Analysis of the tumor-initiating and metastatic capacity of PDX1-positive cells from the adult pancreas

Irene Ischenko[a], Oleksi Petrenko[b,1], and Michael J. Hayman[a,1]

Departments of [a]Molecular Genetics and Microbiology and [b]Pathology, Stony Brook University, Stony Brook, NY 11794

Edited by Douglas R. Lowy, National Cancer Institute, Bethesda, MD, and approved January 22, 2014 (received for review October 22, 2013)

Pancreatic cancer is one of the deadliest human malignancies. A striking feature of pancreatic cancer is that activating Kras mutations are found in ∼90% of cases. However, apart from a restricted population of cells expressing pancreatic and duodenal homeobox 1 (PDX1), most pancreatic cells are refractory to Kras-driven transformation. In the present study, we sought to determine which subsets of PDX1+ cells may be responsible for tumor growth. Using the Lox-Stop-Lox–KrasG12D genetic mouse model of pancreatic carcinogenesis, we isolated a population of KrasG12D-expressing PDX1+ cells with an inherent capacity to metastasize. This population of cells bears the surface phenotype of EpCAM+CD24+CD44+CD133–SCA1− and is closer in its properties to stem-like cells than to more mature cell types. We further demonstrate that the tumorigenic capacity of PDX1+ cells is limited, becoming progressively lost as the cells acquire a mature phenotype. These data are consistent with the hypothesis that the adult pancreas harbors a dormant progenitor cell population that is capable of initiating tumor growth under conditions of oncogenic stimulation. We present evidence that constitutive activation of the mitogen-activated protein kinase (MAPK/ERK) signaling and stabilization of the MYC protein are the two main driving forces behind the development of pancreatic cancer cells with stem-cell–like properties and high metastatic potential. Our results suggest that pancreatic cells bearing Kras mutation can be induced to differentiate into quasi-normal cells with suppressed tumorigenicity by selective inhibition of the MAPK/ERK/MYC signaling cascade.

pancreatic ductal adenocarcinoma | cell of origin

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy characterized by rapid progression, exceptional resistance to all forms of anticancer treatment, and a high propensity for metastatic spread. Paradoxically, despite growing understanding of the genetic causes of PDAC, the mechanism and timing of cancer metastases, the main cause of deaths in pancreatic cancer patients, remain relatively unexplored. A striking feature of pancreatic cancer is that mutations in the Kras gene are found in ∼90% of cases. Genetic studies demonstrated that oncogenic Kras activity is required for the initiation and maintenance of both primary and metastatic pancreatic lesions (1–5). However, apart from a restricted population of pancreatic and duodenal homeobox 1 (PDX1)-expressing cells or subsets thereof, adult pancreatic cells are refractory to transformation by oncogenic Kras even in the context of loss of p53 or INK4A (CDKN2A) tumor suppressors (2–4). PDX1 is a transcription factor expressed in early pancreatic precursor cells, subsequently becoming restricted to insulin-producing islet cells (6). Some exocrine cells express PDX1, albeit at low levels (6). Unlike PDAC, islet cell tumors are relatively rare, and oncogenic Kras mutations are extremely rare in pancreatic islet cell neoplasms (7). Targeting expression of mutant Kras to acinar or other adult cell types induces the formation of tumors reminiscent of PDAC, but only after induction of pancreatitis (3, 4, 8). Although these findings support the notion that the oncogenic potential of mutant Kras is highly dependent on cellular context (9, 10), they raise, but do not resolve, questions as to how pancreatic tumor-initiating cells originate, what are the drivers of pancreatic cancer, and what events are responsible for progression to the malignant (metastatic) phenotype. Finally, do some combinations of mutations make pancreatic cancer a certainty, whereas others make it a stochastic event of some probability?

In this study, we sought to determine which subsets of pancreatic PDX1+ cells may be responsible for tumor growth. We also sought to identify genes that are essential for pancreatic cancer maintenance, and to determine whether targeting these genes can block tumor progression. To that end, we have performed extensive phenotypic and functional characterization of PDX1+ cells from the adult pancreas. We show that PDX1+ cells represent a heterogeneous population composed of cells corresponding to various stages of differentiation that can be discriminated on the basis of SCA1 and CD133 expression. We further demonstrate that endogenous expression of oncogenic KrasG12D induces expansion of a unique subset of PDX1+ cells and endows them with high propensity for metastatic dissemination. We present evidence that the tumorigenic capacity of PDX1+ cells is limited, becoming progressively lost as the cells acquire a mature phenotype. Our results provide support for the hypothesis that the adult pancreas harbors a dormant progenitor cell population that is capable of initiating tumor growth under conditions of oncogenic stimulation. Hyperactivation of the Ras/MAPK/ERK pathway and stabilization of the MYC protein are the two main driving forces behind the development of pancreatic cancer cells with high metastatic potential.

