

EMT and Dissemination Precede Pancreatic Tumor Formation

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SUMMARY

Metastasis is the leading cause of cancer-associated death but has been difficult to study because it involves a series of rare, stochastic events. To capture these events, we developed a sensitive method to tag and track pancreatic epithelial cells in a mouse model of pancreatic cancer. Tagged cells invaded and entered the bloodstream unexpectedly early, before frank malignancy could be detected by rigorous histologic analysis; this behavior was widely associated with epithelial-to-mesenchymal transition (EMT). Circulating pancreatic cells maintained a mesenchymal phenotype, exhibited stem cell properties, and seeded the liver. EMT and invasiveness were most abundant at inflammatory foci, and induction of pancreatitis increased the number of circulating pancreatic cells. Conversely, treatment with the immunosuppressive agent dexamethasone abolished dissemination. These results provide insight into the earliest events of cellular invasion *in situ* and suggest that inflammation enhances cancer progression in part by facilitating EMT and entry into the circulation.

INTRODUCTION

Each step in the metastatic cascade is highly inefficient. Only a small fraction of cells from a primary tumor enter the circulation, and less than 0.01% of these develop into metastases (Gupta et al., 2005). It is thought that tumor cells pass through several stages during which they sequentially acquire the ability to invade through basement membrane(s), enter and exit the bloodstream, and survive and grow in distant organs. Because each of these events is rare, studies of the metastatic process have relied heavily upon cells that have been cultured and

manipulated *in vitro* and reintroduced into recipient animals. As a result, there remains considerable uncertainty regarding the factors that influence each stage *in vivo* as well as the timing of dissemination itself.

Clinical observations, mainly in the field of breast cancer, have given rise to two major metastasis paradigms. The classical model treats metastasis as the final step in a progressive “Darwinian” sequence, in which tumors acquire mutations that promote invasive behavior and dissemination late in tumor evolution (Cairns, 1975). This model has several conceptual problems (Gupta et al., 2005; Klein, 2009) and fails to account for two clinical observations: the appearance of metastatic lesions years after resection of small tumors with no clinically evident metastases at diagnosis (Pantel et al., 2008) and metastases of unknown primary tumors, which account for as many as 4%–5% of all clinical metastases (Greco and Hainsworth, 2009). An alternative model has been proposed that envisions metastasis as an inherent feature of a tumor very early in its natural history (Hellman, 1994; Klein, 2009). Although direct evidence for this model is limited, recent studies of breast cancer are consistent with the notion that metastatic seeding may be mediated by cells that would not meet a standard definition of cancer (Hüsemann et al., 2008; Podsypanina et al., 2008). Furthermore, several small studies concluded that the presence of putative disseminated tumor cells in the bone marrow of patients with low-grade mammary tumors or carcinoma *in situ* correlates with a worse outcome (Ignatiadis et al., 2011; Sängler et al., 2011). The possibility that cellular dissemination leading to metastasis occurs prior to the formation of an identifiable primary tumor has significant clinical and biological implications.

One of the challenges in studying tumor cell dissemination has been the identification of markers that distinguish cancer cells from cells that normally reside in the bloodstream or at sites of seeding. During malignant progression, it has been proposed that carcinoma cells undergo an epithelial-to-mesenchymal transition (EMT), in which they lose epithelial characteristics and acquire invasive properties and stem cell-like features

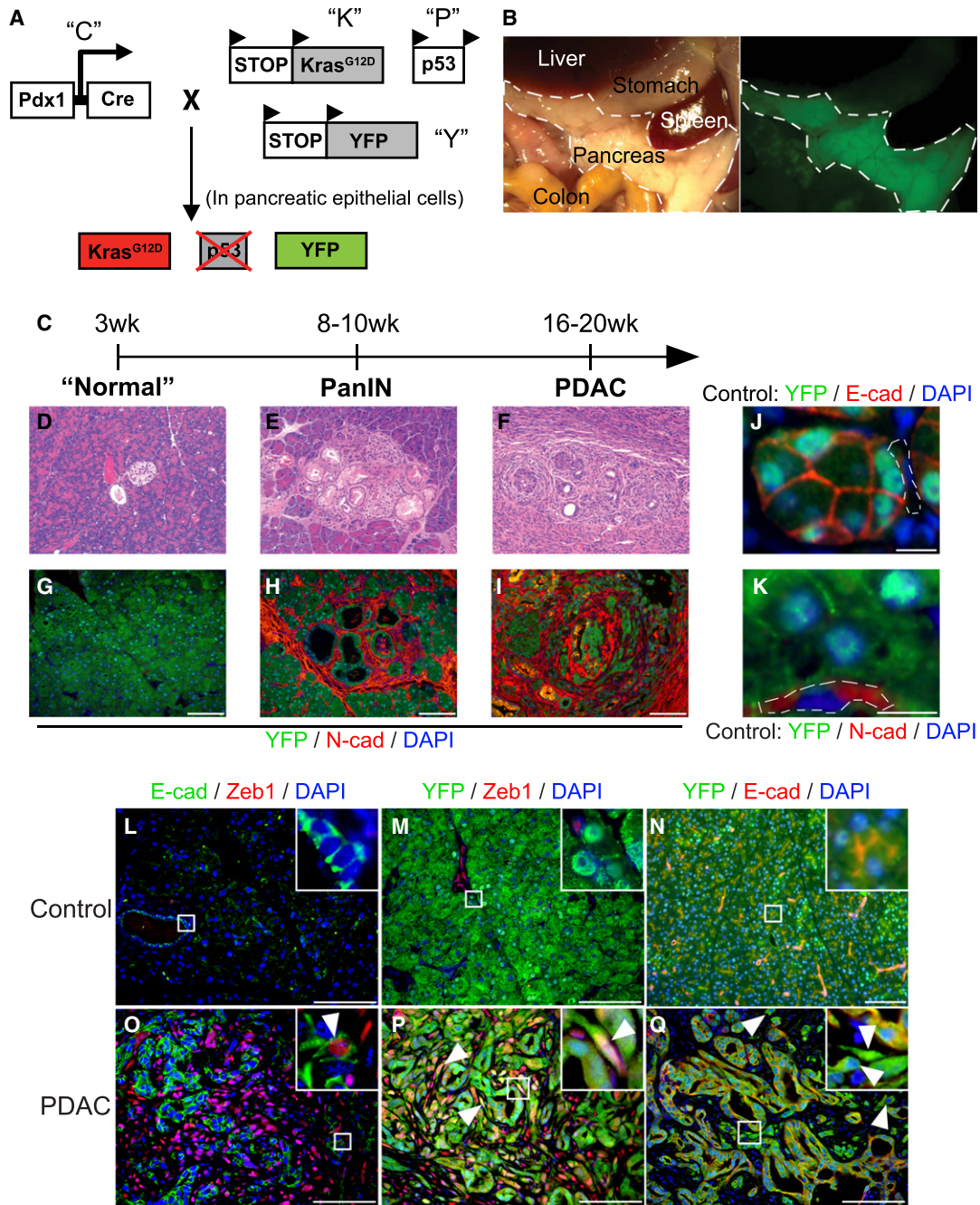


Figure 1. Lineage-Labeled Mouse Models of Pancreatic Cancer and Detection of EMT

(A) Schematic of the PKCY mouse model used in this study, which employs the *Kras^{G12D}* (“K”), *Pdx1-Cre* (“C”), *p53* (“P”), and *Rosa^{YFP}* (“Y”) alleles (see [Experimental Procedures](#)). Cre-mediated activation of *Kras* and deletion of one allele of the *p53* tumor suppressor are accompanied by recombination of the YFP lineage label.

(B) Bright-field and fluorescent images of midgut organs from a CY mouse showing robust and specific fluorescence of the pancreas (outlined); some labeling is also present in the duodenum.

(C) Time course of malignant progression in PKCY mice.

(D–F) Representative images of malignant progression. Prior to weaning, PKCY mice have histologically normal pancreata (D) but develop PanIN lesions (E) and eventually PDAC (F).

(G–I) Images of pancreata from (D)–(F) stained with an antibody against YFP (green) and N-cadherin (N-cad, red); prior to weaning, scant N-cad staining is seen (G).

