as their repair following ionizing radiation exposure.
Dedication

To my late father, Peter Farin, who once dedicated his Ph.D. dissertation to me. I am honored to have the opportunity to dedicate this dissertation to him. To my mother, Charlotte Farin, for her unwavering love and support. My parents have been the most influential mentors and enthusiastic cheerleaders in my life. They introduced me to the amazing world of science, showed me the importance of following your passions, and led by example to demonstrate that family is actually what matters most.

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Abbreviations

AT1, alveolar type 1 cell
AT2, alveolar type 2 cell
BADJ, bronchoalveolar duct junction
BASC, bronchoalveolar stem cell
BrdU, 5-bromo-2’-deoxyuridine
CCSP, club cell secretory protein
DSB, double strand break
IdU, 5-ido-2’-deoxyuridine
IR, ionizing radiation
LET, linear energy transfer
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1. Introduction

Lungs are vital, epithelium-lined organs that function to exchange gasses between the environment and the bloodstream. In 2015, chronic lower respiratory diseases were the third leading cause of death in the United States [1]. Furthermore, lung cancer is the most common type of cancer world-wide and has been the leading cause of cancer-related death for several decades [2]. High mortality rates from lung-related diseases underscore the importance of fully understanding mechanisms that control maintenance and repair of the lung. Lung stem and progenitor cells and their interactions with the stromal microenvironment have been investigated through animal studies, culture techniques, and injury models. One occupationally and therapeutically relevant injury model that can inform the field of lung stem cell biology is exposure to ionizing radiation. The following introduction describes the key scientific advances, paradigm shifts, and current understanding of the lung stem cell and radiation biology fields.

1.1 Lung stem cell biology

1.1.1 Anatomy of the postnatal lung

Proper lung development gives rise to a stereotypically branched tree-like structure beginning in the trachea and ending in the alveoli, where gas exchange occurs. In the adult mouse, the trachea is surrounded by cartilage rings and is the largest, most
proximal airway structure of the lung. This extrapulmonary pseudostratified epithelium is lined with basal cells, club and goblet secretory cells, ciliated cells, brush cells, neuroendocrine cells, and submucosal glands [3,4]. The airway then splits into the mainstem bronchi and continues to branch into the intralobular airway, terminal bronchioles, and finally the respiratory bronchioles and alveolar duct. In the mouse, the simple columnar intralobular epithelium and terminal bronchioles are lined with club cells, very few goblet cells, ciliated cells and neuroendocrine cells [4,5]. In humans, a pseudostratified epithelium consisting of basal cells, club and goblet secretory cells, ciliated cells, and neuroendocrine cells persists into the terminal bronchioles [4,5]. The respiratory bronchioles are the only structure in the human lung that contain a simple cuboidal epithelium lacking basal cells [4]. The airways of both mouse and human end in bronchioalveolar ducts that consist of respiratory bronchioles, consisting of club cells, ciliated cells, and neuroendocrine cells; and alveoli, containing alveolar type 1 and type 2 cells [6]. The diversity in cellular composition along the proximal-distal axis of the airway creates an environment in which region specific differences in the maintenance of the airway epithelium occurs.

1.1.2 Regionally distinct airway and alveolar progenitor cells

The classic definition of an adult tissue stem cell is self-renewing resident cell type that can give rise to all of the specialized functional cells of the tissue [7]. A
growing body of research suggests that the adult airway epithelium is maintained by populations of infrequently dividing stem and progenitor cells that reside in various regional compartments of the lung [8–12]. These regional stem and progenitor cells have been primarily studied in vitro and in vivo through the use of lineage tracing systems. One of the most predominant tools used to date is the Cre-loxP system. In this system, a tissue- or cell-specific promoter drives the expression of Cre recombinase, which can excise a stop sequence flanked by LoxP sites, leading to the expression of a reporter. To achieve temporal control, Cre can be fused to the mutant human estrogen receptor (ER). This mutant ER is unable to bind estradiol, but retains the ability to bind the ligand 4-OHT. In the absence of 4-OHT, heat shock proteins sequester CreER to the cytosol. However, upon delivery of tamoxifen, which is metabolized to 4-OHT, CreER is released from the heat shock proteins and enters the nucleus to excise the stop sequence at the LoxP sites. This induces expression of a reporter construct in a temporal and spatial specific manner. Several other similar methods have also been employed to study stem and progenitor behavior in the lung, such as the Flp-FRT and Tet systems.

The three most widely investigated epithelial progenitor cells that maintain different compartments of the lung: basal cells, club cells, and alveolar type 2 (AT2) cells. Basal cells make up about 30% of the total airway epithelial cell population and fit the definition of stem cells, as they are capable of both self-renewal and differentiation
In humans, basal cells are located the entire length of the conducting airway into the terminal bronchioles, but not in the respiratory bronchioles [11,13]. Markers used to identify basal cells include *Trp63*, *Krt5*, *Krt14*, *Ngfr*, and *Pdpn* [4,13]. Evidence for stemness of basal cells in vivo predominantly comes from lineage tracing studies in the mouse. *Krt5*-expressing basal cells make up the vast majority of the basal cell population and can renew both basal cells and give rise to ciliated and secretory cells [13]. *Krt14* is a proposed marker of a subset of the basal cell population [11,14]. However, recent studies using single cell PCR by Watson et al. have shown that *Krt14* does not faithfully segregate the basal cell population [15]. Additionally, through lineage tracing and single cell gene expression analysis, they revealed that basal cells can be divided into two major subgroups: one expressing *Dlk2*, *Dll1*, *Lmo1*, and *Snai2* and another dedicated luminal progenitor expressing *Krt8* [15,16]. Molecularly and functionally distinct basal cell subpopulations have now been identified in several publications; one of which revealed that intrinsic expression either Notch2 or c-myb in subpopulations of basal cells led to the generation of secretory or ciliated cells, respectively [17,18].

While the mechanisms of basal cell self-renewal and differentiation are not fully understood, multiple developmentally important pathways play roles in determining basal cell fate. As previously mentioned, the Notch pathway is one major determinant of basal cell fate [16,19]. Higher Notch signaling drives the differentiation of secretory and
goblet cells, whereas low levels of Notch signaling encourages ciliated cell
differentiation [16–18,20]. The canonical Wnt/β-catenin pathway has also been
implicated in basal cell fate determination, with stabilization of β-catenin in basal cells
leading to an increase in ciliated cell generation [21,22]. Additionally, IL-6/STAT3
signaling has been shown to promote basal to ciliated cell differentiation [23]. Finally,
the Hippo pathway effector Yap has been shown to regulate self-renewal and
differentiation of basal cells [24,25]. Together, these studies point to a model in which
Notch and Yap signaling promote basal cell self-renewal, high Notch levels promote
secretory cell differentiation, and canonical Wnt signaling, IL-6/STAT3 signaling, and c-myb expression promote ciliated cell differentiation. Further studies are needed to
determine up-stream regulators that coordinate basal cell self-renewal and
differentiation.

Club cells, previously known as Clara cells, are Scgb1a1 (also known as CCSP or
CC10) expressing, nonciliated secretory cells that are found in the conducting airway of
mice and the respiratory bronchioles of humans. Club cells technically fulfill the
definition of a tissue stem cell because they can self-renew the club cell population and
give rise to both ciliated cells and goblet cells [10,26–28]. However, the molecular
phenotype and functional role of club cells in epithelial maintenance varies across the
murine airway. Proximal club cells are replaced by basal cells over time and can
generate both ciliated and goblet cells [10,28]. This is in contrast to club cells residing in the distal airways, which generate ciliated cells during homeostasis [10]. Another progenitor population, presumably basal cells, replaces the club cell population in the trachea and proximal airways, but not in the distal conducting airways and terminal bronchioles [10]. Work by Chen et al revealed that both the molecular and functional phenotype of club cells range along proximal and distal axis of the airway [8]. Together, these studies support the hypothesis that club cells are a heterogeneous population whose function is determined in a region-specific manner.

Tight regulation of the proportions of secretory and ciliated cells in the airway is essential to mucociliary clearance, a critical host defense mechanism. This regulation of club cell self-renewal and differentiation is achieved through pathways similar to those that regulate basal cell fate, including Notch, Wnt, BMP, and Yap signaling [24,29,30]. As with basal cells, upstream regulators that integrate these pathways to dictate club cell quiescence and differentiation have yet to be determined.

Alveoli are lined with alveolar type 1 (AT1) and alveolar type 2 (AT2) cells. AT1 cells, marked by Aqp5 expression, are flat, squamous cells that make up about 95% of the surface area of the lung and facilitate gas exchange [9,31]. AT2 cells, marked by Sftpc expression, contain lamellar bodies and are considered to be progenitors of the alveoli [5]. Both in vitro culture studies and in vivo lineage tracing experiments show that AT2
cells can give rise to AT1 cells during development and homeostasis [32–37].

Dysregulation of alveolar epithelial cell maintenance and repair has been identified as a critical step in the development of lung diseases, including pulmonary fibrosis [5,38].

1.1.3 Lung injury and repair

Diseases of the lung are leading causes of death worldwide and often arise from aberrant differentiation of lung stem and progenitor cell populations [1,2]. For example, chronic obstructive lung disease is characterized by secretory, goblet, and squamous cell hyperplasia [5]. Complete knowledge of the regulation of self-renewal and differentiation of progenitor cells under homeostatic conditions is essential to better understand disease process. However, due to the slow turnover rate in the lung, these regulatory signaling pathways are often only revealed following injury, which induces accelerated rates of proliferation and differentiation and can be used to model disease states. Injury models enable a better understanding of the kinetics of stem cell behavior, which can be extrapolated to predict stem cell behavior during human lung injury, disease, or regeneration.

Toxicants have been extensively used to study the regenerative potential of many progenitor types, particularly basal and club cells in the airways. Exposure of mice to SO2 denudes luminal cells from the trachea and proximal airways, leading to complete repopulation of the pseudostratified epithelium by basal cells [13,23]. Fundamental
work by Stripp and colleagues used the club cell specific toxicant naphthalene to show that a subpopulation of club cells, termed variant club cells, can regenerate the conducting airway [39–42]. These variant club cells can survive cell specific injury due to their unique molecular phenotype, including low levels of the cytochrome p450 enzyme, Cyp2f2, and are located near neuroepithelial bodies (NEB) or bronchiolar-alveolar duct junctions (BADJ) [42,43]. It is still not fully understood whether cell intrinsic or microenvironmental cues are the greatest determinant of survival of variant club cells.

Bleomycin exposure is an injury model that has been influential in understanding alveolar progenitor behavior and modeling fibrosis development [8,34,44,45]. After exposure to either bleomycin or hypoxia AT2 cells differentiate into AT1 cells at an increased rate [34,46]. Additionally, lineage tracing studies using Scgb1a1-CreER; ROSA-Tomato mice have tagged AT1 and AT2 cells in the alveoli after injury with bleomycin, possibly indicating that club cells serve as progenitors for the alveoli following severe injury [34]. However, it is likely that the AT1 cells traced in this study were derived from previously tagged AT2 cells, as this strain of mice is known to tag a small number of AT2 cells [10]. A subpopulation of club cells, which co-express Scgb1a1 and Sftpc, have been implicated as bronchioalveolar stem cells, or BASCs, and were reported to generate both airway and alveolar cell types in vitro [47]. Previous studies show that if Scgb1a1+ cells are lost in the developing and adult lung, hypoplasia
of AT1 and AT2 cells occurs, indicating that club cells either give rise to alveolar cells or participate in the microenvironment essential for alveolar development and maintenance [48,49]. Despite these findings, the in vivo relevance of BASCs has yet to be determined.

Development of genetic mouse models that conditionally ablate progenitors or manipulate signaling pathways have recently revealed that airway epithelial progenitor cells have a greater differentiation potential than previously thought. Rajagopal and colleagues showed that some club cells can dedifferentiate into basal cells in vivo either following the deletion of basal cells or Yap overexpression, indicating that some club cells have an inherent plasticity that is suppressed under homeostatic conditions [25,50]. Cellular plasticity has also been identified in the alveolar epithelium. Following pneumonectomy and in vitro, some AT1 cells, defined by expression of the transcription factor HopX, were able to give rise to AT2 cells [51]. Additionally, exposure to H1N1 influenza virus led to the development of p63+/KRT5+ pods in the alveolar space [52]. The cell-of-origin of these pods have been the source of much debate. Chapman and colleagues previously identified a subpopulation of epithelial cells that express α6β4 integrin molecules, a receptor for the extracellular matrix molecule laminin, which give rise to Scgb1a1- or Sftpc-expressing cells ex vivo [53]. These “lineage negative”
progenitors were a proposed cell-of-origin of the basal-like pods observed post-H1N1 infection [54].

Together, these studies raise several important questions regarding stem cell hierarchy and plasticity. The traditional stem cell hierarchy consists of a multipotent, relatively undifferentiated stem cell that can self-renew and give rise to a transit amplifying population of cells. Transit amplifying cells have a limited ability to divide and function to generate terminally differentiated progeny [55]. Although this tiered system has been extensively considered in theory, it is really only appropriate for describing stem cells in the hematopoietic system. Almost every other organ system is maintained by various populations of stem and progenitor cells that reside in regionally distinct locations. These systems, including the lung, likely fit a different model in which pools of progenitor cells differentially maintain tissues and undergo a regenerative process that is dependent on the type and extent of injury. Extensive injury models have exposed redundant progenitor populations and their inherent plasticity, the limits of which have yet to be fully tested. Stem cell paradigms were largely developed using injury models in mouse. However, many of these injury models are not representative of naturally occurring injuries to humans. More relevant injury models that also effectively exploit stem cell behavior are needed.
1.2 Ionizing radiation: Types, uses, and risks

Radiation exposure is something humans encounter in terrestrial life, cancer treatment, space travel, and radiological warfare or accidents. Therefore, exposure of mice to ionizing radiation is an effective injury model, used not only to study the kinetics of stem and progenitor cell repair in the lung, but also to model a physiologically relevant injury to humans.

1.2.1 Effects of ionizing radiation

Ionizing radiation (IR), or radiation that carries enough energy to remove an electron from an atom, can have a large biological effect when passing through a cell, tissue, or organism. Radiation therapy takes advantage of this fact by using specifically targeted IR to severely damage or kill cancer cells. However, when normal tissue is exposed to IR, such as the case of atomic bomb survivors, astronauts, or patients receiving multiple radiographic studies or radiation therapy, negative biological effects can result, leading to dose-dependent tissue remodeling and/or cancer. The energy carried by various forms of radiation can be generally described using the term linear energy transfer (LET), or the energy transferred per unit length [56]. The two major categories of radiation quality are low- and high-LET radiation [57]. Low-LET radiation deposits energy in a diffuse manner; examples of types of low-LET radiation are medical X-rays, natural terrestrial background radiation, and gamma rays. In contrast, exposure
to high-LET radiation leads to a narrower, more concentrated energy deposition than with low-LET radiation [56]. Therefore, high-LET radiation exposure causes more complex molecular damage and may lead to more severe biological consequences. Types of high-LET radiation include heavy ions and alpha particles. Due to the differences in energy deposition, heavy ions, such as $^{12}$C, are increasingly being considered for use in radiation therapy treatment [57,58]. Additionally, during space travel, astronauts can be exposed to galactic cosmic rays, which contain heavy ions including $^{56}$Fe or $^{28}$Si [59]. A greater understanding of the extent of damage inflicted on normal tissue following high-LET radiation exposure and how this compares to low-LET radiation exposure is essential for therapeutic development and risk assessment.

Exposure to ionizing radiation induces damage to the DNA, as well as other molecules such as RNA and proteins, which can alter cellular signaling [60]. When a cell is traversed by low-LET radiation, 60-70% of DNA damage is due to direct absorption of the radiation by the DNA, while 30-40% of DNA damage is indirect [61]. Indirect biological effects are when radiation interacts with water molecules in a cell to produce free radicals that cause DNA damage. DNA damage is one of the most well studied effects of radiation exposure, considering DNA misrepair can lead to oncogenic mutations. Double strand breaks (DSBs) initiate a cascade of events that begin with the phosphorylation of histone H2AX by ATM, ATR or DNA-PK in regions around the
break [62]. The phosphorylation of H2AX provides a binding site for MDC1, which recruits the remaining downstream DNA repair complex elements, such as MRN, 53BP1 and BRCA1, to the DSB [63]. The DSB is then repaired by non-homologous end joining, a common but error prone method, or homologous recombination repair, a more accurate method [62]. DSBs resulting from exposure to IR can be visualized by immunofluorescence staining for H2AX.