Significance

Pancreatic cancer is characterized by aggressive growth and a high propensity for metastatic spread. Despite growing understanding of the genetic causes of pancreatic cancer, the mechanism and timing of cancer metastasis, the main cause of deaths in pancreatic cancer patients, remain relatively unexplored. In this study, we used experimental mouse models of pancreatic carcinogenesis to show that hyperactivation of the Ras/MAPK/ERK pathway and stabilization of the MYC protein are the two main driving forces behind the development of pancreatic cancer cells with high metastatic potential. Our results suggest that pancreatic cells bearing Kras mutation can be induced to differentiate into quasi-normal cells with suppressed tumorigenicity by selective inhibition of the MAPK/ERK/MYC signaling cascade. These findings may have important therapeutic implications.

Author contributions: O.P. and M.J.H. designed research; I.I. and O.P. performed research; I.I. and O.P. contributed new reagents/analytic tools; O.P. and M.J.H. analyzed data; and O.P. and M.J.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. E-mail: alexei.petrenko@stonybrook.edu or michael.hayman@stonybrook.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1319911111/-/DCSupplemental.

Results

Modeling Metastasis in Pancreatic Cancer. Using genetically engineered models of pancreatic cancer, we recently demonstrated that endogenous expression of oncogenic KrasG12D induces expansion of a distinct subset of immature pancreatic cells and endows them with high propensity for metastatic dissemination (11). This population of metastasizing cells bears the phenotype of EpCAM+CD24+CD44+SCA1− (hereafter referred to as SCA1−) that distinguishes them from a more mature population of EpCAM+CD24+CD44+SCA1+ cells (referred to as SCA1+) (Fig. 1*A*). To identify factors that predispose toward metastatic disease, we generated clonal SCA1− and SCA1+ KrasG12D $p53^{KO}$ cell lines and characterized them for malignant phenotypes. Cell lines were assessed periodically for the expression of pancreatic duct-specific genes (PDX1, SOX9, KRT19) and epithelial cell markers (EpCAM, SCA1, CD133) (Fig. 1 *B* and *C*), and were found to be phenotypically stable during ex vivo expansion. Expression of PDX1 inversely correlated with that of SCA1 and CD133 (Fig. 1 *B* and *C*). When 10^4 SCA1− cells were injected s.c. into nude mice, tumors formed in all of the injections with an average latency of 4.5 wk. Tumor formation by SCA1+ cells was less efficient, occurring in 80% of the injected mice, and the tumors developed with a longer latency (~5.5 wk). Histologically, tumors were classified into two groups. Tumors derived from SCA1− cells showed features of undifferentiated (anaplastic) carcinoma, whereas the histology of tumors derived from SCA1+ cells exhibited a pattern of moderately to well-differentiated adenocarcinoma (Fig. S1*A*). Extensive fibrosis, a hallmark of PDAC, was evident in both tumor types (Fig. S1*A*). Tail vein injections of clonal cells were used to evaluate their metastatic potential. When injected into the tail vein, SCA1− cell lines produced metastasis to lungs or to other parts of the body (lymph nodes, skin) with latencies ranging from 30 to 40 d (Fig. 1*D*). In contrast, SCA1+ cells failed to generate either grossly or microscopically apparent metastatic tumors at 3 mo after inoculation (Fig. 1*D*). Expression of exogenous PDX1 or KrasG12D failed to confer a metastatic phenotype on SCA1+ cells, whereas ectopic MYC or KrasG12D combined with MYC rendered them metastatic (Fig. S1*B*). Likewise, constitutively active MEK2 (an upstream activator of ERK1/2 MAPK) and TGF-β type 2 receptor (TBR2) conferred metastatic capacity to otherwise nonmetastatic SCA1+ cells (Fig. S1*B*). Of note, these metastatic tumors retained the epithelial characteristics observed in PDAC (Fig. S1*C*). These data suggest that cancer metastasis can arise from different cell populations in the pancreas, and that KrasG12D mutation alone is sufficient to confer a full malignant phenotype on SCA1− but not SCA1+ subsets of PDX1-expressing pancreatic cells.

