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REVIEW ARTICLE

Recent advances in preclinical models for lung squamous cell carcinoma

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Abstract

Lung squamous cell carcinoma (LUSC) represents a major subtype of non-small cell lung cancer with limited treatment options. Previous studies have elucidated the complex genetic landscape of LUSC and revealed multiple altered genes and pathways. However, in stark contrast to lung adenocarcinoma, few targetable driver mutations have been established so far and targeted therapies for LUSC remain unsuccessful. Immunotherapy has revolutionized LUSC treatment and is currently approved as the new standard of care. To gain a better understanding of the LUSC biology, improved modeling systems are urgently needed. Preclinical models, particularly those mimicking human disease with an intact tumor immune microenvironment, are an invaluable tool to study cancer development and evaluate new therapeutic targets. Here, we discuss recent advances in LUSC preclinical models, with a focus on genetically engineered mouse models (GEMMs) and organoids, in the context of evolving precision medicine and immunotherapy.

Introduction

Lung cancer remains the most commonly diagnosed malignancy and the leading cause of cancer death worldwide [1]. The majority of lung cancer is categorized as non-small cell carcinoma (NSCLC), of which are mainly lung adenocarcinoma (LUAD) and lung squamous carcinoma (LUSC). LUSC is characterized by its unique squamous appearance; it typically occurs at the proximal part of the lung and originates primarily from basal cells of the bronchi [2]. Genomic analysis of LUSC patient tumors revealed numerous highly altered genes and pathways, but actionable driver mutations are rare [3–5]. Several targeted therapies tested in LUSC patients have demonstrated very limited clinical benefits [6]. This is in contrast to LUAD, which has targetable driver mutations, such as *EGFR*, *ALK* and *ROS1* [7]. In the past decade, treatments enhancing the immune system to target cancer have dramatically shifted the paradigm of cancer therapies [8]. Compared to conventional chemotherapy, immunotherapies such as anti-programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1), lead to a durable response and manageable adverse effects [9]. Of note, several immunotherapy drugs have been approved for LUSC patients based on their substantial clinical benefits [6]. However, there is still a large proportion of LUSC patients who fail to respond to current immunotherapy. Identifying biomarkers for immunotherapy and exploring more effective therapeutics represent an urgent unmet need for LUSC patients.

Preclinical models have been essential in studying lung cancer development and in testing therapeutics [10, 11]. Due to the lack of established driver mutations, the development of LUSC preclinical models that recapitulate human LUSC genetics and pathology remains challenging. Based on the frequently mutated genes in LUSC, multiple genetically engineered mouse models (GEMMs) of LUSC have been successfully characterized [12]. These de novo LUSC models provide instrumental tools to study cell origin, pathogenesis and tumor microenvironment of LUSC. Moreover, recent progress in organoids technology allows culturing and engineering primary tumor cells and normal stem cells in vitro [13, 14]. Several studies

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have utilized the organoids system to successfully generate LUSC models, which has enabled driver mutation evaluation and therapeutic exploration [15–17]. Here, we discuss the latest progress in LUSC preclinical model development, focusing on GEMMs and organoids, in the era of targeted therapies and immunotherapies for lung cancer management.

Genomic hallmarks of lung squamous cell carcinoma

LUSC is strongly associated with smoking and has a relatively high mutational burden, with an average of 261–360 exonic mutations per tumor, which corresponds to a mean mutation rate of 8.1–8.71 mutations per megabase [3, 4]. Comprehensive molecular profiling of LUSC revealed numerous genomic alterations including *TP53*, *CDKN2A*, *PTEN*, *PIK3CA*, *KEAP1*, *KMT2D* and *NFE2L2* [3, 4, 18, 19]. Of note, *TP53* is the most commonly mutated gene (73–81%) and chromosome 3q26 amplification (including *SOX2*, *PIK3CA* and other genes) represents a distinct hallmark for LUSC. In addition, 8p11 (*FGFR1*, *WHSC1L1*), 7p11 (*EGFR*), 11q13 (*CCND1*) and 4q12 (*KDR*, *KIT*, *PDGFRA*), are also the regions of frequent amplification, while 2q37, 4q35 (*CASP3*), 9p21 (*CDKN2A*) and 10q23 (*PTEN*) are the regions of common deletion [3, 4].

These somatic alterations in LUSC are involved in numerous major signaling pathways essential for cancer formation and progression, which include oxidative stress response (*NFE2L2*, *KEAP1* and *CUL3*), squamous differentiation (*SOX2*, *TP63*, *NOTCH1*), cell proliferation/apoptosis (*TP53*, *CDKN2A*, *PTEN*, *PIK3CA*) and chromatin remodeling (*KMT2D*, *KDM6A*) [3, 4]. How these pathways contribute to LUSC formation and progression is being actively investigated. Of note, the genomic landscape of LUSC is quite distinct from LUAD, while it is more similar to other squamous carcinomas such as head and neck squamous cell carcinoma [5, 20, 21]. In contrast to LUAD, driver mutations in LUSC are not clear. The most common drivers in LUAD, *EGFR* and *KRAS*, are rarely mutated in LUSC. Future research is warranted to elucidate the leading, or a combination of, driver mutations in LUSC tumor formation and progression.