(J and K) Fluorescent images of lineage-labeled cells derived from the pancreatic epithelium. In control (CY; *Pdx-Cre*; *YFP*) pancreata, YFP⁺ cells express E-cadherin (E-cad; J) but not N-cad (K). Dotted lines indicate YFP[−] mesenchymal cells.

(Polyak and Weinberg, 2009). Although several studies support a physiologic role during tumor progression (Moody et al., 2005; Trimboli et al., 2008), most studies of EMT in the context of cancer biology have been conducted in vitro, and thus the relevance of EMT to carcinogenesis continues to be debated (Ledford, 2011). If EMT does play a crucial role in cancer cell spread in vivo, then detection methods that rely on cellular expression of epithelial markers alone are likely to provide an incomplete picture of metastasis.

To understand the early events that accompany invasive behavior, we developed a lineage-labeling system to detect and isolate cells of pancreatic epithelial origin during stochastic tumor progression. This system allowed us to determine the kinetics of EMT and hematogenous dissemination during the natural evolution of pancreatic ductal adenocarcinoma (PDAC) in vivo and correlate cell phenotype with the acquisition of invasive and tumor-initiating properties.

RESULTS

Enhanced Detection of EMT using Epithelial Lineage Tracing

We used a Cre-lox-based mouse model of PDAC to study the fate of pancreatic epithelial cells during various stages of tumor progression (Bardeesy et al., 2006). The model relies on the *Pdx1-Cre* transgenic strain (Gu et al., 2003) to generate pancreas-specific mutations in *Kras* and *p53*, genes that are mutated with high frequency in human pancreatic cancers (Hezel et al., 2006). In order to track pancreatic epithelial cells during tumor progression, we introduced a *Rosa^{YFP}* allele into the mutant background, resulting in highly specific and efficient (>95%) labeling (Figures 1A and 1B). Animals containing all four alleles were referred to as PKCY mice. A second model, in which a single allele of *p16^{Ink4a/Arf}* was deleted in place of *p53* (IKCY; Aguirre et al., 2003), was also employed and yielded similar results (data not shown). The lineage-labeled mouse models displayed similar histology as nonlabeled models, including the development of pancreatic intraepithelial neoplasias (PanINs), primary tumors, and metastases, with reproducible kinetics (Figures 1C–1I). Because the *Pdx1* promoter is active only in endoderm-derived pancreatic cells (Gu et al., 2003), only the epithelium was tagged by this method. Importantly, mesenchymal cells were never labeled under control conditions in *Pdx1-Cre; Rosa^{YFP}* (CY) animals (Figures 1J and 1K).

Initially, we looked for cancer cells that coexpressed an epithelial marker and a mesenchymal marker, a standard approach used to detect cells at an “intermediate stage” of EMT. In the course of these studies, we used several mesenchymal markers (Figure S1 available online) but focused our analysis on Zeb1 and Fsp1 as these markers serve as independent predictors of mortality in patients with pancreatic cancer (Brabletz et al., 2011; Wang et al., 2007). Using this method,

we detected tumor cells in tumor-bearing mice (“PDAC mice”) that coexpressed either Zeb1 or Fsp1 and the epithelial marker E-cadherin (E-cad; Figure 1O), indicating that such “biphenotypic” cells exist, albeit at a low frequency (<10%).

We then used the YFP lineage label to identify PDAC cells that had completed an EMT. Because labeling was limited to cells of epithelial origin, we defined EMT as having occurred if a cell coexpressed YFP and either Zeb1 (Figure 1P) or Fsp1 (Figure S1D) and/or lacked E-cad (Figure 1Q) expression. Using this approach, we observed that 42% of the lineage-labeled YFP⁺ cells in PKCY tumors had undergone EMT (Figure 1P); higher rates of EMT (68% of all YFP⁺ cells) were found in the IKCY model (data not shown). EMT was not detected in lineage-labeled CY control mice by either method (Figures 1L–1N). Thus, genetic lineage marking is a sensitive tool for distinguishing cells of epithelial and mesenchymal origin and for the detection of EMT.

EMT in Premalignant Lesions

EMT has been proposed to be a prerequisite for invasion and dissemination of carcinoma cells (Hanahan and Weinberg, 2011). To determine when EMT first occurs during PanIN-to-carcinoma progression, we analyzed 8- to 10-week-old PKCY mice. At these time points, only precancerous PanIN lesions were present, and there was no histological evidence of PDAC based on extensive H&E analysis (n = 18); these animals were referred to as “PanIN mice” solely to reflect the histological state of the pancreas at these time points.

EMT was identified in premalignant lesions from both models (Figures 2B, 2C, S1B, and S1C; data not shown). 2.7% and 6.8% of all PanIN 2 and 3 lesions, respectively, contained at least one YFP⁺Zeb1⁺ cell, whereas EMT was never observed in PanIN 1 lesions (Figures 2A and 2E). Similar results were noted with other mesenchymal markers, including Fsp1, Slug, Snail1, and Sip1 (Figure S1). EMT was also prevalent in areas of acinar-to-ductal metaplasia (ADM), particularly in lesions surrounded by abundant inflammatory cells (Figures 2D and S2A). We refer to these areas as ADMIs (acinar-to-ductal metaplasia with inflammation) and determined that 15.8% of ADMIs had evidence of EMT in 8- to 10-week-old PKCY PanIN mice (Figure 2E).

We sorted YFP⁺ cells and performed qPCR to confirm that epithelium-derived pancreatic cells activated a mesenchymal program at the transcriptional level (Figure 2F). Transcripts for *Zeb1*, *Fsp1*, and *N-cadherin* were found in YFP⁺ cells from tumor-bearing PKCY animals and PanIN animals but not in YFP⁺ cells from CY control mice (Figure 2G; p < 0.01). These data indicate that EMT occurs in PanIN lesions and ADMIs prior to tumor formation.

Pancreatic Epithelial Cells Spread before Tumor Formation

Cells that have undergone EMT acquire an invasive phenotype in vitro (Polyak and Weinberg, 2009). Thus, we hypothesized

(L–Q) Images of sections from control (CY; L–N) or PDAC (PKCY; O–Q) pancreata costained with E-cad and Zeb1 (L and O); YFP and Zeb1 (M and P); and YFP and E-cad (N and Q). Insets and arrowheads in (O)–(Q) show high-magnification views of cells that coexpress an epithelial and mesenchymal marker (O), epithelium-derived (YFP⁺) cells that have acquired expression of the mesenchymal marker Zeb1 (P), or epithelium-derived cells that have lost expression of the epithelial marker E-cad (Q).

Scale bars denote 100 μ m in (G)–(I) and (L)–(Q) and 10 μ m in (J) and (K). See also Figure S1.

