Advances in Small-Cell Lung Cancer (SCLC) Translational Research

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Over the past several years, we have witnessed a resurgence of interest in the biology and therapeutic vulnerabilities of small-cell lung cancer (SCLC). This has been driven in part through the development of a more extensive array of representative models of disease, including a diverse variety of genetically engineered mouse models and human tumor xenografts. Herein, we review recent progress in SCLC model development, and consider some of the particularly active avenues of translational research in SCLC, including interrogation of intratumoral heterogeneity, insights into the cell of origin and oncogenic drivers, mechanisms of chemoresistance, and new therapeutic opportunities including biomarker-directed targeted therapies and immunotherapies. Whereas SCLC remains a highly lethal disease, these new avenues of translational research, bringing together mechanism-based preclinical and clinical research, offer new hope for patients with SCLC.

Small-cell lung cancer (SCLC) afflicts approximately 30,000 patients per year in the United States and carries a 5-year overall survival rate of 6% (American Cancer Society 2020). Because of early and rapid metastasis, SCLC patients rarely benefit from surgery, and responses to chemotherapy and radiation are typically brief. In contrast to non-small-cell lung cancer (NSCLC), in which genotype-directed therapies have dramatically improved treatment outcomes for thousands of patients, there are no clear kinase targets in SCLC, no approved targeted therapies (Shtivelman et al. 2014; Byers and Rudin 2015), and the repurposing of existing drugs has failed to demonstrate meaningful clinical impact (Kalemkerian 2014). Thus, there is a critical need for identification of

novel therapeutic targets and strategies in SCLC. Indeed, SCLC was designated a “recalcitrant cancer” by the National Cancer Institute (NCI) under the Recalcitrant Cancer Research Act (RCRA) of 2013, highlighting the urgency for development of improved preclinical models and therapeutic strategies in this disease.

Although SCLC has been viewed as a monolithic disease with a grim prognosis, this is poised to change thanks to the rapid expansion in research following passage of the RCRA. The purpose of this review is to highlight the advances in six areas of SCLC research:

1. Development of new patient-derived models;
2. SCLC genomic landscapes and oncogenic insights from mouse models;

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3. Investigation of intratumoral heterogeneity;
4. Cell of origin and mechanism of transdifferentiation from adenocarcinoma;
5. Chemoresistance and DNA damage repair; and
6. Immune evasion and immunotherapy.

EXPANSION OF HUMAN DISEASE MODELS FROM BIOPSIES AND CIRCULATING TUMOR CELLS

The basic properties of SCLC growth were elucidated from panels of cell lines developed from 1971 through the early 1990s (Oboshi et al. 1971; Carney et al. 1980; Pettengill et al. 1980; Baillie-Johnson et al. 1985). The largest of these efforts comprised 122 SCLC lines derived from patients treated at the NCI–Department of Vet-

erans Affairs (VA) and NCI Navy Medical Oncology Branches during this time frame (Gazdar and Minna 1996). These cell lines have been distributed globally, and their molecular and clinical features have been extensively annotated. They remain the primary resource for studying this disease.

Questions tied to specific clinical outcomes, such as chemoresistance following relapse, require model fidelity to a relevant clinical history (Fig. 1). Although the clinical histories associated with the NCI series of SCLC cell lines were carefully annotated, staging technology and drug regimens have changed significantly since the 1980s. Only nine NCI cell lines were derived following platinum plus etoposide, while the majority were derived from patients who received cyclophosphamide-based regimens (Johnson et al. 1996). Furthermore, the molec-

Figure 1. Frequently used in vivo models of small-cell lung cancer (SCLC). Cell-line-derived xenografts (CDXs) have been a mainstay of SCLC research for many years. These have advantages including speed, number of available models, and ease of use. Genetically engineered mouse models have greatly expanded in number and complexity. These have advantages including immunocompetence, in situ oncogenesis, and ability to definitively test the role of individual drivers. The number of available patient-derived xenograft (PDX) and CDX models is also rapidly increasing in laboratories around the world. These have advantages including genetic and epigenetic proximity to human disease, heterogeneity, and links to individual patient outcomes. An additional advantage of CDX is the broad availability of blood as a source for model generation.

ular features that distinguish clinical outcomes, such as correlation between in vitro chemosensitivity and whether the line was derived from an untreated or postrelapse patient, were not consistently retained (Polley et al. 2016). In addressing current clinical questions, it is particularly useful to have high-fidelity models from current or recent patients.

Patient-derived xenograft (PDX) models are generated via the direct implantation of tumor material, usually from surgical resections, biopsies, or effusions, into immunocompromised mice with no intermediate in vitro culture (Hidalgo et al. 2014; Aparicio et al. 2015). Clinical development of BH3 mimetics exemplifies their utility as preclinical models of SCLC. BCL2/BCLxL inhibitors showed dramatic efficacy in a series of SCLC cell-line models both in vitro and in vivo (Oltersdorf et al. 2005; Shoemaker et al. 2008). In contrast, SCLC PDX models generally demonstrate minimal single-agent sensitivity to the BCL2/BCLxL inhibitor ABT-737 (Hann et al. 2008). Entirely in keeping with the PDX result, a phase II clinical study of navitoclax, a related compound, in relapsed SCLC patients showed only one partial response in 39 patients (Rudin et al. 2012a). Artifacts of in vitro selection may underlie this difference in clinical fidelity, as marked and irreversible changes in gene expression have been observed during cell-line derivation from SCLC PDX models (Daniel et al. 2009). These studies suggest that PDX models might better recapitulate the expression profiles and drug sensitivities of SCLC patient tumors than xenografts from established cell lines.