Current treatment in advanced LUSC

Targeted therapies in LUSC

Targeted therapies against receptor tyrosine kinases have transformed the treatment in subsets of LUAD patients. For example, *EGFR* is one of the most prevalent oncogenes among LUAD patients and targeting EGFR with tyrosine kinase inhibitors (TKIs) substantially benefits patients with *EGFR* mutations [22–27]. Unfortunately, attempts for targeted treatment in LUSC patients remain mostly unsuccessful [6]. *FGFR1* is frequently amplified in LUSC, which makes it a potential actionable target. Several FGFR inhibitors (BGJ398, AZD4547 and JNJ-42756493) are under investigation in advanced stage LUSC patients. However, data from early phase trials did not show significant clinical benefits [28–30]. Another potential target is PI3K, and early phase studies explored several potential drug candidates including Taselisib, Buparlisib and LY302341 in PI3K-deregulated LUSC patients [31–33]. However, these trials did not meet the primary objective of overall response rate (ORR) and progression-free survival (PFS) [31, 33]. In 2014, the Lung Master Protocol (Lung-MAP), a biomarker-driven protocol supported by the National Cancer Institute (NCI), was initiated to advance the development of targeted therapies for genetically stratified LUSC [34]. Similarly, the National Lung Matrix Trial is another systematic trial aimed to identify genotype-based therapies for NSCLC patients including LUSC [35]. Current data from both studies indicated the clinical benefits for targeted therapy remain low in LUSC patients [35–37]. The umbrella design of these trials enables assessing biomarkers and treatments under a unified protocol, facilitating precision medicine and targeted therapies in LUSC.

Immunotherapies in LUSC

Immunotherapies such as immune checkpoint blockades (ICBs) have revolutionized the treatment of many types of cancer, including LUSC. ICBs promote antitumor response by targeting against immune suppressive pathways modulated by cytotoxic T lymphocyte–associated protein 4 (CTLA-4) or PD-1/PD-L1 signaling [8]. So far, several ICBs have been approved for the first- and second-line treatment for LUSC patients (Table 1). In the first-line treatment, pembrolizumab (an anti-PD-1 antibody) and atezolizumab (an anti-PD-L1 antibody), was approved as monotherapy in LUSC patients with tumor PD-L1 expression ≥50% [38–40]. In addition, the combination of nivolumab (an anti-PD-1 antibody) plus ipilimumab (an anti-CTLA-4 antibody), was approved for these patients [41]. For LUSC patients with median expression of tumor PD-L1 (1–49%), pembrolizumab monotherapy [42] and the combination of nivolumab plus ipilimumab also received approval [41]. Additionally, pembrolizumab plus chemotherapy, as well as nivolumab plus ipilimumab combined with two cycles of chemotherapy was also approved in the first line setting for LUSC patients regardless of tumor PD-L1 expression [43, 44]. As the second-line treatment, nivolumab [45], pembrolizumab

Table 1 Summary of FDA-approved immunotherapies for LUSC.

Approved date	Approved treatment	Clinical trial	Indications	Reference
First-line treatment				
October 24, 2016 & April 11, 2019	Pembrolizumab (Keytruda)	KEYNOTE-024 KEYNOTE-010 KEYNOTE-042	Metastatic NSCLC including LUSC with tumor PD-L1 expression ≥1%	[38, 40, 42]
October 30, 2018	Pembrolizumab (Keytruda) + Chemotherapy	KEYNOTE-407	Metastatic LUSC	[43]
May 15, 2020	Nivolumab (Opdivo) + Ipilimumab (Yervoy)	CheckMate-227	Metastatic NSCLC including LUSC with tumor PD-L1 expression ≥1%	[41]
May 26, 2020	Nivolumab (Opdivo) + Ipilimumab (Yervoy) + limited chemotherapy	CheckMate-9LA	Metastatic or recurrent NSCLC including LUSC	[44]
May 18, 2020	Atezolizumab (Tecentriq)	IMpower110	Metastatic NSCLC including LUSC with high PD-L1 expression (≥50%)	[39]
Second-line treatment				
March 4, 2015	Nivolumab (Opdivo)	CheckMate-017	Metastatic LUSC after chemotherapy	[45]
October 02, 2015	Pembrolizumab (Keytruda)	KEYNOTE-001	Metastatic NSCLC including LUSC after other treatments	[46]
October 18, 2016	Atezolizumab (Tecentriq)	OAK POPLAR	Metastatic NSCLC including LUSC after other treatments	[47, 48]

[46] and atezolizumab [47, 48] have been approved as monotherapy by the FDA based on the substantial clinical benefits.

In summary, ICBs have become the new standard of care and no targeted therapies have been approved for LUSC. Future studies are urgently needed to identify actionable driver targets in genetically stratified patients, and to further explore effective combinational immunotherapies.

Preclinical models for LUSC

Preclinical models of lung cancer are powerful tools to study disease development and evaluate novel therapeutics. The development of LUSC preclinical models has been challenging, partially due to its complex genetics. As seen with their wide application in modeling other cancer types, human cancer cell lines and patient-derived xenografts (PDXs) have also been used to characterize LUSC signature and disease progression [12]. Additionally, several carcinogens including 3-methylcholanthrene (MCA), N-nitroso-methyl-bis-chloroethylurea (NMBCU) or N-nitroso-tris-chlo-oethylurea (NTCU), have been used to induce LUSC formation in mouse lungs [49–52]. The PDXs and carcinogen-induced models have been extensively discussed in several recent reviews [10, 12, 53, 54]. GEMMs in cancer research are de novo tumor mouse models generated through conditionally activating oncogenes or deleting tumor suppressor genes in the targeted tissue regions. For LUAD, a series of oncogene-driven GEMMs have been successfully generated and have contributed significantly to our understanding of LUAD tumorigenesis and discovery of new treatment [10, 55]. In the past decade, there is also a growing interest in utilizing GEMMs for modeling LUSC. Tumors arising from GEMMs develop in the tissue native environment that is immune-proficient. Thus, GEMMs are particularly useful for immuno-oncology research, such as testing immunotherapies. In addition, with the recent development of 3D culture and genetic engineering technologies, organoids have emerged as a promising platform for studying LUSC. Here, we discuss recent progress in LUSC preclinical models, with a focus on GEMMs and organoids models (Fig. 1).