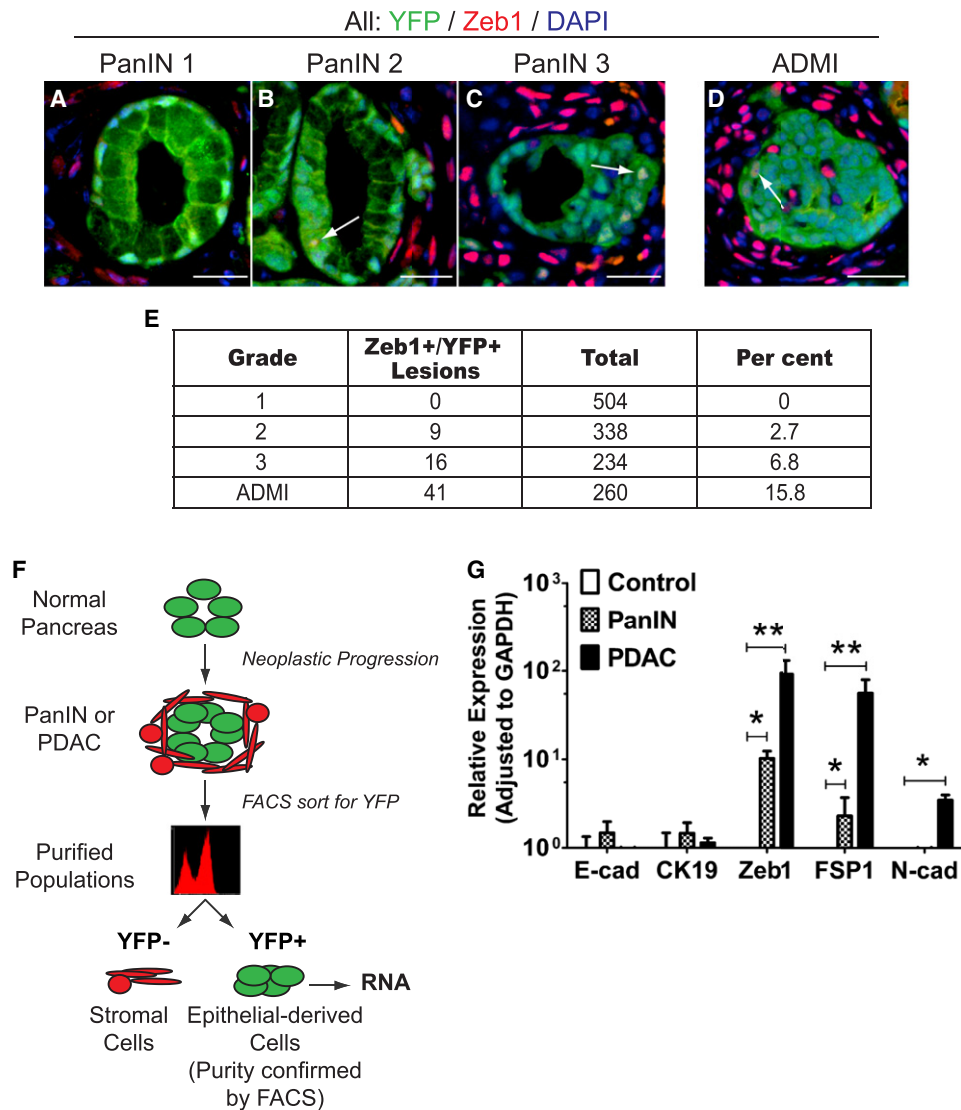


Figure 2. EMT Precedes Tumor Formation

(A–D) In pancreata taken from 8- to 10-week-old PKCY mice, EMT is observed in regions of acinar-to-ductal metaplasia with inflammation (ADMI; D), PanIN 2 (B), PanIN 3 (C), but not in PanIN 1 lesions (A). Arrows show individual YFP⁺ cells (green) that also express Zeb1 (red).

(E) Quantification of observations from (A)–(D), showing the percentage of each type of lesion having at least one cell that has undergone EMT; numbers reflect at least ten medium-powered fields from each of five PanIN mice.

(F) Strategy for isolating YFP⁺ epithelially derived cells from the pancreas; the purity of the YFP⁺ population was confirmed by a repeat FACS analysis.

(G) Transcriptional analysis of sorted YFP⁺ pancreas cells from lineage-labeled CY control (n = 4), PanIN (n = 6), and PDAC (n = 5) pancreata.

Bar graph data are presented as mean ± standard deviation (SD) in this and subsequent figures. *p < 0.01; **p < 0.001 by two-tailed Student's t test in this and subsequent figures, unless otherwise noted. Scale bars, 20 μm. See also Figure S2.

that cells that undergo EMT in PanIN mice might also have invasive properties. Consistent with this notion, we identified individual YFP⁺ cells that had traversed the basement membrane and dissociated from any discernible pancreatic epithelial structure (a process we refer to as “delamination”) in mice bearing PanIN 2 and PanIN 3 lesions (Figures 3A and S2A). Most of these cells expressed Zeb1 (Figure 3A, i–iii) and had acquired a fibroblast-like morphology, making them indistinguishable from surrounding stromal cells by conventional histology (Figures 3A, 3B, and S2); a fraction of the delaminated YFP⁺ cells also

expressed Fsp1 (Figure S2A, insets). To rule out the possibility that the *Pdx1* promoter might be ectopically activated in mesenchymal cells during premalignant progression, we performed lineage tracing in *Mist1*^{CreERT2} mice whose acinar cells had been labeled by tamoxifen pulse. In the setting of experimental pancreatitis (as described in greater detail below), *Mist1*^{CreERT2}; *Kras*^{G12D}; *Rosa*^{YFP} pancreata contained fibroblast-like YFP⁺ cells that lacked E-cad expression (Figure S2B). Because *Mist1*^{CreERT2} mediates labeling solely at the time of tamoxifen administration (Habbe et al., 2008), this experiment demonstrates

unambiguously that the labeled mesenchymal cells were derived from pancreatic acinar cells *in vivo*.

To extend these studies, we performed immunostaining for the Pdx1 transcription factor. Pdx1 is normally expressed at high levels during pancreatic development and in adult β cells, and it is commonly “reactivated” in human PanIN lesions and in PDAC (Park et al., 2011). Pdx1 was widely expressed in PanIN lesions and in a subset of YFP⁺ cells that had delaminated in PanIN mice (Figure S3A). Consistent with these data, sections of human pancreas that contained PanIN lesions (but no tumor) exhibited scattered Pdx1⁺ cells that were separated from any defined epithelial structure (Figures S3B–S3D). Thus, human pancreatic cells may delaminate from PanIN lesions as they do in the mouse model.

Because lineage tracing demonstrated that pancreatic cells can cross the basement membrane before invasive behavior is detectable by standard histology, we asked whether these cells could also enter the bloodstream prior to tumor formation. In tumor-bearing PDAC mice, YFP⁺ circulating pancreatic cells (CPCs) were readily detected in the blood by flow cytometry (Figure 3E). Surprisingly, CPCs were also abundant in the bloodstream of 8- to 10-week-old PKCY PanIN mice (Figures 3C–3G). Sorted YFP⁺ cells contained the recombinant YFP allele (Figure 3H), expressed transcripts for *YFP*, *Pdx1*, and *E-cad* (Figure 3I), and carried the Gly→Asp mutation at codon 12 of the *Kras* cDNA (Figures 3J and 3K). Thus, cells derived from the pancreatic epithelium are present in the circulation of mice with no evidence of carcinoma.

These data raised the possibility that CPCs from PanIN mice might seed distant organs. To assess this possibility, we first examined PDAC mice as a positive control. Bright-field stereomicroscopy permitted detection of peritoneal, liver, or lung metastases in 8/20 animals; use of the YFP lineage label enhanced detection, revealing micrometastases in 16/20 PDAC mice (Figure 3L). Next, we analyzed 8- to 10-week-old PKCY PanIN mice. Although no animals had macro- or micrometastases, liver seeding by YFP⁺ cells was detected in 4/11 PanIN mice (Figures 3M and 3N); most were single cells located near blood vessels and expressed neither Zeb1 nor E-cad (Figures 3M and 3N). By contrast, 0/10 livers from lineage-labeled control CY mice harbored YFP⁺ cells when examined by the same technique.

Characterization of CPCs

The number of YFP⁺ CPCs from PDAC mice depended on the location of blood collection, with a roughly 3-fold increase in abundance in the right side of the heart compared to the left side (Figure 4A). Only 2/9 PDAC mice had evidence of lung metastases, suggesting that the vast majority of CPCs do not survive passage through the pulmonary circulation. To determine whether cells in the circulation exhibited an epithelial, mesenchymal, or mixed phenotype, we stained CPCs with a variety of markers in a flow cytometric assay. The epithelial markers E-cad, CK19, and EpCAM were detected in fewer than 20% of PanIN CPCs and fewer than 40% of PDAC CPCs (Figure 4B). With the exception of Fsp1—which was detected in only 1.1% of all PanIN CPCs compared to 45.2% in PDAC CPCs ($p < 0.01$)—there were no statistically significant differences in cell-surface phenotype between CPCs from the two

groups (Figure 4B). Coimmunofluorescence for Zeb1 and EpCAM revealed that approximately 40% of PDAC CPCs were Zeb1⁺, 27% were EpCAM⁺, and 18% were double positive (Figure S4), suggesting that most CPCs do not exhibit a “mixed” epithelial-mesenchymal phenotype. These data indicate that CPCs from PDAC and PanIN animals are phenotypically similar and that a large fraction maintains a mesenchymal phenotype in the circulation.

