Pancreatic Cancer Metastases Harbor Evidence of Polyclonality

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ABSTRACT

Studies of the cancer genome have demonstrated that tumors are composed of multiple subclones with varied genetic and phenotypic properties. However, little is known about how metastases arise and evolve from these subclones. To understand the cellular dynamics that drive metastasis, we used multicolor lineage-tracing technology in an autochthonous mouse model of pancreatic cancer. Here, we report that precursor lesions exhibit significant clonal heterogeneity but that this diversity decreases during premalignant progression. Furthermore, we present evidence that a significant fraction of metastases are polyclonally seeded by distinct tumor subclones. Finally, we show that clonality during metastatic growth—leading to either monoclonal or polyclonal expansion—differs based on the site of metastatic invasion. These results provide an unprecedented window into the cellular dynamics of tumor evolution and suggest that heterotypic interactions between tumor subpopulations contribute to metastatic progression in native tumors.

SIGNIFICANCE: Studies of tumor heterogeneity indicate that distinct tumor subclones interact during cancer progression. Here, we demonstrate by lineage tracing that metastases often involve seeding by more than one clone and that subsequent cellular outgrowth depends on the metastatic site. These findings provide insight into clonal diversity and evolution in metastatic disease. Cancer Discov; 5(10): 1–12. © 2015 AACR.

INTRODUCTION

Metastasis is thought to occur as a linear sequence of events involving cellular invasion, bloodstream entry, seeding, and colonization of host organs (1). Classically, this process has been conceptualized as the end result of genetic and epigenetic events involving a single cell, leading to clonally derived lesions at distant sites (1–4). Alternatively, it has been proposed that interactions between distinct tumor subclones may promote tumor dissemination (5–8), and recent data from implantable breast cancer models support the view that metastases can arise from collective migration and colonization of tumor cells (6, 9). Importantly, these observations have been based primarily on aggregate sequencing data and cell transplantation assays, and thus the extent to which they reflect the behavior of cancer cells in their native environment (and in solid tumors other than breast cancer) remains unclear.

Pancreatic ductal adenocarcinoma (PDAC) is predicted to soon become the second leading cause of cancer-related death in the United States, with an overall 5-year survival rate of 5.8% (10). Many factors influence this poor outcome, but the presence of metastatic disease at the time of diagnosis is a pivotal contributor. Genetic sequencing studies of human PDAC have demonstrated the presence of extensive clonal heterogeneity in primary tumors (11, 12). However, the manner in which these subclones contribute to metastases is unclear, as sequencing-based approaches are limited in their ability to assess cellular dynamics.

Lineage labeling has proven to be a key tool in addressing questions of cell fate during tumor progression. We previously demonstrated the utility of lineage tracing to understand the role of early cellular dissemination in an autochthonous model of metastatic pancreatic cancer (13). With the advent of multiplexed labeling, it is now possible to track the contributions of multiple distinct cellular populations, including cancer stem cells, to tumor growth (14, 15). We reasoned that these methods could be exploited to study the clonality of metastases in vivo in the native tumor environment. Accordingly, we used a multicolor reporter system to investigate clonal evolution during metastasis in a model driven by stochastic events.