Genetically engineered mouse models (GEMMs)

Sox2 overexpression models

The human LUSC genomics revealed that squamous differentiation signaling is one of the major deregulated pathways in patients [3, 4]. As a key player in squamous differentiation, *SOX2* is amplified in 20–40% and overexpressed in more than 60% LUSC [3, 56, 57]. *Sox2* overexpression alone, however, is not able to drive LUSC formation in mouse models [58]. Overexpression of *Sox2* with *Lkb1* loss promotes LUSC formation in mice [59, 60]. 6–10 months after viral induction, lung tumors with predominantly squamous histology were observed in mice [59]. The $Lkb1^{-/-}$ $Sox2^{OE}$ (Sox2 overexpression) tumors can be further facilitated by loss of *Nkx2-1*, a transcription factor in LUSC with low expression [60]. Moreover, *Sox2*

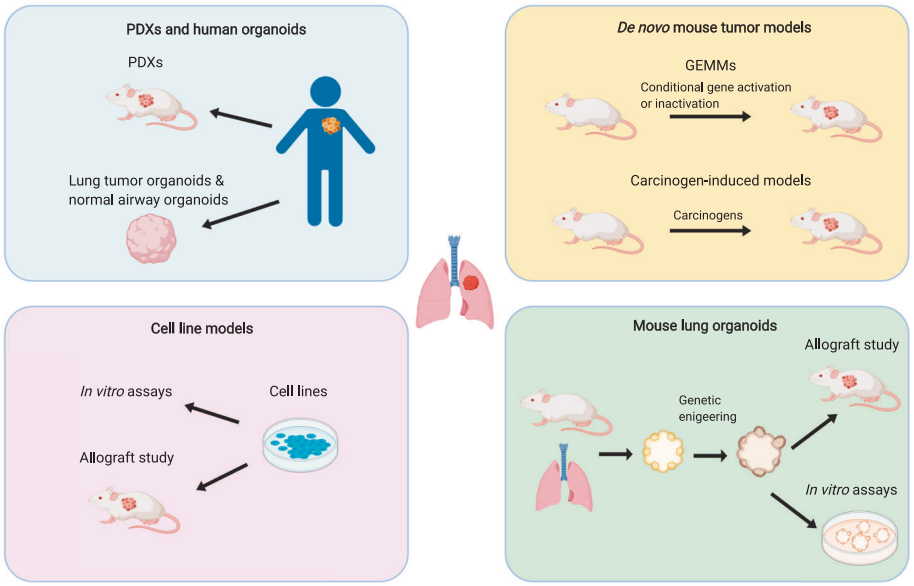

Fig. 1 Preclinical models for LUSC. Schematic summary of different preclinical models for LUSC.

overexpression with *Nkx2-1* inactivation also leads to LUSC formation [61]. Interestingly, *Sox2* overexpression in combination with *Cdkn2a, Cdkn2b* and *Pten* loss, drives pure LUSC formation in multiple cell origins including basal cells, club cells and AT2 cells of the mouse lungs [62]. These studies indicate that *Sox2* plays a major role in LUSC tumorigenesis.

Lkb1 deletion models

LKB1/STK11 serine/threonine kinase is highly mutated in lung cancer [63], and *Lkb1* deletion has been widely used in mouse models of LUSC. It was originally reported in 2007 that simultaneous activation of $Kras^{G12D}$ with deletion of *Lkb1*, results in mixed adenocarcinoma and squamous carcinoma histology in the mouse lung [64]. Subsequent research demonstrated that *Lkb1* is involved in the trans-differentiation from LUAD into LUSC in the $Kras^{G12D}$ driven tumors [65–67]. *KRAS* has relatively low mutation frequency in LUSC and occurs at 0.9%, 3.9%, and 3.6% in the TCGA, GENIE and COSMIC database respectively. Therefore, the $Kras^{G12D}$ $Lkb1^{-/-}$ model only represents a small subset of human LUSC. Other LUSC GEMMs harboring *Lkb1* deletion have also been reported. One is established through *Lkb1* deletion and *Sox2* overexpression as described above [59, 60]. Another model involves biallelic inactivation of *Lkb1* and *Pten* [68], which develops squamous tumors in the lung 40–50 weeks after Ad5-CMV-Cre induction. Similar to human LUSC, these $Lkb1^{-/-}$ $Pten^{-/-}$ mouse tumors have elevated expression of *SOX2*, *P63* and *KRT5*, and are enriched for a squamous differentiation signature [68]. Interestingly, a recent study reported that inactivation of *Lkb1* alone is sufficient to give rise to lung tumors with squamous histology [69]. Instead of using the Cre that targets all cells in the lung, the authors used *CCSPi-Cre*; $Lkb1^{fl/fl}$ mouse to specifically delete *Lkb1* in clara/club cells. *Lkb1* inactivation through this strategy leads to mixed histology lung tumors of squamous carcinoma and adenocarcinoma after one year. The authors also found that the formation of *CCSPi-Cre*; $Lkb1^{fl/fl}$ driven tumors was further accelerated by *Jnk1/2* loss [69]. In summary, these studies highlight the essential role of *Lkb1* in LUSC development and trans-differentiation from LUAD into LUSC in mouse lungs. Despite the pivotal function of *Lkb1* in mouse LUSC, it is worth noting that less than 3% of LUSC patients harbor *LKB1* mutations based on several cancer databases (1.8% in TCGA, 2.3% in GENIE and 2.2% in COSMIC). Given that *LKB1* genetic alterations involve point mutation, exonic loss and deletion of whole gene allele [70], it is possible that *LKB1* mutation frequency is underestimated due to the limitations of current sequencing methods [71]. Indeed, using multiplex ligation-dependent probe amplification (MLPA) analysis, *LKB1* mutation was identified in ~13% (13/101) of LUSC patients [67]. Furthermore, loss of *Lkb1* in mouse lung could recapitulate the biochemical changes and dysregulated signaling observed in human LUSC. For example, mTOR, one of the major downstream pathways of *LKB1* [72], is frequently deregulated in human LUSC by the PTEN-PI3K signaling axis [3, 73, 74]. Future research needs to explore the role of *LKB1* and associated signaling in human LUSC.