CPCs Exhibit Features of Cancer Stem Cells

Cancer stem cells are functionally defined as cells that have enhanced tumor-initiating capacity upon transplantation into a permissive host. In human pancreatic tumors, this activity may be contained within a CD24⁺CD44⁺ population of cells, among others (Hermann et al., 2007; Jimeno et al., 2009; Li et al., 2007). Because EMT in primary cells has been associated with the acquisition of stem cell-like characteristics (Mani et al., 2008), we hypothesized that CPCs might also exhibit features of cancer stem cells. We compared the relative abundance of CD24⁺CD44⁺ cells in pancreata and CPCs from PanIN and PDAC mice. By fluorescence-activated cell sorting (FACS) analysis, 0.11% ± 0.32% and 0.30% ± 0.13% of sorted YFP⁺ cells from PanIN and PDAC pancreata, respectively, expressed both CD24 and CD44 (Figure 4C). By contrast, 23.1% ± 12.9% and 46.4% ± 14.7% of sorted YFP⁺ CPCs from PanIN and PDAC samples were found to be CD24⁺CD44⁺, representing a greater than 100-fold enrichment when compared to the source pancreas (Figure 4D).

We next assessed the survival and self-renewal properties of CPCs directly by employing an *in vitro* pancreatosphere assay, in which single YFP⁺ cells were cultured in attachment-free conditions (Rovira et al., 2010). In both PanIN and PDAC mice, YFP⁺ CPCs had significantly greater rates of clonal survival and growth compared to YFP⁺ pancreas cells from the same animal (Figures 4E and 4F; $p < 0.05$). Taken together, these data suggest that during tumor evolution *in vivo*, bloodstream entry is associated with enrichment of cells that have phenotypic and functional features associated with pancreatic cancer stem cells.

Cells that Have Undergone EMT Have Tumor-Initiating Properties

Although previous work has suggested a link between EMT and increased tumor aggressiveness, most studies have relied on *in vitro* manipulation of cancer cell lines to induce EMT (Weinberg, 2008). Such treatments could have a direct effect on cell behavior apart from their EMT-inducing activity, and thus a direct demonstration of the role of EMT in tumor progression is lacking. We used our lineage-labeling system to isolate cells that had either lost or retained E-cadherin expression to determine whether an EMT *in vivo* is associated with tumor-initiating capacity (Figure 5A).

First, we transplanted 100,000 YFP⁺E-cad⁺ or YFP⁺E-cad⁻ pancreatic cells from PDAC mice into the pancreata of NOD/SCID animals ($n = 5$ for each group). After 3 weeks, all transplants gave rise to large tumors with local invasion and distant metastasis regardless of E-cad status at the time of transplantation; tumors were histologically similar, and YFP⁺ cells coexpressing

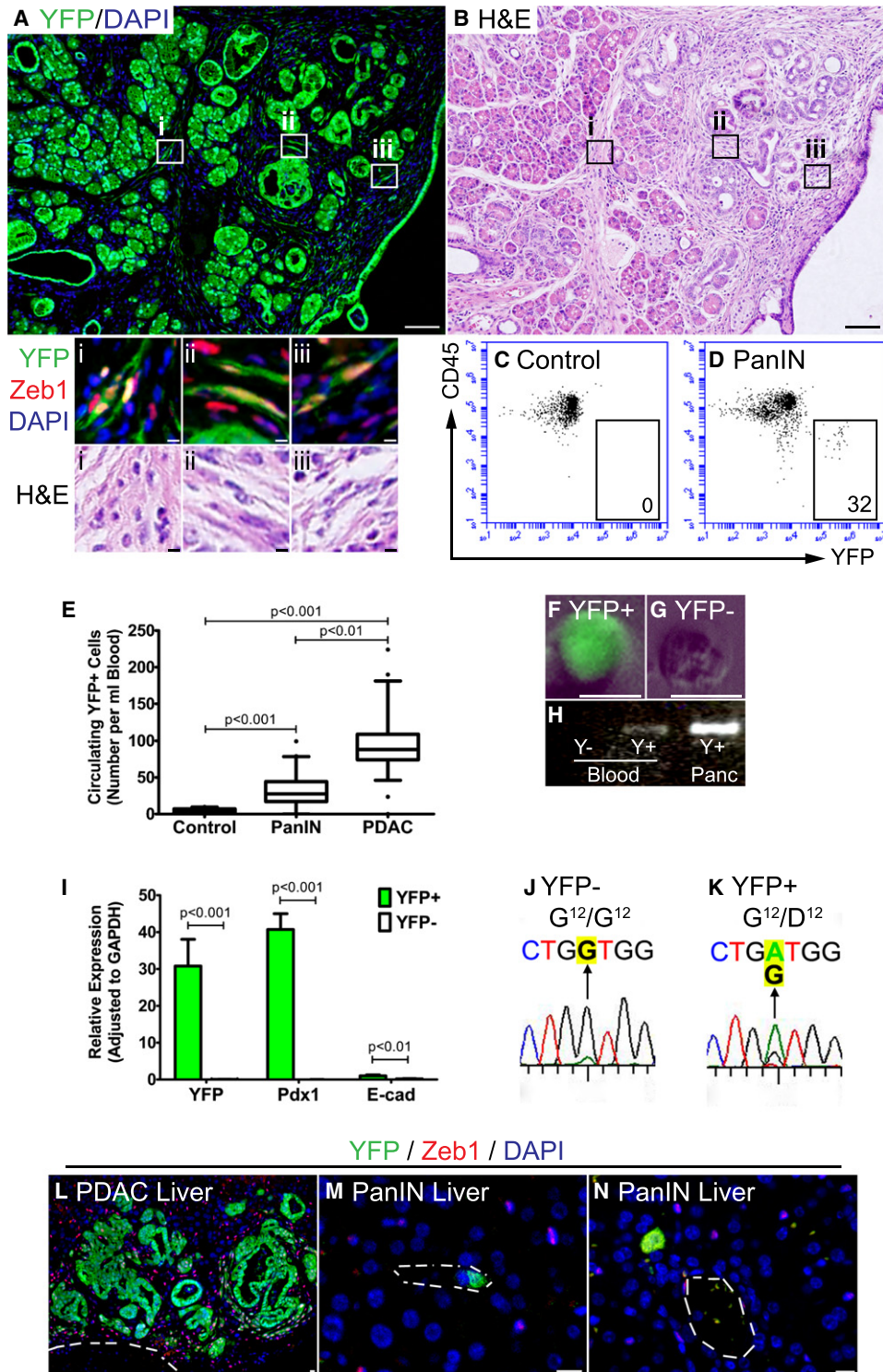


Figure 3. Hematogenous Spread and Liver Seeding Precede Tumor Formation

(A and B) Images showing individual YFP⁺ cells (green) intermingled with stromal cells prior to tumor formation in a 10-week-old PKCY PanIN mouse (A). Delaminated YFP⁺ cells have a spindle-shaped morphology and express Zeb1 (boxes i-iii); they are indistinguishable from surrounding Zeb1⁺YFP⁻ stromal cells by H&E staining of an adjacent section (B).

(C and D) FACS analysis of blood samples from age-matched CY control (C) and PKCY PanIN mice (D). YFP fluorescence and a stain for the leukocyte marker CD45 are depicted on the x and y axes of the FACS plot. YFP⁺CD45⁻ cells were seen in the blood of PanIN (D) and PDAC (not shown) animals (boxed area indicates representative gating and absolute number of YFP⁺ cells).

either Zeb1 or E-cad were found at comparable proportions in both groups (Figures 5B–5E). This result suggests that tumor-derived E-cad⁺ and E-cad⁻ cells can each form tumors and that there is significant plasticity between epithelial and mesenchymal states.