RESULTS

Modeling Tumor Heterogeneity

To track tumor subpopulations during malignant progression, we used the “Confetti” lineage-labeling system (14, 15), in which Cre-mediated recombination leads to stochastic expression of one of four fluorescent proteins in any given cell. A series of crosses were performed to generate “KPCX” mice (Fig. 1A) in which a tamoxifen-inducible Cre recombinase (PDX1-CreER<sup>Tm</sup>; “C”; ref. 16) simultaneously activates an oncogenic KRAS<sup>G12D</sup> allele (“K”; ref. 17), deletes a single p53 allele (“P”; ref. 18), and generates a color-producing recombination event within the Rosa<sup>Confetti</sup> locus (“X”). The efficiency of recombination in PDX1-CreER<sup>Tm</sup>, Rosa<sup>Confetti</sup> (“CX”) mice following tamoxifen administration was found to be approximately 65% (Supplementary Fig. S1A). All four colors were represented in pancreatic parenchymal cells (Fig. 1B), although green fluorescent protein (GFP) cells were significantly underrepresented compared with cyan...
**Figure 1.** A multicolored lineage-labeled model of pancreatic cancer. **A,** schematic of the KPCX mouse model of pancreatic cancer used in this study, which uses the KRAS\textsuperscript{G12D} ("K"), p53\textsuperscript{fl/+} ("P"), PDX1-CreER ("C"), and Rosa\textsuperscript{confetti} ("X") alleles. Tamoxifen-inducible expression of the pancreas-specific Cre leads to expression of activated mutant KRAS\textsuperscript{G12D}, deletion of one allele of the p53 tumor suppressor, and recombination of the multicolor Rosa\textsuperscript{confetti} Cre-reporter. Recombination of loxP sites within the Confetti locus results in labeling of pancreatic cells with one of four possible colors: nuclear GFP (green), cytoplasmic YFP (yellow), membrane CFP (cyan), and cytoplasmic RFP (red). **B,** representative confocal fluorescent image of a section from a 10-week-old CX mouse pancreas depicting expression of the different Rosa\textsuperscript{confetti} fluorescent labels. Labeling is principally seen in acinar cells, with negligible labeling of duct cells. **C,** hematoxylin and eosin (H&E) images of malignant progression in KPCX mice following tamoxifen (TAM) administration at birth. The pancreata of KPCX mice are initially normal but develop acinar-to-ductal metaplasias (ADM), pancreatic intraepithelial neoplasias (PanIN), and PDAC with a reproducible time course. **D,** representative tile scan images of a cross-section through a KPCX pancreas. H&E staining (top) and fluorescence imaging (bottom) demonstrate the presence of two anatomically distinct monochromatic primary tumors within an apparent single pancreatic mass. **E,** magnified fluorescent images from the tumor center (i, iii), periphery (iv), and border between adjacent clones (ii) of the pancreatic tumors depicted in **D.** **F,** a mean of four distinct monochromatic tumor lesions (as depicted in **D** and **E**) were found in each KPCX mouse (•). Data pooled from 26 tumor-bearing KPCX mice. Scale bars, 100 μm for **B,** C, and **E,** and 300 μm for **D.**
fluorescent protein (CFP), red fluorescent protein (RFP), and yellow fluorescent protein (YFP) cells, as has been previously reported (15). Importantly, cell marking was stable, as clonally isolated cells maintained in culture for weeks to months exhibited no evidence of color switching (Supplementary Fig. S1B).

Two other models of PDAC—“KPC” and “KPCY” (which carries a YFP allele instead of the Confetti allele used here)—recapitulate most salient aspects of the human disease (13, 17). Because oncogenic mutations in the KPC model occur postnatally, we compared features of tumor formation in KPC mice to these other, more well-established, PDAC models. Following tamoxifen administration to pups, KPC mice developed pancreatic intraepithelial neoplasia (PanIN) lesions within 8 to 10 weeks and invasive PDAC within 14 to 16 weeks, a time course consistent with that described for KPC and KPCY mice (Fig. 1C and Supplementary Fig. S1C). Moreover, KPCY tumors were indistinguishable from KPC and KPCY tumors at a histologic level (Supplementary Fig. S1D) and exhibited a similar pattern of metastatic spread (predominantly liver, lung, parietal peritoneum, and diaphragm). Thus, KPCY mice behaved similarly to KPC and KPCY mice, even though KRAS and p53 mutations were generated after birth. Despite having what appeared at a gross level to be a solitary dominant tumor mass (Fig. 1D, top), examination of color distributions revealed that most pancreatic lesions were actually a conglomerate of multiple tumors arising from independent initiating events (Fig. 1D, bottom and Fig. 1E). Individual tumor foci contained a single-colored (“monochromatic”) population of cells, which shared borders with adjacent tumor, PanIN, or normal tissue (Fig. 1E). Quantification following serial sectioning revealed that each pancreatic mass contained, on average, four of these monochromatic lesions (Fig. 1F). Retrospectively, it was sometimes possible to recognize these multifocal tumors by virtue of regions with distinctive histology, a phenomenon also observed with KPC and KPCY mice (data not shown).

PanIN Lesions Are Associated with a Clonal Bottleneck

Multiple lines of evidence suggest that PDACs arise from PanINs, which themselves arise from acinar-to-ductal metaplasia (ADM; refs. 13, 19). We thus sought to understand how and when clonality changes during premalignant stages of tumor progression. To this end, we examined serial pancreatic sections from 8- to 10-week-old KPCX mice, a time point at which only PanINs and ADMs were present. We found that roughly a fourth (24%) of all ADMs were polychromatic, indicating that they arose from multiple distinct acinar cells (Fig. 2A and C). In contrast, almost all PanIN lesions (97%) were found to be monochromatic (Fig. 2B and C). In the context of an ADM → PanIN → PDA model, therefore, these results suggest that premalignant progression is associated with a loss of clonal diversity during the transition from ADM to PanIN.