LUSC GEMMs driven by other mutations

In addition to *Sox2* and *Lkb1* alterations, other mutations have also shown promise in generating LUSC mouse

models. Constitutive activation of a dominant negative mutant form of *IKKα* leads to squamous cell carcinoma in the mice lungs [75]. These $IKK\alpha^{KA/KA}$ mice, however, usually die at 6–10 months after induction, which is possibly caused by tumor lesions in the skin and other tissues. Rescuing IKKα expression in the skin of these mice leads to LUSC in all mice [75]. *FBXW7* is mutated in around 5–6% of LUSC. Simultaneous activation of $Kras^{G12D}$ and deletion of *Fbxw7* results in adenocarcinoma and squamous cell carcinoma [76]. Interestingly, using virus that express Cre recombinase under specific promoters, the authors further uncovered that the $CC10^{+}$ club cells, rather than the $CK5^{+}$ basal cells, are likely to be the cell origin of LUSC in this model [76]. Furthermore, Camolotto et al. revealed that transcriptional factors FoxA1 and FoxA2 contribute to squamous identity in lung cancer [77]. They demonstrated that FoxA1/2 are downregulated in the squamous compartment of mouse tumor, as well as in human adeno-squamous carcinoma. Conditionally deleting *Foxa1* or *Foxa2* in $Kras^{G12D}$ $Nkx2\text{-}1^{-/-}$ tumors leads to trans-differentiation of lung tumors toward squamous identity [77].

GEMMs for LUSC immuno-oncology research

As discussed above, lung tumors from GEMMs are developed in the native immunocompetent microenvironment manifested by extensive infiltrating immune populations. Multiple studies have utilized GEMMs to characterize the LUSC immune microenvironment. For example, The $Lkb1^{-/-}$ $Pten^{-/-}$ LUSC tumors display the signature of immune-suppression including high PD-L1 expression in tumor and a large number of tumor-associated neutrophils (TANs) infiltration [68]. Similarly, using the $Lkb1^{-/-}$ $Sox2^{OE}$ model, Mollaoglu et al. reported that mouse LUSC tumors are enriched with TANs [60]. Mechanistically, SOX2 enhances TANs recruitment through repressing NKX2-1 activity, which elevates the expression of chemokine CXCL5. Moreover, the $Sox2^{OE}$ $Cdkn2a^{-/-}$ $Cdkn2b^{-/-}$ $Pten^{-/-}$ LUSC tumors have high percentages of neutrophil infiltration and express high levels of PD-L1 [62]. All together, these findings from LUSC GEMMs harboring different genetic alterations suggest that TANs might play an important role in shaping the immune suppressive environment in LUSC.

In conclusion, LUSC GEMMs (summarized in Table 2) provide a precious platform to study de novo tumor formation and progression in the native tissue environment. Due to the long breeding time and tumor latency, only a few genes have been manipulated to model LUSC GEMMs. Recently, several approaches have been established to facilitate GEMMs development in cancer research. One involves engineering the embryonic stem cells (ESC) from preexisting GEMMs. The ESC derived from established GEMMs can be used to introduce additional genetic modifications [78–80]. For example, this GEMM-ESC strategy has been used to study the role of *MET* in *BRCA1* deficient metaplastic breast cancer [81] and the function of *PTEN* and *MYC* in pancreatic cancer [82]. Another major advancement of GEMMs in cancer research is to generate in situ somatic mutations using CRISPR/Cas9 technology in the targeted tissue regions [83–85]. Recently, the CRISPR/Cas9 system has been extensively used to model different types of cancer including LUAD [84, 86] and small cell carcinoma [87]. Compared with the traditional GEMMs which require laborious breeding, the CRISPR/Cas9-based GEMMs are rapid and less costly for evaluating driver mutations and their roles during tumor progression [88, 89]. Moreover, multiplexed approaches can be incorporated to study a number of genetic alterations at the same time [88, 90–94]. Rogers et al, for example, deciphered the tumor suppressive effects and genetic interactions of 31 commonly mutated genes in LUAD by combining CRISPR/Cas9, tumor barcoding and deep sequencing [93]. Until now, neither GEMM-ESC nor CRISPR/Cas9-based in situ genetic engineering have been reported for LUSC modeling. Considering the complex genetic alterations in LUSC, it would be intriguing to incorporate these rapid and multiplexed strategies in validating potential oncogenic drivers for LUSC. Moreover, only a few studies have utilized GEMMs to evaluate experimental therapeutics for LUSC, while no immunotherapies have been tested. More research is needed to examine mono- and combinational immunotherapies in these immune-competent models.