Tumors with Kras mutations often show loss of the wild-type (WT) Kras allele, consistent with the notion that the proto-oncogenic forms of Ras can function as tumor suppressors (12–14). We found that the recombined KrasG12D and WT Kras alleles were invariably present in both types of premalignant cells (i.e., SCA1+ and SCA1−) and all s.c. tumors (Fig. S2 *A* and *B*). In contrast, nearly all metastatic tumors derived from clonal SCA1− cell lines possessed only the recombined KrasG12D allele, but not the WT Kras allele (Fig. S2*C*), whereas the metastatic tumors derived from MYC-overexpressing SCA1+ cells showed no loss of the WT Kras allele (Fig. S2*D*). These data suggest that seeding metastasis requires mutations (e.g., loss of WT Kras allele or MYC amplification) beyond those observed in solid tumors. Considering the fact that the tumorigenicity of cells depends on the expression levels of mutant Ras (15), these results imply that either SCA1− cells exhibit high levels of genomic instability or SCA1+ cells are less responsive to oncogenic Ras signaling. In other words, both mutational and nonmutational (i.e., epigenetic) events may govern pancreatic cancer metastasis, but in a cell-type–specific manner. We set out to determine to what extent the acquisition of malignant properties depends upon the cell type in which KrasG12D is expressed.

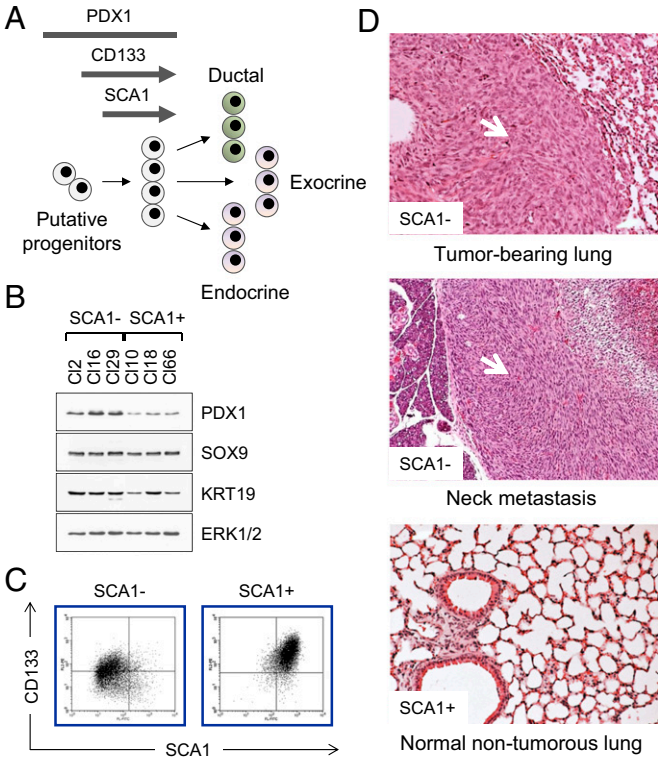

Fig. 1. Modeling metastasis in pancreatic cancer. (*A*) SCA1 and CD133 expression defines distinct PDX1+ cell subpopulations. The population of the presumptive pancreatic progenitors bears the phenotype of EpCAM+CD24+CD44+SCA1− that distinguishes them from more mature EpCAM+CD24+CD44+CD133+SCA1+cells. (*B*) Western blot analysis of PDX1, SOX9, and KRT19 expression in clonal SCA1− and SCA1+ KrasG12D $p53^{KO}$ pancreatic cell lines. ERK1/2 (MAPK) is a loading control. (*C*) FACS analysis of clonal KrasG12D $p53^{KO}$ pancreatic epithelial cells using the indicated antibodies. (*D*) Metastatic tumor formation in nude mice injected with 10^4 of SCA1− but not SCA1+ KrasG12D $p53^{KO}$ cells.

