ABSTRACT

Despite its clinical importance, very little is known about the natural history and molecular underpinnings of lung cancer dissemination and metastasis. Here, we used a genetically engineered mouse model of metastatic lung adenocarcinoma in which cancer cells are fluorescently marked to determine whether dissemination is an inherent ability or a major acquired phenotype during lung adenocarcinoma metastasis. We find very little evidence for dissemination from oncogenic KRAS-driven hyperplasias or most adenocarcinomas. p53 loss is insufficient to drive dissemination but rather enables rare cancer cells in a small fraction of primary adenocarcinomas to gain alterations that drive dissemination. Molecular characterization of disseminated tumor cells indicates that downregulation of the transcription factor Nkx2-1 precedes dissemination. Finally, we show that metastatic primary tumors possess a highly proliferative subpopulation of cells with characteristics matching those of disseminating cells. We propose that dissemination is a major hurdle during the natural course of lung adenocarcinoma metastasis.

SIGNIFICANCE: Because of its aggressively metastatic nature, lung cancer is the top cancer killer of both men and women in the United States. We show that, unlike in other cancer types, lung cancer dissemination is a major initial barrier to metastasis. Our findings provide insight into the effect of p53 deficiency and downregulation of Nkx2-1 during lung adenocarcinoma progression.

INTRODUCTION

Metastasis is a multistep process, and the acquisition of metastatic ability has traditionally been considered a late step in cancer evolution (1). Recent evidence in mouse models of breast and pancreatic cancer suggests that cancer cells within premalignant lesions may already possess the ability to disseminate (1–4). These observations support a model in which cancer cells leave primary tumors at an early stage of tumor growth, thereby allowing the primary tumor and disseminated cells to accrue genomic alterations independently (5–7). This paradigm of metastatic progression is consistent with findings of disparate DNA copy-number alterations in primary tumors and related disseminated cancer cells and metastases in patients (5–7).

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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Lung adenocarcinoma is the most common subtype of lung cancer, with more than 90,000 patients diagnosed each year in the United States alone (8). Several factors contribute to the poor outcome of patients with lung cancer but, as in most solid tumors, the ability of cancer cells to leave the primary tumors and establish inoperable metastases is a major impediment to successful therapy (9). Whether early premalignant lesions in human lungs have an inherent capacity to give rise to disseminated tumor cells (DTC) is a question of both clinical and biological importance. Despite its significance, systems to address the question of whether lung adenocarcinoma metastatic progression conforms to a traditional late-evolution model or a parallel progression model have not been developed. Although these questions would ideally be studied in humans, early lesions are by definition small and exist in undiagnosed patients, making their study difficult if not impossible. Hence, accurate and tractable genetic models are required to investigate this process.

Human lung adenocarcinoma frequently harbors oncogenic mutations in KRAS and inactivation of the p53 tumor-suppressor pathway (10, 11) and has been modeled using transgenic systems, stochastic intrachromosomal recombination, or Cre-mediated deletion of the transcriptional/translational Stop element in the R26-Stop-loxP-KrasG12D knock-in mice (Kras\textsuperscript{LSL-G12D/+}). This single event initiates the stepwise development of lung lesions that closely resemble early-stage atypical adenomatous hyperplasias (AAH), adenomas, and early adenocarcinomas (12–14). Concomitant mutation of the p53 tumor suppressor allows the development of overtly invasive and metastatic cancer (15–17). Notably, these genetically engineered mouse models of human lung cancer recapitulate the genetic events, histologic appearance of lesions at both the early and late stages, and metastatic ability of the human disease (12–17).

Here, we use a series of \textit{in vivo} mouse models to uncover the kinetics of lung tumor cell dissemination, investigate the importance of p53 loss in this process, and characterize the gene expression changes associated with this critical step of the metastatic cascade.

RESULTS

To analyze cancer cell dissemination at defined time points after tumor initiation, we used a system to induce fluorescently labeled lung tumors. To stably mark all cancer points after tumor initiation, we used a system to induce conditional alleles in mice. Oncogenic KrasG12D has been expressed in lung epithelial cells using transgenic systems, stochastic intrachromosomal recombination, or Cre-mediated deletion of the transcriptional/translational Stop element in the R26-Stop-loxP-KrasG12D knock-in mice (Kras\textsuperscript{LSL-G12D/+}). This single event initiates the stepwise development of lung lesions that closely resemble early-stage atypical adenomatous hyperplasias (AAH), adenomas, and early adenocarcinomas (12–14). Concomitant mutation of the p53 tumor suppressor allows the development of overtly invasive and metastatic cancer (15–17). Notably, these genetically engineered mouse models of human lung cancer recapitulate the genetic events, histologic appearance of lesions at both the early and late stages, and metastatic ability of the human disease (12–17).