1.2.2 Ionizing radiation and stem cells

The biological effects of IR on lung stem cells are largely unknown. However, work in other tissues has shown tissue-specific differences in mechanisms of repair and regeneration following IR exposure. Melanocyte stem cells are eliminated from the progenitor pool following exposure to IR due to premature terminal differentiation [64]. In contrast, bulge stem cells in the skin are resistant to apoptosis post-radiation due to a radiation-induced upregulation of the anti-apoptotic gene Bcl-2 and stabilization of p53 [65]. Mammary stem cells are also radioresistant due to induction of the canonical Wnt signaling pathway [66]. Further evidence for tissue-specific regulation of repair following IR exposure comes from the hematopoietic system, which is a highly radiosensitive tissue and one of the first organ systems to fail following high dose exposure to IR [67]. The propensity for radiation-induced apoptosis in the hematopoietic system can be mediated by factors such as VEGF, MCL1 and aPC [68–71]. Human
Hematopoietic stem cells have an increased susceptibility to p53-dependent apoptosis compared to their differentiated progeny, which is mediated by both ASPP1 and BCL-2 [72]. However, species-specific differences may occur, as other studies demonstrated that mouse hematopoietic stem cells are resistant to radiation-induced apoptosis due to a strong upregulation of anti-apoptotic genes and p53-dependent DNA damage repair following radiation exposure [73,74].

As previously discussed, many tissues have multiple redundant progenitor populations, which can complicate interpretation of progenitor cell radiosensitivity. Early studies of the effects of IR on intestinal stem cells by Grant and colleagues indicated that the slow cycling +4, Bmi1+ intestinal stem cells are radiosensitive [75]. Recent studies indicate that the Lgr5+/Sox9low crypt base columnar stem cells are radioresistant and are the major progenitor cell that regenerates the small intestine following exposure to IR [76–78]. However, contradictory to these findings, two groups reported that Bmi1+ cells are actually resistant to apoptosis and responsible for regenerating the intestine post-radiation [79,80]. These studies all heavily rely on lineage tracing in the mouse and their differences could lie in the susceptibility of Bmi1+ cells to apoptosis following tamoxifen delivery [80]. The response of lung progenitor cells following ionizing radiation has yet to be fully explored. However, based on evidence from the aforementioned studies, a cell type and region specific effect of IR is expected.
1.3 p53: a master regulator of cell fate

Trp53 is a tumor suppressor that is one of the most commonly mutated genes in cancer [81]. The most well studied role for p53 is in response to DNA damage. Under steady-state conditions, p53 is held at relatively low levels, which is mediated by ubiquitination by MDM2 and subsequent degradation [82]. However, following DNA damage, p53 is phosphorylated at serine or threonine residues by proteins such as ATM, ATR, DNA-PK, CHK1, and CHK2, leading to the activation of p53 and its accumulation in the nucleus [83]. p53 can also be stabilized through inhibition of MDM2 by ATM and p14ARF [84]. Through nuclear accumulation, p53 can bind to downstream target genes and activate various cellular responses. While the DNA damage is being repaired, p53 can induce a temporary G1/S, S, or G2/M cell cycle arrest through downstream targets, such as p21 [85–87]. However, if the DNA damage cannot be repaired, p53 will activate targets such as PUMA, NOXA, and BAX to induce apoptosis or p21CIP1 and p16INK4A to induce senescence [88].

In addition to its classical functions of regulating cell fate following cellular stress, p53 can also regulate migration, autophagy, metabolism, and tumor microenvironment signaling [89]. Interestingly, previous studies in the nervous and mammary systems have shown that p53 regulates stem and progenitor cell self-renewal and terminal differentiation [90–93]. However, these studies were almost entirely
performed \textit{in vitro}. Studies in the hematopoietic system reveal that germ line knockout of p53 results in a decrease in stem cell quiescence and an increase in self-renewal [94]. Additionally, p53 has recently been shown to play a role in regulating progenitor cell self-renewal in the developing kidney [95]. These studies highlight an essential role for p53 in various organ systems \textit{in vitro} and during development. However, how p53 controls progenitor cell differentiation under homeostatic conditions in a quiescent tissue such as the lung has not been studied.

Given that p53 mutations are present in about 70\% of small cell lung carcinoma and 50\% in non-small cell lung carcinoma and that recent literature has demonstrated that stem and progenitor cells are often the cell-of-origin of cancers, there is likely a link between p53 mutations in lung progenitor cells and cancer development [85,96–98]. However, very few studies have examined the role for p53 in regulating progenitor cells in the lung during homeostasis or in response to ionizing radiation. Manipulation of p53 levels \textit{in vivo} and \textit{in vitro} will enhance our understanding of p53’s role in normal tissue both prior to and following IR exposure.

\textbf{1.4 Conclusion}

The following dissertation is focused on understanding the molecular mechanisms underlying the maintenance and repair of airway epithelial progenitor cells during homeostasis and after injury, particularly following ionizing radiation exposure.
Exposure to IR can lead to tissue remodeling, fibrosis, and potential cancer progression. However, the effects of various qualities of IR on airway epithelial progenitor cells are largely unknown. A greater understanding of these effects would improve both therapeutic development and risk assessment. The working hypothesis for Chapters 2 and 3 of this dissertation is that radiation exposure injures airway epithelial cells in a dose- and quality-dependent manner. Studies presented in Chapter 2 demonstrate that exposure to ionizing radiation leads to dose-dependent effects on the clonal expansion airway epithelial progenitor cells. Chapter 3 expands on these findings to show that clonal expansion occurs in a quality-dependent and distal airway specific manner. Additionally we found that clonal expansion occurs due to p53-dependent senescence, apoptosis, and mitotic defects. Working with p53 deficient mice in the context of radiation injury led to the discovery that p53 regulates progenitor cell behavior in the homeostatic lung, which is presented in Chapter 4. Chapter 5 summarizes the work presented in this dissertation, examines the immediate and broad implications, and discusses future directions.
2. Low- and high-LET radiation drives clonal expansion of lung progenitor cells *in vivo*

2.1 Introduction

Exposure of lung tissue to ionizing radiation results in DNA damage and altered cellular signaling, leading to tissue remodeling, fibrosis, and cancer [60,63,99]. Humans are exposed to ionizing radiation (IR) in a variety of situations including cancer treatment, high altitude flight or space travel, and radiological warfare or accidents. However, these different radiation exposures differ in the quality of ionizing radiation delivered. Radiation quality can be characterized by the amount of energy deposited as it traverses a tissue, a property referred to as linear energy transfer (LET). Low-LET radiation, such as X-rays, deposits energy in a diffuse manner and is commonly used in radiation therapy [56]. High-LET radiation, such as heavy ions found in cosmic radiation, deposit large, concentrated amounts of energy along the path taken by the high energy ion [56]. Studying low- and high-LET radiation is important for optimization of regimens for therapeutic radiation and to better understand occupational exposure effects such as that encountered by astronauts.

Many studies have investigated the effects of ionizing radiation on stem and progenitor cells in a number of organs, including the epidermis, mammary gland, intestine, and hematopoietic system [65,66,73,77,100]. A consensus from this work is that tissue stem and progenitor cells have differential radiosensitivity as compared to their
differentiated progeny. Despite the wide knowledge base in these systems, less is known of the effects of ionizing radiation on progenitors in the pulmonary system. Club cells, previously known as Clara cells, are non-ciliated secretory progenitors that express the protein Scgb1a1 (also known as CCSP and CC10) [101]. Experiments using an inducible Cre recombinase, CreER, driven by the Scgb1a1 promoter to lineage-label Scgb1a1-expressing cells with a fluorescent reporter, have demonstrated that these non-ciliated secretory cells function as self-renewing progenitors that maintain the mouse bronchiolar airway [10]. Additionally, Scgb1a1-expressing cells are the primary progenitors responsible for repopulating the airway epithelium following injury [8,10,39,40]. Not only are these secretory progenitors capable of maintenance and repair in vivo, they can also self-renew to form colonies when placed in a 3D matrix and co-cultured with lung fibroblasts [8,102].

The goals of the present study were to define the effect of ionizing radiation on maintenance and renewal of the airway epithelium. We used lineage tracing approaches coupled with evaluation of progenitor cell behavior in vitro and in vivo to determine the effects of low- and high-LET radiation on lung epithelial progenitor cells in mice using the bronchiolar epithelium as a model. We found that airway epithelial progenitors isolated from mice exposed to whole-body ionizing radiation lost their ability to form colonies in vitro in a dose-dependent manner. Additionally, we observed expansion of
Scgb1a1-expressing progenitor cells in vivo following exposure to either low- or high-LET radiation. However, exposure to radiation did not increase the lung epithelial proliferative index. These data suggest that radiation-resistant progenitor cells clonally expand for normal epithelial maintenance after functional loss of radiation-sensitive progenitors.

2.2 Materials and methods

2.2.1 Mice

The Scgb1a1-CreER; Rosa26R-Confetti mice were established by crossing Scgb1a1-CreER™ mice (kindly provided by Brigid L.M. Hogan, Duke University) with Rosa26R-Confetti mice (The Jackson Laboratory) as previously reported by Chen et al [8]. Scgb1a1-CreER mice heterozygous for the Rosa26R-Confetti allele were injected i.p. 3 times every other day with 0.2mg/g body weight tamoxifen in Mazola corn oil to randomly introduce one of four genetic tags into the Scgb1a1-expressing epithelial cells. Mice constitutively expressing either RFP or GFP under the Rosa promoter (U-RFP and U-GFP, respectively) were obtained from the Jackson Laboratory. All mice were maintained in pathogen-free conditions in AAALAC approved animal facility at Duke University. Mice were exposed to a 12-hour light/dark cycle and had free access to food and water. Adult mice between the ages of 2–4 months were sacrificed for experiments according to IACUC approved protocols.
2.2.2 IdU drinking water

5-Iodo-2'-deoxyuridine (IdU; Sigma-Aldrich, St.Louis, MO) was resuspended in sterile drinking water at a concentration of 1 g/L. Fresh IdU drinking water was provided weekly, for 4 weeks, in light protected water bottles.

2.2.3 Radiation exposure

Mice, eight to ten weeks old, were either exposed to either X-rays or $^{56}$Fe radiation. For low-LET irradiation, unanesthetized mice were placed in plexiglas restraining tubes, and irradiated with 1, 2, 4, 6, or 8 Gy of 320 kVp X-rays (X-RAD 320 Biological Irradiator, Precision X-ray, Filter#4: 2.5 mm aluminum + 0.1 mm copper, dose rate = 1.95 Gy/min) delivered only to the thorax by shielding the head and abdomen with lead. For high-LET irradiation, mice were exposed whole body to 0.2, 0.5, 1, and 2.5 Gy of 600 MeV/nucleon $^{56}$Fe ions (NASA Space Research Laboratory’s linear accelerator at Brookhaven National Laboratory, dose rate 0.1 Gy/min).

2.2.4 Lung cell isolation and sorting

Eighteen hours post irradiation, suspensions of primary lung cells were isolated by elastase digestion and subsequently flow sorted for epithelial cells using cell specific surface markers, as previously described [8]. Following euthanasia, the chest cavity was opened and the lungs were perfused via the heart with PBS. The trachea was cannulated and lavaged with PBS. The heart and lungs were then removed en block and
the lungs instilled with elastase (Worthington Biochemical, Lakewood, NJ) for 10 minutes in a 37°C water bath followed by 3 additional 0.5 mL instillations with a 5 minute incubation period between each instillation. After elastase digestion, the lung lobes were dissected away from the heart and extrapulmonary airways, minced with scissors and further digested by the addition of DNase I (Promega, Madison, WI) for 15 minutes at 37°C. The cell suspension was passed through a 70 µm cell strainer, gently centrifuged (600 × g, 6 min, 4°C), and briefly resuspended in a red blood cell lysis solution (eBioscience Inc., San Diego, CA), then staining buffer (HBSS, 10 mM HEPES, 2% FBS) and centrifuged as above.

Cells were sorted using a FACS Vantage cell sorter (BD Biosciences, San Jose, CA). For lung epithelial cells from ubiquitous-RFP (Rosa26-mT/mG) or -GFP mice, cell populations were positively selected for EpCAM (PE-Cy7 anti-EpCAM; eBioscience, San Diego, CA) and negatively selected for 7-aminoactinomycin D (7-AAD, Biolegend) and biotin labeled antibodies to anti-CD34, -CD31, -CD45 (eBioscience) labeled with APC-Cy7 anti-biotin (eBioscience). For GFP labeled Scgb1a1+ cells (Scgb1a1-Cre; Rosa26-mT/mG), GFP positive cells were positively selected for EpCAM and negatively selected for 7-AAD.
2.2.5 Matrigel cell culture

Flow sorted epithelial cells were cultured with mouse lung fibroblasts (MLg cell line) and Matrigel (BD Biosciences) seeded onto Transwell filter inserts (BD Biosciences) as previously described [8]. For ubiquitous-RFP + ubiquitous-GFP mixing experiments, red and green epithelial cells were mixed in equal amounts, $2.5 \times 10^3$ each, with $1 \times 10^5$ mouse lung fibroblasts. The epithelial/fibroblast mixture was then added to an equal volume of growth factor reduced Matrigel (BD Biosciences) and seeded to the apical surface of 24-well transwell filter inserts (BD Biosciences) placed in 24-well flat-bottom culture plates. The solution was allowed to polymerize for $>30$ min at $37^\circ C$, then basic medium was added to the basal compartment. Cell cultures were maintained for 14 days at $37^\circ C$ in a humidified incubator (5% CO2). Colony-forming efficiency was calculated as the percentage of seeded cells that give rise to colonies, visualized on a Zeiss Axiovert40 inverted fluorescent microscope.

2.2.6 Immunofluorescence and morphometric analysis

Immunofluorescence imaging was performed on fixed lung tissue embedded in paraffin and processed as previously described [8]. The following primary antibodies were used: Rabbit anti-GFP (1:1000, Abcam, Cambridge, MA), Rabbit anti-RFP (1:300, Rockland Immunochemicals, Gilbertsville, PA), Mouse anti-C-terminus $\beta$-catenin (1:400, BD Biosciences), Rabbit anti-SCGB1a1 (1:15000, in house), Mouse anti-IdU/BrdU (1:200,
BD Biosciences). Primary antibodies were labeled with the appropriate corresponding fluorescently conjugated secondary antibody (Alexa Fluor 488 or Alexa Fluor 594; Invitrogen, Carlsbad, CA). Images were acquired using a Zeiss 780 confocal inverted confocal microscope with Zen Software.

Morphometric analysis of confetti-labeled airway epithelial patches and epithelial proliferation was performed using a Zeiss Axiovert40 inverted fluorescent microscope. Sections were selected that contained the greatest representation of the airway epithelial tree and stained on serial sections for IdU/Scgb1a1, β-catenin/RFP, and GFP-variants (YFP, CFP, GFP)/β-catenin. All contiguous patches of epithelial cells in a section that share the same lineage tag (cytoplasmic RFP, nuclear GFP, cytoplasmic YFP, and membrane CFP) and share the same lateral membrane (β-catenin) were counted. For proliferative index, all IdU+CCSP+ were counted and represented over the total number of DAPI+ airway epithelial cells. Patch size and frequency, and proliferative index were plotted using Microsoft Excel and GraphPad Prism.

2.3 Results

2.3.1 Low-LET radiation results in cell autonomous and dose-dependent decrease in epithelial colony-forming ability

Previous work by us and others have shown that the murine airway epithelium possess the potential to proliferate and repair after injury as well as the ability to clonally expand and differentiate when cultured in a 3D-in vitro culture system [8,102]. To
determine the effects of low-LET radiation exposure on airway epithelial progenitor cells, the colony forming ability of primary mouse lung epithelial cells was assessed in \textit{vitro} after whole-body exposure of mice to X-rays (Fig. 1A). Epithelial cells were isolated from the lungs of mice that ubiquitously expressed RFP (U-RFP) 18 hours after whole-body exposure to X-rays. Primary epithelial cells were also isolated from the lungs of unirradiated mice ubiquitously expressing GFP (U-GFP) and mixed in equal proportion with the irradiated epithelial cells. These cells were co-cultured in Matrigel along with unirradiated immortalized mouse lung fibroblasts and assessed for colony forming efficiency at culture day 14. X-ray dose-dependent decreases were observed in colony-forming ability of isolated epithelial progenitor cells (Fig. 1B). Colony forming efficiency decreased to 44\% and 14\% of control after exposure to 2 Gy and 4 Gy of X-rays, respectively, and was completely lost after exposure to 8 Gy of X-rays. No differences were observed in the colony forming efficiency of un-irradiated U-GFP progenitor cells that were co-cultured with irradiated progenitor cells.