By contrast, we observed a dramatic effect of E-cad status on tumor formation when cells from PanIN mice were transplanted: 4/6 animals transplanted with 100,000 YFP⁺E-cad⁻ cells formed tumors after 2 months, whereas 0/6 animals transplanted with 100,000 YFP⁺E-cad⁺ cells formed tumors over the same time period (Figures 5F–5I). Tumors derived from E-cad⁻ PanIN cells were heterogeneous with respect to E-cad and Zeb1 expression (Figures 5H and 5I). By comparison, E-cad⁺ transplanted animals had few detectable YFP⁺ cells at the 2 month time point, and almost all of the YFP⁺ cells detected were Zeb1⁻ (Figure 5F). Mice transplanted with E-cad⁺ PanIN cells eventually developed tumors (with a mean latency of 4 months after transplantation) that were indistinguishable from the PDAC-derived tumors. Thus, functional analysis of tumor-initiating capacity revealed that E-cad⁻ cells that have undergone EMT have a marked advantage at the PanIN stage.

Inflammation Promotes EMT, Invasiveness, and Dissemination

The emergence of PanIN lesions is associated with the appearance of an inflammatory stroma characterized by activated fibroblasts and myeloid-derived cells (Aguirre et al., 2003; Clark et al., 2007). Inflammation is commonly correlated with tumor initiation and progression (Coussens and Werb, 2002; Grivnikov et al., 2010) and accelerates pancreatic carcinogenesis in adult *Kras* mutant mice (Guerra et al., 2007, 2011). Our observation that ADMIs have a high prevalence of EMT (Figure 3) led us to hypothesize that inflammation contributes to EMT and dissemination at the PanIN stage.

To address this possibility, we induced acute pancreatitis with cerulein, a cholecystokinin analog that induces acinar cell death (Siveke et al., 2008). Initially, KCY mice were analyzed to determine whether expression of oncogenic *Kras* alone facilitated EMT and dissemination in response to acute pancreatitis. At 2 months of age, KCY mice had few PanIN lesions or inflammation at baseline; maximum PanIN grade found at this stage was PanIN 1 (Figure 6B). As expected, cerulein treatment resulted in the formation of ADMIs and accelerated PanIN formation (compare Figures 6B and 6E; Figures S5A–S5D). Surprisingly, cerulein treatment also resulted in a marked elevation in circu-

lating cells, such that CPCs in 8-week-old cerulein-treated KCY mice were almost as abundant as CPCs in vehicle-treated PKCY mice of the same age (Figure 6G). Stated otherwise, cerulein pancreatitis had nearly the same effect on CPC number as the addition of a single floxed *p53* allele. Similar changes were observed in PKCY PanIN and PDAC mice treated with cerulein (Figures 6C, 6F, S5E, and S5F), as well as control CY animals treated with cerulein (Figures 6A and 6D), resulting in a significant increase in CPC number across all groups following cerulein treatment ($p = 0.014$ by two-way ANOVA; Figure 6G). CPCs from cerulein-treated KCY mice exhibited a nearly 100-fold enrichment of CD24⁺CD44⁺ cells compared to the source pancreas, as had been observed for PanIN CPCs, although this did not result in an increase in clonogenic growth for KCY-derived CPCs in the pancreatosphere assay (Figures S4B and S4C).

To confirm that inflammation promotes EMT and bloodstream entry, we employed a second paradigm of pancreatitis and performed pancreatic duct ligation (PDL) on 8- to 10-week-old PKCY PanIN mice. One week after PDL, the portion of the pancreas distal to the ligation was enlarged and nodular compared to the proximal portion from the same mouse or sham-treated PanIN mice (Figures S6A and S6D). This resulted in inflammation, more advanced PanINs, and a loss of epithelial markers (Figures S6B, S6C, S6E, and S6F), as well as an increase in CPCs (Figure S6G; $p = 0.042$, $n = 7$). Thus, both chemical and surgical methods for inducing pancreatitis result in an increase in EMT and CPC number.

Finally, we sought to determine whether inflammation is necessary for EMT and bloodstream entry. We treated 10-week-old PKCY mice with dexamethasone (Dex), a potent anti-inflammatory drug that has no effect on pancreatic histology in control animals (Figures 6H and 6K). As expected, vehicle-treated mice at this age had PanIN lesions with an inflammatory stroma and evidence of EMT but no tumors (Figures 6I and 6J). Daily treatment with Dex (10 mg/kg for 7 days) resulted in a marked reduction in inflammation (Figures 6L and 6M; $n = 6$ each group). Remarkably, PanINs and ADMIs were almost undetectable in these pancreata (Figures 6K–6M), and this change in histology was associated with a significant drop in CPC number compared to vehicle-treated controls (Figure 6N). Importantly, neither cerulein nor Dex treatment of PanIN-derived epithelial cells in vitro had any effect on morphology, proliferation, or expression of epithelial or mesenchymal markers (Figures S6H–S6N).

(E) Quantification of circulating YFP⁺ pancreatic cells (CPCs). Mean CPC numbers (per ml blood) were 3.65 ± 3.76 (CY control, $n = 13$), 32.8 ± 26.2 (PanIN, $n = 17$), and 97.3 ± 48.9 (PDAC, $n = 18$) ($p < 0.001$).

(F and G) Phase-fluorescent images showing epifluorescence of a sorted YFP⁺ cell.

(H) Genomic PCR showing the presence of the recombined *YFP* allele in YFP⁺ cells but not YFP⁻ cells. Pancreatic DNA containing the recombined allele was included as a positive control.

(I) Expression of transcripts encoding *YFP*, *Pdx1*, and *E-cad*, comparing sorted YFP⁺ and YFP⁻ cells and measured by qPCR (\pm SD).

(J and K) Sanger sequencing after PCR amplification of cDNA showing that YFP⁺ CPCs express a mutant *Kras* allele that harbors an altered codon 12 (G \rightarrow A, highlighted).

(L–N) CPCs from 8- to 10-week-old PKCY animals seed the liver. (L) Micrometastasis in a liver from a tumor-bearing mouse ("PDAC Liver"). (M and N) Individual CPCs seed the liver at the PanIN stage ("PanIN Liver"); vascular lumens are outlined.

Scale bar, 40 μ m for (A and (B); 5 μ m for (L)–(N). See also Figure S3.