Organoids models

The recent development of 3D culture technologies has enabled long-term culture of adult stem cells and primary cancer cells in more physiological conditions in vitro. These stem cells or cancer cells can self-organize into organotypic structures, known as organoids [13, 95, 96]. Organoids are increasingly appreciated as an important tool for basic and translational cancer research and success has been made in culturing many mouse and human epithelia organoids including colon, liver, pancreas, prostate, stomach and lung (reviewed in [13, 97]). Many living biobanks of human healthy and cancer organoids have been generated and served as a useful platform for personalized cancer treatment testing and drug screening [98–102]. Furthermore, genetic engineering in organoids allows de novo cancer modeling to examine the role of driver mutations in cancer

Table 2 Genetically engineered mouse models (GEMMs) for LUSC.

Genetic alterations	Mouse genotype	Tumor induction method	Histology	Descriptions	Reference
Sox2^{OE} *Lkb1*$^{-/-}$	*Lkb1*$^{fl/fl}$	Intranasal delivery of Lenti-ACTIN-Sox2-PGK-Cre	SCC	Latency 6–10 months. The majority of tumors (8/9) show SCC histology. One mouse (1/9) shows ADC pathology.	[59]
Sox2^{OE} *Lkb1*$^{-/-}$ & *Sox2*^{OE} *Lkb1*$^{-/-}$*Nkx2.1*$^{-/-}$	*Rosa26-LSL-Sox2-IRES-GFP*; *Lkb1*$^{fl/fl}$ & *Rosa26 LSL-Sox2-IRES-GFP*; *Nkx2-1*$^{fl/fl}$; *Lkb1*$^{fl/fl}$	Intranasal /Intratracheal delivery of Ad5-CMV-Cre	SCC	*Sox2*^{OE} *Lkb1*$^{-/-}$ tumor latency is around 11 months; the majority is SCC. Loss of *Nkx2.1* with *Sox2*^{OE} *Lkb1*$^{-/-}$significantly facilitates SCC formation, as early as 8 weeks tumors can be detected.	[60]
Sox2^{OE} *Cdkn2a*$^{-/-}$ *Cdkn2b*$^{-/-}$ *Pten*$^{-/-}$	*Cdkn2ab*$^{fl/fl}$; *Pten*$^{fl/fl}$; *LSL-Sox2*	Intratracheal delivery of Ad5-K5/K14-Cre, Ad5-CC10-Cre and Ad5-SPC-Cre to target basal cells, club cells and AT2 cells	SCC	Latency 6–10 months. Combinations of *Sox2*^{OE} with *Cdkn2a, Cdkn2b* and *Pten* loss drive SCC formation in basal cells, club cells and AT2 cells.	[62]
Sox2^{OE} *Nkx2.1*$^{-/-}$	*Sox2-CreER*; *Rosa26R-LSL-Sox2-IRES-GFP*; *Nkx2-1* $^{fl/fl}$	Tamoxifen administration to induce Cre under Sox2 promoter	SCC	After 30 days of tamoxifen induction, LUSC was observed in 6 of 8 mice.	[61]
Kras^{G12D} *Lkb1*$^{-/-}$	*LSL-Kras*^{G12D}; *Lkb1*$^{fl/fl}$	Intranasal delivery of Ad5-CMV-Cre	SCC, ADC	Latency is short (4–6 weeks). More than half of mice have squamous or mixed pathology. These tumors progressively transdifferentiate from ADC to SCC.	[64, 67]
Pten$^{-/-}$ *Lkb1*$^{-/-}$	*Pten*$^{fl/fl}$; *Lkb1*$^{fl/fl}$	Intranasal delivery of Ad5-CMV-Cre	SCC	Latency is 40–50 weeks. All tumors analyzed display SCC histology. High expression of PD-L1.	[68]
Lkb1$^{-/-}$ & *Lkb1*$^{-/-}$ *Jnk1/2*$^{-/-}$	CCSP-Cre; *Lkb1*$^{fl/fl}$ CCSP-Cre; *Lkb1*$^{fl/fl}$; *Jnk1*$^{fl/fl}$; *Jnk2*$^{fl/fl}$	Cre expressed in clara/club cells under CCSP promoter	SCC, ADC	*Lkb1* inactivation is sufficient to drive SCC and ADC. This *Lkb1*$^{-/-}$ driven tumor formation is further facilitated by loss of *Jnk1/2*.	[69]
IKKα (K44A) overexpression	Lori.IKK*α; Ikkα*$^{KA/KA}$	IKKα (K44A) is expressed in all mice tissues except the epidermis IKKα expression is rescued	SCC	A dominant negative mutant form of IKKα leads to lung tumors formation with squamous histology. Most tumors were observed at 4–6 months.	[75]
Kras^{G12D} *Fbxw7*$^{-/-}$	*LSL-Kras*^{G12D}; *Fbxw7*$^{fl/fl}$	Intratracheal delivery of Ad5-CMV-Cre, Ad5-CC10-Cre and Ad5-Sftpc-Cre or Lenti-Foxj1-Cre	SCC, ADC	After 9–12 weeks induction with Ade-CMV-Cre, both ADC and SCC were detected in the lungs (around 1:1 ratio). Using Cre under different promoters, the authors find that the CC10 + club cells but not basal cells are likely to be the cell origin of SCC in this model.	[76]
Kras $^{LSL-G12D}$, *Nkx2.1*$^{-/-}$ with *Foxa1*$^{-/-}$*or Foxa2*$^{-/-}$	*Kras* $^{LSL-G12D}$; *Nkx2.1*$^{fl/fl}$; *Foxa1*$^{fl/fl}$ & *Kras* $^{LSL-G12D}$; *Nkx2-1*$^{fl/fl}$; *Foxa2*$^{fl/fl}$	Intratracheal delivery of Lenti-PGK-Cre Ad5-CMV-Cre or Ad5-SPC-Cre	SCC, ADC	20 weeks after tumor induction, macroscopic adenosquamous carcinomas are observed.	[77]