We considered the possibility that changes in the colony-forming ability of epithelial cells isolated from irradiated mice may have resulted from either direct or indirect (microenvironmental non-targeted) effects of radiation exposure. To distinguish between these possibilities, epithelial cells were isolated from un-irradiated mice and irradiated to the same doses of X-rays \textit{ex-vivo} prior to evaluating colony-forming ability.
in 3D epithelial-fibroblast co-cultures (Fig. 2A). Radiation dose-dependent decreases in epithelial colony forming ability were observed following ex-vivo irradiation that were identical to those observed following whole-body in vivo radiation exposure, for example, colony forming ability was 13% after 4 Gy whole body exposure as well as after 4 Gy ex-vivo exposure (Fig. 2B). Together these data suggest that X-ray exposure induces direct alterations in lung epithelial proliferative capacity, resulting in dose-dependent decrease in colony forming efficiency.
Figure 1. Dose-dependent decrease in lung epithelial colony formation following whole body X-ray irradiation.

(A) Isolated and fractionated primary epithelial cells from X-ray irradiated U-RFP and unirradiated U-GFP mice were co-cultured with unirradiated mouse lung fibroblasts for 14 days. (B) Colonies were imaged and quantitated and the colony forming efficiency calculated as the percentage (± SEM) of seeded cells that give rise to colonies after 14 days in culture. Graph was plotted as the percent of colony forming efficiency normalized to the control. Significance to unirradiated controls indicated by: * (p ≤ 0.05), ** (p ≤ 0.001), *** (p ≤ 0.0001).
Figure 2. Dose-dependent decrease in lung epithelial colony formation following ex vivo X-ray irradiation.

(A) Isolated and fractionated primary epithelial cells were irradiated with X-rays ex vivo. (B) Colony forming efficiency was calculated as the percentage (± SEM) of seeded cells that give rise to colonies after 14 days in culture. Graph was plotted as the percent of colony forming efficiency normalized to the control. Significance to unirradiated controls indicated by: * (p ≤ 0.05), *** (p ≤ 0.001), **** (p ≤ 0.0001).
2.3.2 Clonal expansion of epithelial progenitor cells in airways of mice following exposure to low-LET radiation

To understand the effect of low-LET radiation exposure on airway epithelial progenitors in vivo, we used a lineage tracing method to assess changes in the clonal behavior of Scgb1a1-expressing progenitor cells of conducting airways. Prior to whole body X-ray exposure, Scgb1a1-CreERTM; Rosa26R-Confetti mice were given tamoxifen to randomly tag airway progenitor cells with one of four lineage reporters. Reporters introduced into Scgb1a1-expressing cells could be distinguished by native fluorescence and/or subcellular distribution. Their expression is under the control of a ubiquitous promoter, which allowed visualization of progenitor-progeny relationships as a function of time after exposure to ionizing radiation (Fig. 3A). The frequency of large epithelial patches composed of 3 or more touching cells carrying the same lineage tag significantly increased greater than 2-fold at both 30 and 70 days following low-LET radiation exposure (Fig. 3B). Interestingly, only 6.12% of lineage traced club cells undergo increased patch expansion post-IR, indicating that only a small proportion of progenitors are contributing to epithelial maintenance and regeneration after low-levels of radiation-induced progenitor cell depletion.

We next sought to determine whether clonal expansion of epithelial progenitor cells resulted from increased radiation-induced turnover of the epithelium. We measured epithelial cell proliferative responses to X-ray exposure as a surrogate to
follow epithelial turnover. Immunofluorescent detection of nuclear 5-iodo-2-deoxyuridine (IdU) was used to determine the cumulative labeling index after 7 or 30 days of continuous IdU labeling in either control mice or mice exposed to 8 Gy whole body X-rays (Fig. 4A). Cumulative IdU labeling indices were not different between control and X-ray exposed mice at either time evaluated (Fig. 4B). IdU labeling indices were 9.7% and 8.3% at the 7 day time point and 21.2% and 21.7% at the 30 day time point, for control and X-ray exposed mice, respectively. However, contiguous patches of IdU-labeled epithelial cells were more frequently observed in X-ray exposed mice and were correlated with the clonogenic patches (data not shown). These data suggest that X-ray exposure does not increase the rate of epithelial turnover. Together with lineage tracing data, our results suggest that radiation reduces the pool of epithelial progenitor cells available for proliferation, leading to increased cycling of the remaining progenitors to maintain the epithelium.
Figure 3. X-ray irradiation induces clonal expansion of club progenitor cells.

(A) Schematic describing experimental design and immunofluorescence staining to visualize 4 distinct fluorescent protein reporters. Tamoxifen treated $Scgb1a1$-CreER$^{TM}$; $Rosa26R$-Confetti mice recombine to induce one of four distinct fluorescent markers that differ in their subcellular distribution (nuclear GFP, cytoplasmic YFP, cytoplasmic RFP, membrane CFP) in $Scgb1a1$-expressing club cells. RFP lineage tagged airway epithelial cells were assessed by immunofluorescence for RFP; lineage tags of GFP variants (GFP, YFP, CFP) within club progenitor cells were assessed by immunofluorescence of GFP and distinguished by subcellular localization. (B) Patches of lineage tags assessed by immunofluorescence in mice exposed to 0 or 8 Gy X-rays and recovered for 30 or 70 days (RFP not shown, arrows indicate tagged cells, asterisks indicate patches). Quantitation of patches (3+ cells that share the same lineage tag) from unirradiated and X-ray irradiated mice. Data is presented as the relative frequency (± SEM) of patches (3+) per all lineage tagged club cells. Significance to unirradiated controls indicated by: * (p ≤ 0.05), ** (p ≤ 0.01).
Figure 4. Proliferative kinetics of airway epithelium following X-ray irradiation.

(A) Proliferative index of the airway epithelium post-IR was assessed at 7 and 30 days using continuous IdU labeling. (B) Proliferative kinetics data are expressed as the percent (± SEM) IdU+ airway epithelial cells per total DAPI+ airway epithelium. No significant changes were found.
2.3.3 Epithelial colony-forming ability and expansion of progenitor cells in airways of mice following exposure to high-LET radiation

High-charge and energy (HZE) nuclei, such as $^{56}$Fe, are high-LET radiation and a potentially highly damaging component of space radiation. Since evolutionary pressure may have led to protection of progenitor cell pools against terrestrial sources of low-LET radiation, no such selective pressure exists to develop defenses against high-LET radiation. To determine the effects of high-LET radiation on airway epithelial progenitor cells, we used whole body exposure to 600 MeV/nucleon $^{56}$Fe ionizing radiation in conjunction with lineage tracing and either in vitro or in vivo analysis of progenitor cell behavior. $Scgb1a1$-expressing cells were lineage tagged, isolated following whole body exposure to high-LET radiation, placed in co-culture with unirradiated mouse lung fibroblasts, and assessed for colony forming ability at 14 days (Fig. 5A). No significant decrease in colony forming ability occurred following exposure to low doses of high-LET radiation; however, after exposure to 2.5 Gy $^{56}$Fe, we observed a significant decrease of 79% in the ability of $Scgb1a1$ lineage-labeled progenitor cells to form colonies (Fig. 5B). Compared to results from X-ray exposure (Fig. 1B), we show that, when exposed to the same dose, the airway progenitor cells exposed to high-LET radiation had a lower colony forming efficiency, with a relative biological effectiveness of approximately 2.

We next evaluated the effect of high-LET exposure on airway epithelial progenitor cells in vivo. $Scgb1a1$-$CreER^{TM}$; Rosa26R-Confetti mice were given tamoxifen to
lineage label airway progenitor cells and exposed to 0 or 2.5 Gy $^{56}$Fe (Fig. 6A). No significant differences in lineage patch size distribution were observed between control mice and mice exposed to whole-body 0.5 Gy $^{56}$Fe (data not shown). However, after whole body irradiation with 2.5 Gy $^{56}$Fe, we observed a significant increase in the abundance of large lineage-labeled patches indicative of increased clonal expansion of airway progenitor cells (Fig. 6B). Taken together, airway epithelial cells showed greater sensitivity when exposed to high-LET radiation, resulting in decreased colony forming ability. However, after in vivo high-LET radiation exposure, the remaining pool of proliferation-competent epithelial progenitors was able to divide and maintain the epithelium.
Figure 5. Dose dependent changes in lung epithelial colony formation following 600 MeV/nucleon $^{56}$Fe irradiation.

(A) Primary GFP lineage tagged club epithelial cells were isolated from unirradiated mice or mice exposed to 600 MeV/nucleon $^{56}$Fe (0, 0.2, 0.5, 1.0, and 2.5 Gy) mice (Scgb1a1-Cre; Rosa26-mT/mG), fractionated, and co-cultured with mouse lung fibroblasts for 14 days. (B) Colonies were quantitated and the colony forming efficiency calculated as the percentage (± SEM) of seeded cells that give rise to colonies after 14 days in culture. Graph was plotted as the percent of colony forming efficiency normalized to the control. Significance to unirradiated controls indicated by: * (p ≤ 0.05).
Figure 6. $^{56}$Fe irradiation induces clonal expansion of club progenitor cells.

(A) Patches of GFP lineage tags (cytoplasmic RFP, nuclear GFP, cytoplasmic YFP, membrane CFP) assessed by immunofluorescence of GFP in formalin-fixed lung tissue from tamoxifen treated $Scgb1a1$-CreERTM; $Rosa26R$-Confetti mice exposed to 2.5 Gy 600 MeV/nucleon $^{56}$Fe and recovered for 70 days. (B) Quantitation of lung epithelial patches (1-2 or 3+ cells that share the same lineage tag) from unirradiated and $^{56}$Fe irradiated mice. Arrows indicate tagged cells and asterisks indicate patches. Data is presented as the relative frequency (± SEM) of patches (1-2 vs 3+) per all lineage tagged airway epithelial cells. Significance to unirradiated controls indicated by: *** ($p \leq 0.001$).
2.4 Discussion

In the present study we used an *in vitro* epithelial-stromal co-culture assay in combination with *in vivo* lineage tracing to better understand the effects of radiation on progenitor cells that maintain the airway epithelium. Whole body exposure to low-LET radiation resulted in a dose-dependent decrease in *in vitro* colony forming efficiency of airway progenitors removed from lung tissue 18 hours after radiation injury. Following *ex vivo* exposure of isolated lung epithelial progenitor cells to low-LET IR, a similar dose-dependent decrease in *in vitro* colony forming efficiency was observed. These data suggest that radiation-induced decreases in epithelial *in vitro* colony forming ability reflect the direct effects of radiation on progenitors without a significant contribution made by either systemic or local non-target effects. In accordance with the results using low-LET radiation, exposure to high-LET radiation resulted in a significant decrease in colony forming ability after 2.5 Gy $^{56}$Fe compared to unexposed controls, without significant effects at 0.5 Gy. When comparing results from low- and high-LET exposure we calculate a relative biological effectiveness of approximately 2 for $^{56}$Fe particles compared to X-rays. In contrast to the radiation-induced decrease in colony forming ability *in vitro*, a significant increase in clonal expansion of progenitor cells was observed *in vivo* following exposure to either low- or high-LET radiation. Taken together, these findings suggest that radiation exposure results in a dose-dependent reduction of
proliferation-competent progenitor cells leading to clonal expansion of remaining progenitors \textit{in vivo}.

We have previously shown that ablation of mouse airway progenitor cells through parenteral exposure to naphthalene is repaired through expansion of chemically resistant progenitors that localize to neuroepithelial bodies (NEB) and bronchoalveolar duct junctions (BADJ) of terminal bronchioles [39,40]. Chemical resistance of NEB-associated progenitors results from the differentiation-modulating properties of the local microenvironment [40]. However, it is not clear what mechanisms lead to preservation of progenitor cells that survive after exposure to radiation. Epithelial progenitor cells undergoing clonal expansion following exposure to radiation were not associated with NEB and BADJ microenvironments that harbor naphthalene-resistant airway progenitors. Furthermore, whereas resistance of progenitor cells to naphthalene-induced airway injury has been associated with reduced expression of genes involved in bioactivation of toxicants [42], it is not clear whether progenitor cells that retain proliferative capacity following radiation necessarily represent a resistant population versus progenitors that survive the damaging effects of radiation by chance. Further studies are needed to explore mechanisms by which epithelial progenitor cells of airways retain their proliferative capacity following exposure to radiation.
A surprising finding from our study was that the doses of radiation exposure that were sufficient to decrease lung progenitor in vitro colony formation was not sufficient to increase the normally slow rate of epithelial turnover in airways, as indicated by cumulative IdU labeling. Nevertheless, this radiation exposure was sufficient to increase progenitor cell clonal expansion \textit{in vivo}. Epithelial cell damage and lung injury resulting from exposure to inhaled or systemically delivered toxicants typically results in the activation of repair responses including proliferation of surviving epithelial progenitor cells. This is evident in models of progenitor cell depletion such as naphthalene-induced lung injury, which results in the clonal expansion of naphthalene-resistant progenitors to replenish depleted epithelium [39,40]. However, whole-body exposure of mice to doses of X-rays up to 8 Gy and \textsuperscript{56}Fe radiation up to 2.5 Gy resulted in no apparent loss of epithelial integrity. Exposure to these doses of radiation appeared to cause some epithelial progenitors to lose their ability to proliferate, as was made evident by the decreased colony-forming ability \textit{in vitro}. This interpretation is also consistent with the observation of clonal expansion of some epithelial progenitor cells into patches \textit{in vivo} in the absence of an associated increase in the overall epithelial proliferative index.

Taken together, our results suggest that, within the dose range we studied, radiation to the lung inhibits proliferation of some epithelial progenitor cells, which
places an additional proliferative demand on the pool of progenitors that remain
competent for proliferation after radiation for epithelial maintenance (Fig. 7). Together
our data suggest that airway progenitor cells are functionally compromised following
radiation exposure up to 8 Gy of X-rays or 2.5 Gy of $^{56}$Fe, but are retained within the
epithelium rather than being eliminated by cell death pathways. Other changes to the
function of radiation-damaged epithelial cells, such as acquiring a senescent phenotype,
remain to be determined. Our data contrast those observed in frequently cycling
progenitors of rapidly replacing epithelia, such as in the gut [75,100,103], and suggest
that radiation-induced epithelial damage has the potential to accumulate in lung tissue.
These findings have potentially important implications for lung homeostasis and repair
among individuals receiving long-term low dose radiation or higher dose radiotherapy.
By impairing the ability of some progenitor cells to divide with the resulting clonal
expansion of remaining progenitors, radiation has the potential to impair normal repair
and promote aberrant tissue remodeling.
Following exposure to ionizing radiation, a dose-dependent decrease in the pool of available progenitor cells occurs (indicated by grey club cells). Therefore, as the lung epithelium undergoes normal turnover, fewer progenitors have the capacity to divide, so that the progenitors that retain proliferate capacity must divide more frequently to maintain the airway epithelium, giving rise to patches. This proliferative response following exposure to radiation could contribute to carcinogenesis.
2.5 Acknowledgements

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3. Distal airway epithelial progenitor cells are radiosensitive to high-LET radiation.