Expression Signatures of SCA1− and SCA1+ Pancreatic Cancer Cells. Activating Ras mutations can induce proliferation, senescence, and/or differentiation depending on signal intensity, duration, and cellular context (16). In all of the cases, the biological outcome of Ras signaling is determined in large part by the involvement of the ERK1/2 pathway and activation of nuclear transcription factors, most notably MYC (17). Thus, a mere twofold increase in MYC protein levels results in a major difference for the malignant properties of Ras-transformed cells (18). This may explain why MYC amplification and overexpression are common events in PDAC (19, 20). When analyzed by Western blot, precancerous SCA1− and SCA1+ cell lines showed no significant differences in levels of total and activated nuclear phospho-ERK1/2 (pERK1/2) (Fig. 2*A*). However, tumors derived from SCA1− cell lines tended to contain substantially higher levels of nuclear pERK1/2 than tumors derived from SCA1+ cells (Fig. 2*B*). We also observed "normal" or even reduced levels of activated protein kinase B (PKB/AKT) in tumors formed by SCA1+ cells (Fig. S3). As a reflection of these differences (21), MYC protein levels with elevated S62 phosphorylation were increased about two- to threefold in SCA1− cell lines and the respective tumors compared with premalignant and cancer-derived SCA1+ cell lines (Fig. 2 *A* and *B* and Fig. S3).

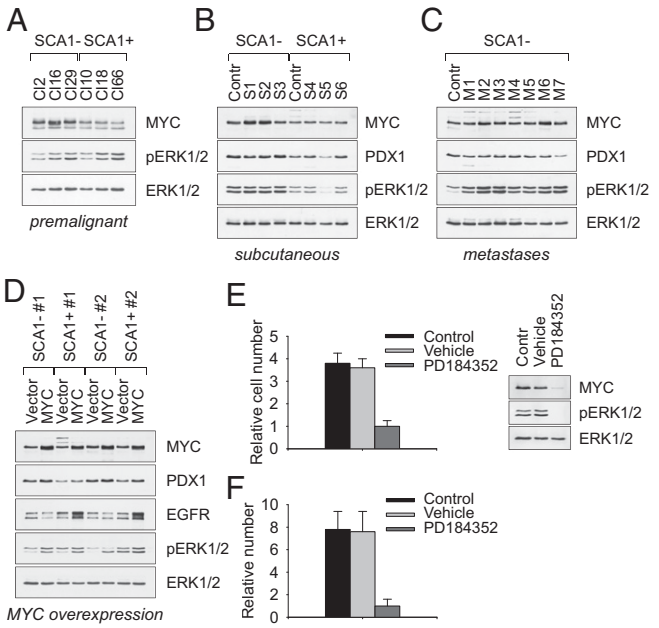

Fig. 2. Expression profiling of pancreatic cancer cells. (*A*) Western blot analysis of nuclear extracts from clonal SCA1− and SCA1+ KrasG12D p53^{KO} pancreatic cell lines. Total nuclear ERK1/2 is a loading control. Note that ERK1/2 can translocate into the nucleus in both the phosphorylated and unphosphorylated states. (*B* and *C*) Western blot analysis of nuclear extracts from premalignant KrasG12D p53^{KO} cell lines (control) and s.c. tumors (S1–S6) (*B*) or metastatic tumors (M1–M7) (*C*). (*D*) Western blot analysis of nuclear extracts from KrasG12D p53^{KO} cell lines transduced with MYC-expressing retroviruses. (*E* and *F*) SCA1− KrasG12D p53^{KO} cells were incubated for 3 d with or without 0.2 μM MEK inhibitor PD184352 and then analyzed by Western blotting. PD184352 blocked the phosphorylation of ERK1/2 (*E*, *Inset*), produced a strong growth inhibitory response (*E*), and impeded the ability of cells to form pancreatospheres in suspension culture (*F*).

Importantly, high levels of nuclear pERK1/2 and MYC expression were maintained in metastatic lesions formed by SCA1− cell lines (Fig. 2*C*). Moreover, overexpression of MYC in SCA1+ cells also led to increased ERK1/2 phosphorylation with a concomitant increase in levels of EGF receptor (EGFR) (Fig. 2*D*). Although EGFR is known to signal upstream of Ras, it paradoxically is required for Kras-driven PDAC formation (22, 23). Most pancreatic cancers result from alterations of genes that function through a relatively small number of signaling pathways (e.g., Ras/MAPK, JAK/STAT, TGF-β/SMAD, and Wnt/Notch) (24). We found no evidence of SMAD4 inactivation in SCA1− or SCA1+ tumors (Fig. S3). However, the SCA1+ but not SCA1− phenotype cosegregated with low TBR2 expression (Fig. S3). Genetic inactivation of TGF-β signaling in pancreatic cells promotes progression of PDAC in the presence of activated Kras (25, 26). However, reduced TBR2 expression leads to acquisition of a well-differentiated tumor phenotype associated with fewer metastases (25, 26).