Here, we use a series of \textit{in vivo} mouse models to uncover the kinetics of lung tumor cell dissemination, investigate the importance of p53 loss in this process, and characterize the gene expression changes associated with this critical step of the metastatic cascade.
Figure 1. Titratable induction of fluorescently labeled lung tumors enables the analysis of cancer cell dissemination at defined time points after tumor initiation. 

A. Infection of Kras^{G12D/LSL}p53^{flox/flox}R26^{LSL-tdTomato/+} (KPT) mice with the indicated viral titers generates KPT-Early and KPT-Late mice. A time course of tumor progression is shown.

B. Representative light and fluorescent images of KPT-Early (~1 month after infection) and KPT-Late (~5 months after infection) lung lobes. Scale bar, 5 mm.

C. KPT-Early lungs have diffuse hyperplasia throughout the alveolar space. KPT-Late lungs have solid tumors of variable histologic presentation among areas of normal lung. Top scale bar, 2 mm. Bottom scale bar, 50 μm.

D. Expanding Tomato-positive cells have histologic features of atypical AAHs and express lung epithelial markers [Nkx2.1 and cytokeratins (pan-CK)]. Scale bar, 50 μm.

E. KPT-Early and KPT-Late mice have dramatically increased lung weight relative to age-matched normal lung. Each dot represents one mouse, and the bar indicates the mean.

F. Quantification of the number of Tomato-positive tumor cells per lung section in KPT-Early and KPT-Late mice normalized to the average number of Tomato-positive cells per lung section in KPT-Early mice. Each dot represents one mouse, and the bar indicates the mean. H&E, hematoxylin and eosin; n.s., not significant.

pleural lavage and flow cytometry to quantify Tomato^{positive} DTCs. Despite the very high numbers of neoplastic cells, we detected very few if any DTCs in the pleural cavity of KPT-Early mice (Fig. 2A and B). To exclude the possibility that we failed to detect DTCs in the KPT-Early mice for technical reasons related to the flow cytometry preparation, we directly transplanted unfractionated pleural cavity cells from these mice into syngeneic recipient animals. None of the recipient mice injected with pleural cavity cells from KPT-Early mice developed tumors (Fig. 2F).
We and others have previously shown that not all KrasLSL-G12D+/p53fl ox/fl ox late-time point tumor-bearing mice have macrometastases (15, 16). However, we were surprised to find that not all KPT-Late mice had DTCs, and that the number of DTCs was very diverse across mice within this group (Fig. 2B and Supplementary Fig. S3). The presence and number of DTCs was not related to the total tumor burden or time after tumor initiation but did correlate with the number of metastases (Fig. 2B–D and Supplementary Fig. S4). The presence of metastases seeded through hematogenous spread suggests that circulating tumor cells (CTC) in the blood should also be detectable using flow cytometry. We identified CTCs in two KPT-Late animals that also exhibited high numbers of pleural cavity DTCs (Supplementary Fig. S4). Despite extremely high total tumor burden, large numbers of tumors, and relatively long times after tumor initiation, approximately half of the KPT-Late mice had no DTCs in their pleural cavity (Fig. 2B–D).

To determine whether DTCs from KPT-Late mice have tumor-seeding potential, we transplanted a fraction of the pleural cells into syngeneic recipient mice. Tomato positive tumor-seeding potential, we transplanted a fraction of the KPT-Late pleural cavity cells. Scale bar, 2 mm.