Polyclonality in Peritoneal and Diaphragmatic Metastases

We next sought to understand how clonality evolves during metastasis. To this end, we used the multifocal nature of KPCX tumors to model subclonal heterogeneity. In human cancers, subclones are defined genetically as distinct tumor populations that share a set of identical founder mutations (2, 11, 20). In this regard, each fluorescent KPCX clone shares an identical KRAS and p53 mutation but otherwise evolves independently and can thus be used to represent a distinct subclonal population. Using the fluorescent marker in each lesion, we then tracked their contribution to metastasis formation.

We initially examined metastases to the peritoneal wall and diaphragm. Each KPCX animal had, on average, two to four distinct large metastatic foci at each of these sites (Supplementary Fig. S2A–S2C). Importantly, these metastases were spatially well separated from the pancreas and from each other, eliminating the possibility of direct local invasion as a route of spread. Surprisingly, nearly 80% of these large lesions were polychromatic (Fig. 3A–C), indicating that they originated from more than one source in the primary tumor. Likewise, examination of peritoneal and diaphragmatic micrometastases also revealed the presence of polychromatic lesions (Fig. 3D and Supplementary Fig. S2A), demonstrating that polyclonality is present in small as well as large metastatic lesions. Interestingly, polychromatic metastases were composed of at most two distinct populations (i.e., tricolor metastases were not observed even if the pancreas harbored three distinctly colored tumors). Thus, pancreatic cancer metastases to the peritoneum and diaphragm are frequently polyclonal.

Evidence That Polyclonal Diaphragmatic Metastases Come from Polyclonal Clusters

Polyclonal metastases could arise from the outgrowth of lesions that were polyclonal at the time of seeding or through a two-step mechanism involving seeding by one clone and subsequent recruitment (reseeding) by another (21). Examination of ascites fluid from tumor-bearing KPCX mice revealed the presence of bichromatic cellular aggregates ranging in size from 2 to 50 cells in 5 out of 5 KPCX mice (Fig. 4A), suggesting that polyclonality might arise through the former mechanism.

To further understand the dynamics of polyclonal metastasis formation, we performed a series of in vivo cell-mixing experiments in which low-passage cell lines were derived from a multicolor (RFP/YFP) diaphragmatic metastasis and then FACS sorted into their RFP (458d_R) and YFP (458d_Y) components (Supplementary Fig. S3A and S3B). We then injected 30,000 cells intraperitoneally into 6- to 8-week-old NOD.SCID mice, either as a 1:1 suspension of single 458d_R and 458d_Y cells or after the cells were allowed to form multicolored clusters (Fig. 4B and Supplementary Fig. S3B). After 3 weeks, diaphragmatic tissue was harvested and the number of gross monochromatic and polychromatic lesions was examined using fluorescent stereomicroscopy. Significantly, although polychromatic lesions constituted the majority (70%) of metastases following injection of cell clusters, only monochromatic lesions were seen following injection of the single-cell suspension (Fig. 4B and C). Furthermore, cell clusters were more efficient than single cells at forming metastases (Fig. 4D). Taken together, these data suggest that polyclonal peritoneal metastases develop from multiclonal aggregates shed from the primary tumor.

Polyclonality in Liver and Lung Metastases

We then turned our attention to the liver and lung, the most common sites of metastasis in human PDAC (12), which are thought to arise through hematogenous dissemination.

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Figure 2. Clonal selection occurs early in tumor progression. **A** and **B**, fluorescent images of ADM (**A**) and PanIN (**B**) of 10-week-old KPCX mice. In **A**, ADMs are positive for the ductal marker KRT19 (white). Representative examples of monochromatic (top) or polychromatic (bottom) ADMs are shown. In **B**, representative images of monochromatic PanINs are shown. **C**, quantification of monochromatic and polychromatic ADMs and PanINs in 10-week-old KPCX mice. Data were pooled from *n* = 3 mice, and the total number of lesions counted is shown. The bar graph on the right shows the mean percentage of ADMs and PanINs in each group. Error bars represent 95% confidence intervals. *, *P* < 0.001 by the Fisher exact test between lesion type in ADMs and PanINs. Scale bars, 25 μm for **A** and 50 μm for **B**.