3.1 Introduction

Humans are exposed to radiation during circumstances such as medical diagnostic or therapeutic treatment, high altitude or space travel, and radiological warfare or accidents. In each of these situations, the extent of damage to normal tissues varies according to radiation dose and quality. Radiation quality can be categorized according to linear energy transfer (LET), or the amount of energy deposited as a particle traverses the tissue [57]. X-rays and γ-rays, types of low-LET radiation, deposit energy in a diffuse manner, whereas heavy ions, types of high-LET radiation, deposit energy along more concentrated tracks [56]. Due to the differences in energy deposition and subsequent DNA damage, heavy ions, such as $^{12}$C, are increasingly being utilized in radiation therapy treatment [57,58]. Additionally, during space travel, astronauts can be exposed to galactic cosmic rays, which contain high charge and energy (HZE) ions including $^{56}$Fe and $^{28}$Si [59]. A greater understanding of the extent of damage inflicted on normal tissue following high-LET radiation exposure and how this compares to low-LET radiation exposure is important for further development of heavy ions for therapy and for risk assessment of normal tissue damage during radiotherapy or deep space travel.
The carcinogenic effects of ionizing radiation exposure are well established [59,105]. Recent literature has shown that tumors frequently arise from resident tissue progenitor cells [96,97]. However, the relationship between progenitor cell injury by radiation and cancer development is unknown. Progenitor cell sensitivity and response to radiation exposure has been studied in a number of organs, including the epidermis, mammary gland, intestine, and hematopoietic system, and is largely tissue-specific [65,66,73,76,77,106]. Yet, less is known about the effects of radiation on progenitor cells in the pulmonary system. Club cells, previously known as Clara cells, specifically express the protein Scgb1a1 and function as regional progenitors that maintain the distal conducting airway of the murine lung [101]. We previously reported that immediately following whole-body radiation exposure, these club progenitor cells exhibit a dose-dependent decrease in \textit{in vitro} colony forming ability, but that a subset of these cells undergo radiation-induced clonal expansion without an increase in the overall rate of epithelial cell proliferation \textit{in vivo} [104]. However, in this study we were unable to resolve differences arising from radiation quality in low- and high-LET radiation. Additionally, we did not identify a mechanism by which radiation depletes the pool of progenitor cells in the lung and leads to clonal expansion.

Here we show that radiation exposure injures airway epithelial club progenitor cells in a region-specific and quality-dependent manner. High- but not low-LET
exposure resulted in the persistent loss of colony-forming progenitors. Using a novel whole mount imaging and quantification technique, we reveal that high-LET radiation exposure causes substantial progenitor cell expansion in the distal, but not proximal airways. This expansion is induced by apoptosis, senescence, and defects in mitotic progression among neighboring progenitor cells that are regulated in a p53-dependent manner. Together, these data indicate that high-LET, but not low-LET radiation leads to prolonged impairment of distal airway epithelial progenitor cells, leaving fewer progenitors to maintain the airway during homeostasis.

3.2 Materials and Methods

3.2.1 Mice

The Scgb1a1-CreER; Rosa26R-Confetti mice were established by crossing Scgb1a1-CreER™ mice (kindly provided by Brigid L.M. Hogan, Duke University) with Rosa26R-Confetti mice (JAX stock number 017492) as previously reported [8]. These mice were crossed to p53<sup>flox</sup> (JAX stock number 008462) and p53<sup>−/−</sup> (JAX stock number 002101) mice to generate Scgb1a1-CreER; Rosa26R-Confetti,p53<sup>flox−/−</sup> mice. Confetti mice were injected i.p. 3 times every other day with 200mg/kg body weight tamoxifen in corn oil to randomly introduce one of four genetic tags into the Scgb1a1-expressing progenitor cells and recombine the p53 allele to generate progenitor cells deficient for p53. In vitro experiments used mice heterozygous for Rosa26R-<i>mTmG</i> (JAX stock number 007576). All
mice were maintained and treatments were carried out according to IACUC approved protocols.

3.2.2 Radiation exposure

Mice, eight to twelve weeks old, were either exposed to γ-rays, $^{56}$Fe, or $^{28}$Si radiation. For low-LET irradiation, unanesthetized mice were exposed whole body to 1 or 5 Gy of γ-rays (Gammacell 40 Exactor, dose rate 1 Gy/min). For high-LET irradiation, mice were exposed whole body to 0.2, 0.5, 1, or 2.5 Gy of 600 MeV/nucleon $^{56}$Fe ions or 300 MeV/nucleon $^{28}$Si ions (NASA Space Research Laboratory’s linear accelerator at Brookhaven National Laboratory, dose rate 0.2 Gy/min).

3.2.3 In vitro cultures

Airway epithelial cell isolation and flow cytometry was performed as previously described by Farin et al [104]. Briefly, 5,000 sorted EpCAM⁺,CD31/34/45⁻,7AAD⁻ epithelial cells were mixed with 100,000 unirradiated immortalized MLg (ATCC) mouse fibroblasts. The mixture was added to an equal volume of growth factor reduced Matrigel (BD Biosciences) and seeded to the apical surface of 24-well transwell filter inserts (BD Biosciences) placed in 24-well flat-bottom culture plates. The solution was allowed to polymerize for 30 min at 37°C, then basic medium was added to the basal compartment of the well. Cell cultures were maintained for 14 days at 37°C in a humidified incubator (5% CO₂). Colony-forming efficiency was calculated as the
percentage of seeded cells that give rise to colonies, imaged on a Zeiss Axiovert 40 fluorescent microscope and quantitated using FIJI. Enrichment for CD24<sup>med</sup>, Sca-1<sup>-</sup> distal and CD24<sup>med</sup>, Sca-1<sup>+</sup> proximal airway epithelial cells was performed as previous described [8].

### 3.2.4 Whole mount imaging and quantification

All images were taken using a Zeiss 780 confocal microscope. For patch expansion experiments, accessory lobes from at least 3 independent animals were treated with Scale to clarify tissue [107]. The lobes were microdissected to expose the airways and the native fluorescent proteins were imaged. Patch size was quantified using Imaris (Bitplane), with the number of contiguous cells defining a patch.

### 3.2.5 Immunofluorescence staining and quantification

Five-micrometer sections were collected from lung tissue fixed with 10% NBF. De-waxing, antigen retrieval, and blocking was performed and the sections were incubated with primary antibodies at 4°C overnight. The following antibodies were used: Mouse IgG1 anti-phospho-Histone H2AX (Ser139) (1:500, Milipore), Rabbit anti-CCSP (1:10,000, in house), Mouse IgG2a anti-E-Cadherin (1:1000, BD Biosciences), Rabbit anti-Cleaved Caspase-3 (Asp175) (1:500, Cell Signaling Technology). Sections were washed with PBS and incubated with fluorochrome-conjugated secondary antibody and DAPI for 30 minutes at room temperature. Sections were washed again and mounted in
Fluormount G. Sections of tissue from 3-5 independent animals were imaged and between 500 and 1000 cells per animal was quantitated using FIJI. For proximal and distal analysis, distal airways were categorized as 100 cells from a bronchioalveolar duct junction and proximal airways were categorized as the cells along a primary airway branch.

3.2.6 Statistical analysis

Data were analyzed and compared between groups using a one-way ANOVA or two-way ANOVA with post-hoc analysis (Prism, GraphPad). p < 0.05 was considered statistically significant and is presented as *p < 0.05, **p < 0.01, ***p < 0.001 or ****p < 0.0001.

3.3 Results

3.3.1 Airway progenitors exposed to high-LET radiation have a prolonged decrease in colony forming ability

To determine the long-term effect of radiation exposure on club progenitor cells, we used an in vitro colony-forming assay to measure their clonogenic capacity. Mice that ubiquitously expressed a membrane localized RFP were irradiated with either 5 Gy γ-rays, 2.5 Gy 600 MeV/nucleon $^{56}$Fe, or 2.5 Gy 300 MeV/nucleon $^{28}$Si. Fluorescent lung epithelial cells were isolated at various times post-radiation exposure and were plated in a 3D co-culture system containing unirradiated non-fluorescent fibroblasts. Whole-body exposure to ionizing radiation led to an acute loss of progenitor cell colony-forming
ability at 1 day post-exposure regardless of radiation quality. However, the magnitude of this decline differed between radiation qualities. Exposure to 2.5 Gy $^{28}$Si resulted in the greatest decrease in colony-forming ability (15% of control), followed by 2.5 Gy $^{56}$Fe (20% of control), with 5 Gy $\gamma$-rays showing the least reduction (25% of control), to yield an relative biological effectiveness (RBE) of about 2 between $\gamma$-rays and $^{56}$Fe and approximately 4 between $\gamma$-rays and $^{28}$Si (Fig. 8e and 9). After exposure to 5 Gy $\gamma$-rays the initial decline in epithelial colony-forming ability recovered and was not different from that of un-irradiated controls when isolated 70 days after exposure (Fig. 8a-b and 8e). However, the colony forming ability of progenitors from mice exposed to high-LET radiation, either $^{56}$Fe or $^{28}$Si, remained significantly decreased compared to control at the 70 day recovery time point (Fig. 8c-e). Taken together, these results suggest that exposure to high-, but not low-LET radiation leads to prolonged defects in the ability of club progenitor cells to proliferate and contribute to maintenance of the airway epithelium.
Figure 8. Epithelial progenitor cells of mice exposed to low- but not high-LET radiation recover colony forming ability over time.

Mice were exposed to 2.5, or 5 Gy high- or low-LET radiation respectively and epithelial cells were isolated at 1, 7, 14, 30, or 70 days post-radiation exposure. (a-d) Fluorescent images of 3D colonies grown from RFP+ epithelial cells of mice that were irradiated 70 days prior to isolation. (e) Colony forming efficiency of epithelial cells isolated from mice at various time points following radiation exposure. Colony forming efficiency is expressed as a percent of the unirradiated control. Significance of differences relative to unirradiated control is indicated by: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 9. $^{28}$Si ions reduce colony forming ability more than $^{56}$Fe ions.

Mice were exposed to 0, 0.5, 1, or 2.5 Gy $^{56}$Fe or $^{28}$Si ions and epithelial cells were isolated 1 day post-radiation exposure. Colony forming efficiency of epithelial cells isolated from mice at various time points following radiation exposure. Colony forming efficiency is expressed as a percent of the unirradiated control. Significant differences between radiation type is indicated by: *$p < 0.05$; **$p < 0.01$. 

![Graph showing colony forming efficiency after radiation exposure ranging from 0 to 2.5 Gy for $^{56}$Fe and $^{28}$Si ions.](image-url)
3.3.2 Low-LET radiation induces moderate airway progenitor expansion in vivo

Given our findings that radiation induces quality-dependent differences in club progenitor cell behavior in vitro, we hypothesized that quality-dependent differences in progenitor cell dynamics existed in vivo as well. To assess progenitor cell dynamics, we developed a sensitive whole mount imaging system to detect clonal expansion. *Scgb1a1-CreER*; *Rosa26R-Confetti* mice were exposed to tamoxifen one week before radiation exposure. This induced recombination and stable expression of one of four fluorescent proteins (cytoplasmic RFP, nuclear GFP, cytoplasmic YFP, or membrane CFP) specifically in club progenitor cells. The fluorescent proteins genetically tag individual cells and their descendants. Therefore, progenitor cell expansion over time leads to the formation of a patch of daughter cells carrying a common fluorescent color tag (Fig. 10a).

To test the effect of low-LET radiation exposure on progenitor cell dynamics, *Scgb1a1-CreER*; *Rosa26R-Confetti* mice were treated with tamoxifen and exposed to 1 or 5 Gy γ-rays. 70 days following radiation, moderate patch expansion was observed (Fig. 10b and 10c). Exposure to both 1 and 5 Gy γ-rays resulted in a significant increase in the number of cells per patch (Fig. 10e). Exposure to 5 Gy γ-rays resulted in a significant increase in the proportion of medium patches, containing 6-10 cells per patch (Fig. 10d). This indicates that exposure to low-LET radiation causes a subpopulation of club progenitor
cells to undergo successive rounds of cell division and clonal expansion, as reflected by patch formation.
Figure 10. Low-LET radiation induces moderate patch expansion.

5cgb1a1-CreER; Rosa26R-Confetti mice were exposed to 0, 1, or 5 Gy γ-rays and lungs were harvested after 70 days. (a-c) Tiled image of the native YFP fluorescence (white) in a whole mount, microdissected, Scale treated accessory lobe 70 days post-γ radiation exposure. Red arrows indicate medium or large patches. Scale bar represents 500 µm.

(a’-c’) Whole mount 4-color fluorescence images of native confetti fluorescence (cytoplasmic RFP, nuclear GFP, cytoplasmic YPF, and membrane CFP) 70 days post-γ radiation exposure corresponding to the tiled image above. Scale bar represents 50 µm.

(d) Relative frequency of YFP patches containing various numbers of cells 70 days post-γ-ray exposure. (e) Number of YFP cells per patch 70 days post-γ-ray exposure.

Significance is indicated by: *p < 0.05; **p < 0.01, ****p < 0.0001.
3.3.3 High-LET radiation is a more potent inducer of *in vivo* airway progenitor expansion

The impact of radiation quality on club progenitor cell expansion was determined by exposure of *Scgb1a1-CreER; Rosa26R-Confetti* mice to high-LET radiation. We previously reported a relative biological effectiveness of 2 between X-rays and $^{56}$Fe ions [104]. Therefore, one week after tamoxifen treatment we exposed the *Scgb1a1-CreER; Rosa26R-Confetti* mice to 2.5 Gy of high-LET radiation to induce comparable lung injury as mice exposed to low-LET radiation. Exposure to 2.5 Gy of either $^{56}$Fe or $^{28}$Si ions resulted in a significant increase in both the proportion of medium and large patches, as well as the total patch size compared to unirradiated controls (Fig. 11a-e). Interestingly, $^{28}$Si ion exposure induced larger and more numerous patches than $^{56}$Fe ion exposure (Fig. 11d and 11e). To test the effect of low dose high-LET radiation exposure on progenitor cell expansion, mice were exposed to 0.2 Gy of $^{56}$Fe or $^{28}$Si ions. Although this low dose exposure increased moderate patch expansion, this difference did not reach statistical significance (Fig. 12). Together these data suggest that high-LET radiation induces greater clonal expansion than low-LET radiation and that this differences was further impacted by ion type.
Figure 11. High-LET radiation induces dramatically potentiates expansion of subsets of lineage-labeled airway progenitor cells.

Scgb1a1-CreER; Rosa26R-Confetti mice were exposed to 0 or 2.5 Gy $^{56}$Fe or $^{28}$Si ions and lungs were harvested 70 days post-radiation exposure. (a-c) Tiled image of the native YFP fluorescence (white) in a whole mount, microdissected, Scale treated accessory lobe 70 days post-$^{56}$Fe or $^{28}$Si radiation exposure. Red arrows indicate medium or large patches. Scale bar represents 500 µm. (a’-c’) Whole mount 4-color fluorescence images of native confetti fluorescence (cytoplasmic RFP, nuclear GFP, cytoplasmic YFP, and membrane CFP) 70 days post-$^{56}$Fe or $^{28}$Si radiation exposure corresponding to the tiled image above. Scale bar represents 50 µm. (d) Relative frequency of YFP patches containing various numbers of cells 70 days post-$^{56}$Fe or $^{28}$Si exposure. (e) Number of YFP cells per patch 70 days post-$^{56}$Fe or $^{28}$Si exposure. Significance of differences between groups is indicated by: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 12. No significant increase in patch expansion after low dose high-LET radiation exposure.

Scgb1a1-CreER; Rosa26R-Confetti mice were exposed to 0 or 0.2 Gy $^{56}$Fe or $^{28}$Si ions and tissue was collected 70 days post-radiation exposure. Relative frequency of YFP patches containing various numbers of cells 70 days post-$^{56}$Fe or $^{28}$Si exposure.
3.3.4 Distal airway progenitors show enhanced sensitivity to apoptosis following high-LET radiation compared to proximal airway progenitors

An unexpected finding from our analysis was that patch size was not uniformly impacted as a function of airway location; larger patches generated following radiation exposure were located predominately in terminal bronchioles of the distal conducting airway. We quantitated the location of large patches post-high-LET radiation exposure and found that a significant increase in the percent of large patches occurred only in the terminal bronchioles (Fig. 13a). These data suggest that clonal expansion occurs preferentially in the distal airways, indicating increased radiosensitivity in this region. We hypothesized that either progenitor cell apoptosis or senescence was driving clonal expansion and that these responses would vary by region. To determine if apoptosis was occurring post-radiation, we exposed mice to either 2.5 Gy $^{56}$Fe or 5 Gy $\gamma$-rays and quantified the number of airway epithelial cells positive for cleaved-caspase 3 at 1 day post-radiation. No significant increase in cleaved-caspase 3 positive cells was observed along the entire airway epithelium (Fig. 14). However, when cleaved-caspase 3 positive cells were evaluated according to airway location, the distal terminal bronchioles contained significantly more cleaved-caspase 3 positive cells than proximal airways (Fig. 13b).
3.3.5 High-LET radiation induces persistent DNA damage in distal airway epithelial progenitor cells

To evaluate DNA damage repair, we quantified the percent of airway epithelial cells containing γ-H2AX foci at 1, 30, and 70 days post-radiation (Fig. 13c). As expected, the abundance of γ-H2AX foci increased acutely following radiation exposure and decreased over time, remaining elevated compared to baseline controls (Fig. 15). Interestingly, there was no difference in the percent of persistent γ-H2AX foci in all epithelial cells in the airways between low- or high-LET exposures. We hypothesized that the differences in clonal expansion between radiation qualities may be due to altered repair rates of γ-H2AX foci specifically in progenitor cells. To assess this, we evaluated the proportion of club progenitor cells within γ-H2AX foci-containing cells. γ-H2AX positive club cells in mice exposed to γ-rays had an accelerated recovery rate compared to those in mice exposed to ⁵⁶Fe (Fig. 13d). To see if club progenitor cells containing persistent γ-H2AX foci following high-LET radiation resided in a particular airway region, we evaluated γ-H2AX positive club cells according to airway location. Club cells in the terminal bronchioles contained significantly more persistent γ-H2AX foci following high-LET radiation exposure (Fig. 13e). We next sought to determine if this response was specific to progenitor cells or if it could be observed in differentiated cells as well. To assess this, we quantified the number of persistent γ-H2AX foci following high-LET radiation exposure in post-mitotic ciliated cells. Although a greater
proportion of ciliated cells contained γ-H2AX foci compared to club cells, there were no differences between airway locations (Fig. 16). This suggests that club progenitor cells in the distal airways of mice have persistent DNA damage and may either undergo senescence or a prolonged cell cycle arrest following high-LET radiation exposure.