SCC squamous cell carcinoma, *ADC* adenocarcinoma, *Sox2*^{OE} *Sox2* overexpression.

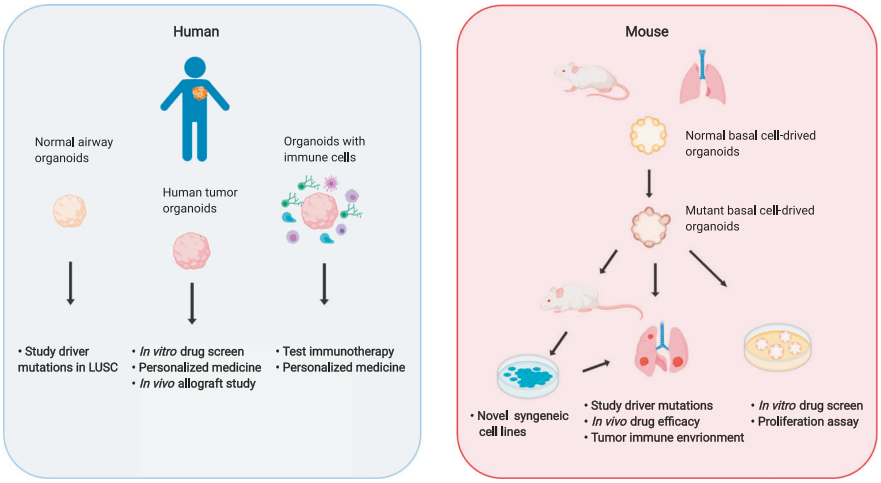

Fig. 2 Organoids for LUSC research. Schematic summary of organoids for LUSC study. Human lung normal and tumor organoids can be used for genetic cancer modeling, personalized medicine and testing immunotherapy. Mouse basal cell-derived organoids are ideal for testing LUSC driver mutations and generating novel syngeneic models.

Table 3 Human organoids models for LUSC.

Sources	No. of organoids (success rate)	Tumor histology	In vitro culture time	Applications	Reference
Normal & tumor tissues of lungs	18 (28%) tumor 18 (94%) normal	Adenocarcinoma, squamous carcinoma, large cell carcinoma	>1 year	In vitro drug screen; In vivo orthotopic transplantation	[103]
Normal & tumor tissues of lungs	80 (~70%) tumor 5 normal	Adenocarcinoma, squamous carcinoma, large cell carcinoma, small cell carcinoma	>6 months	In vitro drug screen; In vivo S.C. transplantation	[104]
Tumor tissues of lungs & PDX	57 (~88%) from tumor and PDX	Adenocarcinoma, squamous carcinoma	72% short-term (1–3 months) 15% long term (>3 month)	In vitro drug screen; In vivo S.C. transplantation	[105]

S.C. subcutaneously.

initiation and progression [96]. Here we discuss the recent development in organoids for LUSC modeling and characterization (Fig. 2).

Human lung cancer organoids

Several groups have successfully generated human lung cancer organoids [103–105] (Table 3). These organoids recapitulate the genetic and pathologic characteristics of the original tumors. When transplanted into mice, some cancer organoids maintain their tumorigenic capability. Sachs et al. first reported the long-term expansion of human airway organoids from both healthy and cancer tissues [103]. They generated different subtypes of human NSCLC organoids, including LUSC organoids. Subsequent orthotopic transplantation of these cancer organoids into immunocompromised mice enabled lung cancer formation in vivo. In addition, Kim and colleagues established the largest and most inclusive biobank of lung organoids to date, comprising of 80 human lung cancer organoids and 5 normal bronchial organoids [104]. The banked lung cancer organoids include five subtypes of lung cancer: LUAD, LUSC, adenosquamous carcinoma, large cell carcinoma, and small cell carcinoma. Similarly, Shi et al. successfully established human NSCLC organoids from both primary lung tumor and PDX tumor tissues [105]. Using organoids derived from PDXs, the authors further revealed that the combination of FGFR and MEK inhibition suppressed the growth of a *FGFR1* amplified LUSC organoid line in vitro and in vivo.

Mouse lung cancer organoids

Mouse organoids are ideal to study de novo transformation and identify novel oncogenic drivers, as normal adult stem cell organoids can be cultured and passaged for long term in vitro and are easy to manipulate using genetic approaches. For instance, driver mutations such as *Kras*, *Apc*, *Trp53* and

Table 4 Mouse organoids models for LUSC.