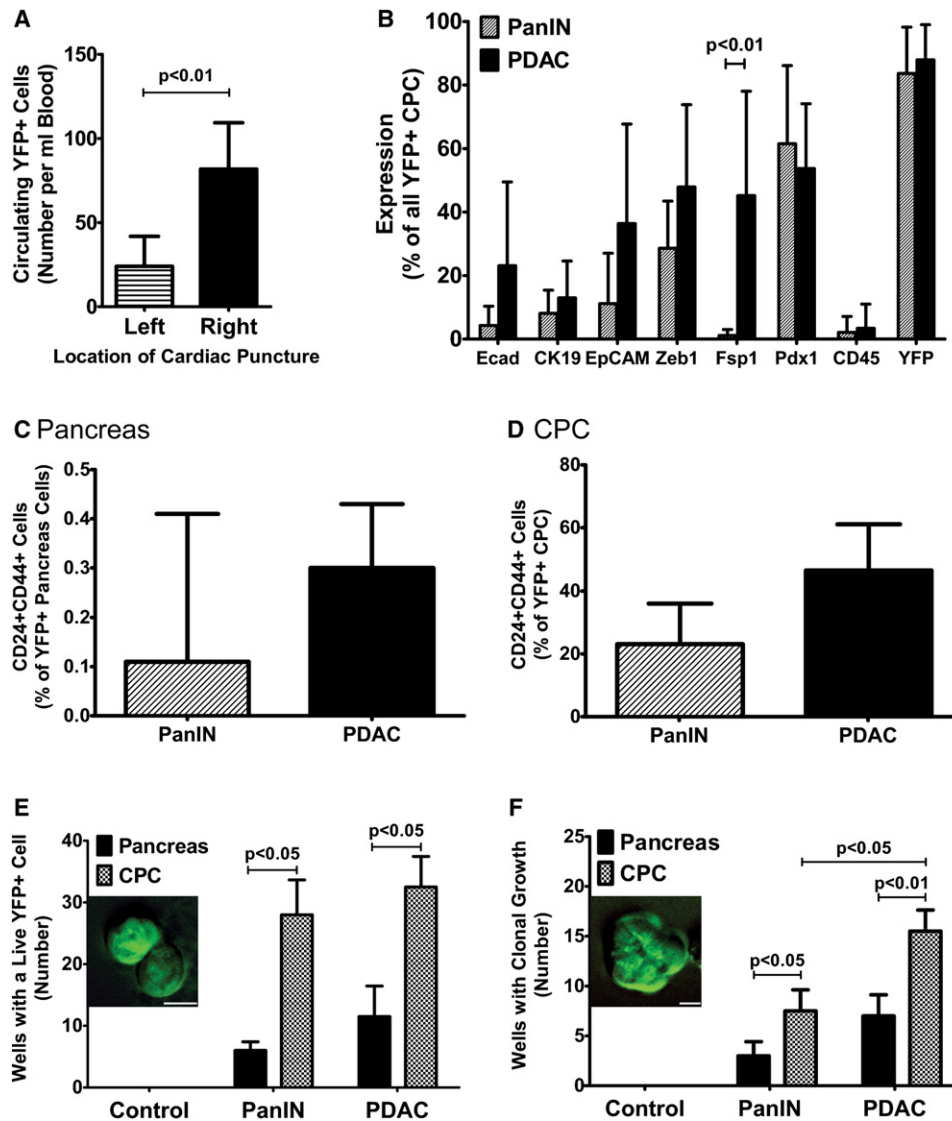


Figure 4. CPC Characterization

(A) Quantification of CPCs after sampling from the left atrium or ventricle ("Left") or right atrium or ventricle ("Right") of the same animal (n = 3).

(B) Quantification of FACS staining for epithelial and mesenchymal markers in CPCs obtained from PanIN or PDAC mice (n = 6–8 for each data point).

(C and D) Quantification of YFP⁺ cells from the pancreas (C) and circulation (D) in PKCY PanIN and PDAC mice that stained positive for the putative pancreatic cancer stem cell markers CD24 and CD44.

(E and F) Quantification of survival (E) or clonal growth (F) of YFP⁺ cells obtained from lineage-labeled control (CY), PanIN, and PDAC mice in ultra-low attachment wells. Bar graphs show the number of wells (out of 96 wells seeded with a single cell) exhibiting any live YFP⁺ cells (E, inset) or evidence of clonal growth (F, inset) after 7 days.

p values for paired two-tailed Student's t tests are shown. Scale bars, 10 μ m. See also Figure S4.

DISCUSSION

Invasive Behavior Precedes Frank Tumorigenesis

Using *in vivo* lineage tracing, we found that EMT, migration of epithelially derived cells into the stroma, bloodstream entry, and seeding of the liver occur at a stage of pancreatic adenocarcinoma progression previously thought to be preinvasive based on standard histological examination. The relevance of these findings to patients is supported by our detection of delaminated

Pdx1⁺ cells adjacent to PanIN lesions in sections of human pancreata. Thus, our data support a model for pancreatic cancer progression in which the seeding of distant organs occurs before, and in parallel to, tumor formation at the primary site. Such an interpretation is especially applicable to PDAC, as the vast majority of patients with pancreatic cancer have metastatic disease at the time of diagnosis. More than 75% of patients who undergo surgical resection of small pancreatic tumors with clear surgical margins and no evidence of metastasis die from

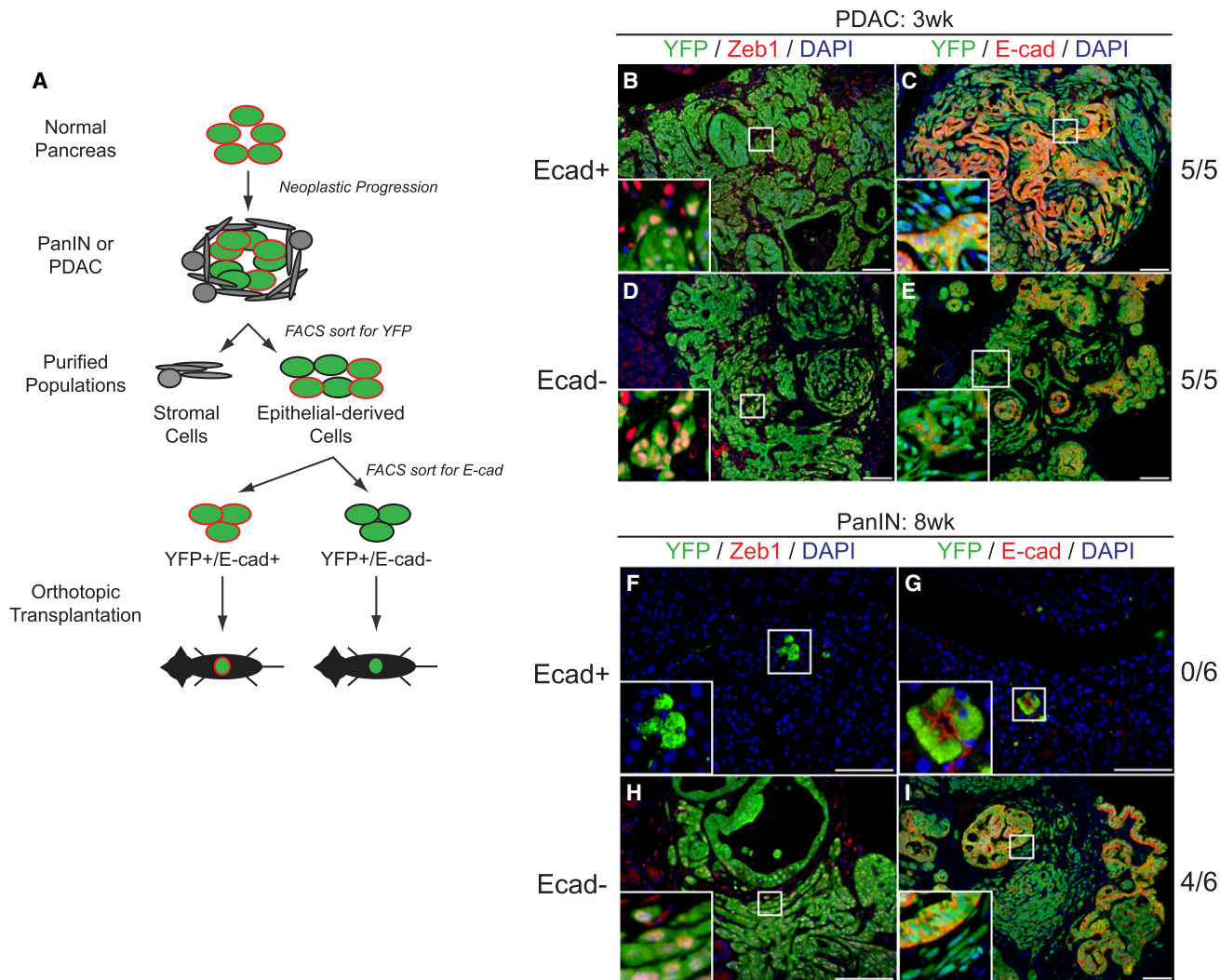


Figure 5. Epithelial and Mesenchymal States Are Plastic

(A) Schematic of orthotopic transplantation experiments.

(B–E) Fluorescent images taken 3 weeks after transplantation of YFP⁺ cells from PDAC mice into NOD/SCID hosts. Tumors form in all mice regardless of E-cad status ($n = 5$ for each condition). YFP⁺E-cad⁺ and YFP⁺E-cad⁻ cells are present in both conditions (C and E), as are YFP⁺Zeb1⁺ and YFP⁺Zeb1⁻ cells (B and D). (F–I) Fluorescent images taken 8 weeks after transplantation of YFP⁺ cells from PanIN mice into NOD/SCID hosts. After transplantation of YFP⁺E-cad⁺ cells, no tumors are found ($n = 6$); the few transplanted YFP⁺ cells that remain are Zeb1⁻ and E-cad⁺ (F and G). Transplantation of YFP⁺E-cad⁻ cells results in tumor formation (H and I). Tumors contain both E-cad⁺ and E-cad⁻ cells (I) as well as Zeb1⁺ and Zeb1⁻ cells (H), providing direct evidence for MET.

metastatic disease within 5 years (Neoptolemos et al., 2004), a finding that is consistent with early spread. Moreover, metastatic PDAC has been documented in a cohort of patients who underwent pancreatectomy for chronic pancreatitis and in whom histologic analysis of the resected pancreas revealed only PanIN lesions (Sakorafas and Sarr, 2003).