Figu re 2. Lung cancer dissemination is a rare late event. A, DTCs are detected in the pleural cavity of KPT-Late mice. Representative plots of FSC/SSC gated, viable (DAPI<sup>−</sup>), lineage<sup>−</sup> cells are shown. The percentage of Tomato<sup>+</sup> DTCs is indicated. B, DTCs are present in the pleural fluid of some but not all KPT-Late mice. The number of mice in each group is indicated. Each dot represents a mouse. C, quantification of the number of DTCs in KPT-Late mice illustrates that not all late-stage tumors have acquired the ability to metastasize, and that DTC number does not correlate with time after tumor initiation in KPT-Late mice. D, representative KPT-Late mice analyzed at the same time point after tumor initiation and with comparable tumor mass have dramatically different numbers of DTCs. Hematoxylin and eosin (H&E) staining is shown. Scale bar, 0.5 cm.

Transplantation of bulk pleural cavity cells from the indicated donor mice into syngeneic recipient mice. Tomato positive metastases that formed from KPT-Late pleural cavity cells are not detected in the pleural cavity of KPT-Late mice; Supplementary Fig. S5). Consistent with published studies, KPT-Late mice developed large primary tumors (with total tumor burden approaching that of KPT-Late mice), but did not develop macrometastases (12, 16). Flow cytometry and direct fluorescent microscopy indicated that KPT-Late mice express tdTomato but remain p53-proficient. As with KPT mice, we generated cohorts of KT mice infected with high-titer Adeno-Cre (3 × 10<sup>9</sup>; KT-Early mice) or low-titer Adeno-Cre (10<sup>7</sup>; KT-Late; Supplementary Fig. S5). Consistent with published studies, KT-Late mice developed large primary tumors (with total tumor burden approaching that of KPT-Late mice), but did not develop macrometastases (12, 16). Flow cytometry and direct fluorescent microscopy indicated that KT-Late mice had neither micrometastases nor cancer cells in the blood (Supplementary Fig. S4 and data not shown). In addition, pleural DTCs were not detected in KT-Early mice.

Inactivation of the p53 tumor suppressor is a common event in human lung adenocarcinoma and is associated with more advanced disease, metastatic spread, and poor patient outcome. p53 could function to inhibit dissemination or could strictly limit metastatic seeding and/or growth in distant organs. To clarify how p53 inactivation contributes to cancer progression and metastasis and therefore patient outcome, we generated KrasLSL-G12D+/p53<sup>fl ox/fl ox</sup> autochthonous model (data not shown).

We confirmed that these metastases were epithelial in origin, with the characteristic poorly differentiated histology of the metastases that typically form in the KrasLSL-G12D+/p53<sup>fl ox/fl ox</sup> autochthonous model (data not shown).

To determine whether DTCs from KPT-Late mice have tumor-seeding potential, we transplanted a fraction of the pleural cells into syngeneic recipient mice. Tomato<sup>+</sup> metastases grew in approximately 40% of recipient mice (2–19 metastases/mouse; Fig. 2E and F). Histologic analyses confirmed that these metastases were epithelial in origin, with the characteristic poorly differentiated histology of the metastases that typically form in the KrasLSL-G12D+/p53<sup>fl ox/fl ox</sup> autochthonous model (data not shown).
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Figure 3. Fluorescent and genetic tumor lineage marking confirms that only rare tumors gain the ability to disseminate. A, representative light and fluorescent images of a Kras<sup>SL-G12D<sub>+</sub></sup>:p53<sup>fl ox/fl ox</sup>:R26<sup>Motley/+<sub></sub></sup> (KPM) lung lobe documents monochromatic tumors. Scale bar, 2 mm. RFP, red fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein. B, quantification of the number of pleural cavity DTCs of each color in seven KPM mice. C, schematic of the barcoded lentiviral-Cre vector. LTR, long terminal repeat. D, barcode sequencing of purified cancer cells from primary tumors and DTCs. The arrow indicates relationship between primary tumor T4<sup>(Fluo)</sup> and DTCs. The sequencing chromatogram shows barcode homogeneity in the DTCs. WRE, woodchuck hepatitis post-transcriptional regulatory element. E, cloning and sequencing of the barcode region confirm that DTCs are largely from a single primary tumor. Barcode sequencing results from three mice are shown as the frequency of that barcode/total number of clones sequenced.