Historically, generation of SCLC PDX models has been hindered by scarcity of viable tumor samples. SCLC is almost never surgically resected, and invasive tumor sampling is usually not clinically indicated following diagnosis. Circulating tumor cells (CTCs) can be sampled noninvasively (Yu et al. 2011, 2013; Haber and Velculescu 2014), and are highly abundant in SCLC patients (Bevilacqua et al. 2009; Hou et al. 2009, 2012; Naito et al. 2012). Change in CTC number correlates closely with response to chemotherapy, suggesting that CTCs mirror SCLC tumor biology. However, isolation tech-

nologies often rely on either chemical fixation, which would render CTCs nonviable, or positive enrichment by EpCAM expression, which would exclude nonepithelial CTCs (Calbo et al. 2011; Yu et al. 2014). Enrichment of live unmanipulated SCLC CTCs was first accomplished by depletion of nonmalignant nucleated cells with an antileukocyte antibody cocktail, and for SCLC patients with high tumor burdens, these CTC-enriched blood products yielded PDX models with high efficiency (Hodgkinson et al. 2014). CTC-derived PDX models were histologically similar to patient tumors, and copy number alterations in the models mirrored the parent CTCs. A similar approach using a microfluidic-based live CTC capture platform, the CTC-iChip (Ozkumur et al. 2013; Karabacak et al. 2014), was used to generate a large cohort of PDX models with high efficiency (35% per blood draw) (Drapkin et al. 2018). In contrast with established SCLC cell lines (Polley et al. 2016), PDX sensitivity to first-line cisplatin/etoposide (EP) was strongly correlated with observed tumor response in the corresponding patient (Drapkin et al. 2018), suggesting that these models had sufficient clinical fidelity to study chemoresistance.

Scarcity of clinical samples was previously an obstacle to PDX establishment, but this has been overcome by development of new techniques, and recognition of the capacity of even small numbers of SCLC cells to engraft in foreign environments. At least 66 PDX models of SCLC have been reported (Némati et al. 2010; Saunders et al. 2015; Gardner et al. 2017; Drapkin et al. 2018; Lallo et al. 2018), and many more have been established. Their molecular and functional fidelity to the originating patient's disease makes them important tools for preclinical testing. However, PDX models have significant limitations, most notably: (1) they are inherently low throughput, as short-term cultures still require an originating xenograft (Lallo et al. 2019); (2) they are difficult to manipulate genetically, although a recently reported CRISPR-Cas9 system in NSCLC PDX models offers a strategy to address this obstacle (Hulton et al. 2020); (3) they require a severely immunosuppressed microenvironment to grow; and (4)

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their clinical relevance may decline over time as management of SCLC advances. The standard of care first-line therapy in the United States is now EP + atezolizumab (Horn et al. 2018). No models have yet been reported that represent relapse following durable response to this regimen, and the immune checkpoint blockade will be a challenge to study without development of at least partially immunocompetent PDX models.

SCLC GENOMIC LANDSCAPES AND ONCOGENIC INSIGHTS FROM MOUSE MODELS

The genomic hallmarks of SCLC, biallelic loss of *RB1* and *TP53*, are so prevalent within the disease that they cannot define subclasses, and drugs have not been developed to target the absence of either tumor suppressor. Recent genomic profiles of primary patient samples have revealed additional recurrent alterations. These have been recapitulated using genetically engineered mouse models (GEMMs) to assess functional significance, with the ultimate goal of developing subtype-specific targeted therapies.

Genomics

The first SCLC genome, reported in 2010, identified a mutation signature associated with tobacco smoking in the cell line NCI-H209 (Pleasant et al. 2010). This was rapidly followed by two whole-exome sequencing studies, representing 36 and 29 patients, respectively (Peifer et al. 2012; Rudin et al. 2012b). The largest effort to date scoured global tissue repositories to obtain 110 fresh-frozen samples suitable for whole-genome sequencing (George et al. 2015). For these studies, tumor samples were obtained almost exclusively from untreated patients and enriched for early-stage disease.

Loss of *RB1* and *TP53* were first identified as common recurrent alterations in SCLC in established cell lines (Harbour et al. 1988; Hensel et al. 1990, 1991), and this has been confirmed in genome-sequencing studies of primary tumors. Whole genome sequencing revealed universal biallelic inactivation of *TP53* (110/110

cases), and loss of *RB1* was more common than previously reported (108/110 cases) due to detection of complex genomic rearrangements (George et al. 2015). The two exceptional *RB1*^{WT} cases harbored chromothripsis events that resulted in massive overexpression of cyclin D1, underscoring the requirement for RB inactivation in SCLC. Recurrent mutations were identified in the RB-related tumor suppressors *RBL1* (p107) and *RBL2* (p130), both of which can also bind E2F transcription factors (Cao et al. 1992; Dyson et al. 1992; Cobrinik et al. 1993; Schwarz et al. 1993).

In addition to loss of *RB1* and *TP53*, genome sequencing revealed recurrent amplification of *BCL2* and the MYC family of proto-oncogenes (*MYC*, *MYCL1*, and *MYCN*) and loss of the tumor suppressor *PTEN* in a small but significant fraction of cases (Peifer et al. 2012; Rudin et al. 2012b; George et al. 2015). Although these genomic features had been previously described in cell lines (Little et al. 1983; Nau et al. 1985, 1986; Ikegaki et al. 1994; Kim et al. 1998; Olejniczak et al. 2007), their prevalence in primary samples had not been evaluated. New recurrent alterations were identified in the histone-modifying enzymes *CREBBP*, *EP300*, and *MLL*, amplifications of *SOX2* and *FGFR1*, amino-terminal dominant-negative truncations in *TP73* (Jost et al. 1997; Flores et al. 2002; Stiewe et al. 2002), and mutually exclusive inactivating mutations in the extracellular domains of *NOTCH* receptors. The functional significance of these recurrent genomic alterations has been established primarily through conditional knockout or expression in autochthonous mouse models (murine SCLC [mSCLC]).

RBL2 (p130), *PTEN*, and *NFIB*

The original mSCLC model carried conditional homozygous knockout alleles of *Rb1* and *Trp53* (*Rb1*^{fl/fl}; *Trp53*^{fl/fl}, herein “RP”), and tumors were generated in lung epithelial cells by intratracheal instillation of Adeno-Cre (Meuwissen et al. 2003). These mice develop dysplastic precancerous lesions that slowly progress to mSCLC over 10–15 months, and expression of a single wild-type allele of either *Rb1* or *Trp53* results instead

in adenocarcinoma formation after 1–2 years. During the long latency, *RP* tumors can acquire *Mycl* or *Nfib* amplifications, or *Pten* loss (Calbo et al. 2005, 2011; Dooley et al. 2011; McFadden et al. 2014). All three alterations are recurrent in human SCLC, with *NFIB* expression enhanced in metastatic lesions (Denny et al. 2016). Additional knockout of either *Rbl2* (p130) or *Pten*, or constitutive expression of *Nfib*, within the *RP* background accelerates mSCLC tumorigenesis significantly (Schaffer et al. 2010; Cui et al. 2014; Wu et al. 2016), with *RP-Pten* tumors containing mixed adenocarcinoma components (Gazdar et al. 2015). These experiments demonstrated tumor-suppressor functions for *p130* and *Pten* and an oncogenic function for *Nfib* in mSCLC.