Recent genetic studies examining low-passage cell lines or microdissected primary tumors and matched metastases have concluded that metastasis is a late event in human PDAC (Campbell et al., 2010; Yachida et al., 2010). In these studies, a large proportion of mutations were shared among primary and metastatic lesions, leading to the conclusion that metastasis constituted a terminal event in the disease process. However,

mathematical modeling of such phylogenetic relationships relies on assumptions about proliferation and mutation rates at stages of metastatic progression (e.g., micrometastasis) that are not measured easily. Indeed, the notion that colonization occurs early in PDAC is supported by the observation that proliferation is significantly lower in metastatic lesions (Okimura et al., 2009) compared to the primary tumor (Yachida et al., 2010), and yet their sizes at the time of diagnosis are similar.

Our *in vivo* studies do not provide direct evidence that CPCs from PanIN mice (and the corresponding liver-seeding cells) ultimately give rise to metastases. Nevertheless, several lines of evidence indicate that at least some of these cells may be capable of doing so. First, CPCs found in the blood of

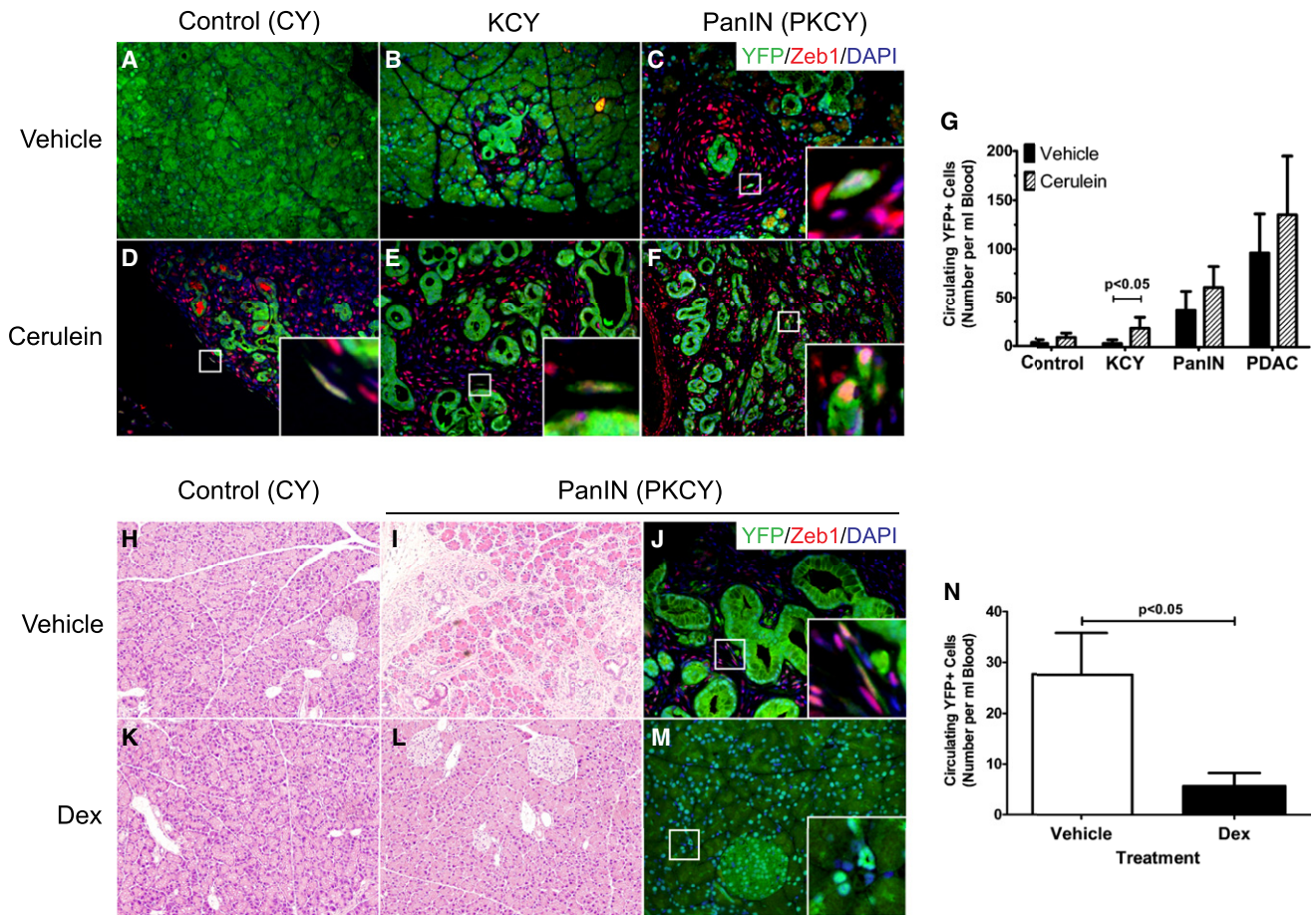


Figure 6. Inflammation Augments EMT and Dissemination

(A–F) Fluorescent images of 8-week-old control *Pdx1-Cre; Rosa^{YFP}* (CY; A and D), *Kras^{G12D}; Pdx1-Cre; Rosa^{YFP}* (KCY; B and E), and PanIN (C and F) pancreata 3 days after injection with vehicle (A–C) or cerulein (D–F) to induce acute pancreatitis (n = 3 for each condition). YFP⁺Zeb1⁺ cells present in PanIN mice or observed following cerulein treatment of CY and KCY mice are shown (C–F, insets).

(G) Quantification of CPC number after 3 days of treatment with vehicle or cerulein for mice with the indicated genotypes (n = 3 for each group; p < 0.05 comparing the two groups of KCY mice by Student’s t test; p = 0.014 for the effect of cerulein versus control across all groups by two-way ANOVA).

(H–M) Images of 10-week-old control (H and K) and PanIN pancreata (I and J, L and M) after 7 days of treatment with vehicle (DMSO; H–J) or dexamethasone (Dex; K–M) and analyzed 24 hr after the last injection. YFP⁺Zeb1⁺ cells are seen in vehicle-treated controls (J, inset) but not in Dex-treated animals (M, inset).

(N) Quantification of CPC number in vehicle- and Dex-treated PanIN mice (p = 0.029, n = 6 for each group).

See also Figure S6.

PanIN-bearing mice exhibit increased survival and self-renewal properties *in vitro*, suggesting that they may be able to persist for long periods of time in a foreign environment such as the liver. Second, most PanIN-derived CPCs exhibit a YFP⁺E-cad⁻ cell-surface phenotype, which our transplantation experiments showed was associated with enhanced tumor-initiating capacity. Finally, a wealth of clinical and experimental data from other systems is consistent with early spread (Weinberg, 2008). Additional experiments will be needed to prove that cells that enter the circulation prior to the development of frank malignancy have metastatic potential. Nevertheless, the finding by routine histology that delaminating cells are indistinguishable from surrounding stromal cells indicates that current histological criteria to diagnose invasive PDAC may be inadequate. Our data also suggest that the location of sampling within the peripheral blood may influence the detection rate of circulating cells.

EMT, MET, and the Acquisition of Stem Cell Characteristics

Lineage tracing enabled us to distinguish pancreatic cells that had acquired mesenchymal characteristics from those that retained an epithelial phenotype. The majority of labeled cells that delaminated from the epithelium (i.e., locally invasive cells) expressed Zeb1, indicating that they had undergone EMT. This strong correlation between invasive behavior and the acquisition of mesenchymal characteristics *in vivo* suggests that EMT is not merely an epiphenomenon but rather represents a critical hurdle that cells must clear to escape from their epithelial neighbors. At present, the signals that initiate EMT in advanced PanINs and ADIMs *in vivo* remain to be determined.