Genotype	Generation method	Tumor histology	Donor mouse background	Applications	Reference
Trp53$^{-/-}$ *Keap1*$^{-/-}$	FACS sorting tracheal basal cell plus Ade-Cre transfection, imbedded in Matrigel	SCC	Mixed	In vitro growth assay, pathways analysis and therapeutic test; In vivo S.C. transplantation (NSG mice) and therapeutic test	[15]
Bcl11a overexpression & *Bcl11a*$^{-/-}$	FACS sorting tracheal basal cells, imbedded in Matrigel	NA	Mixed	In vitro growth assay	[111]
Trp53$^{-/-}$ *Sox2*, *Prkci*, and *Ect2* overexpression	FACS sorting tracheal basal cells; Ade-Cre and Lenti-viral overexpression, imbedded in Matrigel	SCC	C57BL/6	In vitro growth assay and pathways analysis; In vivo orthotopic transplantation (C57BL/6 mice)	[17]
Sox2^{OE} *Trp53*$^{-/-}$ *Pten*$^{-/-}$ *Cdkn2a*$^{-/-}$	Enzymatic digestion trachea; Lenti-Cre and Lenti-CRISPR in vitro, imbedded in Matrigel	SCC	C57BL/6	In vitro growth assay, pathways analysis and therapeutic test; In vivo S.C. transplantation (Nude and C57BL/6 mice) and immunotherapy test	[16]

S.C. subcutaneously, *SCC* squamous cell carcinoma.

Smad4, were introduced into mouse colon organoids followed by transplantation into mice to study colon cancer progression [106–108]. To date, genetic engineering of normal mouse organoids has been performed to study driver mutations and disease progression in several cancer types including pancreatic, gastric, brain and ovarian [96].

Mouse lung organoids derived from basal cells, secretory cells and alveolar type-II cells (AT2 cells), have been reported [109]. In 2009, Rock et al. first reported mouse lung basal cell-derived organoids (basal epithelia cells or lung basal cell spheres/tracheospheres described in some literature) [110]. When cultured in vitro, these basal cells can self-renew and differentiate into luminal club cells and ciliated cells [109, 110]. As basal cells are proposed to be the main cell of origin for LUSC, these basal cell-derived organoids are optimal to study LUSC in vitro and in vivo (Table 4). Jeong et al. discovered that inactivation of *Keap1* or *Trp53* promotes airway basal stem cell self-renewal in vitro and in vivo [15]. By transducing Ade-Cre virus in vitro, the authors generated the *Trp53*$^{-/-}$ *Keap1*$^{-/-}$ basal cells. Interestingly, these genetically modified basal cells formed LUSC subcutaneously in mice. Furthermore, *BCL11A* is a potential oncogenic driver amplified in a subset of LUSC patients. Utilizing the mouse organoids system, Lazarus et al. found that overexpressing *Bcl11a* in mouse basal cell-derived organoids leads to a hyperproliferative and abnormal structure, while *Bcl11a* knockout organoids were unable to form the 3D structure in vitro [111]. However, whether the *Bcl11a* overexpressing organoids can form LUSC in vivo was not studied. Chromosome 3q26 copy number gain (CNG) is a genetic hallmark of LUSC, but its functional significance in LUSC formation is not well understood. Liu et al. discovered that overexpression of *Sox2, Prkci* and *Ect2* in the context of *Trp53* loss, significantly promotes mouse lung basal stem cells growth in vitro [17]. Intriguingly, the transformed basal cells formed LUSC when orthotopically injected into mouse lungs. Using CRISPR/Cas9 genomic engineering technology, our laboratory recently characterized the potential of tumor formation from multiple mouse lung basal organoids with the deletion of LUSC specific tumor suppressor genes [16]. Our findings revealed that *Sox2*^{OE} *Trp53*$^{-/-}$ *Pten*$^{-/-}$ *Cdkn2a*$^{-/-}$ organoids, but not the *Sox2*^{OE} *Trp53*$^{-/-}$ *Cdkn2a*$^{-/-}$ organoids, are able to efficiently form LUSC in vivo which closely mimics the human disease.

Organoids for LUSC immuno-oncology research

Human lung organoids are a promising tool for personalized medicine and drug screening [103–105]. However,

one of the main drawbacks is that most of the current human lung organoids lack tumor infiltrating immune cells and stroma, thus they are not suitable for immuno-oncology research. Recent studies from our group and others attempted to incorporate tumor-associated immune cells to mimic the immune tumor microenvironment in human lung organoid culture systems [112]. One approach is using the patient-derived organotypic tumor spheroids (PDOTS) from human primary tumors [113, 114]. PDOTS contain the autologous lymphoid and myeloid cell populations and they respond to immunotherapy and other therapies in vitro. Another way involves the utilization of an air-liquid interface method to culture patient-derived organoids with native embedded immune cells (T, B, NK cells and macrophages). Thus far, this method has enabled the establishment of organoids with immune microenvironment originated from different types of cancer, including lung cancer [112, 115]. The preservation of tumor infiltrating immune cells enables evaluation of the response to ICBs such as anti-PD-1 and/or anti-PD-L1 treatment. Currently, the in vitro maintenance of infiltrating immune cells is challenging, therefore future research is needed to improve the lifespan of immune cells and to illustrate how effective these organoids are at predicting an immunotherapy response.