MYCL and MYC

SCLC tumors harbor recurrent amplifications of *MYC*, *MYCL*, or *MYCN*, as well as inactivating mutations in *MYC*-regulatory factors *MAX*, *MGA*, and *BRG1* (Romero et al. 2014). Although these alterations are mutually exclusive, implying some redundancy, they result in tumors with distinct morphologies, expression profiles, and gene dependencies. Constitutive expression of *Mycl1* in the *RP* background accelerates mSCLC growth (Kim et al. 2016), resulting in tumors with a “classic” SCLC morphology first identified in cell-line-derived xenografts: small round cells with scant cytoplasm, fine granular chromatin, and indistinct nucleoli (Gazdar et al. 1985, 2015). In contrast, expression of a stabilized *Myc* allele (*Myc^{T58A}*) results in highly aggressive mSCLC with variant morphology similar to *MYC*-amplified human SCLC, including reduced expression of neuroendocrine (NE) markers (Mollaoglu et al. 2017) and increased dependence on both Aurora kinase (Hook et al. 2012; Sos et al. 2012) and arginine biosynthesis (Chalishazar et al. 2019).

CREBBP

Conditional knockout of *Crebbp* in the *RP* background (*RP-Crebbp*) also accelerated mSCLC tumorigenesis (Jia et al. 2018). Although *RP-*

Crebbp tumors have classic SCLC histology, loss of histone acetyltransferase (HAT) activity leaves histone deacetylases (HDACs) unopposed, leading to partial epithelial-to-mesenchymal transition (EMT). Treatment with HDAC inhibitors reversed this effect and caused *RP-Crebbp* regression.

ASCL1, NEUROD1, NOTCH, and POU2F3

ASCL1 and NEUROD1, two basic-loop-helix transcription factors with roles in neuronal development, are differentially expressed in SCLC cell lines and act as essential, lineage-defining oncogenes (Guillemot et al. 1993; Yasunami et al. 1996; Borges et al. 1997; Osada et al. 2005; Jiang et al. 2009; Osborne et al. 2013; Augustyn et al. 2014; Borromeo et al. 2016). *ASCL1^{high}* lines and xenografts demonstrate classic morphology, high NE expression, and frequent *MYCL* amplification, whereas *NEUROD1^{high}* SCLC demonstrates variant morphology, *MYC* amplification, and selective tropism for the oncolytic Seneca Valley Virus (Carney et al. 1985; Gazdar et al. 1985; Poirier et al. 2013; Borromeo et al. 2016). *RP-p130* mSCLC is dependent on *Ascl1* but not *Neurod1* (Borromeo et al. 2016), whereas *RP-Myc^{T58A}* tumors preferentially express *Neurod1* (Mollaoglu et al. 2017). During neuronal development, NOTCH represses *ASCL1* to restrict progenitor differentiation (Meredith and Johnson 2000; Sriuranpong et al. 2002), and in lung development NOTCH suppresses both NE differentiation and expression of *ASCL1* and *NEUROD1* (Chen et al. 1997; Ito et al. 2000). Primary SCLC tumors reflect this relationship, as *ASCL1* expression correlates with *DLK1*, an inhibitory NOTCH ligand (George et al. 2015). In the *RP-p130* model, expression of the NOTCH intracellular domain (NICD) delays tumorigenesis, directly demonstrating tumor-suppressor function. An inhibitor of the histone demethylase LSD1 has shown selective activity in SCLC models, and acts through NOTCH reactivation and repression of *ASCL1* (Augert et al. 2019).

A subset of SCLC lacks expression of either *ASCL1* or *NEUROD1* (George et al. 2015; Borromeo et al. 2016), and some of these double-

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negative tumors are highly dependent on *POU2F3*, a transcription factor required for chemosensory tuft-cell development (Huang et al. 2018). *POU2F3*^{high} SCLC tumors lack typical NE markers, and their divergent expression profile and gene dependencies suggest that this subtype may arise from a distinct cell of origin. A classification scheme for SCLC has been proposed based on dominant expression of one of four transcriptional regulators: *ASCL1*, *NEUROD1*, *POU2F3*, and *YAP1* (Rudin et al. 2019). Prospective evaluation of these classes to predict responses to therapy may pave the way for subtype-specific clinical interventions.

Need for More Genomic Sequencing

Although significant progress has been made, there may be important recurrent genomic alterations in SCLC yet to be discovered. After *RB1* and *TP53*, no recurrent alterations have been reported in more than 25% of SCLC, with the majority present in 2%–10% of the population (Peifer et al. 2012; Rudin et al. 2012a; Umemura et al. 2014; George et al. 2015; Iwakawa et al. 2015; Sundaresan et al. 2017). Given a somatic mutation burden of ~10 per Mb, ~900 samples would be required to saturate for mutations with 5% recurrence, and more than 3000 samples to saturate at 2% (Lawrence et al. 2014). The largest genome-sequencing study published to date encompasses 110 samples, which may not fully saturate for mutations with 30% recurrence. To accumulate this, many samples will likely require routine CTC collection and expansion, or the advent of clinical rebiopsy, as in NSCLC.

FLOATERS, STICKERS, AND INTRATUMORAL HETEROGENEITY

Despite clonality, most tumors harbor significant cellular heterogeneity, which has been implicated in tumorigenesis, metastasis, and therapeutic resistance. Challenges in assessing the roles and importance of intratumoral heterogeneity include distinguishing stochastic variation from recurrent subpopulations of clinical

significance, and developing strategies to selectively target key subpopulations (Table 1).

Floater and Stickers

The initial efforts at SCLC cell-line derivation revealed subpopulations within individual tumors that were not just molecularly but mechanically distinct (Carney et al. 1985; Gazdar et al. 1985). Cell lines grew as either suspension aggregates or adherent monolayers, and in some cases as mixtures of both floating and adherent cells (Carney et al. 1985). For mixed cell lines, subculturing of these components revealed that suspension cells expressed high levels of NE markers, whereas adherent cells were non-neuroendocrine (non-NE) (Doyle et al. 1990). In parallel, a study of clonally derived cell lines revealed antigenic heterogeneity that would regenerate upon expansion from a single cell (Fargion et al. 1986). These studies suggest that within individual SCLC tumors there are characteristic and heritable patterns of differentiation into subpopulations.