To characterize the size distribution of metastatic foci, we counted individual lesions and binned them according to size: single cell, nanometastases (2–10 cells), micrometastases (11–100 cells), and macrometastases (>100 cells; Supplementary Fig. S4A and S4B). This analysis revealed that the majority of liver and lung metastases in KPCX mice were single cells or nanometastases, with only 10% to 26% having more than 10 cells (Supplementary Fig. S4C). We then analyzed metastatic deposits in each size category for number and color composition. This revealed that 11% to 14% of nanometastases and micrometastases contained two clones (Fig. S5A–C); tricolored metastases were never observed. Surprisingly, and in contrast to our findings in peritoneum and diaphragm, large polyclonal metastases were also not observed (Fig. S5B and C).

This result raised the possibility that the development of large metastases in the lung and liver might involve preferential...
Clonality of Metastases

Figure 3. Polyclonal metastasis is a frequent event in murine pancreatic cancer. A, representative fluorescent images and H&E stains of adjacent sections depicting polychromatic metastasis in the peritoneum. B, representative fluorescent images and H&E stains of adjacent sections depicting polychromatic metastasis in the diaphragm. C, total counts and percentages of metastases in each site. Data pooled from n = 7 (peritoneum) and n = 9 (diaphragm) 14- to 16-week-old tumor-bearing KPCX mice. D, fluorescent images of microscopic metastases from the peritoneal lining (top) and diaphragm (bottom). Scale bars, 100 μm for A and B, and 25 μm for D.

outgrowth of one clone over another. To test this possibility, we quantified changes in the color composition of polychromatic liver and lung metastases as a function of cell number. Consistent with the notion of selective outgrowth, we found that lesions became increasingly dominated by a single clonal population as they increased in size (Fig. 5D and Supplementary Fig. S5A). This process was evident early, with approximately 90% of each polychromatic lesion dominated by one clone by the 10-cell stage (Fig. 5E and Supplementary Fig. S5B). These results indicate that although small metastatic lesions in the lung and liver may be polyclonal, there is an early drift toward monoclonality during metastatic growth in these tissues.

Evidence That Polyclonal Clusters Seed Polyclonal Lung Metastases

As with diaphragmatic and liver metastases, polyclonal liver and lung metastases could arise via seeding of polyclonal lesions present in the circulation or by seeding of one clone followed by subsequent seeding by a second clone. Examination of blood smears from tumor-bearing KPCX mice revealed that clusters of fluorescent tumor cells were abundantly present in the bloodstream, where they comprised approximately 7% to 20% of all circulating tumor cells (Fig. 6A). Furthermore, in 2 of 6 mice, multicolor clusters were detected in the circulation (Fig. 6B).

To further resolve the cellular mechanisms by which polyclonal metastases form, we again performed a series of cell-mixing experiments with the 458d_R and 458d_Y cells in a retro-orbital injection model of lung metastasis (22). First, we injected 458d_R and 458d_Y cells simultaneously (either as a single-cell suspension or as clusters) and then examined the lungs 24 hours after injection (Fig. 6C). Similar to our results with peritoneal metastasis, metastatic burden was markedly higher following injection of tumor cell clusters compared with single cells when the same number of cells was introduced (Supplementary Fig. S6A and S6B).

We then examined the color makeup of the metastatic lesions following injection of single cells or clusters (Fig. 6C). Although roughly 36% of metastatic lesions following the injection of clusters were polychromatic, all lesions seen following injection of single cells were monochromatic (Fig. 6D). This result suggests that cellular aggregates capable of giving rise to polychromatic metastases do not readily form in the bloodstream. Finally, to assess whether multicolored lung metastases could arise via recruitment, we injected 20,000...
**Figure 4.** Polyclonal diaphragm metastases are seeded by polyclonal clusters. A, bright-field (i) and fluorescent images (ii) of a multicolored cluster of disseminated tumor cells in the ascites. B, intraperitoneal injection of 458d_R and 458d_Y cells (30,000) either as a suspension mixture of single cells (top) or multicolor clusters (bottom) into NOD.SCID mice. Images are paired bright-field (left) and fluorescent (right) stereomicroscope images from mice 3 weeks following injection with \( n = 4 \) mice for each group. Monochromatic lesions are either YFP- or RFP-positive and polychromatic lesions are both YFP- and RFP-positive. C, bar graph depicting mean percentage of total gross monochromatic (RFP or YFP only) or polychromatic (positive for both YFP and RFP) metastases between single-cell and cluster injection groups. Data pooled from \( n = 4 \) mice for each group (a total of 24 lesions were counted in the single-cell group and 50 lesions were counted in the cluster group). No lesions from the single-cell injection group were polychromatic. *, \( P < 0.001 \) by the Fisher exact test comparing multicolor metastases between single-cell and cluster injections. D, bar graph depicting the mean number of gross metastases in the single-cell and cluster injection groups (\( n = 4 \) mice in each group). Error bars represent SEM. *, \( P = 0.0026 \) by the Student t test. Scale bars, 25 \( \mu \)m for A and 1 mm for B.