(Fig. 2G). Although most KPT-Late mice did not have cancer cells in their pleural cavity, we did detect Tomato<sup>+</sup> DTCs in 1 of 14 KPT-Late mice (Fig. 2G). This suggests that even in the absence of engineered tumor-suppressor loss, very rare tumors can independently evolve mechanisms that allow dissemination within the timeframe of their growth in vivo in this model. Consistent with the general absence of Tomato<sup>+</sup> cancer cells from KPT mice, transplantation of pleural cavity cells from these mice into syngeneic recipients did not generate metastases (Fig. 2H). These results indicate that at least one major function of p53 is to directly or indirectly regulate phenotypes associated with cancer cell dissemination. In addition, our observation that not all KPT-Late mice have DTCs suggests that, contrary to work in other systems (21, 22), p53 loss per se is insufficient to drive dissemination but rather enables rare cancer cells in a small fraction of primary tumors to gain the required alterations that drive dissemination.

Fluorescent marking in KPT mice allows for the accurate detection of DTCs but does not uncover how many individual tumors are the source of disseminating cancer cells at late time points. Systemic effects, including the ill health of late-stage animals, minor differences in individual's genetic background, and a single advanced tumor's ability to alter the host in such a way as to induce malignant progression of otherwise benign primary tumors, could induce a large number of tumors to synchronously gain the capacity to disseminate (23). In addition, local paracrine effects, including the inflammatory environment, could induce multiple tumors in close proximity to each other to disseminate. On the basis of these considerations, we used two separate systems to determine whether most or all of the cancer cells in the pleural cavity of each KPT-Late mouse are from a single advanced tumor, or whether large numbers of tumors synchronously gain the capacity to disseminate.

To fluorescently mark distinct tumors, we generated a Cre-regulated multicolor Rosa26 knock-in reporter allele based on the recombination of heterotypic lox sites surrounding a stop cassette and three unique fluorescent proteins (R26<sup>Motley+</sub>; Supplementary Fig. S6; ref. 24). Infection of Kras<sup>SL-G12D<sub>+</sub></sup>:p53<sup>fl ox/fl ox</sup>, R26<sup>Motley/+<sub></sub></sup> (KPM) mice generated red, cyan, and yellow fluorescent tumors (Fig. 3A and Supplementary Fig. S6). Despite the presence of multiple large tumors with typical adenocarcinoma features, including high nuclear-to-cytoplasmic ratio, giant cells, and dedifferentiated solid structure, DTCs in each animal were generally only one or two of the three possible colors (Fig. 3B).

To complement our three-color KPM system, we infected KPT mice with a pool of lentiviral vectors that express Cre and carry a unique nucleotide barcode to generate tumors that each have a stably integrated nucleotide barcode (Fig. 3C; ref. 16). To determine whether cancer cells in the pleural cavity were from one or more tumors, we isolated these DTCs and sequenced their barcode region. PCR amplification and Sanger sequencing of the barcode region from eight DTC samples indicated that cancer cells in the pleural cavity are seeded from one dominant parental tumor (Fig. 3D). Cloning and sequencing of the barcode amplicon confirmed that DTCs are largely derived from a single primary tumor (Fig. 3E). Collectively, our fluorescent and genetic clonal marking experiments indicate that in lung adenocarcinoma...
the ability to disseminate is uniquely gained in a cell-intrinsic manner by rare tumors.

The difficulty in identifying and purifying DTCs from patients and the paucity of methods to isolate these cells from autochthonous mouse models have limited their molecular analysis. We and others have previously identified the transcription factor Nkx2-1 as a critical suppressor of lung adenocarcinoma progression and metastatic ability (16, 25, 26). In our initial study, we were unable to determine whether both Nkx2-1

protein B (Sftpb) by qRT-PCR on cancer cells from each stage of metastatic progression. In all but one of the families both Nkx2-1 and Sftpb were downregulated 10-fold or more in DTCs compared with primary tumors (Fig. 4A–C).

Our ability to identify the metastatic primary tumors (TMet) also allowed us to estimate the fraction of the primary tumor composed of the metastatic subclone. Nkx2-1 and Sftpb expression was variable in the TMet tumors with three of four TMet tumors being nearly indistinguishable from Tumor/lineage tumors (Fig. 4B and C). Immunohistochemical staining also indicated that the Nkx2-1

Figure 4. Acquisition of an Nkx2.1

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DISCUSSION

These studies were initiated to gain insight into whether lung tumors have an inherent ability to disseminate or whether this is a major limiting step of lung adenocarcinoma metastasis. Our results indicate that lung epithelial hyperplasias nor most tumors that exist 4 to 8 months after tumor initiation have any ability to shed cancer cells. Our data indicate that the expression of oncogenic Kras and loss of p53 are insufficient to enable lung tumor cells to disseminate, but rather create a permissive genomic context that allows rare tumors to transition to an invasive and metastatic state characterized by downregulation of the lung differentiation transcription factor Nkx2-1. Importantly, some KPT-Late mice with approximately 1 g of tumor mass have no DTCs, whereas others have ≈1,000 pleural DTCs derived from a single tumor. We propose that dissemination is a major hurdle during the natural course of lung adenocarcinoma metastasis.