NE versus Non-NE

Since these early descriptions of intratumoral heterogeneity, several recurrent SCLC subpopulations have been described. Most distinguish NE and non-NE populations, but they may not all be mutually exclusive. In the *RP* mSCLC GEMM, it was observed that single cells from the same tumor could give rise to either NE suspension or non-NE adherent mesenchymal cell lines that retained these morphologies upon expansion to xenografts (Calbo et al. 2011). However, co-culture increased proliferation in vitro, and mixed xenografts gave rise to metastases due to paracrine Fgf2 signaling from non-NE cells (Kwon et al. 2015). These mixing studies suggested that cooperativity between subpopulations is an essential feature of SCLC biology.

NOTCH

Although genomic and functional studies suggest that *NOTCH* acts as a tumor suppressor (Sriuranpong et al. 2001; Wael et al. 2014;

Table 1. Recurrent subpopulations in small-cell lung cancer (SCLC)

Functional phenotype	Cell type	Neuroendocrine expression	In vitro growth	Molecular phenotype	References
Tumor-propagating cells (TPCs)	Epithelial	Positive	Suspension	CD44 ⁺ Mycl1 ⁺	Jahchan et al. 2016
Paracrine proliferation, chemoresistance	Epithelial	Negative	Adherent	CD44 ⁺ Notch ⁺	Lim et al. 2017
Paracrine proliferation, metastasis	Mesenchymal	Negative	Adherent	CD44 ⁺ Fgf2 ⁺	Calbo et al. 2011; Kwon et al. 2015
Vasculogenic mimicry, chemoresistance	Epithelial	Negative	Unknown	VE-cadherin ⁺ CD31 ⁺	Williamson et al. 2016

George et al. 2015), SCLC cell lines and tumors demonstrate variable but significant levels of NOTCH expression and activity (George et al. 2015; Kaur et al. 2016; Zhang et al. 2018). To investigate intratumoral heterogeneity of Notch signaling, the *RP-p130* GEMM was engineered with a single-cell reporter for *Notch* activity: *GFP* under control of the endogenous *Hes1* promoter (Lim et al. 2017). Notch^{active} cells were adherent, slow-growing, and non-NE, but expressed epithelial rather than mesenchymal markers (Calbo et al. 2011). Rapidly proliferating NE cells in suspensions were Notch^{inactive}, but could be irreversibly converted to the Notch^{active} state by Notch ligand on neighboring cells. Mixing of the cell types increased survival, and Notch^{active} cells were relatively resistant to chemotherapy. These results suggest a second intratumoral cooperative relationship that promotes growth and chemoresistance, with Notch signaling inducing non-NE differentiation.

Tumor-Propagating Cells

Two subpopulations of non-NE cells have been described that established a favorable microenvironment for the NE majority. Within the NE cells, a CD24^{high} CD44^{low} EpCAM^{high} subpopulation was identified that was 100-fold more efficient at tumorigenesis than other immunophenotypes (Jahchan et al. 2016). These tumor-propagating cells (TPCs) expressed high levels of *Mycl1*, and although knockdown of *Mycl1* did not significantly affect proliferation, it did reduce tumorigenicity.

Vasculogenic Mimicry

A fourth functional subpopulation was discovered in SCLC PDX models that expressed the endothelial marker VE-cadherin (Williamson et al. 2016). In these xenografts, vascular networks were negative for murine CD31, suggesting that they were tumor-derived. Vasculogenic mimicry (VM), in which cancer cells undergo endothelial differentiation to form neovasculature, was first reported in uveal melanoma and has since been identified in a variety of solid tumors (Maniotis et al. 1999; Hendrix et al. 2003; Seftor et al. 2012). In human SCLC tumors with VM⁺ vasculature, knockdown of VE-cadherin impaired growth and increased sensitivity to chemotherapy.

Subclonal Mutations

Despite the high mutational burden in SCLC, estimates of intratumoral genomic heterogeneity have been surprisingly low (Imielinski et al. 2012; George et al. 2015). These estimates were corroborated by comparison of PDX model genomes with originating patient tumors, which were nearly superimposable whether derived from biopsies or CTCs (Drapkin et al. 2018). However, recent efforts to compare pretreatment and postrelapse samples of circulating tumor DNA (ctDNA) (Nong et al. 2018) or CTCs (Su et al. 2019) have suggested significant clonal heterogeneity and emergence of genomically distinct subclones with therapy. More genomic studies will be required to elucidate the contri-

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butions of clonal heterogeneity to chemoresistance.

Significance of Subpopulations

Cooperative subpopulations with divergent expression of NE markers have been identified in multiple studies, but their relationship to intertumoral heterogeneity remains unclear. Global differences between SCLC tumors may be the consequence of varying ratios of recurrent cell types, which may be influenced by clonal mutations such as those in *NOTCH1-4*. Identification of subpopulations responsible for drug resistance will be particularly important. The next steps to investigate intratumoral heterogeneity, such as multilineage tracing experiments in GEMMs (Yang et al. 2018) and single-cell catalogues of multiple cases to define recurrent subpopulations (Su et al. 2019; Stewart et al. 2020) are now being reported. It will also be important to determine whether an underlying feature of SCLC is responsible for its intrinsic intratumoral heterogeneity. Loss of *RB1* may be partially responsible, as RB can regulate lineage commitment (Ku et al. 2017; Mu et al. 2017) and inhibit pluripotency networks (Kareta et al. 2015; Dyson 2016; Dick et al. 2018); and in lung adenocarcinoma, deletion of *RB1* has been shown to promote lineage plasticity (Walter et al. 2019).

CELL OF ORIGIN AND TRANSDIFFERENTIATION

In contrast to NSCLC, current early detection strategies are ineffective for SCLC even among high-risk populations. Most SCLC cases detected by computed tomography (CT) screening are already late stage, and many progress so rapidly that they manifest within the screening interval (National Lung Screening Trial Research Team et al. 2011; Aberle et al. 2013; Pinsky et al. 2013; Silva et al. 2016; Thomas et al. 2018). This explosive growth and early dissemination precludes detection of precancerous lesions that could reveal the cell of origin, which remains unknown. Loss of *RB1* and *TP53* are necessary

for tumorigenesis, but without knowing the cellular context their specific roles in driving oncogenesis remain incompletely defined.