3.3.6 Distal airway epithelial progenitor cells have defects in mitotic progression

To determine if progenitor cells that repaired their DNA damage could successfully divide, we evaluated the number of cells in the airway in mitotic arrest, as indicated by binucleated cells. The number of binucleated airway epithelial cells in vivo progressively increased over time following high-LET radiation, suggesting defects in mitotic progression (Fig. 13f). We categorized these binucleated cells by airway location and found that they also predominately resided in the terminal bronchioles of the distal airway (Fig. 13g). To evaluate if radiation exposure resulted in functional impairment of distal progenitor cells, we used a previously described sorting strategy to enrich for proximal and distal lung progenitor cells [8]. Sca-1^CD24^{med}CD326^+ distally enriched cells had a significant decrease in colony forming ability 1 day after low-LET radiation exposure when compared to Sca-1^CD24^{med}CD326^+ proximally enriched cells (Fig. 17). This suggests that distal lung epithelial progenitors are more radiosensitive than their proximal airway counterparts. Together, these data show that following high-LET radiation exposure, club progenitor cells in the distal airways undergo apoptosis,
senescence, and mitotic defects, which reduces the pool of progenitor cells, leading to clonal expansion.
Figure 13. Distal airway progenitor cells undergo apoptosis, senescence, and defects in mitosis following high-LET radiation exposure.

(a) Patches containing more than 11 cells were categorized by airway location. (b) The percent of cleaved-caspase 3 positive cells in the distal and proximal airway epithelium was quantitated. (c) Immunostaining for γ-H2AX in green, CCSP (Scgb1a1) in red, E-Cadherin in white, and DAPI in blue at 70 days post-radiation. Fluorescent channels are overlaid with a DIC image to show cilia. Scale bar represents 10 µm. (d) Percent of cells containing γ-H2AX foci that are club cells, as marked by CCSP (Scgb1a1), at various time points post-radiation exposure. (e) Percent of club cells containing γ-H2AX foci in the distal and proximal airway epithelium was quantitated. (f) Percent of binucleated cells, visualized using DAPI, in the airway epithelium. Solid line marks the percent of binucleated cells in unirradiated control. (g) Percent of binucleated cells in the distal and proximal airway epithelium was quantitated. Significant differences between radiation type or location indicated by: *p < 0.05, ***p < 0.001, ****p < 0.0001.
Figure 14. No significant increase in apoptosis is observed in the airway epithelium following radiation exposure.

Mice were exposed to 2.5, or 5 Gy high- or low-LET radiation respectively and tissue was collected 1 day post-radiation exposure. ns = not significant.
Figure 15. No significant difference in DNA damage following low- or high-LET radiation exposure.

Mice were exposed to 2.5, or 5 Gy high- or low-LET radiation and sacrificed at 1, 30, or 70 days. No difference in the number of cells containing γ-H2AX foci was observed. Graphed as a percent of control, as indicated by the solid line.
Figure 16. No significant difference in persistent DNA damage in ciliated cells between airway locations.

Mice were exposed to 2.5 Gy $^{56}$Fe and sacrificed at 70 days. No difference in the number of ciliated cells with persistent $\gamma$-H2AX foci residing in proximal or distal airways was observed. ns = not significant.
Figure 17. Distally enriched progenitors are more radiosensitive than proximally enriched progenitors.

Mice were exposed to various doses of low-LET radiation and CD24^med, Sca-1^ proximally enriched cells or CD24^med, Sca-1^- distally enriched cells were isolated 1 day post-radiation exposure. Colony forming efficiency was evaluated at day 14 in vitro. Colony forming efficiency is expressed as a percent of the unirradiated control. Significance to unirradiated control is indicated by: *p < 0.05, ****p < 0.0001.
3.3.7 Patch expansion post-high-LET radiation is p53-dependent

We hypothesized that enhanced clonal expansion in subsets of club cells following exposure to high-LET ionizing radiation was the result of chronic progenitor cell deficiency. Therefore, we sought to determine whether p53, a key regulator of cell fate post-DNA damage, played role in mediating these effects. To determine if radiation-induced senescence and mitotic defects in club cells were indeed driving patch formation, we developed a conditional p53 loss-of-function mouse model. Scgb1a1-CreER; Rosa26R-Confetti,p53flox/- mice were treated with tamoxifen to yield club cells that are deficient in p53 (p53Δ/-) and lineage traced by expression of one of the four fluorescent proteins from the recombined Rosa26-Confetti allele (Fig. 18a). In contrast to radiation-induced patch expansion observed in p53-sufficient mice, we observed no evidence for patch expansion 70 days post-2.5 Gy ⁵⁶Fe exposure of mice with conditional p53 loss-of-function (Fig. 18b-c and 19). We next assessed if p53 loss rescued the persistent DNA damage and binucleated cell phenotypes observed post-high-LET radiation exposure. Contrary to p53 sufficient samples, we found no significant change in the number of γ-H2AX foci in the airway epithelium of p53Δ/- mice at 70 days following ⁵⁶Fe exposure (Fig. 18d). Additionally, the number of binucleated cells did not change in p53 deficient mice 70 days after exposure to ⁵⁶Fe ions (Fig. 18e). These results
suggest that high-LET radiation induces alterations in progenitor function that are p53-dependent.
Figure 18. Patch expansion following high-LET radiation is p53-dependent.

*Scgb1a1-CreER; Rosa26R-Confetti,p53<sup>−/−</sup>* mice were exposed to 0 or 2.5 Gy <sup>56</sup>Fe ions and tissue was collected 70 days post-radiation exposure. (a and b) Tiled image of the native YFP fluorescence (white) in a whole mount, microdissected, Scale treated accessory lobe 70 days post-<sup>56</sup>Fe radiation exposure. Scale bar represents 500 µm. (a’ and b’) Whole mount 4-color fluorescence images of native confetti fluorescence (cytoplasmic RFP, nuclear GFP, cytoplasmic YPF, and membrane CFP) 70 days post-<sup>56</sup>Fe radiation exposure corresponding to the tiled image above. Scale bar represents 50 µm. (c) Relative frequency of YFP patches containing various numbers of cells 70 days post-<sup>56</sup>Fe exposure. (d) Percent of airway epithelial cells containing at least one H2AX foci at 70 days post-<sup>56</sup>Fe exposure. (e) The percent of binucleated cells in the airway epithelium. p53 deficient mice showed differences in steady state compared to wild type, so graphs are reported as a percent of the unirradiated genotypic control. Significance to the genotypic control is indicated by: *p < 0.05; **p < 0.01.
Figure 19. No significant change in patch size occurs in high-LET exposed p53-deficient mice.

*Scgb1a1-CreER; Rosa26R-Confetti,p53Δ* mice were exposed to 0 or 2.5 Gy $^{56}$Fe ions and tissue was collected 70 days post-radiation exposure. Number of YFP cells per patch 70 days post-$^{56}$Fe exposure. ns = not significant.
3.4 Discussion

High-LET radiation exposure can occur during charged particle radiotherapy, radiological accidents, and space travel. However, the risks of exposure to high-LET radiation are largely unknown. Although several studies have evaluated the effect of radiation on progenitor cell behavior using low-LET radiation [65,66,73,77,79,106,108,109], very few studies have compared the effects of high-LET and low-LET radiation on stem cell behavior in any organ system. Here we show that airway epithelial progenitor cell response to radiation is quality-dependent and impacted by ion type. High-LET radiation exposure leads to a prolonged decrease in colony forming ability of club progenitor cells and a more pronounced clonal expansion than low-LET radiation. Additionally, we show that the relative biological effectiveness for injury to the distal airway progenitors between 300 MeV/nucleon $^{28}\text{Si}$ and 600 MeV/nucleon $^{56}\text{Fe}$ is approximately 2. This is consistent with previous studies that show $^{28}\text{Si}$ exposure increases tumor development compared to $^{56}\text{Fe}$ and highlights the importance of investigating radiation response in a quality-dependent manner [110,111]. The finding that high-LET radiation, specifically $^{28}\text{Si}$ induces more damage to progenitor cells than low-LET radiation should be considered when determining risk assessment from radiation exposure in both a therapeutic and occupational setting.
Many lung diseases, including radiation-induced fibrosis and tumorigenesis, involve pathological remodeling of the distal lung. Our data demonstrate that progenitor cells residing in distal airways are more radiosensitive than those in proximal airways, which may contribute to radiation-induced distal lung pathologies. Together our data point to a model in which high-LET radiation activates p53, leading progenitor cells in the distal airway epithelium to undergo apoptosis, senescence, and defects in mitotic progression. This decreases the pool of progenitor cells able to successfully proliferate and forces the remaining proliferative-competent progenitors to clonally expand in order to maintain homeostatic levels of turnover. One intriguing question that remains is the identity of the proliferative-competent cells. Studies in the skin, mammary gland, hematopoietic system, and intestine have revealed that resident progenitor populations have varying levels of radiosensitivity due to differential expression of p53, Bcl-2, and canonical Wnt signaling [65,66,73,77,79,106,108,109]. Further research is needed to determine if the club progenitors undergoing clonal expansion in the distal airway epithelium are inherently radioresistant or represent stochastically determined surviving cells.

Evidence from other organ systems suggests that cell fate decisions following radiation-induced p53 activation are tissue- and cell-type dependent [103,112–114]. Here we show that mice lacking p53 in club cells do not form patches in the airway following
high-LET radiation and do not retain persistent DNA damage or show defects in mitotic progression. This finding is consistent with the role of p53 as a major regulator of cell fate; it can activate DNA repair pathways, cell cycle checkpoints through its transcriptional target p21, and can initiate apoptosis if the DNA damage is irreparable [85–87]. However, previous studies have shown that tissue responses to radiation can occur through p53-dependent and -independent pathways [67,83]. Our results suggest that airway epithelial progenitor cell response to radiation exposure is p53-dependent. Additionally, the finding that p53 loss abrogates patch formation further supports our hypothesis that clonal expansion in the distal airways results from progenitor cells undergoing apoptosis, senescence, and defects in mitotic progression.

Given that progenitor cells are thought to be the predominant cell-of-origin of cancer [96,98], clonal expansion likely describes an early event along the path to radiation-induced carcinogenesis. Previous studies have implicated clonal expansion as an important step in carcinogenesis [115–118]. One hypothesis about the mechanism of how this occurs is that clonal expansion of a progenitor cell carrying a mutation in an oncogene or tumor suppressor would lead to the generation of a field of cells all carrying the same mutation. This field effect might lead to further mutations and eventually oncogenic transformation. However, precisely how this leads to tumor development is still unknown.
In conclusion, this work reveals that distal airway epithelial cells have prolonged functional impairments and altered clonal dynamics following high-LET, but not low-LET radiation exposure. This knowledge deepens our understanding of the extent of normal tissue damage following various qualities of radiation, which has implications for high-LET radiation therapy and for more accurately predicting the risk to patients and astronauts exposed to high energy and charge (HZE) radiation.

3.5 Acknowledgements

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4. p53 regulates progenitor cell quiescence and differentiation in the airway

4.1 Introduction

Epithelial tissues consist of closely packed functional cells that are replaced by resident stem or progenitor populations at rates that vary between tissues. Here, we investigate how these progenitor cells are regulated in a slowly replacing tissue, the airway epithelium of the lung. The mammalian airway epithelium varies in composition between species and according to airway location. Intralobar conducting bronchi and bronchioles of the mouse lung are composed of secretory and ciliated cells. Tight control over the proportions and abundance of these cell types is essential for effective mucociliary clearance of inhaled particulates and microorganisms.

Club cells, previously known as Clara cells, are secretory cells that express the protein Scgb1a1 (also known as CCSP or CC10) [101]. They function as regional progenitors due their ability to self-renew and differentiate into ciliated cells [5]. Club cells have low level of turnover during homeostasis, yet how this quiescence is maintained is still poorly understood [10]. Developmentally important pathways, such as Wnt, BMP, Notch, JAK/STAT, and Hippo/Yap, have been shown to regulate various populations of lung stem and progenitor cells, directly influencing epithelial composition [5]. However, very few studies have identified novel integrators of these
pathways that control the critical balance between progenitor cell renewal and differentiation.

Trp53 (p53) is a tumor suppressor that is one of the most commonly mutated genes in cancer [81]. In addition to its classical functions in regulating cell fate following cellular stress, there is growing evidence that p53 regulates progenitor cells in a variety of developing and adult tissues [90–95]. These studies highlight an essential role for p53 using rapidly dividing models. However, it is not clear what role, if any, p53 plays in regulating progenitor cell behavior in a quiescent tissue, such as the lung.

Here, we show that p53 regulates the composition of postnatal airway epithelium by maintaining quiescence and regulating differentiation of resident progenitor cells during homeostasis. Genetic manipulation of p53 copy number altered both the density and cellular composition of the airway epithelium. Furthermore, p53 regulated multipotency of club progenitors. Finally, using single cell RNA-Seq, we discovered that p53 loss changes the proportions of progenitor cells and alters expression of cell cycle regulators. Together, our findings reveal new roles for p53 in the homeostatic regulation of airway epithelial progenitor cells.
4.2 Materials and Methods

4.2.1 Mouse Strains

In order to test the effect of p53 loss on isolated club cells *in vitro*, we used
Scgb1a1-CreER<sup>Tm</sup>; Rosa26-<i>m</i>T/<i>m</i>G; p53<sup>flox</sup>/<i>flox</i> mice. Tamoxifen treatment of these mice yields
club cells that express GFP and are deficient for p53, due to the recombination of the lox-
P sites flanking exons 2-10 of the p53 allele. The recombination of the p53 allele is
described using the symbol Δ. We also tested the effect of p53 loss on proliferation and
differentiation using the p53<sup>flox</sup>/<i>flox</i> allele. Tamoxifen treated Scgb1a1-CreER<sup>Tm</sup>; Rosa26R-
Confetti; p53<sup>flox</sup>/<i>flox</i> mice were used for these experiments and are described in the text and
figures as p53<sup>Δ/Δ</sup> to indicate the recombination of the p53 allele. However, given that
these mice are heterozygous for p53 in all cells, we could not exclude the possibility that
the proliferation and differentiation effects were due to interactions with surrounding
cells. Therefore, we used the p53<sup>flox/flox</sup> allele to determine the effect of p53 loss only on
club cells. These mice, crossed to either Scgb1a1-CreER<sup>Tm</sup>; Rosa26-<i>m</i>T/<i>m</i>G or Scgb1a1-
CreER<sup>Tm</sup>; Rosa26R-Confetti mice were indicated in the figures and text by p53<sup>Δ/Δ</sup>. To test
the effect of an extra copy of p53 on proliferation and differentiation, we used either
Super p53 transgenic mice alone or in combination with the Scgb1a1-CreER<sup>Tm</sup>; Rosa26R-
Confetti alleles to follow the behavior of club cells over time.
4.2.2 Immunofluorescence staining

Five-micrometer sections were collected from either lung tissue fixed with 10% NBF or cultures fixed with 4% PFA and embedded in Histogel. De-waxing and antigen retrieval was performed and the sections were incubated with primary antibodies at 4°C overnight. Sections were washed with PBS and incubated with fluorochrome-conjugated secondary antibody and DAPI for 45 minutes at room temperature. Sections were washed again and mounted in Fluormount G. The following antibodies were used:

Table 1: Antibody Information

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4.2.3 Imaging and quantitation

All images were taken using a Zeiss 780 confocal microscope. Sections of tissue from 3-5 independent animals were imaged and between 500 and 1000 cells per animal was quantitated using FIJI. For patch number and clonal expansion experiments, accessory lobes from at least 3 independent animals were treated with Scale [107] to clarify tissue. The lobes were microdissected to expose the airways and the native
fluorescent proteins were imaged. Patch size was quantified using Imaris (Bitplane), with the number of contiguous cells defining a patch. To determine the number of patches per unit area, the number of YFP patches as quantified by Imaris (Bitplane) was divided by the area of tissue as quantified by FIJI in square pixels. To determine the density of cells using whole mount analysis, accessory lobes from 3 mice per genotype were stained with DAPI. At least 1000 cells were quantitated using FIJI from five images per mouse taken at 80X zoom along the proximal to distal axis of the airways.