458d_R or 458d_Y cells retro-orbitally on day 0, followed by injection of 20,000 cells of the other population on day 3, and then assessed the color makeup of the resulting metastases (Fig. 6E). Consistent with our previous results, all resulting metastases were monochromatic (Fig. 6E), suggesting that multicored metastases in this model are likely to come from seeding by polyclonal clusters of tumor cells rather than sequential rounds of seeding.

**DISCUSSION**

Efforts to understand the clonal origins of metastases often invoke cell-autonomous factors as primary determinants of spread (1). Using an unbiased lineage-labeling approach, we have observed that metastases can have polyclonal origins and that further outgrowth can differ based on the location of the lesion. Importantly, such evidence comes from an autochthonous model, making it likely that this phenomenon reflects cellular behavior in the context of naturally evolving tumors. Taken together, our results are consistent with a model in which heterotypic interactions between tumor subclones, in conjunction with site-dependent selective pressures, influence metastatic initiation and progression (Fig. 6F).

Genomic sequencing of human primary pancreatic tumors and their associated metastases has failed to identify a consistent prometastatic gene signature or "metastatic driver"
metastases could arise, including (i) seeding by polyclonal cell clusters forming cooperative invasion, facilitating cell survival in the bloodstream, and/or cooperatively enhancing the fitness of different tumor subsets (6, 7, 9, 23). Our data are consistent with this notion of cooperativity, as the high frequency of polyclonal metastasis in the KPCX model (11%–14% in lung and liver and 80% in peritoneum and diaphragm) and the enhanced metastatic phenotype associated with disseminated cell clusters observed by us and others (9) suggest that clonal interactions may provide a metastatic advantage. Although the molecular nature and functional consequences of such putative interactions remain to be determined, it is enticing to imagine that clonal collaboration improves the efficiency of metastatic spread by enhancing cellular invasion, facilitating cell survival in the bloodstream, and/or improving seeding/colonization at distant sites.

One can envision several mechanisms by which polyclonal metastases could arise, including (i) seeding by polyclonal clusters that form within ascites fluid or the circulation, or (ii) independent seeding by distinct clones at the same location, either simultaneously or sequentially. Using low-passage cell lines derived from a polyclonal YFP/RFP metastasis, we found that injection of polychromatic clusters, but not single cells, resulted in polychromatic metastases. Because either of the latter mechanisms should have resulted in polychromatic lesions following the injection of single cells, the failure to find such lesions supports the first possibility (i.e., polychromatic lesions are unique to each metastatic site (4)). This interplay is reflected in our system as evidenced by the differences in clonal outgrowth between organ sites, whereby peritoneal and diaphragmatic

mutations (11, 12). These findings suggest a role for non-genetic mechanisms during metastasis formation, and recent data from implantable tumor models suggest that cell-cell interactions may facilitate metastasis development by enhancing collective invasion, altering the tumor microenvironment, and/or cooperatively enhancing the fitness of different tumor subsets (6, 7, 9, 23). Our data are consistent with this notion of cooperativity, as the high frequency of polyclonal metastasis in the KPCX model (11%–14% in lung and liver and 80% in peritoneum and diaphragm) and the enhanced metastatic phenotype associated with disseminated cell clusters observed by us and others (9) suggest that clonal interactions may provide a metastatic advantage. Although the molecular nature and functional consequences of such putative interactions remain to be determined, it is enticing to imagine that clonal collaboration improves the efficiency of metastatic spread by enhancing cellular invasion, facilitating cell survival in the bloodstream, and/or improving seeding/colonization at distant sites.

