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. Cancer Discov; 4(7); 1–9. © 2014 AACR.
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 onco-
genic mutations in KRAS and inactivation of the p53 tumor-suppressor pathway (10, 11) and has been modeled using 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 loxP-Stop-loxP KrasG12D knock-in mice (KrasLSL-G12D/+). This single event initiates the step-wise 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 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 cells, we incorporated a Rosa26 knock-in Cre-reporter allele (R26LSL-tTomato+/−; ref. 18) into the KrasLSL-G12D/+; p53flox/flox lung adenocarcinoma mouse model to generate KrasLSL-G12D/+; p53flox/flox; R26LSL-tTomato+/− (KPT) mice (Supplementary Fig. S1). In KPT mice, lung tumors initiated by inhaled viral-Cre express oncogenic KrasG12D, delete p53, and express the red fluorescent protein tdTomato (Fig. 1A and B and Supplementary Figs. S1 and S2). We have documented excellent specificity and sensitivity to detect Tomatopositive micro- and macrometastases using fluorescence microscopy and IHC and quantifying single Tomatopositive disseminated cancer cells using flow cytometry, confirming stable marker expression in even the most advanced cancer cells (Figs. 1B and 2A; data not shown).

Tumors in KPT mice are initiated synchronously, thus allowing the analysis of cancer cell dissemination at defined time points after tumor initiation (15, 16). To most accurately compare the ability of early-stage and late-stage lung cancer cells to disseminate, we generated two separate groups of mice, which we called KPT-Early and KPT-Late mice. Tumor initiation with either 5 × 106 Adenoviral-Cre or 2 × 106 Lentiviral-Cre induced 10 to 50 tumors that included small and large adenomas as well as adenocarcinomas with varying degrees of invasion (Fig. 1A–C and Supplementary Fig. S1; data not shown). These KPT-Late mice survived 4 to 8 months. To generate KPT-Early mice, we infected KPT mice with 3 × 109 Adeno-Cre (600-fold more than for KPT-Late mice), which induced high numbers of early-stage hyperplastic lesions such that the total number of neoplastic cells within the lungs was approximately equal to that in KPT-Late mice. KPT-Early mice became tachypneic approximately 1 month after tumor initiation, and histologic analysis indicated that AAHs had replaced almost the entire lung parenchyma (Fig. 1A–D and Supplementary Fig. S1). The Tomatopositive cells that expanded in the lungs of KPT-Early mice displayed histologic and cytologic features of atypical epithelial hyperplasias and expressed lung epithelial markers (Fig. 1D).

The relative tumor burden in KPT-Early and KPT-Late mice was assessed by measuring total lung weight, semiquan-
titative genotyping of total lung DNA for recombination of the KrasLSL-G12D and p53flox alleles, and direct quantification of Tomatopositive tumor cells by IHC (Fig. 1E and F and Supplementary Fig. S2). Collectively, these analyses indicate that similar numbers of neoplastic cells are present in KPT-
Early mice with massive numbers of hyperplastic lesions and in KPT-Late mice with distinct solid tumor masses within larger areas of normal lung. We have additionally confirmed a recombination efficiency of >95% for the KrasLSL-G12D and p53flox alleles in purified Tomatopositive cells from both KPT-Early and KPT-Late mice (Supplementary Fig. S2). By matching both the neoplastic cell number and core genetic alterations in both subsets of mice, this system enables the comparison of DTCs between the two groups.

Dissemination of cancer cells into the blood and lymphatic systems as well as directly into the pleural cavity represents an early stage of metastatic spread. The presence of lung cancer cells in the pleural cavity of human patients with lung adenocarcinoma has been assessed by intraoperative pleural lavage cytology (19, 20). Cancer cells in the pleural cavity correlate with lymphatic invasion and are a strong predictor of distant metastatic relapse, even in patients with stage I disease (19, 20). Malignant pleural effusions as well as the high prevalence of pleural metastases at diagnosis and relapse further suggest that metastatic lung cancer cells frequently invade into the pleural cavity.

The high levels of epithelial hyperplasia in KPT-Early mice represent a large reservoir of “premalignant” tumor cells that, if capable of disseminating, should be detectable in the pleural cavity and vascular circulation of these mice. To determine whether lung epithelial tumor cells are inherently endowed with the ability to disseminate, we performed...
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\(^{LSL-G12D/+}\);p53\(^{fl ox/fl ox}\);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 Kras\(^{LSL-G12D/+}\);p53\(^{fl ox/fl ox}\) 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 circumventing 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\(^{positive}\) 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 Kras\(^{LSL-G12D/+}\);p53\(^{fl ox/fl ox}\) autochthonous model (data not shown).