4.2.4 BrdU

BrdU was injected at a concentration of 50mg/kg body weight at 12 and 2 hours before sacrifice. Sorted epithelial cells were fixed and stained using the FITC BrdU Flow Kit (559619, BD Biosciences). Cells were analyzed on an LSRFortessa (BD Biosciences) flow cytometer.

4.2.5 IdU

Mice were given 5-Iodo-2′-deoxyuridine (IdU; Sigma-Aldrich) was resuspended in sterile drinking water at a concentration of 1 g/L. Mice had access to fresh IdU drinking water in light protected water bottles for 1 week. Mice were sacrificed at 1 week and cells were stained, imaged, and quantitated as described above.
4.2.6 *In vitro* cultures

Five thousand flow sorted epithelial cells were cultured with one hundred thousand immortalized mouse MLg lung fibroblasts and Matrigel (BD Biosciences) seeded onto Transwell filter inserts (BD Biosciences) as previously described [8]. Briefly, the epithelial/fibroblast mixture was added to an equal volume of growth factor reduced Matrigel (BD Biosciences) and seeded to the apical surface of 24-well transwell filter inserts (BD Biosciences) placed in 24-well flat-bottom culture plates. The solution was allowed to polymerize for 30 min at 37°C, then basic medium was added to the basal compartment of the well. Cell cultures were maintained for 7-14 days at 37°C in a humidified incubator (5% CO₂). Colony-forming efficiency was calculated as the percentage of seeded cells that give rise to colonies, imaged on a Zeiss Axiovert40 fluorescent microscope and quantitated using FIJI.

4.2.7 Serial passaging

After 7 days *in vitro*, cultures were treated with 5U/mL dispase in Ham’s F12 and incubate at 37°C for 30 minutes. Halfway through incubation, cultures were disrupted by pipetting. Cells were collected and resuspended in 0.05% trypsin and incubated at 37°C for 30 minutes. Samples were vortexed several times during incubation. Cells were then filtered through a 70µm filter stained with 7AAD and sorted for GFP\(^{+}7AAD\). One
thousand sorted epithelial cells were cultured with one hundred thousand mouse lung fibroblasts in 50% Matrigel as described previously [104].

4.2.8 Single cell RNA-Seq

GFP⁺, EpCAM⁺,CD31/34/45⁻,7AAD⁻ cells were sorted at 70 days after tamoxifen exposure and run on the C1 chip (Fluidigm).

4.2.9 Single cell sequencing library preparation

Cell capture, lysis, reverse transcription and cDNA amplification was performed on the C1 IFC for mRNA-seq on Fluidigm C1 Single-Cell Auto Prep System following the manufacturer’s protocol. Medium sized C1 mRNA-Seq chips were used to capture each cell-cycle fraction. The C1 Auto-prep system captured the dissociated single cells across 96 wells and performed cell lysis, cDNA synthesis with reverse transcription and PCR reaction using the SMARTer Ultra Low Input RNA Kit. Cells captured across the 96 wells are manually inspected as a quality control measure to remove empty well, doublets or debris containing wells. 92 ERCC spike-ins of known concentration were added during the lysis step to assess and quantify technical variation. cDNA from several representative cells were checked by High Sensitivity DNA chips using Fragment Analyzer (Advanced Analytical). Libraries for each of the 96 captured cells were prepared using the Illumina Nextera XT DNA sample preparation kit with 96 dual barcoded indices. Single cell libraries were multiplexed and sequenced across 4 lanes of
a NextSeq 500 platform (Illumina) using 75 single-end sequencing. On average, about 6-7 million reads would be generated from each single cell library.

4.2.10 Quality control, processing and analysis of single-cell RNA-Seq data

Raw reads were aligned to the transcriptome using Bowtie (version 1.1.1) [119] / RSEM (version 1.2.20) [120] with default parameters, using a custom reference containing both 92 ERCC sequences and the mouse transcriptome GRCm38 reference downloaded from http://www.gencodegenes.org, containing all protein coding and long non-coding RNA genes based on Release M3 annotation. Expression counts for each gene in all samples were normalized by the total number of reads mapped to the transcriptome in order to account for sequencing depth (transcripts per million; TPM) with the correction of size factors derived from ERCC spike-in. We imposed additional quality control criteria to remove low quality cells: 1) total number of raw sequencing reads <1M; 2) among all sequencing reads, percentage of total mapped read is <50%; 3) among mapped reads, percentage of exonic reads <50% and 4) percentage of ERCC spike-in reads >40% to eliminating majority of the dying or dead cells. The resultant 64 WT cells and 64 p53 cells passed quality control and were included for the subsequent analysis. The expression counts were transformed into log scale. To identify the subpopulation among the samples in an unsupervised way, principle component analysis (PCA) was performed to select most varied genes across all single cells based on
their eigenvectors [121,122] with FactoMineR v1.31 in R/Bioconductor v3.2. The top 250 most varied genes from PCA was clustered with Euclidean distance matrix with ggplot2 v1.0.1 in R/Bioconductor.

4.2.11 Population RNA-Seq

Scgb1a1-CreERTM; Rosa26-mT/mG; p53floxflox or p53+/+ mice were tamoxifen treated and lineage labeled GFP+,EpCAM+,CD31/34/45-,7AAD- cells cells were FACS isolated from their lungs at 30 days. RNA was extracted from the sorted cells using the RNeasy Micro Kit (Quiagen). Total RNA was quantified using both the NanoDrop to assess sample contamination by proteins or carryover reagents from RNA isolation, and the Qubit fluorometer (Invitrogen). Samples were then qualified using the Fragment Analyzer (Advanced Analytical Technologies) that analyzes the integrity of the total RNA by measuring the ratio between the 18S and 28S ribosomal peaks. Libraries were constructed using SMARTer Ultra Low Input RNA v3 kit (Clontech), indexed using Nextera indices (Illumina). Final libraries were again quantitated with the Qubit fluorometer and checked for size via the Fragment Analyzer. Libraries were diluted to 4nM and pooled in equal volumes for denaturation, hybridization, and sequencing on an Illumina NextSeq 500 (Illumina) with single end 75bp sequencing chemistry. On average, about 20 million reads were generated from each sample. Raw reads were aligned using TopHat (Bowtie2) aligner (Basespace). Differential gene expression
between wild type and p53 deficient samples was determined using the Cufflinks Assembly and Differential Expression application (Basespace).

4.3 Results

4.3.1 p53 regulates proliferation and cell death in vitro

To examine the contribution of p53 in regulating club progenitor cells, we used a loss-of-function model coupled with lineage tracing to assess in vitro clonogenic potential. Scgb1a1-CreERTM; Rosa26-mT/mG mice with either a wild type or p53Δ/+ allele were treated with tamoxifen, yielding club cells that are genetically tagged with GFP and are either sufficient (p53+/+) or deficient (p53Δ/−) for p53. Lineage-labeled club progenitors were sorted and co-cultured with p53 sufficient fibroblasts in a 3D Matrigel culture system (Figure 20A). After 7 days, p53 deficient cells generated significantly more colonies than p53 sufficient cells (Figure 20B and 20C). Additionally, p53 deficient cells generated significantly more colonies across multiple passages (Figure 20D and 20E). Considering that p53 is a well-known regulator of cell proliferation and survival, we assessed the impact of altered p53 status on in vitro colony forming ability. p53 loss resulted in significantly more Ki67 positive lineage tagged cells (Figure 20F and 20G). Additionally, we found significantly fewer cleaved caspase 3 positive apoptotic cells in cultures of p53 deficient cells (Figure 20H and 20I). Furthermore, using flow cytometry at the time of passage, we detected significantly more 7AAD positive lineage tagged
cells in p53 sufficient cultures than p53 deficient cultures at late passages (Figure 20J and 20K). These results suggest that p53 plays a role in regulating progenitor cell proliferation and survival \textit{in vitro}. 
Figure 20. p53 deficient colonies have greater colony forming ability.

(A) Flow plot from the FACS sorting of tamoxifen treated Scgb1a1-CreERTM; Rosa26-mT/mG mice. 7AADneg,CD31/34/45neg were selected for EpCAMpos,GFPpos. (B) GFP+ primary colonies (P0). (C) Colony forming efficiency (percentage of colonies of total number of cells seeded) at P0. (D) GFP+ colonies after 7 days in culture at passage 1 and 4. (E) Colony forming efficiency at various passages. (F) Immunofluorescence (IF) of a p53 sufficient and deficient colony with Ki67 in red, GFP in green, and DAPI in blue. (G) Quantification of the percent of GFP+ Ki67+ cells. (H) IF of a p53 sufficient and deficient colony with cleaved-caspase 3 (CC3) in red, GFP in green, and DAPI in blue. White arrowhead points to CC3 positive cell. (I) Quantification of the percent of GFP+ CC3+ cells. (J) Flow plots from p53 sufficient and deficient cultures at passage 4. (K) Bar graph depicting the percent of dead cells (7AADpos) that are lineage labeled with GFP at various passages after 7 days in culture. Scale bars represent 1mm in (B) and (D), 50 µm in (F) and 10 µm in (H). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
4.3.2 p53 maintains quiescence of club cells in vivo

We next sought to determine if altering p53 level would affect epithelial proliferation in vivo in a normally quiescent tissue such as the lung. FACS enriched total epithelial cells from tamoxifen treated Scgb1a1-CreERTM; p53floxflox or p53floxflox mice (p53Δ− or p53Δ/Δ mice respectively) were stained with propidium iodide and analyzed for DNA content to measure cell cycle phase via flow cytometry (Figure 21A). p53 deficient mice had significantly more epithelial cells in G2/M at 3 days following tamoxifen exposure compared to p53 sufficient controls (Figure 21B). No change in proliferation existed in non-tamoxifen treated p53Δ/Δ mice (Figure 21C). Additionally, Scgb1a1-CreERTM; Rosa26-mT/mG; p53floxflox mice were treated with a single dose of tamoxifen then given IdU drinking water for 7 days to assess proliferation. p53 deficient mice had significantly more GFP lineage labeled cells that incorporated IdU as compared to controls (Figure 21D and 21E). We also assessed cell cycle in mice carrying one transgenic p53 allele in addition to the two endogenous alleles, referred to as “Super p53” mice (García-Cao et al., 2002). In contrast to our findings with p53 deficient mice, Super p53 mice with three copies of p53 had significantly fewer cells in G2/M (Figure 21F). These findings were further reinforced when BrdU incorporation was used to measure proliferation. Flow cytometry for BrdU revealed that Super p53 mice had significantly fewer proliferating cells compared to control (Figure 21G). Together, these results indicate that altering p53
copy number directly controls quiescence of epithelial progenitor cells in the homeostatic airway.
Figure 21. p53 regulates proliferation

(A) Histograms of propidium iodide (PI) staining of isolated EpCAM$^{pos}$ epithelial cells from Super p53, p53$^{+/+}$, or p53$^{Δ/-}$ mice. The cells falling in the G2/M phase of the cell cycle are indicated. (B) Percent of isolated epithelial cells in G2/M relative to control. (C) Percent of epithelial cells from non-tamoxifen treated mice in G2/M relative to control as indicated by PI. (D) IF staining for GFP lineage tag and IdU. Arrowheads indicate IdU$^+$ cells; dashed line at basement membrane. (E) Percent of IdU positive lineage tagged cells. (F) Percent of isolated epithelial cells in G2/M relative to control. (G) Bar graph depicting the number of BrdU positive cells, as detected by flow cytometry. Graphed as a percent of control. Scale bars represent 10 µm in (D). *p < 0.05; **p < 0.01, ****p < 0.0001.
4.3.3 Airway epithelial cell density is regulated by p53 in a dose-dependent manner

Considering that p53 gene dose regulates proliferation in the airway epithelium, we next sought to determine if altered proliferation led to changes in cell density. To test this, we assessed the number of lineage tagged cells per unit basement membrane at 2, 30, and 70 days post-tamoxifen exposure in Scgb1a1-CreERTM; Rosa26R-Confetti; p53ΔΔ- or p53ΔΔ/ΔΔ mice (p53ΔΔ- or p53ΔΔ/ΔΔ mice respectively). p53 loss significantly increased the number of lineage tagged cells per unit basement membrane (Figure 22A and 22B). Additionally, total cell density was significantly increased in p53ΔΔ- and p53ΔΔ/ΔΔ mice (Figure 22D). Similar findings were observed using whole-mount imaging, in which p53 loss significantly increased both the number of lineage-labeled patches per unit area as well as the total airway epithelial cell density (Figure 22E-22I). Next, we assessed the number of lineage tagged cells per unit basement membrane in Scgb1a1-CreERTM; Rosa26R-Confetti; Super p53 mice at 70 days post tamoxifen. Super p53 mice had significantly fewer lineage tagged cells per unit basement membrane as well as significantly fewer nuclei per unit area (Figure 22C, 22G, and 22I). Taken together, these data demonstrate that p53 copy number determines the density of airway epithelial cells during homeostasis.
Figure 22. p53 regulates cell density.

(A) IF staining for confetti lineage tag (nGFP, cYFP, and mCFP in green and cRFP in red) and DAPI in blue 70 days post-tamoxifen. (B) Number of lineage tagged cells per unit basement membrane (uBM) post-tamoxifen. (C) Number of lineage tagged cells per uBM. (D) Quantification of the total nuclei per unit basement membrane in p53Δ/Δ or p53Δ/Δ mice. (E) Tiled image of the native YFP fluorescence (white) in a whole mount, microdissected, Scale treated accessory lobe from p53 sufficient or deficient mice. (F) Whole mount 4-color fluorescence of airways. (E) Number of cYFP patches per unit area. (F) Whole mount DAPI staining along airways. (G) Number of nuclei per uBM. BM and area are measured in pixels and square pixels, respectively. Scale bars represent 20 µm in (A), 500 µm in (E), 50 µm in (F), and 10 µm in (G). *p < 0.05; **p < 0.01, ****p < 0.0001.
4.3.4 p53 levels influence clonal behavior of progenitor cells

To assess the ability of a single progenitor cell to clonally expand, we administered a low dose of tamoxifen (1 x 5mg/kg) to Scgb1a1-CreER\textsuperscript{TM}; Rosa26R-Confetti; p53\textsuperscript{floxed/floxed} mice, referred to as p53\textsuperscript{Δ/Δ} mice, and quantified the size of GFP, RFP, or YFP patches. p53 loss resulted in a significant increase in the number of clones containing 3 or more cells (Figure 23A and 23B). Additionally the number of cells per patch was significantly increased, with p53 deficient cells generating patches twice as large as control (Figure 23C). Scgb1a1-CreER\textsuperscript{TM}; Rosa26R-Confetti; Super p53 mice had a significant decrease in the number of cells per patch, indicating that tight control over p53 levels is essential to maintain correct progenitor pool size (Figure 23D-23G).
Figure 23. p53 controls clone size in vivo.