Pulmonary Neuroendocrine Cells

In the *RP* GEMM, SCLC arises most efficiently from pulmonary NE cells (PNECs), but may arise in other contexts as well (Fig. 2; Sutherland et al. 2011). Tissue-specific promoters were used in an attempt to restrict deletion of *Rb1* and *Trp53* in three cell types: (1) club cells, which line the trachea, bronchi, and small airways; (2) PNECs, which are dispersed throughout intrapulmonary airways and cluster at airway junctions; and (3) type 2 alveolar cells (AT2). SCLC arose with greatest efficiency from PNECs, but could also arise more slowly from AT2 cells and very rarely from club cells. The true specificity of these promoters remains unclear, as a complementary study using different constructs revealed a similar high efficiency of mSCLC tumorigenesis from PNECs but not from AT2 cells (Park et al. 2011).

In a powerful complementary approach, human embryonic stem cells (hESCs) were induced to differentiate into lung precursors (LPs), and combinations of putative SCLC drivers were tested (Chen et al. 2019). NOTCH inhibition could induce LPs to differentiate into PNECs, and in these cells knockdown of *RB1* induced nonmalignant proliferation. Combined inactivation of NOTCH, *RB1*, and *TP53* drove formation of xenografts that grossly resembled SCLC, establishing a minimal set of oncogenic events. This mechanism of tumorigenesis was strongly reinforced by the recent finding that following mouse lung epithelial injury, a subset of cells in neuroendocrine bodies (NEBs) activate *Notch* signaling, proliferate, migrate to the site of injury, and differentiate to restore the surrounding normal epithelium (Ouadah et al. 2019). This subset of NE stem cells (NE^{stem}) can be prevented from reentering quiescence with ablation of *Rb1* and *Trp53*, and remain both proliferative and migratory. Taken together, these mouse and hESC studies suggest that PNECs are the principal cell of origin for SCLC.

Figure 2. Cell of origin versus destination phenotype. Multiple cells of origin have been implicated as contributing to small-cell lung cancer (SCLC). As described in detail in the text, current models point to pulmonary neuroendocrine cells (PNECs) as a primary cell of origin; recent data also implicates a second cell, the tuft cell, as a putative progenitor of the POU2F3-driven subtype of SCLC. Lung adenocarcinoma can transdifferentiate to an SCLC phenotype through lineage plasticity under targeted therapy selection, and what appears to be an analogous pathway to small-cell neuroendocrine cancers has been defined for extrapulmonary adenocarcinomas, most notably prostate cancer.

Lineage Plasticity and Transdifferentiation

Normal healthy lung does not undergo rapid cell turnover. However, following epithelial injury, robust tissue regeneration occurs, and during this process there is a high degree of lineage plasticity (Tata and Rajagopal 2017). Examples from diverse lung injury models include restoration of the multipotent basal cell compartment from club cells (Tata et al. 2013), repopulation of AT2 progenitors from AT1 cells (Jain et al. 2015), and regeneration of club and ciliated cells from PNECs in a Notch-dependent process (Yao et al. 2018). Lineage plasticity within nonmalignant pulmonary epithelium raises the possibility that for SCLC, NE differentiation could occur during or even after tumorigenesis. This possibility is reinforced by the phenome-

non of transdifferentiation of adenocarcinomas to small-cell neuroendocrine cancers (SCNCs).

EGFR tyrosine kinase inhibitors (TKIs) are highly effective therapies for lung adenocarcinomas with activating *EGFR* mutations, but they are not curative (Mok et al. 2009, 2017; Soria et al. 2018). In 3%–10% of cases, TKI-resistant tumors assume the histologic characteristics of SCLC (Sequist et al. 2011; Yu et al. 2013a; Piotrowska et al. 2018; Bordi et al. 2019), and, in these cases, *RB1* and *TP53* are inactivated and mutant *EGFR* expression is low-to-absent (Niederst et al. 2015; Lee et al. 2017). Clinical outcomes following transdifferentiation are similar to de novo SCLC, with initial chemosensitivity followed by relapse and progressive chemoresistance (Marcoux et al. 2019). *EGFR*-mutant SCLC mirrors the molecular, phenotypic, and

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clinical characteristics of smoking-related *EGFR*^{WT} SCLC, including lack of dependence on the *EGFR* oncogene.

However, it is not clear when transdifferentiation occurs. Comparison of serial biopsies before and after transdifferentiation suggests that the SCLC subclone diverges before TKI therapy, and loss of *RB1* and *TP53* are early events (Lee et al. 2017). Consistent with early divergence, the majority of SCLCs that emerge following third-generation TKIs for *EGFR*^{T790M} resistance lack the T790M gatekeeper mutation (Marcoux et al. 2019). Furthermore, SCLC with activating *EGFR* mutations can also arise de novo in non-smokers (Varghese et al. 2014; Le et al. 2015; Sun et al. 2015). A similar phenomenon has been observed in prostate cancer that becomes castrate-resistant through NE differentiation (CRPC-NE) and loses androgen receptor expression (Beltran et al. 2011, 2016; Tzelepi et al. 2012; Tan et al. 2014), *RB1* and *TP53* (Ku et al. 2017; Mu et al. 2017). As with lung adenocarcinoma, reconstructed clonal evolution trees from serial biopsies suggests early divergence of the CRPC-NE precursor (Beltran et al. 2016).

Origin versus Destination

SCNCs arise frequently in lung, prostate, and bladder, and have been reported in nearly every organ (Frazier et al. 2007). The histopathology of these cancers appears very similar despite diverse and anatomically distinct cells of origin. Recent pancancer bioinformatic analyses have extended this observation to integrate genomic, transcriptional, and epigenetic data (Smith et al. 2018; Balanis et al. 2019). Principal component analysis revealed that while transcriptomes from normal lung, prostate, and bladder form widely separated clusters, adenocarcinomas are more similar and SCNCs overlap completely (Balanis et al. 2019). SCNCs are distinguished by the expression of adult stem cell markers, and at the core of this expression signature are targets of DNA methyltransferases (DNMTs) that regulate cell fate and apoptosis, including *ASCL1* (Smith et al. 2018). Convergence of gene expression suggests that a common set of oncogenic events may lead to SCNCs. This was tested di-

rectly in primary cell culture transformation assays, in which inactivation of *RB1* and *TP53* combined with activation/overexpression of *AKT1*, *MYC*, and *BCL2* could induce either prostate basal cells or bronchial epithelial cells to form SCNC xenografts (Park et al. 2018). Although derived from different organs, the transcriptomes and chromatin landscapes of these SCNCs were nearly indistinguishable. Together, these studies raise the question of whether SCNCs are best understood as the consequence of oncogenic transformation of a single cell of origin, or as a destination cancer phenotype that can be reached from multiple tissues.