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**Figure 6.** Polyclonal lung metastases are seeded by polyclonal circulating tumor cell (CTC) clusters. **A,** bar graph depicting the mean percentage of single-cell CTC and CTC cluster events per mL of blood in 6 individual KPCXY tumor-bearing mice. Total CTC events/mL are shown for each mouse are listed to the right of the graph. Percentage of CTC clusters ranged from 7% to 20%. **B,** bright-field (top) and fluorescent (bottom) image of a multicolor CTC cluster isolated from the blood of a KPCXY mouse. **C,** retro-orbital injection of 458d_R and 458d_Y cells (20,000) either as mixture of single cells (top) or multicolor clusters (bottom) into NOD.SCID mice. Right, representative fluorescent images of resulting metastatic lung lesions in the two injection groups. **D,** quantification of the data in C. The mean percentage of monochromatic (RFP or YFP only) or polychromatic (positive for both RFP and YFP) metastases are indicated in stacked graph format for each injection group (single cell or cluster). Data are pooled from n = 4 mice for each group (a total of 208 lesions were counted for the single-cell group and 607 lesions were counted for the cluster group). *, P < 0.001 by the Fisher exact test comparing the frequency of polychromatic metastases between single-cell and cluster injections. **E,** retro-orbital injection of 458d_R and 458d_Y cells as sequential injections of single cells into NOD.SCID mice separated by 3 days. Right, representative fluorescent images of lung metastatic lesions detectable 21 days later. Table shows total metastatic counts and the percentage of monochromatic and polychromatic lesions. Data pooled from n = 4 mice. *, P < 0.001 by the Fisher exact test. **F,** model for the development of polyclonal metastases. Polyclonal cell clusters derived from the primary tumor give rise to polyclonal seeding events, followed by either monoclonal or multiclonal outgrowth depending on tissue site. Scale bars, 25 μm for B, C, and E.
metastases remain polyclonal and lung and liver lesions drift toward monoclonality. Although this observation may in part reflect differences in the behavior of lesions derived from early versus late dissemination, it also suggests that the peritoneum and diaphragm provides a more permissive state for multiclonal expansion. Conversely, factors intrinsic to lung and liver may exert significant selective pressures during metastatic growth.

One advantage of lineage tracing is that it permits the recognition of selective “bottlenecks” at discrete stages during the life of a stochastically evolving tumor. Specifically, we observed that nearly a quarter of ADMs are polyclonal, whereas almost all PanINs are monoclonal. As ADMs are widely viewed as the precursors to PanINs (19), this observation suggests that PanIN formation is associated with clonal expansion. For example, such bottlenecks could reflect the outgrowth of a KRAS/p53-mutant cell from a field of wild-type cells that have undergone ADM. Alternatively, this reduction in clonal diversity might reflect the outgrowth of a cell with additional genetic and/or epigenetic changes from a field of cells that are already mutant for KRAS and p53. Likewise, the transition to frank carcinoma is associated with a further decrease in clonal diversity, as only a few dominant lesions emerge from the large number of PanIN precursors present in each KPCX pancreas. These results indicate that selective pressures acting throughout tumorigenesis can influence the clonal progression through each of these bottlenecks. In the future, it should be possible to use this system to delineate the mechanistic underpinnings of these hurdles to progression in native tumor environments, providing new therapeutic avenues.

**METHODS**

**Mice**

To perform lineage labeling, a series of backcrosses were performed to introduce the Rosa<sup>Confetti</sup> (“X”) reporter allele into mutant strains bearing PDX1CreER (“C”), KRAS<sup>G12D</sup> (“K”), and p53<sup>fl/+</sup> (“P”) alleles to obtain PDX1CreER; KRAS<sup>G12D</sup>; p53<sup>fl/+</sup>; Rosa<sup>Confetti</sup> (“KPCX”) and PDX1CreER; Rosa<sup>Confetti</sup> (“CX”) mice. For some experiments, animals were homozygous for the reporter allele or contained the Rosa<sup>YFP</sup> (“KPCXY”) in lieu of the second Confetti allele. To induce recombination, a suspension of tamoxifen (MP Biomedicals) in corn oil (Sigma-Aldrich) was administered to pups via lactation following oral gavage of the mother. A single dose of 6 mg of the drug on postnatal days 0, 1, 2, and 4. All experiments involving control CX mice were performed at 8 to 10 weeks of age following tamoxifen administration as described above. For studies involving PanIN lesions, KPCX mice were sacrificed at 10 weeks of age, following tamoxifen (MP Biomedicals) in corn oil (Sigma-Aldrich) was administered to pups via lactation following oral gavage of the mother. With 6 mg of the drug on postnatal days 0, 1, 2, and 4. All experiments involving control CX mice were performed at 8 to 10 weeks of age following tamoxifen administration as described above. For studies involving PanIN lesions, KPCX mice were sacrificed at 10 weeks of age, a time point at which there was no evidence of carcinoma based on histologic evaluation. PDAC-bearing KPCX mice were examined three times per week for evidence of morbidity and euthanized accordingly. On average, tumor-bearing KPCX mice were 14 to 16 weeks of age at time of sacrifice (as shown in Supplementary Fig. S1C). PDX1Cre; KRAS<sup>G12D</sup>; p53<sup>fl/+</sup>; Rosa<sup>YFP</sup> (“KPCXY”) mice used in this study were generated as previously described (13). All experiments were performed in accordance with the NIH policies on the use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Philadelphia, PA).