(A) Whole mount image of native confetti fluorescence at 70 days after 5mg/kg tamoxifen. (B) Quantification of the percent of patches according to the number of cells per patch at 70 days post-5mg/kg tamoxifen (n=3-5). (C) Quantification of the number of cells per patch at 70 days post-tamoxifen. A total of 1328 patches were counted in 3 p53<sup>−/−</sup> mice and 7080 patches were counted in 4 p53<sup>Δ/Δ</sup> mice. (D) Whole mount image of native confetti fluorescence at 70 days after 5mg/kg tamoxifen. (E) Quantification of the percent of patches according to the number of cells per patch at 70 days post-5mg/kg tamoxifen (n=3-5). (C) Quantification of the number of cells per patch at 70 days post-tamoxifen. A total of 4021 patches were counted in 4 p53<sup>−/−</sup> mice and 4567 patches were counted in 5 Super p53 mice. (G) Schematic representing clonal expansion following p53 manipulation. Scale bars represent 50 µm. *p < 0.05; **p < 0.01, ***p < 0.001.
4.3.5 p53 regulates differentiation of club progenitor cells

Given that club cells generate ciliated cells, we tested the hypothesis that p53 regulates club to ciliated cell differentiation in the airway epithelium. To do this we assessed the number of lineage tagged ciliated cells, indicated by FoxJ1 staining, following tamoxifen exposure in Scgb1a1-CreERTM; Rosa26R-Confetti; p53flx− or p53flxflx mice (p53Δ/Δ mice respectively). Loss of p53 in club cells led to the generation of significantly fewer ciliated cells as compared to control (Figure 24A and 24B). To test if increasing p53 gene dose would influence ciliated cell differentiation, we quantified the number of FoxJ1+ cells in the airway epithelium of Super p53 mice and found significantly more ciliated cells compared to controls (Figure 24C and 24D). Conversely, we found that p53 loss led to an increase in club cell generation, while an extra copy of p53 decreased club cell numbers (Figure 24E and 24F). These results demonstrate that the number of p53 gene copies directly regulates club cell to ciliated cell differentiation in vivo.
Figure 24. p53 regulates ciliated cell differentiation.

(A) IF for confetti lineage tag (nGFP, cYFP, and mCFP in green and cRFP in red) and FoxJ1 in white 70 days post-tamoxifen. (B) Percent of FoxJ1 positive lineage tagged cells at various times post-tamoxifen. (C) Immunostaining for FoxJ1 in red and DAPI in blue are overlayed on a DIC image to highlight cilia in p53<sup>+/−</sup> and Super p53 mice. (D) Percent of FoxJ1 positive nuclei. (E) Percent of Scgb1a1 positive lineage tagged cells at 70 days post-tamoxifen. (F) Percent of Scgb1a1 positive cells. Scale bar in (A) and (C) represents 10 µm. **p < 0.01, ****p < 0.0001.
4.3.6 p53 loss of function alters differentiation potential

We hypothesized that p53 loss might also affect differentiation \textit{in vitro}. Cultures were stained for markers of various airway cell types, including p63 and keratin 5 (K5), which traditionally mark basal cells located in the trachea and proximal conducting airway and are not derived from \textit{Scgb1a1}-expressing cells under homeostatic conditions in the postnatal mouse. Surprisingly, culture of lineage labeled GFP+ cells from tamoxifen treated \textit{Scgb1a1-CreER}^{TM}; \textit{Rosa26-mT/mG}; \textit{p53}^{flox/-} mice yielded organoids composed of a pseudostratified epithelium containing a significant number of p63 and K5 positive lineage tagged cells, indicating that an \textit{Scgb1a1}-expressing progenitor was able to generate a basal-like cell \textit{in vitro} following p53 loss (Figure 25A-25G). Interestingly, only about 40\% of colonies containing p63 positive cells also contained K5 positive cells (Figure 25C). Taken together, these data indicate that p53 loss alters the differentiation potential of club cells \textit{in vitro} (Figure 25H).
Figure 25. p53 loss alters differentiation potential in vitro.

(A) IF of a colony with p63 (red) and GFP (green) after 14 days in culture; higher magnification in (A’). (B) Percent of lineage tagged colonies with p63+ cells (14 days in culture). (C) Immunostaining of a colony with keratin 5 in white, p63 in red, GFP in green after 14 days in culture. Inset shows higher magnification of area indicated by white box. (D) IF of a colony after 14 days in culture with GFP (red) and α-tubulin (green); higher magnification of area in white box (D’). (E) IF of K5 (red) and α-tubulin (green); adjacent serial section corresponding to box in (D). (F and G) Percent of lineage tagged colonies containing K5 positive cells (F) or ciliated cells (G) (14 day cultures). (H) p53 controls ciliated cell differentiation in vivo and expands differentiation potential in vitro. DAPI is in blue. Scale bars in (A), (C), (D) and (E) represent 50 µm for low magnification and 10 µm for high magnification. *p < 0.05; **p < 0.001.
4.3.7 p53 regulates the proportion of progenitors in the airway

To better understand the cellular and molecular mechanisms driving p53-dependent regulation of progenitor cell fate, we employed single cell RNA-Seq. Lineage tagged GFP+ cells from tamoxifen treated Scgb1a1-CreER\textsuperscript{TM}; Rosa26-mT/mG mice with a p53\textsuperscript{lox/lox} or p53\textsuperscript{+/+} allele (p53\textsuperscript{Δ/Δ} and p53\textsuperscript{+/+} respectively) were sorted at day 70 and single cell sequencing was performed using a Fluidigm C1 system. After quality control and normalization, transcriptomes of 64 p53\textsuperscript{+/+} and 64 p53\textsuperscript{Δ/Δ} cells were analyzed using principle component analysis and an unsupervised heat map of the top 250 protein coding genes with the highest expression variation across all cells (Figure 26A and 26B). Surprisingly, clustering patterns observed in the principle component analysis and heat map suggest that cellular heterogeneity between Scgb1a1 lineage labeled cells, rather than p53 status, represent the principal determinants of molecular variability (Figure 26C). We saw segregation between four clusters of cells; each cluster containing various markers of proximal or distal club cell sub-types. p53 loss altered the proportion of cells that fell in each cluster (Figure 26D).

Considering p53 loss expanded differentiation potential \textit{in vitro}, RNA-Seq data were mined for alterations in other progenitor types. Bronchioalveolar stem cells (BASCs) are a controversial progenitor type marked by the co-expression of Scgb1a1 and Sftpc and were proposed to generate both airway and alveolar cell types [47]. We
identified airway epithelial cells (Sox2\(^{+}\) [124]) that express both *Scgb1a1* and *Sftpc*, which increased in abundance when p53 was lost (Figure 26E). Additionally, the number of lineage labeled *Scgb1a1*\(^{-}\)\*Sftpc\(^{+}\) cells per terminal bronchiole is significantly increased at 70 days post-tamoxifen in p53\(^{-/-}\) mice; further supporting the notion that p53 regulates multipotency of club progenitors (Figure 26F and 26G). These data show that p53 loss does not radically change the gene expression profile of club progenitor cells, but instead may regulate the proportions of regionally distinct sub-types of progenitor cells within the *Scgb1a1* lineage labeled population.
Figure 26. p53 status does not account for the largest variability in gene expression between Scgb1a1-lineage labeled cells.

(A) Unsupervised principle component analysis of single-cell gene expression patterns for 64 p53Δ/Δ cells (black points) and 64 p53+/+ cells (red points). Black and red 95% confidence ellipses contour the p53Δ/Δ cells and p53+/+ cells, respectively. (B) Unsupervised heatmap showing the top 250 genes that vary among cells. (C) Unsupervised heatmap showing cell subtype specific genes, segregated by 4 secondary clades (clusters). (D) Pie chart depicting the percentage of cells that fall into the four clusters in (C). (E) Percent of single cells that co-express Sox2, Scgb1a1, and Sftpc. (F) IF for Sftpc in red, Scgb1a1 in green, and GFP in white at 70 days post-tamoxifen. Arrowheads indicate co-expressing cells; dashed line at basement membrane. (G) Number of lineage tagged Scgb1a1‘Sftpc’ cells per terminal bronchiole. Scale bar represents 10 µm in (P). *p < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001.
4.3.8 p53 loss increases the number of cycling cells

To gain further insights into mechanisms by which p53 regulates progenitor behavior, we analyzed pathways that were altered in p53Δ/Δ cells using the DAVID online pathway analysis tool [125,126]. The most significantly enriched pathway in p53Δ/Δ cells was cell cycle (Figure 27A). Expression levels of genes frequently enriched in the G2/M cell cycle phase were increased in p53 deficient cells (Figure 27B). Both the total number and expression levels of cell cycle promoting genes were significantly increased in p53Δ/Δ cells (Figure 27C and 27D). Conversely, p53Δ/Δ cells expressed significantly reduced levels of cell cycle inhibitor genes compared to p53-sufficient cells (Figure 27E and 27F). Notably, we found reduced expression of the cell cycle inhibitor p21 (Cdkn1a), whose expression is normally upregulated following stress-induced p53 activation (Figure 27G). As a control for random variability in gene expression between genotypes, we evaluated the number of housekeeping genes expressed in each cell and found no significant difference between genotypes (Figure 27K). Finally, the expression of pro-apoptotic genes was also significantly lower in p53Δ/Δ cells as compared to control (Figure 27I and 27J). Additionally, we performed RNA-Seq on sorted GFP+ cells from Scgb1a1-CreERT²; Rosa26-mT/mG; p53fl/flu or p53+/+ mice and verified that the expression of p53 and traditional p53 pathway members decreased in p53Δ/Δ cells (Figure 27H). Together, these findings reinforce the notion that p53 maintains quiescence of club
progenitor cells by regulating genes that are normally associated with stress-activated p53 signaling.
Figure 27. p53 loss activates the cell cycle.

(A) DAVID KEGG pathway analysis of genes that are more highly expressed in p53Δ/Δ cells compared to control. (B) Supervised heatmap showing cell cycle promoting genes. (C) Expression of cell cycle promoting genes are significantly higher when p53 is lost (two-way ANOVA, *p=0.0157). (D) Scatter plot depicting the total number of cell cycle promoters expressed (Log₂(TPM) > 1) per cell. (E) Expression values of cell cycle inhibitors are lower when p53 is lost. (F) Scatter plot depicting the number of cell cycle inhibition genes expressed (Log₂(TPM) > 1) per cell. (G) Scatter plot depicting the expression values (Log₂(TPM) > 1) of Cdkn1a per cell. (H) Unsupervised heatmap showing p53 pathway genes. (I) Expression values of pro-apoptotic genes are lower when p53 is lost. (J) Scatter plot depicting the total number of pro-apoptotic genes expressed (Log₂(TPM) > 1) per cell. (K) Scatter plot depicting the total number of housekeeping genes expressed (Log₂(TPM) > 1) per cell. ns = not significant, **p < 0.01.
4.3.8 p21 regulates proliferation, but not differentiation of airway progenitors

To further explore roles for p21 as a downstream target of p53 in regulating progenitor cell quiescence, we determined whether p21 deletion phenocopied cell cycle and density phenotypes observed with p53 loss of function. To assess proliferation, the number of FACS isolated epithelial cells from p21+/− and p21−/− mice in G2/M phase was measured by PI staining. p21−/−, but not p21+/− mice contained significantly more cells in G2/M (Figure 28A and 28B). We quantified the number of nuclei per unit basement membrane in p21+/− and p21−/− mice and found that p21 loss resulted in increased cell density in both genotypes compared to control (Figure 28C and 28D). To determine if p21 also regulates differentiation, we quantified the percent of FoxJ1 positive ciliated cells in the airway epithelium. Interestingly, there was no significant change in the number of ciliated cells in p21 deficient mice (Figure 28E and 28F). These results suggest that quiescence, but not differentiation, of club progenitor cells is regulated by p21 (Figure 28G). This finding partially phenocopies p53 loss of function and suggests that other p53 targets regulate differentiation of club progenitor cells.
Figure 28. p21 deficient mice have increase airway epithelial cell proliferation and cell density, but not altered differentiation.

(A and B) Percent of isolated epithelial cells in G2/M relative to control as indicated by propidium iodide (PI) staining in p21⁻/⁻ (A) or p21⁺/⁺ (B). (C and D) Number of nuclei per unit basement membrane in the airway epithelium in p21⁻/⁻ (C) or p21⁺/⁺ (D). (E and F) Percent FoxJ1 positive nuclei in the airway epithelium in p21⁻/⁻ (E) or p21⁺/⁺ (F). (G) p53 controls proliferation and density through p21. ns = not significant, *p < 0.05.
4.4 Discussion

In this study we demonstrate that p53 critically regulates progenitor cell behavior to control cell density and composition in airways (Figure 29). We present novel data using cell type specific p53 knockouts as well as Super p53 mice to show that regulation of progenitor cell behavior occurs in a gene dose-dependent manner. These data illustrate that changes in baseline expression of p53 are important determinants of progenitor cell fate.

Previous studies show that p53 regulates self-renewal and differentiation of neural, mammary, hematopoietic, and nephron stem and progenitor cells [90–95]. However, these previous studies have been performed under conditions of rapid cell expansion in vitro or during development. As such, these studies leave gaps in our understanding of roles for p53 in regulating stem and progenitor cells in quiescent tissues under homeostatic conditions. Our finding that p53 controls quiescence and differentiation in the homeostatic lung provides novel insights into progenitor cell regulation and complements previously published results.

We show that an extra copy of p53 promoted ciliated cell differentiation and decreased proliferation, leading to decreased epithelial cell density. Furthermore, we found a dose-dependent effect on p53 levels in the ability of club progenitors to clonally expand. This demonstrates that tight control of p53 is essential to maintain the proper
number of progenitor cells that contribute to epithelial maintenance under homeostatic conditions. Precise regulation of secretory to ciliated cell ratios is essential for effective mucociliary clearance and host defense.

Surprisingly, club cells with p53 loss showed altered differentiation potential in vitro, yielding organoids composed of a pseudostratified epithelium containing basal and ciliated cells. Previous reports have described the dedifferentiation of club cells to basal cells following basal cell deletion and Yap overexpression, indicating that club cells have an inherent plasticity that is suppressed under homeostatic conditions [25,50]. Although we never observed lineage labeled basal cells in vivo, our in vitro data indicate that the differentiation potential of club cells is suppressed by both p53-dependent cell autonomous and microenvironmental factors. The finding that ciliated cells only arose in cultures containing K5 positive cells and the appearance of K5 positive cells preceded that of ciliated cells (not shown) suggests ciliated cells are basal cell derived. These data also suggest that club to basal to ciliated cell differentiation is permissive in the absence of p53, despite our finding that direct club to ciliated differentiation was abrogated when p53 was lost in vivo.

*Scgb1a1-*expressing cells were previously thought to be a relatively uniform pool of quiescent progenitors [10]. However, using single cell RNA-Seq, we observed heterogeneity between various sub-types of cells within the *Scgb1a1*-lineage labeled...
population. We identified lineage-traced cells that co-expressed Scgb1a1 and Sftpc, the molecular phenotype described for putative BASCs. Furthermore, the number of Scgb1a1+Sftpc+ cells increased with p53 loss, implicating p53 as a regulator of this progenitor state. However, the functional role of these progenitor cells in repairing the airway and alveoli has yet to be determined. The potential for p53 loss to expand the pool of “active” progenitors was supported by the observation of an increase in the number of cycling cells and a decrease in cell cycle inhibitors, particularly p21, following p53 loss. We found that p21 loss phenocopied effects of p53 loss on epithelial cell proliferation, but not differentiation, suggesting that these processes are regulated by distinct downstream targets of p53. Candidate pathways that may mediate the effects of p53 on cellular differentiation include Notch, a known target of p53 and a pathway that has been shown to modulate club to ciliated cell transdifferentiation [127].

Recent literature has demonstrated that cancers often arise from resident stem or progenitor cells [96,97]. Here, we show that the loss of a tumor suppressor leads to an increase in self-renewal and proliferation of progenitor cells. These conditions could lead to preneoplastic lesions, either alone or in combination with additional injuries or mutations. Interestingly, a high number of p53 copies in elephants has been correlated with a lower cancer risk [128,129]. We discovered that an additional copy of p53 reduced the proliferation rate and increased terminal differentiation, both of which likely
promote tumor suppression. Together, our data demonstrate that p53 plays an essential role as a tumor suppressor by regulating progenitor cell behavior in the lung.

Figure 29. p53 regulates proliferation and differentiation in the homeostatic airway epithelium.