CHEMORESISTANCE AND DNA DAMAGE REPAIR

DNA-damaging chemotherapy and ionizing radiation are the mainstays of both local and systemic control of SCLC. Untreated SCLC is highly sensitive to first-line platinum plus etoposide, with clinical response rates that nearly double those of NSCLC (Sandler et al. 2006; Scagliotti et al. 2008; Rossi et al. 2012; Socinski et al. 2012; Patel et al. 2013; Horn et al. 2018; Owonikoko et al. 2019). However, relapse and resistance inevitably arise, and response rates to second-line agents are far lower because of the emergence of cross-resistance (Owonikoko et al. 2012). To discover new therapeutic targets, protein expression was compared between SCLC and NSCLC cell lines by reverse phase protein array (RPPA), and high expression of PARP1 and EZH2 were identified as features of SCLC (Byers et al. 2012). These expression features have contributed to two strategies to exploit the vulnerability of SCLC to DNA damage: (1) combination of DNA-damaging agents with inhibitors of repair, such as PARP inhibitors; and (2) direct reversal of chemoresistance through EZH2 inhibition (Fig. 3).

PARP Inhibitors

PARP1/2 activity mediates both single- and double-stranded DNA break repair (Ray Chaudhuri and Nussenzweig 2017), and PARP inhibitors are effective in tumors with impaired

Figure 3. DNA damage checkpoints and vulnerabilities in small-cell lung cancer (SCLC). The universal inactivation of the tumor-suppressor genes *TP53* and *RB1* in SCLC disrupts several key cell-cycle checkpoints, affecting cell-cycle entry from G₁ to S, response to DNA damage in S phase, and commitment to mitotic entry in G₂. The loss of these checkpoints may increase dependence on remaining checkpoint pathways, creating a synthetic vulnerability in SCLC to inhibitors of DNA damage repair pathways. Critical factors lost in SCLC are indicated in red, additional notable factors controlling cell-cycle progression are indicated in blue, and targeted therapies being explored in SCLC are boxed in black.

homologous recombination (Turner et al. 2004; Bryant et al. 2005; Farmer et al. 2005; Fong et al. 2009; Audeh et al. 2010; Tutt et al. 2010; Li and Yu 2013). SCLC cell lines are highly sensitive to PARP inhibitors despite little evidence of defective DNA repair (George et al. 2015; Lok et al. 2017). In addition to catalytic inhibition, PARP inhibitors can block the release of PARP complexes from single-stranded breaks, resulting in “trapped” complexes that interfere with both DNA replication and transcription (Murai et al. 2012, 2014; Hopkins et al. 2015). PARP inhibitors vary in trapping efficiency, and the most efficient, such as talazoparib, synergize strongly with both DNA-damaging agents and ionizing radiation in SCLC (Murai et al. 2014; Lok et al. 2017; Laird et al. 2018). Clinical trial data reflects this combinatorial efficacy. Whereas single-agent PARP inhibitors were ineffective in SCLC patients, addition of the PARP inhibitor veliparib to first-line cisplatin/EP resulted in a modest but significant increase in progression-free survival (PFS) (de Bono et al. 2017; Woll et al. 2017; Owonikoko et al. 2019). In relapsed SCLC, combination of veliparib with temozolo-

mid (TMZ) showed a marked increase in response rate (39% veliparib/TMZ vs. 14% placebo/TMZ) (Pietanza et al. 2018), and a similarly high response rate was observed with olaparib/TMZ (42%) in a single-arm study (Farago et al. 2019). PARP inhibitor combinations are active in unstratified SCLC patients, but predictive biomarkers will likely be required to maximize clinical efficacy.

SLFN11 and Chemosensitivity

An unbiased cell line screen for biomarker candidates for topoisomerase I (TOP1) revealed that Schlafen 11 (SLFN11) expression strongly correlated with sensitivity to a broad spectrum of DNA-damaging agents and inhibitors of repair (Zoppoli et al. 2012). This correlation has since been confirmed in both pancancer and SCLC-specific cell line screens (Barretina et al. 2012; Garnett et al. 2012; Sousa et al. 2015; Polley et al. 2016; Reinhold et al. 2017). Suppression of SLFN11 confers resistance to DNA damage (Nogales et al. 2016; Tang et al. 2018), and in the setting of replication stress SLFN11 appears to

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irreversibly block stalled replication forks, resulting in cytotoxic double-stranded breaks (Murai et al. 2018). SLFN11 is expressed in approximately half of all cancer cell lines, including many SCLCs (Barretina et al. 2012; Murai et al. 2016; Lok et al. 2017). This bimodal expression pattern has facilitated development of an immunohistochemistry (IHC) assay, and among SCLC PDX models sensitivity to talazoparib correlated with positive IHC staining (Lok et al. 2017). Retrospective analysis of the randomized phase II trial of veliparib plus TMZ revealed a substantial increase in PFS for SLFN11 IHC⁺ patients receiving the combination (mPFS 5.7 vs. 3.6 months), but not TMZ plus placebo (Pietanza et al. 2018). If SLFN11 is prospectively validated for patient selection, veliparib/TMZ may represent the first biomarker-targeted therapy for SCLC.

EZH2 and Chemoresistance

SLFN11 expression correlates with sensitivity to a broad spectrum of DNA-damaging agents, including EP, and its expression is frequently silenced via promoter methylation, histone methylation, and histone deacetylation (Barretina et al. 2012; Zoppoli et al. 2012; Nogales et al. 2016; Stewart et al. 2017; Reinhold et al. 2017; Tang et al. 2018). Expression of the histone methyltransferase EZH2 is a distinguishing feature of SCLC (Byers et al. 2012; Poirier et al. 2015). To discover mediators of acquired chemoresistance, 10 PDX models derived from chemosensitive SCLC patients were treated in vivo with EP until full resistance emerged (Gardner et al. 2017). Decreased SLFN11 expression was among the most significant changes, and EZH2 inhibition both restored SLFN11 expression and prolonged efficacy of EP. Among the PDX models with unchanged SLFN11 levels, recurrent up-regulation of TWIST was observed, consistent with a mesenchymal expression profile reported in chemoresistant SCLC cell lines (Stewart et al. 2017). In PDX models derived from both chemo-naïve and postrelapse patients, high levels of MYC-regulated transcripts and low basal expression of inflammatory-response genes correlated with intrinsic resistance to EP as well as

other DNA-damaging agents (Drapkin et al. 2018; Farago et al. 2019), and single-cell analysis of PDX models revealed increasing intratumoral heterogeneity with resistance (Stewart et al. 2020).