Recent studies indicate that oncogenic Kras accelerates tumor progression by imposing on cells an immature stem-like state in which differentiation is inhibited (27, 28). We therefore assessed to what extent constitutive activation of the MAPK/ERK pathway affects differentiation and proliferation of KrasG12D-expressing cells. To that end, SCA1− KrasG12D p53^{KO} cell lines were incubated with pharmacological MEK1/2 inhibitor PD184352. As expected, treatment with PD184352 inhibited the phosphorylation of ERK1/2 in all cell lines tested and drastically reduced MYC protein levels (Fig. 2*E*). When cultured in the presence of drug, the cells displayed considerably slower growth rates (Fig. 2*E*), impaired ability to form self-renewing "pancreatospheres" in suspension culture (Fig. 2*F*), with a concomitant increase in differentiation and apoptotic cell death (Fig. S4). Given that cells isolated from such pancreatospheres have a multilineage differentiation potential (11, 29), these data indicate that activated Ras and ERK1/2 are essential for survival, proliferation, and maintenance of the undifferentiated state of PDX1+ cells.

Phenotypic Plasticity of Pancreatic Cancer Cells. Cancer stem cells (CSCs) are defined by their multipotential capabilities and are suggested to play a key role in driving tumor growth. In pancreatic tumors, EpCAM, CD24, CD44, and CD133 were proposed to represent the surface markers for CSCs (30, 31). However, SCA1 and CD133 have also been reported to mark mature pancreatic ductal cells (32–34). Flow cytometric (FACS) analysis of our SCA1+ (i.e., EpCAM+CD24+CD44+SCA1+) KrasG12D p53^{KO} cell lines showed that they were ≥90% CD133+/high (hi). Moreover, all tumors derived from such cell lines were phenotypically identical to the injected precancerous cells (Fig. 3*A* and Fig. S5*A*). In contrast, their less differentiated SCA1− (i.e., EpCAM+CD24+CD44+SCA1−) cell line counterparts were only 25–50% positive for CD133, and all tumors derived from such cell lines showed a large number (up to 80%) of CD133-negative cells (Fig. 3*A* and Fig. S5*B*). In an effort to clarify which populations are enriched in tumorigenic cells, we FACS-sorted such clonal CD133+/low cell lines into four subpopulations defined as (*i*) CD133–single positive (CD133-SP), (*ii*) CD133/SCA1–double positive (DP), (*iii*) SCA1–single positive (SCA1-SP), and (*iv*) CD133/SCA1–double negative (DN) (Fig. 3*B*). When

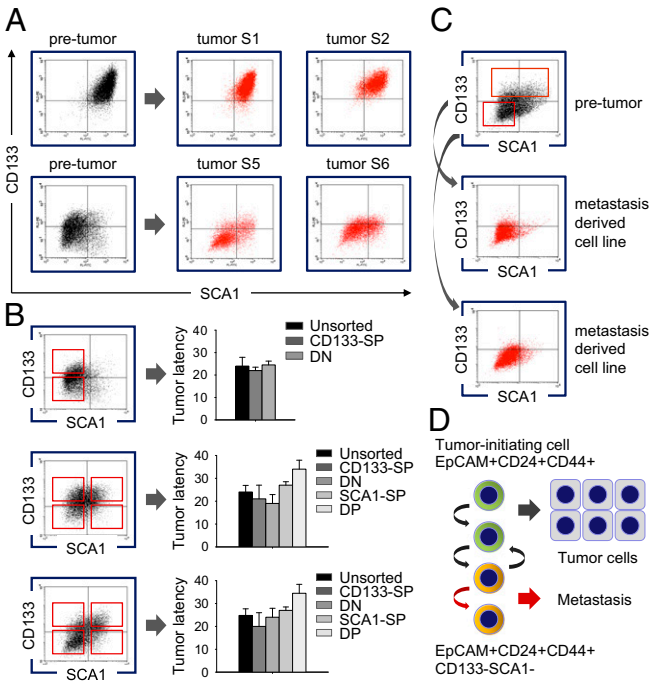

Fig. 3. Phenotypic plasticity of pancreatic cancer cells. (*A*) FACS analysis of clonal SCA1+ (*Upper* panels) and SCA1− KrasG12D p53^{KO} cell lines (*Lower* panels) and tumors derived from such lines. (*B*) KrasG12D-expressing cell clones (nos. 2, 16, and 29) were FACS-sorted into four populations based on expression of CD133 and SCA1: CD133-SP, DP, SCA1-SP, and DN. Latencies of tumor formation in nude mice induced by 10^4 cells from each sorted subpopulation are shown. (*C*) Metastatic tumors generated by KrasG12D p53^{KO} cells (*Top*) are composed, in large part of DN cells (*Middle* and *Bottom*). (*D*) KrasG12D p53^{KO} cells adopt the phenotypic properties of CD133−/SCA1− cells before becoming metastatic.