On the other hand, the main advantage of mouse lung organoids is its application in in vivo immuno-oncology research, when the recipient animals are in the same genomic background with the organoids. As described previously, $Sox2^{OE}$ $Trp53^{-/-}$ $Pten^{-/-}$ $Cdkn2a^{-/-}$ organoids were successfully established from C57BL/6J mice in our laboratory. Upon injection back into C57BL/6J mice, LUSC tumors were formed and LUSC syngeneic cell lines were subsequently generated. We further examined the effect of WEE1 inhibition in enhancing immunotherapy in immune-competent C57BL/6J mice. Our study uncovered that WEE1 inhibition induces DNA damage and enhances the immune response of anti-PD-1 therapy in LUSC [16].

In summary, organoids have become an important component of preclinical LUSC models in recent years (Fig. 2), although lung organoids are still at an early stage of development. It is worth noting that the current culturing method has limitations to generate pure human lung cancer organoids. For example, the growth of normal airway organoids could outcompete the tumor organoids in vitro [116]. In addition, developing a better organoids system for long-term culturing lung cancer organoids with the tumor infiltrating immune cells, would greatly facilitate LUSC immuno-oncology research. Genetic engineering in normal human lung organoids is currently ongoing in our laboratory and we foresee this will be a powerful strategy to study driver mutations, disease progression and to evaluate treatment of LUSC in vitro. Likewise, mouse basal cell-derived organoids are a powerful tool for genetic LUSC modeling; they are optimal for efficiently testing genetic drivers and performing genome-wide screens. Moreover, mouse organoids can be used to develop novel syngeneic allograft models with defined genetic alterations. Given the long and variable latency of LUSC GEMMs (6–10 months) [12], these basal cell-derived organoids hold great promise to model LUSC and facilitate immuno-oncology studies in an efficient manner.

Conclusion and future direction

The past decade has witnessed substantial progress in LUSC genetics and therapeutics, as comprehensive genomic sequencing has begun dissecting the genetic mutational landscape and immunotherapy has transformed treatment in patients with advanced stage disease. In parallel, characterization of preclinical models mimicking human LUSC has proven to be an invaluable tool in understanding tumor biology and developing better treatment strategies. In particular, GEMMs harboring key genetic mutations that recapitulate human tumor physiology and pathology have shed light on the essential role of functional driver mutations in tumor intrinsic signaling as well as on how genetic characteristics affect the tumor microenvironment. With the help of emerging approaches such as GEMM-ESC and CRISPR/Cas9, GEMMs are positioned to continue playing pivotal roles in preclinical LUSC modeling. Combining CRISPR/Cas9 with next generation sequencing will be a powerful approach to decipher LUSC driver mutations and their genetic interactions. Furthermore, the evolving organoids technology is an encouraging and complementary ex vivo and transplantable model system to study LUSC biology and test therapeutics.

Utilizing these preclinical models through genetic engineering and 3D culturing technologies, future research is needed to characterize the role of underappreciated driver genetic alterations in tumorigenesis and to develop combination therapy to target dysregulated genes/pathways identified in LUSC patients. It is worthwhile highlighting that from GEMMs to organoids, and transplantable mouse models, each system has its strengths and shortcomings (Table 5). In LUSC immuno-oncology research, selecting an appropriate model is the first and crucial step in investigating the interactions between cancer cells and immune cells, as well as evaluating immunotherapies. Combining these different toolsets in a complementary manner will greatly advance our research in this field. Ultimately, improved understanding of tumor immune microenvironment and identifying biomarkers of

Table 5 Comparisons of preclinical models for LUSC.

Models	Cell lines	PDX	Carcinogen induced	GEMMs	Human organoids	Mouse organoids
Cost	+	+++	+++	+++	++	++
Time	+	++	+++	+++	+	+
Driver mutations validation	Yes	No	No	Yes	Model dependent[a]	Yes
In vitro						
High throughput drug screen	Yes	NA	NA	NA	Yes	Yes
Genetic Manipulation	Yes	NA	NA	NA	Yes	Yes
In vivo						
Tumor model type	Allograft	Allograft	De novo	De novo	Allograft	Allograft
Tumor latency	+	++	+++	+++	+	+
Drug test (non-immune)	Yes	Yes	Yes	Yes	Yes	Yes
Immuno-oncology						
Characterize immune environment	Yes, if syngeneic	Yes[c]	Yes	Yes	Maybe[b]	Yes, if syngeneic
Immune environment type	Transplant[d]	Reconstituted	Native	Native	Native	Transplant[d]
Testing Immunotherapy	Yes	Yes[c]	Yes	Yes	Maybe[b]	Yes, if syngeneic

[a]Normal human airway organoids can be used to test driver mutations.

[b]Human lung organoids can be used for immuno-oncology research if co-cultured with immune cells.

[c]PDXs could be used for immuno-oncology research if reconstituted by human immune cells.

[d]Tumor immune environment would differ depending on methods (S.C. or orthotopic) of inoculation.

response to immunotherapy will have significant translational impact in tailored treatment for LUSC patients.

Compliance with ethical standards

Conflict of interest KKW is a founder and equity holder of G1 Therapeutics and has consulting and sponsored research with: AstraZeneca, Janssen, Pfizer/Array Biopharma, Novartis, Merck, Zentalis; as well as sponsored research (only) with: Takeda, BMS, Mirati, Alkermes, Merus, Amgen, Ansun Biopharma, Enliven Therapeutics, Tvardi Therapeutics, Delfi Diagnostics, and Dracen Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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