Relapsed SCLC

Following diagnostic biopsy, SCLC is rarely sampled, and as a result most information about the biology of human tumors has been gleaned from untreated patients (Peifer et al. 2012; Rudin et al. 2012b; George et al. 2015). This is beginning to change. In a single-institution study, postrelapse tumor samples were obtained from 30 patients, including 12 patients with paired pretreatment samples (Wagner et al. 2018). Recurrent WNT pathway alterations were observed, and APC inactivation conferred resistance to EP in SCLC cell lines. A CTC-based classifier that distinguishes platinum-sensitive from resistant patients remains unchanged in paired postrelapse samples, suggesting that de novo and acquired resistance mechanisms may be distinct (Carter et al. 2017). CTC and ctDNA analyses may be the fastest way to assemble a well-powered cohort for a comprehensive survey of relapsed SCLC.

The rapid progress in targeting DNA damage sensitivity in SCLC has inspired numerous ongoing clinical trials, but important questions remain. Although SCLC has a high mutational burden, and loss of RB can contribute to genomic instability (Manning et al. 2010, 2014; Cook et al. 2015; Vélez-Cruz et al. 2016), it is not clear that SCLC tumors are genomically unstable. Low clonal heterogeneity and infrequent acquisition of new mutations with passaging of PDX models or in serially collected primary tumor samples argue for a surprising degree of genomic stability (George et al. 2015; Ben-David et al. 2017; Drapkin et al. 2018; Wagner et al. 2018). In light of these observations, an explanation for the extreme sensitivity of untreated SCLC to DNA damage remains to be elucidated, as DNA damage repair appears to be generally intact. Direct comparison of pre- and post-treatment models from patients with significant clinical responses may provide insight into

the relevant mechanisms. Finally, it is unclear whether resistance to DNA damage arises from a preexisting subpopulation of cells or is acquired stochastically during therapy. Discovery of resistant subpopulations may be facilitated by classification of SCLC tumors at the single-cell level.

IMMUNOSUPPRESSIVE PHENOTYPE AND IMMUNOTHERAPY

T-cell activation requires both antigen presentation and a costimulatory signal (Bretscher 1999). Activation by self-antigens is averted by coinhibitory signals through receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Tivol et al. 1995; Waterhouse et al. 1995) and programmed death protein 1 (PD-1) (Nishimura et al. 1999; Freeman et al. 2000; Latchman et al. 2001). Tumors may co-opt the machinery of self-tolerance by expressing PD-1 ligand (PD-L1) (Dong et al. 2002; Iwai et al. 2002), and reactivation of costimulatory signaling with immune checkpoint inhibitors (ICIs) such as humanized antibodies against CTLA-4 (e.g., ipilimumab, tremelimumab), PD-1 (pembrolizumab, nivolumab), and PD-L1 (atezolizumab, durvalumab) (Abril-Rodriguez and Ribas 2017) has proven to be highly effective at enhancing antitumor immunity (Allison et al. 1995; Leach et al. 1996).

ICIs have revolutionized the management of NSCLC. For metastatic NSCLC without a targetable oncogenic driver, PD-(L)1-directed ICIs are superior to chemotherapy alone (Langer et al. 2016; Reck et al. 2016a, 2019; Gandhi et al. 2018; Paz-Ares et al. 2018; Socinski et al. 2018; Mok et al. 2019) and have become standard-of-care first-line therapy. In this setting, PD-L1 IHC is an approved biomarker (Reck et al. 2016a; Gandhi et al. 2018; Mok et al. 2019), and tumor mutational burden (TMB) may further refine patient selection (Rizvi et al. 2015; Carbone et al. 2017; Hellmann et al. 2018a). With additional indications following relapse in ICI-naïve patients (Borghaei et al. 2015; Brahmer et al. 2015; Fehrenbacher et al. 2016; Herbst et al. 2016; Rittmeyer et al. 2017), and as consolidation therapy following chemo-

radiation for unresectable stage III disease (Antonia et al. 2018), ICI antibodies have rapidly become part of the backbone of systemic therapy for NSCLC.

Many of the ICI susceptibility features in NSCLC are even more pronounced in SCLC. SCLC occurs almost exclusively in patients with heavy tobacco exposure (Pesch et al. 2012), carries a higher median mutational burden (9.9 mutations/Mb vs. 6.3–9 NSCLC) (Chalmers et al. 2017), and lacks the recurrent driver alterations in *EGFR* or *ALK* that correlate with poor response to ICIs (Gainor et al. 2016; Lisberg et al. 2018; Hastings et al. 2019). Furthermore, SCLC tumors can spontaneously provoke strong immune responses. In a prospective study of 264 patients, 9.4% developed paraneoplastic neurologic syndromes (PNSs) associated with expression of neuronal antigens on tumor cells (Gozzard et al. 2015). Lambert-Eaton myasthenic syndrome (LEMS) is the most common PNS, developing in 3%–4% of SCLC patients (Elrington et al. 1991; Payne et al. 2010; Gozzard et al. 2015), and in both retrospective and prospective studies LEMS correlates with a significant increase in overall survival (Chalk et al. 1990; Maddison et al. 1999, 2017; Payne et al. 2010). For some patients with anti-Hu-associated primary sensory neuropathy (PSN) diagnosis of occult SCLC was only made at autopsy (Dalmau et al. 1992), suggesting disease control despite the devastating neurologic consequences. The burden of smoking-associated mutations and frequency of spontaneous antitumor immune reactions provide a rationale for the potential activity of ICIs.