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 Kras\(^{LSL-G12D/+}\);R26\(^{SL-TdTomato/+}\) (KT) mice, in which the tumor cells express tdTomato but remain p53-proficient. As with KPT mice, we generated cohorts of KT mice infected with high-titer Adeno-Cre (3 \times 10^9; KT-Early mice) or low-titer Adeno-Cre (10^8; 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.
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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>LSL-G12D/+; p53<sup>flox/fox; R26<sup>Motley/+</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 pure tumor cells from primary tumors and DTCs. The arrow indicates the relationship between primary tumor T4 (T<sub>4</sub>) and DTCs. The sequencing chromatogram shows barcode homogeneity in the DTCs. **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 each 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. 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>M</sup>; Supplementary Fig. S6; ref. 24). Infection of Kras<sup>LSL-G12D/+; p53<sup>flox/fox; R26<sup>Motley/+</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 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<sup>positive</sup> and Nkx2-1<sup>negative</sup> cells are equally capable of disseminating but differentially able to seed or grow at distant sites, or whether Nkx2-1<sup>negative</sup> cells have a unique ability to lead the primary tumor (Supplementary Fig. S7; ref. 16).

To address this question, we purified Tomato<sup>positive</sup> cancer cells from primary tumors, disseminated cancer cells, as well as metastases from lymph nodes and pleura. We used the lentiviral barcode sequences to determine the relationship between the primary tumors, DTCs, and metastases (Fig. 4A). This allowed us to segregate primary tumors into those that had seeded metastases (T<sub>Met</sub>) and those that had not (T<sub>nonMet</sub>) and allowed us to choose samples based on the known relationship of the T<sub>Met</sub> tumors, DTCs, and metastases in individual mice (Fig. 4A). This barcode analysis indicated that these data represent the gene expression state of nine individual metastatic families (Fig. 4A). We determined the expression of Nkx2-1 and its canonical target gene surfactant 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 metastatic areas compared with primary tumors (Fig. 4A–C).

Our ability to identify the metastatic primary tumors (T<sub>Met</sub>) 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 T<sub>Met</sub> tumors with three of four T<sub>Met</sub> tumors being nearly indistinguishable from T<sub>nonMet</sub> tumors (Fig. 4B and C). Immunohistochemical staining also indicated that the Nkx2-1<sup>negative</sup> areas comprised diverse proportions in T<sub>Met</sub> tumors (Supplementary Fig. S8). Given the variable size in the metastatic subclones, we investigated whether Nkx2-1<sup>negative</sup> areas have a selective advantage that drives their expansion within the primary tumor. IHC for BrdUrd incorporation, phosphorylated histone H3 (H3P), and Ki67 all uncovered increased proliferation of Nkx2-1<sup>negative</sup> areas (Fig. 4D–F and Supplementary Fig. S8). Importantly, Nkx2-1<sup>negative</sup> areas were consistently more proliferative than the adjacent Nkx2-1<sup>positive</sup> areas within the same tumor, and metastases were also more proliferative than Nkx2-1<sup>positive</sup> primary lung tumors (Fig. 4D–F). Conversely, cell death was low and unaltered in Nkx2-1<sup>negative</sup> primary tumor areas and metastases, suggesting that increased proliferation rather than evasion of cell death contributes to the competitive advantage of cancer cells within Nkx2-1<sup>negative</sup> areas (Supplementary Fig. S8).
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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 neither lung epithelial hyperplasias nor most tumors that exist 4 to 8 months after tumor initiation have any ability to seed 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 differentiation transcription factor Nkx2-1. Importantly, some static state characterized by downregulation of the lung differentiation transcription factor Nkx2-1. These tumors, rather than creating 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, 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>flox</sup> and Rosa26<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 Rosa26<sup>Mklb</sup> knock-in construct was generated using components of the Brainbow-1.1”M” and Brainbow-1.0”L” plasmids (24). The Rosa26<sup>Mklb</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 (Ad5-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>Tomato</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>flox</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 cavity 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. Cell analysis and sorting were performed on BD LSR II analyzers and FACS Aria 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 PCR and qRT-PCR were performed to track cells and confirm metastatic spread.
sequences were PCR amplified from genomic DNA and directly sequenced or TOPO cloned (Invitrogen) and sequenced. Fluidigm 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 (Mm0047558_m1) and Tbp (Mm00455672_g1; Applied Biosystems) using standard methods. Samples were run in quadruplicate and normalized to the average of three housekeeping genes (Gusb[Mm01245874_g1], Rps29[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 paraffin embedding. IHC was performed on 4-μm sections with the ABC Vectastain Kit (Vector Laboratories) with antibodies to Nkx2.1 (Epitomics; EP1584Y), Tomato (Rockland Immunochemicals; 600-401-379), cleaved caspase-3 (Cell Signaling; 9661), BrdUrd (BD Pharmingen; 555627), Ki67 (BD Pharmingen; 550609), SP-B (Santa Cruz Biotechnology; sc-13978), H3P (Scope 10; Millipore; 06–570), 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; 11684817910). 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 test was categorical, 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
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