Unlike in pancreatic and breast cancers in which tumor cells have been suggested to disseminate from premalignant lesions (2, 3), lung adenocarcinomas begin as benign proliferative lesions with a very limited ability to initiate the metastatic cascade. Our data suggest that even once tumors lose p53 and progress to exhibiting the canonical features of adenocarcinoma, they still require additional alterations to physically escape the primary tumor. Although different combinations of oncogenic and tumor-suppressor alterations could drive metastasis through unique mechanisms, disruption of the p53 pathways can allow cancer cells to acquire alterations that allow loss of differentiation before dissemination.

Our data suggest that the ability to seed metastases early during tumor growth is not a general feature of carcinomas that develop in different organs, but rather that the early dissemination from breast and pancreatic cancer likely represents cell type–specific responses to the initiating oncogenic events or the specific environmental context within those organs or early lesions. We speculate that distinct cancer types and subtypes will have dramatically different thresholds for each step of the metastatic cascade. Although pancreatic cancer cells may disseminate from early pancreatic intraepithelial neoplasms, these DTCs seem to have limited-to-no metastasis seeding ability (3). These primary pancreatic tumors thus require the acquisition of additional traits to allow their DTCs to overcome at least one later step of metastasis. Alternately, for cancers in which dissemination requires a considerable change in cell state, including the fraction of human lung adenocarcinomas driven by the molecular mechanisms described in this autochthonous mouse model, disseminated cancer cells should be viewed as clinically meaningful agents of metastatic spread. These initial disseminated cancer cells may already possess many, if not all, of the attributes required to form metastases. As the molecular characterization of CTCs in patients becomes more feasible, these types of studies will help in interpreting the clinical significance of these cancer cells.

Most patients with lung adenocarcinoma with sub-1-cm lung tumors do not present with lymph node metastases or recur with metachronous distant metastatic disease (27–30). Our data suggest that this is likely due to the inability of cells to disseminate, rather than the general inefficiency of each step of the metastatic cascade. Unfortunately, lung adenocarcinoma often presents as overtly metastatic disease, and even patients who have potentially curative surgery often relapse due to the presence of metastases that were below the detection level at the time of diagnosis. Defining the molecular mediators of lung adenocarcinoma metastasis in this and other genomic contexts will be an important area of future investigation.

METHODS

Mouse Strains

Kras<sup>LSL-G12D</sup>, p53<sup>fl ox</sup>, and Rona26<sup>LSL-tdTomato</sup> mice have been described previously (12, 15, 18, 31). We generated mice with the Kras<sup>LSL-G12D</sup> and the R26<sup>LSL-tdTomato</sup> in cis on chromosome 6, guaranteeing retention of the R26<sup>LSL-tdTomato</sup> allele even in genomically unstable tumors. Wild-type male 6- to 10-week-old B6129SF1/J recipients were from The Jackson Laboratory (Stock number: 101043). The Rona26<sup>Moeley</sup> knock-in construct was generated using components of the Brainbow-1.1"M" and Brainbow-1.0"L" plasmids (24). The Rona26<sup>Moeley</sup> allele was generated from V6.5 ES cells using standard methods. Long-range PCR and Southern blotting identified correctly targeted clones. The MIT and Stanford Institutional Animal Care and Use Committees approved all animal studies and procedures.

Tumor Initiation and Quantification

Tumors were initiated by intratracheal injection of mice with adenoviral and lentiviral vectors expressing Cre recombinase as previously described (32). Adenoviral-Cre (AdS-CMV-Cre) was from the University of Iowa Gene Transfer Core. Lentiviral-barcode-Cre (Lenti-BC-Cre) was generated as previously described (16). Tumor area, lung area, as well as Nkx2-1<sup>-positive</sup> and Nkx2-1<sup>-negative</sup> areas, were determined using ImageJ. Tomato<sup>positive</sup> cancer cell number was determined using IHC staining and direct counting. The recombination efficiency of the p53<sup>fl ox</sup> and Kras<sup>LSL-G12D</sup> alleles and the ratio of tumor cells to normal cells at early and late lung adenocarcinoma tumor stages were determined using semiquantitative PCR.