When PDAC cells were separated according to EMT status (i.e., YFP⁺E-cad⁺ or YFP⁺E-cad⁻) and transplanted orthotopically into the pancreas, the resultant tumors were similar with

respect to their epithelial and mesenchymal composition. This result demonstrates that an epithelial or mesenchymal phenotype is not a stable property of a malignant cell and provides direct evidence for a mesenchymal-to-epithelial transition (MET) *in vivo*. We also noted that orthotopically transplanted YFP⁺E-cad⁻ cells from 8- to 10-week-old PKCY PanIN mice gave rise to tumors with a much shorter latency than the same number of YFP⁺E-cad⁺ cells. One interpretation of this result is that cells that have undergone EMT at the PanIN stage are more tumorigenic; alternatively, the YFP⁺E-cad⁻ population may be enriched for a subset of cells with greater or more rapid tumor-initiating properties. In either case, our findings highlight the striking degree of plasticity that exists between epithelial and mesenchymal states during tumor progression *in vivo*.

In the PKCY model, cells with a CD24⁺CD44⁺ phenotype—a population possessing tumor-initiating properties by xenograft assay (Li et al., 2007)—were significantly enriched in the circulation compared to the pancreas. Accordingly, circulating cells exhibited increased survival and self-renewal in low-attachment conditions. Thus, our findings provide *in vivo* support for the notion that EMT is associated with the initiation of a stem cell program (Mani et al., 2008) and indicate that acquisition of a CD24⁺CD44⁺ phenotype facilitates entry into the circulation and/or survival within the bloodstream.

The development of technologies to identify circulating tumor cells (CTCs) from patients represents an enormous advance in metastasis research (Pantel et al., 2008). CTC number correlates with clinical outcome and response to chemotherapeutics in many cancers (Cristofanilli et al., 2004), and isolated disseminated cells can be interrogated for molecular changes that are associated with an increased risk of death (Stoecklein et al., 2008). CTC biology thus has both clinical utility and the potential to advance our understanding of the metastatic cascade. However, standard techniques for isolating CTCs rely heavily upon the use of epithelial markers, particularly EpCAM, for detection (Pantel et al., 2008). The presence of EpCAM-negative YFP⁺ CPCs in our studies raises several possibilities: (1) standard methods that employ epithelial epitopes may not capture all CTCs; (2) cells may enter the circulation after undergoing an “incomplete” EMT, in which residual expression of EpCAM is maintained but not detected by flow cytometry; or (3) CTCs may enter the bloodstream with a mesenchymal phenotype and subsequently “revert” to an epithelial phenotype through a process of MET.

Inflammation Promotes EMT and Dissemination

Inflammation has a well-established role in promoting tumor progression (Grivennikov et al., 2010). Using two models of pancreatitis, we have demonstrated that inflammation induces EMT in CY and KCY animals and results in an increase in CPC number across all genotypes tested. Although mice bearing a single mutant *Kras* allele seemed to have the greatest increase in CPC number following cerulein treatment, even nonmutant pancreatic cells underwent EMT and entered the circulation in the setting of inflammation. This latter observation is quite surprising, as hematogenous spread has not been thought to occur in adult animals in the absence of a tumor. Phenotypically normal cells injected into the bloodstream can seed distant

organs and persist for long periods of time until stimulated to grow (Podsypanina et al., 2008), and in both mice and humans, chronic pancreatic inflammation is strongly associated with pancreatic cancer (Grover and Syngal, 2010; Guerra et al., 2007). Our study suggests that inflammation may promote cancer progression through two independent mechanisms: by facilitating changes in the microenvironment at the primary site of neoplasia and by facilitating invasion and dissemination by increasing cellular access to the circulation.

There is likely to be heterogeneity among pancreatic epithelial cells at the PanIN stage, and it is possible that bloodstream entry prior to the development of a recognizable tumor is facilitated by loss of the second allele of *p53* in PKCY mice. However, the increase of CPCs in KCY and CY mice (which bear no cancer-promoting mutations) following experimental pancreatitis suggests that loss of this tumor suppressor gene is not required for bloodstream entry. Interestingly, although pancreatitis augmented CPC number in KCY animals, these cells did not have the same clonal growth properties exhibited by PKCY CPCs (compare Figure S4C with Figure 4F), supporting the notion that *p53* loss enhances the survival and/or self-renewal of circulating cells.

Finally, we have demonstrated that the inflammatory stroma is necessary for EMT and dissemination. Treatment of 10-week-old PKCY mice with dexamethasone for 1 week resulted in an almost complete elimination of PanIN lesions in the pancreas and loss of YFP⁺ cells from the blood, underscoring a critical requirement for the inflammatory stroma in the maintenance of premalignant PanIN lesions. Similar regression of murine PanIN disease was also seen after treatment with the cyclooxygenase-inhibitor sulindac (Guerra et al., 2011). Our findings have implications for the management of individuals at high risk for pancreatic cancer, including patients with hereditary pancreatitis or kindreds with inherited pancreatic cancer. If dissemination and seeding of pancreatic epithelial cells precede the detection of a tumor in humans, as they do in the mouse model, a window of opportunity may exist for prophylactic therapy in high-risk patients. Indeed, anti-inflammatory drugs have proven moderately successful at reducing mortality due to several cancers, including PDAC (Rothwell et al., 2011).

EXPERIMENTAL PROCEDURES

Mouse Strains and Experimental Treatment

The behavior of mutant strains bearing various allele combinations of *Pdx1-Cre*, *Kras*^{G12D}, *p16/p19^{fl}*, and *p53^{fl}* has been described previously (Aguirre et al., 2003; Bardeesy et al., 2006; Hingorani et al., 2003). To perform lineage tracing, we introduced a *Rosa*^{YFP} reporter allele into these mutant backgrounds to generate a panel of compound mutant strains: *Pdx1-Cre; Rosa*^{YFP} (“CY”), *Pdx1-Cre; Kras*^{G12D}, *Rosa*^{YFP} (“KCY”), *Pdx1-Cre; Kras*^{G12D}, *p16/p19^{fl/+}; Rosa*^{YFP} (“IKCY”), and *Pdx1-Cre; Kras*^{G12D}, *p53^{fl/+}; Rosa*^{YFP} (“PKCY”). All experiments involving the KCY model employed mice between 8 and 10 weeks of age. For studies involving mice harboring only PanIN lesions by histologic analysis (“PanIN” mice), PKCY animals were sacrificed at 8–10 weeks of age based on prior observations regarding tumor progression (Bardeesy et al., 2006); no PKCY mice (out of 18 examined) had evidence of carcinoma at this time point (see Extended Experimental Procedures for details of histological analysis by a pancreatic pathologist [A.M.]).

For PDAC mice, animals were examined three times per week for evidence of morbidity and sacrificed when they exhibited limited physical activity,

depressed response to toe pinch, dehydration, and/or abdominal enlargement from ascites. More than 90% of IKCY and PKCY mice were 34–38 weeks of age or 16–20 weeks of age, respectively, at the time of sacrifice.

Experimental pancreatitis was elicited with cerulein as described (Siveke et al., 2008), and specimens were obtained after 3 days of treatment. Three to four CY, KCY, PanIN PKCY, and PDAC PKCY were used for cerulein and vehicle (PBS) treated cohorts. Orthotopic transplantations were performed on NOD/SCID mice by injecting 1×10^5 sorted pancreas cells as previously described (Mohammad et al., 1998). PKCY PanIN mice aged 10 weeks were treated with seven daily injections of dexamethasone or vehicle (DMSO) as described (Stairs et al., 2011) and analyzed 24 hr later.

Cell Staining

For experiments involving flow cytometry and sorting of pancreatic cells, at least 25% of the tissue was saved for histologic analysis and was processed and stained as described in the [Extended Experimental Procedures](#).

Pancreatosphere Assay

YFP⁺ blood or pancreatic cells from the same mouse were sorted into ultra-low attachment 96-well plates (Corning) at 1 cell per well, confirmed by microscopy. Cells were grown as previously described (Rovira et al., 2010) and assayed at 5 days for clonal growth of fluorescent cells (defined as clusters of cells >3 cell widths in diameter) or presence of live YFP⁺ cells (singlets or doublets). Three to four mice were analyzed for each category.

Additional information can be found in the [Extended Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and two tables and can be found with this article online at doi:10.1016/j.cell.2011.11.025.

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