**Cell Sorting and Culture**

Pancreatic tumors were dissociated into single-cell suspensions through mechanical separation and enzymatic digestion as described previously (13). FACS sorting was performed using the FACSAriaII (BD Biosciences) sorter at the Penn PathBioResource Flow Cytometry Core. Excitations of Confetti colors were performed using the 488-nm argon laser for YFP, 405-nm violet laser for CFP, and the 532-nm red laser for RFP. Detection was performed using bandpass filters at 530/40 nm for YFP, 450/50 nm for CFP, and 575/25 nm for RFP. Cells were collected into a 2% BSA/PBS solution and cultured in pancreatic ductal cell media (13). For monitoring of fluorescent cell marker stability, >5,000 cells were counted from passage 1 and passage 5 cultures following a 3- to 4-week interval. The total number of each fluorescent cell type was counted and averaged from five replicates.

For in vivo cell-mixing experiments, cells were isolated as described above from a polychromatic diaphragmatic metastasis (mouse 458b) containing RFP and YFP populations. Cells were FACS sorted and cultured as described. No cell line authentication was performed, as all cell lines used in this article were obtained from primary cultures of tumors derived from KPCX mice.

**Image Acquisition and Quantification**

Immunofluorescent (IF) images were acquired using both confocal and inverted fluorescent microscopes. The Zeiss LSM 710 confocal microscope used the argon laser 488-nm line to excite nuclearGFP (nGFP), 514-nm line for YFP, 561-nm red diode laser for RFP, laserline at 458 nm for membraneCFP (mCFP), and 405-nm laser line for DAPI. Fluorescence was collected between 495 and 514, airy 0.41 for nGFP; 524–563, airy 0.41 for YFP; 585–622, airy 0.41 for RFP; 462–498, airy 0.41 for mCFP; and 417–494, airy 0.41 for DAPI. Images were taken with Zen 2011 software and spectral imaging coupled with image analysis using linear unmixing was performed. Imaging was performed with the Olympus IX71 inverted multicolor fluorescent microscope equipped with the following dry objective lenses: ×4 (UPlanFLN NA0.13), ×10 (UPlanFLN NA0.30), ×20 (UPlanFLN NA0.50), and ×40 (UPlanFLN NA0.75). A mercury short arc lamp source (Oxram) and the following excitation/emission filter sets (Chroma) were used to detect fluorescence: 535/500 nm for YFP/nGFP, 480/436 nm for CFP, 645/560 nm for RFP, 406/350 nm for DAPI, and 690/640 nm for Far Red. Images were captured with the DP71 camera (Olympus). Cytoplasmic YFP was distinguished from nuclear GFP based on cellular localization; GFP recombination events were rare compared with YFP. Histologic images were captured using the Olympus BX1 light microscope attached to the DP25 camera (Olympus). Montage hematoxylin and eosin (H&E) images were taken with the EVOS FL auto cell-imaging system (Thermo Fischer) with a x10 objective. Stereomicroscope images were obtained using the Lecia M216FA fluorescent microscope. Assembly and analysis of all CMYK images were performed using the ImageJ 1.47v software.