Cell Isolation, Flow Cytometry, and Cell Sorting

Cells within the pleural cavity were collected immediately after euthanasia by making a small incision in the ventral aspect of the diaphragm followed by introduction of 1 mL of PBS. One third of the recovered pleural cancer cells were used for flow cytometry. Approximately 0.5 mL of blood was collected from the carotid artery. Primary tumors and metastases were dissociated as previously described (16). Cells were stained with antibodies to CD45 (30-F11), CD31 (390), F4/80 (BM8), and Ter119 (all from BioLegend) to exclude hematopoietic and endothelial cells. DAPI was used to exclude dead cells. Cells analysis and sorting were performed on BD LSR II analyzers and FACSAria sorters (BD Biosciences).

Transplantation

To assess the tumor-seeding ability of cancer cells from the pleural cavity, one fourth of the total unsorted pleural cells were injected intravenously into the lateral tail vein of syngeneic recipient animals. Recipient mice were analyzed 3 months after transplantation.

Barcode PCR and qRT-PCR on Sorted Cancer Cells

RNA and genomic DNA were extracted from sorted cancer cells using the AllPrep DNA/RNA Micro Kit (Qiagen). Lentiviral barcode
sequences were PCR amplified from genomic DNA and directly sequenced or TOPO cloned (Invitrogen) and sequenced. Fluorescent qRT-PCR was performed on 2 ng of total RNA from each sample. Site-specific target amplification and real-time PCR was performed on the Fluidigm BioMark HD using Taqman primer probe sets for Nkx2.1 (Mm00447558_m1) and SftpB (Mm00455672_g1; Applied Biosystems) using standard methods. Samples were run in quadruplicate and normalized to the average of three housekeeping genes (Rplp0(Mm01245874_g1), Rp29(Mm02342448_gH), and Actb(Mm01205647_g1)).

Histologic Preparation and IHC

Samples were fixed in 4% formalin in PBS overnight and transferred to 70% ethanol until paraﬁn embedding. IHC was performed on 4-μm sections with the ABC Vectastain Kit (Vector Laboratories) with antibodies to Nkx2.1 (Epitomics; EP1584Y), Tomodak Immunochromics; 600-401-379; cleaved caspase-3 (Cell Signaling; 9661), BrdUrd (BD Pharmingen; 555627), Ki67 (BD Pharmingen; 550609), SP-B (Santa Cruz Biotechnology; sc-19378), H3P (Serine 10; Millipore; 96-570), and cytokeratin (Sigma-Aldrich; clone PCK-26). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using the In Situ Cell Death Detection Kit (Roche; 1168417910). Sections were developed with DAB and counterstained with hematoxylin. For in vivo BrdUrd labeling, mice were injected intraperitoneally with 50 mg/kg of BrdUrd 24 hours before analysis. The number of BrdUrd, Ki67, H3P, CC3, and TUNEL-positive cancer cells was quantiﬁed by IHC and direct counting, taking into account the morphologic feature of cancer cells and excluding areas directly adjacent to necrotic areas.

Statistical Analysis

The Mann–Whitney test was used except when the outcome of the experiment was a categorical variable, in which case the Fisher exact test was used.

Disclosure of Potential Conﬂicts of Interest

No potential conﬂicts of interest were disclosed.

Authors’ Contributions

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.R. Caswell, C.-H. Chuang, D. Yang, S.-H. Chiou, C. Kim-Kiselak
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.R. Caswell, C.-H. Chuang, D. Yang, A. Connolly, M.M. Winslow
Writing, review, and/or revision of the manuscript: D.R. Caswell, C.-H. Chuang, D. Yang, C. Kim-Kiselak, A. Connolly, M.M. Winslow
Study supervision: M.M. Winslow
Genotyping animals (mouse): S. Cheemalavagu

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RESEARCH BRIEF

**Obligate Progression Precedes Lung Adenocarcinoma Dissemination**

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