Our data support a model in which p53 controls both proliferation and differentiation in a dose-dependent manner. Under homeostasis, p53 inhibits proliferation via p21. Additionally, p53 likely regulates differentiation through multiple downstream targets, including the Yap pathway. Tight control of p53 must be maintained to regulate stemness, proliferation, and progenitor pool size.
4.5 Acknowledgements

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5. Conclusion and Future Directions

5.1 Conclusions

Resident club progenitor cells in the airway epithelium maintain a low level of turnover during homeostasis. However, despite their quiescence, these progenitor cells are capable of regenerating the epithelium following injury. One type of injury that has been relatively unexplored in the field of lung stem cell biology is ionizing radiation. This physiologically relevant injury model is known to induce fibrosis and cancer, two of the most deadly lung diseases [105,130,131]. Yet little is known about the effects of ionizing radiation exposure on progenitor cells in the lung. In Chapter 2, we show that club progenitor cells exhibit a dose-dependent decrease in colony forming ability immediately following radiation exposure and that a subset of these progenitors undergo clonal expansion without an increase in proliferation in vivo. Chapter 3 expands on these findings to reveal that the clonal expansion is specific to the distal lung and occurs due to p53-dependent apoptosis, senescence, and mitotic defects in club progenitors residing in the terminal bronchioles. Additionally we found that this phenotype is radiation quality-dependent. High-LET radiation exposure induces greater clonal expansion than low-LET radiation due to the inability of progenitor cells to recover their DNA-damage and ability to proliferate to renew the airway long-term.
Together, these results reveal dose- and quality-specific alterations in progenitor cell dynamics in the airway epithelium following ionizing radiation exposure.

In addition to uncovering a role for p53 in radiation-induced clonal expansion, we discovered that p53 regulates progenitor cell behavior under homeostatic conditions as well. In working with p53 deficient mice, we found that p53 loss decreased ciliated cell differentiation and increased the proliferative capacity of club progenitors, thus increasing epithelial cell density. p53 deficient club progenitors generated a pseudostratified epithelium containing basal-like cells in vitro and putative bronchioalveolar stem cells in vivo, suggesting that p53 regulates multipotency. Conversely, an additional copy of p53 increased quiescence and ciliated cell differentiation, highlighting the importance of tight regulation of p53 levels during homeostasis. Using single cell RNA-Seq, we found that loss of p53 altered the molecular phenotype of progenitors and decreased cell cycle regulatory genes, particularly p21. These findings reveal that p53 is an essential regulator of progenitor cell behavior, which shapes our understanding of stem cell quiescence during homeostasis and in cancer development. Together, this work reveals molecular mechanisms regulating airway epithelial progenitor cells during homeostasis, as well as following ionizing radiation exposure.
5.2 Future Directions

5.2.1 Radiation-induced clonal expansion and cancer

As previously discussed, the carcinogenic effects of ionizing radiation exposure are well known [105]. The current dogma in the field is that radiation exposure leads to DNA damage, which can either induce mutations in tumor suppressors or oncogenes directly or promote cancer progression through secondary changes in the epigenetic landscape, cellular metabolism, or microenvironmental signaling [59,60]. Considering cancer is a complex, multistep disease, the latency in radiation-induced tumor development is expected. However, few studies have closely examined the early changes in normal cell behavior in vivo following radiation exposure.

In Chapters 2 and 3, we show that ionizing radiation exposure increases the size of patches formed by club progenitor cells in the airway epithelium. The appearance of these patches indicates that clonal expansion occurred in a small number of progenitor cells in response to radiation exposure. However, a high dose of tamoxifen was used in these studies to attain maximal recombination of the confetti allele in club cells. Due to the limited number of fluorescent tags and high tagging efficiency, the possibility of clonal misassignment, or the incorrect assumption that a patch is clonally derived, must be acknowledged. One way to identify if the radiation-induced patches are indeed clonal is to treat with a low dose of tamoxifen. However, this method is not without
caveats. The cell specific gene driving CreER may not be expressed at the same level in every cell, yielding cells with variable levels of CreER. Therefore, when giving a low dose of tamoxifen, lineage tracing could be biased towards cells expressing the highest levels of CreER. Regardless of the dose of tamoxifen used, the results from experiments using the confetti reporter should be carefully interpreted. An alternative method to determine radiation-induced clonal expansion is to increase the color combinations used in lineage tracing. This has been done in previous studies and often requires the use of powerful imaging modalities and processing software [132,133]. Experiments using these techniques are required to truly visualize radiation-induced clonal expansion on an individual cell level.

Given that progenitor cells are thought to be the predominant cell-of-origin of cancer [96,98], clonal expansion likely describes an early event along the path to radiation-induced carcinogenesis. Previous studies have implicated clonal expansion as an important step in carcinogenesis [115–118]. However, precisely how this leads to tumor development is still unknown. One hypothesis is that clonal expansion of a progenitor cell carrying a mutation in an oncogene or tumor suppressor would lead to the generation of a field of cells all carrying the same mutation. This field effect might lead to further mutations and eventually oncogenic transformation. Furthermore, secondary injuries could play a role in tumor development. Work by Manning et al
showed that viral infection after radiation exposure exacerbated radiation-induced lung injury [134]. Additionally, exposure to both radiation and naphthalene enabled substantial engraftment of embryonic lung cells in mice, indicating that injury with two modalities decreases the resident progenitor pool in an synergistic manner [135]. A fertile area for future investigation would be to determine how clonal expansion of epithelial progenitor cells leads to cancer development and if secondary injuries play a role in neoplastic progression.

5.2.2 Regional radiosensitivity of progenitor cells

In Chapter 3, we show that exposure to ionizing radiation leads to clonal expansion specifically in the terminal bronchioles, due to apoptosis, senescence and mitotic defects in progenitor cells. Many lung diseases, including radiation-induced fibrosis and tumorigenesis, involve pathological remodeling of the distal lung. Our findings demonstrate that the distal airways are more radiosensitive than proximal airways, which may contribute to radiation-induced distal lung pathologies. However, both fibrosis and adenocarcinoma, the most predominant form of lung cancer, primarily occur in the alveolar space [99,136,137]. Further investigation is needed to determine how radiation affects progenitor cells in the alveoli and how this relates to the development of fibrosis and cancer.
The idea of varying levels of progenitor cell radiosensitivity is not unique to the lung. Hematopoietic stem cells, bulge stem cells of the skin, mammary stem cells, and Lgr5+/Sox9low crypt base columnar stem cells are more radioresistant than their differentiated progeny due to differentially expressed levels of p53, Bcl-2, and canonical Wnt signaling [65,66,73,77,100]. Radiosensitive stem cell populations include melanocyte stem cells and slow cycling +4 Bmi1+ intestinal stem cells [64,75]. The effects of radiation on progenitor cell behavior in the lung, particularly how regional progenitors respond to various radiation qualities, had not been previously studied. Our finding that club progenitors residing at terminal bronchioles are more susceptible to apoptosis, senescence, and mitotic defects than proximal progenitors is intriguing, but more investigation is needed to determine why this radiosensitivity occurs. Taking into account previous literature from other organ systems, it is likely that distal progenitor cells have increased levels of pro-apoptotic or pro-senescence proteins, such as p53.

However, the contribution of the microenvironment in region-specific progenitor cell radiation response cannot be ruled out. Club progenitors are unable to form colonies in vitro without either the support of fibroblasts or endothelial cells, or a large number of growth factors. This indicates that distal airway progenitor cells require more stromal derived factors than their proximal airway counterparts and highlights the importance of the stromal microenvironment in normal progenitor function. DNA damage induces
the upregulation of cytokines, growth factors, and chemokines [60]. These factors can initiate remodeling of the extracellular matrix (ECM), which can affect the stem cell niche. One signaling pathway that is upregulated following exposure to IR is the TGFβ pathway. TGFβ is a multifunctional secreted protein that acts as a signaling molecule to control proliferation, apoptosis, and extracellular matrix deposition [60]. It is possible that TGFβ is increased due to radiation-induced stromal damage, which could affect the clonal behavior of epithelial cells. The role of the microenvironment in progenitor cell response to radiation is an important consideration and should be further explored.

5.2.3 Cell autonomous and non-autonomous roles for p53

In Chapter 4, we present the novel finding that p53 regulates differentiation, proliferation, and cell density under homeostatic conditions in the quiescent airway epithelium. We use multiple genetic manipulations of p53, including comparing p53 loss with p53Δ/Δ and p53Δ/Δ mice. We initially used p53Δ/Δ mice to ensure complete loss of p53 in club cells. However, all of the cells in these mice are heterozygous for p53. Given that the microenvironment plays a role in determining club cell fate, the contribution of p53 heterozygous neighboring cells on the club cell phenotype could not be excluded. Therefore, we used p53Δ/Δ mice to delete p53 specifically in the club cells. Interestingly, both the ciliated cell differentiation and proliferation phenotypes were more exaggerated in p53Δ/Δ mice as compared to p53Δ/Δ mice. This suggests that the difference
in p53 levels between neighboring cells plays an important role in regulating club cell proliferation and differentiation into ciliated cells. Alternatively, given that the number of functional copies of p53 in club cells dropped from 2 to 0 following tamoxifen exposure, it is possible that the magnitude of change in p53 levels within a cell influences club cell differentiation and proliferation. Further genetic manipulations of p53 in stromal cells and ciliated cells are necessary to gain a better understanding of the role for p53 in the microenvironment and how this influences club cell proliferation and differentiation.

Assessing clonal expansion using low doses of tamoxifen in p53Δ/Δ mice allowed us to visualize the potential of individual progenitor cells in vivo. We found that p53 loss increased both the size and frequency of large patches, indicating clonal expansion occurs in p53 deficient cells. Using a low dose of tamoxifen to infrequently tag cells also creates a situation in which a single p53 deficient cell lies in a field of p53 sufficient cells. This raises interesting questions about cell competition. Previous studies by Bondar and Medzhitov found that hematopoietic stem and progenitor cells with higher levels of p53 undergo growth arrest and senescence following DNA damage and are outcompeted by cells with lower levels of p53 [138]. Interestingly, this response is distinct from classical p53-mediated DNA damage response in that it persists for months and depends on the relative rather than absolute level of p53 in competing cells. Additionally, studies with
p53 deficient cells in the developing kidney reveal that p53 mediates competition via metabolism and growth arrest in nephron progenitor cells [95]. The question of whether the level of p53 controls progenitor cell competition in the lung has yet to be addressed. Experiments using various doses of tamoxifen as well as in vitro cultures containing cells with varying levels of p53 could be employed to answer questions such as this one.

5.2.4 p53, stem cells, and cancer

p53 is one of the most commonly mutated genes in lung cancer [85]. As previously discussed, stem and progenitor cells have been shown to be the cell-of-origin in several types of cancer [96,98]. Given that p53 is one of the most commonly mutated genes in lung cancer, our finding that p53 regulates proliferation and differentiation in progenitor cells reveals a mechanism for how p53 loss alters epithelial architecture and is likely related to the role for p53 in cancer. p53 loss results in increased proliferation, cell density, and progenitor cells, all conditions potentially leading to a pre-neoplastic epithelium. However, we found that cell density plateaued following p53 loss, which is indicative of a negative feedback mechanism. One pathway that may be providing negative feedback is the Hippo pathway, which is a known regulator of proliferation in response to altered density [139]. Further studies are required to determine the precise mechanism of negative feedback and if deletion of such pathways result in neoplastic transformation.
Although p53 loss in mice is known to induce cancer in many organs [89], tumors were not observed at the time points analyzed in Chapter 4. As previously discussed, the link between early changes in progenitor cell behavior and tumor development is complex and likely involves factors such as secondary mutations or injuries. Some of the p53 loss-of-function models used in this study may be representative of Li-Fraumeni syndrome, a cancer predisposition disorder due to germline loss-of-function mutations of p53 [140,141]. However, many of p53 mutations found in tumors result in a gain-of-function [89]. Gain-of-function p53 mutant protein has altered transcriptional binding and can co-opt chromatin pathways to drive cancer growth [89,142]. Several mouse strains have been developed to model such mutations and should be used as a more physiologically relevant model to assess the role of p53 in homeostatic regulation of stem and progenitor cells.

5.2.5 p53 and Notch

The study presented in Chapter 4 identified p53 as a regulator of progenitor cell proliferation and differentiation in the homeostatic lung. We discovered that p21 is a downstream target of p53 that regulates proliferation and cell density. Yet, downstream targets of p53 that control differentiation are still unknown. The Notch pathway is a well known regulator of proliferation and differentiation in many tissues, including the developing and adult airway epithelium [16,20,29,30,127,143–145]. In the adult airway
epithelium, loss of Notch signaling promotes ciliated cell differentiation, whereas high levels of Notch signaling promote secretory cell differentiation [16,30,145]. Interestingly, Notch is a known downstream target of p53 and the two pathways have been shown to interact at both the DNA and protein levels [146–152]. When further evaluating the single cell RNA-Seq data presented in Chapter 4, p53 deficient cells had an increase in the number of Notch pathway genes expressed (Fig. 33A and 33B). Additionally, mice harboring a Notch reporter were crossed with p53Δ/Δ and p53Δ/+ mice to evaluate the effect of p53 loss on Notch signaling in vivo. Although evaluation of more animals is needed, preliminary data suggest p53Δ/Δ and p53Δ/+ mice have elevated levels of reporter activation, indicating increased Notch signaling (Fig. 33C). Previous studies have shown that elevated levels of Notch in club cells promotes differentiation into goblet cells [16,145]. Accordingly, the percent of cells expressing Muc5ac, a goblet cell marker, increased in p53 deficient samples (Fig. 33D). This correlative data suggests that p53 loss may be activating the Notch pathway in club cells, which prevents ciliated cell differentiation and promotes goblet cell differentiation. However, two caveats that must be acknowledged are the infancy of the single cell RNA-Seq technology and the dependency on bioinformaticians for analysis of the single cell RNA-Seq data. Although their analysis was thorough and contained the appropriate normalization procedures, biologists are largely dependent on the accuracy of bioinformaticians when analyzing
expression data. Therefore, gain- and loss-of-function in vivo models are required to fully understand the connection between the p53 and Notch pathways and their relevance to differentiation of airway epithelial progenitor cells.

A recent paper showed that club cells can transdifferentiate to ciliated cells without an increase in proliferation following inhibition of Jagged, a Notch pathway ligand [127]. We found that p21 regulates proliferation, but not differentiation in the airway epithelium; raising the possibility that proliferation and differentiation need not be coupled and could be regulated by various downstream targets of p53. The idea that cell division may not be necessary for differentiation is intriguing and, if proven, would alter the current dogma in the field.
Figure 30. Notch signaling increases when p53 is lost.

(A) Scatter plot depicting the total number of Notch pathway genes expressed (Log$_2$(TPM) > 1) per cell. (B) Heat map of Notch pathway genes from RNA-Seq performed on isolated, lineage labeled cells. (C) Percent of Notch reporter positive airway epithelial cells (n=1). (D) Percent of single cells expressing Muc5ac (Log$_2$(TPM) > 1). ***p < 0.001.
5.3 Final Remarks

This dissertation probes the molecular mechanism by which airway epithelial progenitor cells are maintained during homeostasis and repair of the epithelium following ionizing radiation exposure. The novel finding that high-LET radiation exposure results in clonal expansion of progenitor cells in the distal airways in a p53-dependent manner is relevant to both patients undergoing radiation therapy and astronauts exposed to the space environment. Hopefully these results provide a basis for others to use this physiologically relevant injury model to further explore the effect of ionizing radiation exposure on progenitor cells in the lung. Additionally, work with p53 deficient mice led to the discovery of a novel role for p53 in regulating proliferation and differentiation to maintain quiescence in the airway epithelium. Together, the work presented in this dissertation advances both the radiation and stem cell biology fields and raises intriguing questions central to cell and cancer biology.
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Biography

Alicia Marie Farin was born in Columbia, Missouri on September 12, 1989. Her parents, Charlotte and Peter Farin, moved to Raleigh, North Carolina less than a year after her birth, where Alicia and her sister, Elizabeth Farin, were raised. Alicia attended Athens Drive High School where she became fascinated with biology and anatomy. Her interest in animals motivated her to enroll as an Animal Science major with a minor in Genetics at North Carolina State University. In addition to participating in research in several labs, two courses in developmental biology sparked her interest, leading her to apply to the Duke Developmental and Stem Cell Biology graduate program after receiving her bachelors in 2010. In her second year, Alicia moved with the Stripp lab to Cedars-Sinai Medical Center to complete her dissertation research.

Publications


Honors

Spielberg Young Investigator Award in Cancer Biology at Cedars-Sinai Medical Center
First place oral and poster presentation winner, Cedars-Sinai Graduate Symposium Lung Repair and Regeneration Consortium Young Investigator Scholar Award
NASA Space Radiation Summer School Fellow
Duke University Student Science Education Outreach Grant