This potential was partially realized in 2018, as the addition of atezolizumab to first-line carboplatin/plus etoposide (EC) significantly prolonged PFS and overall survival for patients with extensive-stage disease (mOS 12.3 months vs. 10.3 months, mPFS 5.2 months vs. 4.2 months) (Horn et al. 2018). This trial represented a landmark achievement as the first significant improvement in systemic therapy for untreated SCLC since the advent of platinum-containing combinations in the 1980s (Chute et al. 1999; Pujol et al. 2000). However, the gain in survival was modest relative to those achieved in NSCLC,

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and response rate was unaffected. Similar results were subsequently observed with addition of durvalumab to first-line therapy, with a modest increase in response rate and overall survival, but no difference in median PFS (overall response rate [ORR] 79% vs. 70%, mOS 13 months vs. 10.3 months, mPFS 5.1 months vs. 5.4 months) (Paz-Ares et al. 2019). Addition of ipilimumab to first-line chemotherapy did not significantly prolong PFS (Reck et al. 2016b). In patients with recurrent metastatic SCLC, nivolumab has demonstrated clinical activity both alone and in combination with ipilimumab (Antonia et al. 2016; Ready et al. 2019), as has pembrolizumab (Ott et al. 2017; Chung et al. 2019), and both have been granted accelerated Food and Drug Administration (FDA) approvals for this indication. However, most SCLC patients receive two lines of therapy or fewer (Steffens et al. 2019), and second-line nivolumab demonstrated a lower response rate and PFS relative to chemotherapy (Reck et al. 2018). Thus, despite some features that suggest SCLC should be more vulnerable to ICIs than NSCLC, the clinical data to date has been less compelling.

The determinants of ICI activity in SCLC may differ from those of NSCLC and other solid tumors. Expression of PD-L1 is much lower in SCLC (Borghaei et al. 2015; Brahmer et al. 2015; Schultheis et al. 2015; Antonia et al. 2016; Yu et al. 2017; Socinski et al. 2018), and cancer cell expression of PD-L1 has not correlated with ICI efficacy. Although TMB as measured in ctDNA did not predict benefit of addition of atezolizumab to first-line therapy (Horn et al. 2018), tissue-based TMB may be a promising biomarker for ICI efficacy following relapse (Hellmann et al. 2018b; Ricciuti et al. 2019). However, even if ICI efficacy for SCLC patients correlates with TMB, the increase in sensitivity per nonsynonymous mutation is significantly lower than for most other tumors (Yarchoan et al. 2017). The disparity between ICI efficacy and TMB, as well as the lack of correlation with PD-L1 staining, suggest that coinhibitory signaling may be less important for evasion of immune surveillance in SCLC than in NSCLC.

Antigen presentation in SCLC may be defective due to suppressed inflammation and expression of MHC class I pathway components. Surface expression and messenger RNA (mRNA) levels of HLA-A,B,C and β 2 microglobulin are orders of magnitude lower in SCLC cell lines compared with NSCLC (Doyle et al. 1985; Yazawa et al. 1999), and these cell lines are less immunogenic when injected into immunocompetent mice (Doyle et al. 1987). SCLC tumor microenvironments are notable for scant immune infiltrates (Kellish et al. 2019), and both the presence of effector T cells and expression of immune-related genes such as *B2M* correlate with earlier stage and better prognosis (Koyama et al. 2008; Wang et al. 2013; Bonanno et al. 2018; Muppa et al. 2019). Exogenous interferon can restore MHC class I expression (Funa et al. 1986; Fisk et al. 1994; Traversari et al. 1997), and although interferon treatment did not improve survival in SCLC (Jett et al. 1994; Kelly et al. 1995; van Zandwijk et al. 1997), these trials were conducted before the advent of ICIs and could have been confounded by interferon-stimulated expression of PD-L1 (Tseng et al. 2001; Dong et al. 2002; Kim et al. 2005; Garcia-Diaz et al. 2017). A recent screen for mediators of MHC-I transcriptional silencing identified multiple components of the polycomb repressor complex 2 (PRC2), and pharmacologic inhibitors of either EED or EZH2 restored MHC-I expression and antigen presentation (Burr et al. 2019). SCLC is characterized by high expression of *EZH2* (Byers et al. 2012), and in the *EZH2* inhibition overcomes resistance to T-cell-mediated killing in the *RP* GEMM. ICI combination with PRC2 inhibition constitutes a promising experimental approach that merits testing in clinical trials.

Alternative strategies for enhancing immunoresponsiveness of SCLC are being explored. Induction of RAS signaling in SCLC cell line NCI-H69 results in production of type I interferon and expression of both MHC class I and PD-L1 (Cañadas et al. 2014, 2018). This inflammatory phenotype was traced to derepression of a subclass of endogenous retroviruses (ERVs) embedded in the 3'-UTRs of interferon-stimulated genes. ERV transcription activates both the

RLR/MAVS and cGAS/STING pathways, which in turn stimulate further interferon production and ERV transcription in a feedforward loop. Inhibitors of DNA repair, such as PARP and CHK1 inhibitors, can directly activate the cGAS-STING pathway through release of damaged genomic DNA into the cytosol, and combination of PARP inhibition and PD-L1 blockade resulted in dramatic regressions in mSCLC tumors (Sen et al. 2019). A similar phenomenon was observed for a novel selective inhibitor of CKD7, which induced a DNA replication stress response and subsequent stimulation of dormant immune surveillance (Zhang et al. 2020). Although a trial of combination durvalumab and olaparib did not meet the pre-set bar for efficacy, responses were observed in all tumors with significant CD8-positive T-cell infiltrates (Thomas et al. 2019).

Evasion of immune surveillance appears to be different for SCLC from NSCLC, and will require a distinct therapeutic approach, but rapid progress is being made in elucidating these mechanisms. Development of patient-derived models that can faithfully recapitulate clinical response and resistance to immunotherapy would accelerate progress, but this is a challenging technical hurdle. Alternatively, the development of GEMMs with a similar TMB to the human disease may better simulate response to immune checkpoint blockade.

CONCLUSION

SCLC research has accelerated over the past decade. Advances in genomics and development of diverse mouse- and patient-derived models have facilitated insights into the cell(s) of origin, intratumoral heterogeneity, gene dependencies, immune evasion, and chemoresistance mechanisms of this disease. SCLC is now being subdivided into functional and molecular categories, a prerequisite for development of biomarker-directed therapy. Pancancer studies suggest greater biological similarity with small-cell NE cancers arising from other organs than with NSCLC, and the therapeutic advances made in SCLC will likely benefit the broader population of solid tumor patients whose can-

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cers become resistant to therapy through small-cell transformation.

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