**Multicolor Immunofluorescence and Histologic Analysis**

Tumors and tissue samples were fixed in 4% paraformaldehyde at room temperature for 30 to 60 minutes, followed by an overnight incubation in 30% w/v sucrose solution. Samples were then embedded in optimum cutting temperature (OCT; Tissue-Tek) and frozen on dry ice. Once solid, 10-μm sections were cut with a Microm HM550 cryostat (Thermo Scientific). Serial sectioning was performed on all tissue samples with 70 to 100 μm between sections. All sections were laid out in a similar orientation on each slide, so that any given lesion could be tracked over multiple levels based on anatomic tissue landmarks and XY coordinates on the slide. Sections were stained with the nuclear marker DAPI (Life Technologies; 1:1,000) and the ductal marker cytokeratin-19 (KRT-19, rabbit, 1:1,000; ref. 24). For immunostaining, frozen tissues were blocked in StarvingBlock (Thermo Scientific) with 2% donkey serum and 0.1% Tween-20 for 1 hour, and incubated for 1 hour in primary antibody diluted in blocking buffer in a humidified chamber. Sections were washed three times in PBS containing 0.1% Tween-20 and incubated with DAPI and Alexa Fluor 633–conjugated antibodies at a 1:250 dilution for 1 hour at room temperature, followed by an additional three washes, and mounted on slides with Aqua polymount mounting reagent (Polysciences). Histologic analyses...
were performed on adjacent frozen sections with H&E staining using standard protocols. Recombination efficiency of the Rosa<sup>Confetti</sup> allele was examined in CX mice by counting the fluorescent cells of each color and dividing by the total number of DAPI-positive cells. Data were gathered from five random pancreas fields in five adjacent levels. DAPI-positive cells were counted using the ImageJ 1.47v software, resulting in >16,000 DAPI-positive cells counted per pancreas. ADMs and PanINs were identified in pancreatic tissue based on typical histologic features (13) and positive staining for KRT-19. Quantification was performed by counting the total number of ADMs and PanINs in one section from each level throughout the entirety of the pancreas. Lesions were categorized as monochromatic if >95% of the cells in the lesion were of a single color and polychromatic if they contained cells of more than one color. To ensure accuracy, all PanINs that were identified as monochromatic were tracked in adjacent levels in their entirety. Analysis of metastases was performed by counting all distinct fluorescent lesions in one section from each level throughout the organ. To avoid counting larger lesions more than once, all lesions >50 cells in size were tracked in all adjacent levels in their entirety. Lung and liver metastases were binned on the basis of the number of DAPI-positive fluorescent cells in each lesion. Groupings included single (1 cell), nano (<10 cells), micro (11–100 cells), and macro (>100 cells). Lesions were scored as monochromatic if >95% of the cells in a lesion were the same color; otherwise they were considered polychromatic. Counting of lung metastases from <i>in vivo</i> mixing assays was performed on multiple sections taken from each organ. Diaphragnostic lesions were assessed based on stereomicroscopic images. To quantitate the number of distinct lesions within each primary tumor mass, we defined a “tumor clone” as an anatomically contiguous region of monochromatic cells that shared distinct histologic and IF borders with adjacent clones as examined in multiple levels throughout the pancreatic mass.

In Vivo Cell-Mixing Experimental Metastasis Assay

For intraperitoneal injections, an equal number of cells (30,000) were injected either as a mixture of single cells or as clusters of 458d_R and 458d_Y cells into the peritoneal cavity of 6- to 8-week-old NOD. SCID mice using a 27-gauge needle. For lung metastasis, an equal number of cells (20,000) were injected retro-orbitally, as previously described (22), either as a mixture of single cells or as clusters of 458d_R and 458d_Y cells, into 6- to 8-week-old NOD.SCID mice. Clusters were generated by mixing an equal number of RFP and YFP cells in a low-attachment petri dish (Corning) and placed on a rocker for 8 to 12 hours at a 37°C incubator.

Analysis of Cell Clusters from Ascites and Blood

Ascitic fluid was isolated from the abdominal cavity of euthanized tumor-bearing mice with a 3-ml insulin syringe containing 100 μl of 1 mg/ml heparin sulfate (Sigma-Aldrich, H3149) to prevent coagulation. Fluid was immediately placed on a 100-cm plate (BD Falcon) containing PBS with minimal manipulation. Monochromatic and polychromatic disseminated tumor cell clusters were identified by direct visualization on a fluorescent microscope and imaged. Similarly, blood was obtained via cardiac puncture and immediately placed on a 100-cm plate (BD Falcon) containing PBS with minimal manipulation and then visually examined for cell clusters.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: R. Maddipati, B.Z. Stanger

Development of methodology: R. Maddipati, B.Z. Stanger

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Maddipati, B.Z. Stanger

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Maddipati, B.Z. Stanger

Writing, review, and/or revision of the manuscript: R. Maddipati, B.Z. Stanger

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Maddipati

Study supervision: R. Maddipati, B.Z. Stanger

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Clonality of Metastases

# CANCER DISCOVERY

## Pancreatic Cancer Metastases Harbor Evidence of Polyclonality

Ravikanth Maddipati and Ben Z. Stanger

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