sorted cells were maintained in culture, some DP and CD133-SP cells lost their respective phenotype and converted to DN cells, whereas a proportion of DN cells converted to CD133-SP or DP cells, indicating that there exists an equilibrium between well-differentiated (i.e., SCA1+) and poorly differentiated (i.e., SCA1−) KrasG12D-expressing cells. To pin down at which point during the tumorigenic process it still matters that cells are less differentiated and at which point it stops mattering, 10^4 cells of each sorted subpopulation were injected s.c. or i.v. into nude mice. When injected s.c., SCA1− (DN and CD133-SP) cells initiated tumor growth earlier and sustained a faster growth rate than the respective SCA1+ (SCA1-SP and -DP) subsets (Fig. 3*B*). When injected into the tail vein, DN cells formed tumors more rapidly than other cell subpopulations. Cell lines established from the resected tumors showed a preponderance of DN cells regardless of the type of tumor (s.c. or metastatic) or phenotype of the parental precancerous cells (Fig. 3*C* and Fig. S5*B*). Moreover, secondary tumors arising from such cancer-derived cell lines had shorter latency periods than the respective primary tumors, reflecting their increased tumor-initiating ability. Collectively, these data indicate that (*i*) pancreatic cancer can arise from different CD133/SCA1 subsets of PDX1+ cells; (*ii*) precancerous PDX1+ cells are phenotypically plastic, reversibly turning on and off expression of SCA1 and CD133; and (*iii*) other cell populations revert to the CD133−/SCA1− (DN) phenotype before becoming metastatic (shown schematically in Fig. 3*D*). However, the phenotypic plasticity and tumorigenic capacity of PDX1+ cells are limited, becoming progressively lost as the cells acquire the CD133hi/SCA1hi phenotype (Fig. S5*C*).

Differentiation of Tumorigenic Pancreatic Cells into Quasi-Normal Cells with Suppressed Tumorigenicity. To identify pathways that play either positive or negative roles during differentiation of PDX1+ cells, SCA1+ and SCA1− KrasG12D $p53^{KO}$ cell lines were retrovirally transduced with Ras-responsive genes representative of Ras/MAPK, TGF-β/SMAD, and Wnt/Notch signaling pathways, and subsequently analyzed by flow cytometry to assess their SCA1 and CD133 expression (Fig. 4*A*). Constitutively active MEK2 K71W, MYC, and TBR2 showed the strongest ability to convert SCA1+ cells into a DN-like state (Fig. 4 *A* and *B*). In contrast, ectopic expression of a dominant-negative TBR2 mutant (DN TBR2) induced robust conversion of DN cells into mature DP cells (Fig. 4 *A* and *B*), confirming that inhibition of TGF-β signaling enhances the differentiation of PDAC-initiating cells. The MEK inhibitor PD184352 and the BET bromodomain inhibitor JQ1, which both target MYC at the transcriptional and posttranscriptional levels (35), also stimulated the differentiation of SCA1− cells into quasi-normal SCA1+ cells with suppressed tumorigenicity (Fig. S6 *A* and *B*). We found that the efficiency of conversion of SCA1+ cells was low (<15%) compared with that of SCA1− cells (>30%), indicating that in vitro differentiation of premalignant pancreatic cells into more mature phenotypes is more easily attainable than dedifferentiation (Fig. 4 *A* and *B*). In contrast, the efficiency of conversion of tumor-derived SCA1− cell lines was relatively low (<20%), implying that the most resilient subpopulations of cells contribute to tumor growth in vivo (Fig. S7*A*), whereas the efficiency of conversion of tumor-derived SCA1+ cell lines was high (>40%), implying that phenotypic plasticity is an inherent attribute of differentiated cancer cells (Fig. S7 *B* and *C*). Of particular interest, we observed cell-type–specific responses to the expression of reprogramming transcription factors. Although OCT4 induced partial dedifferentiation of SCA1+ cells (Fig. 4*B* and Fig. S7 *B* and *C*), ectopic SOX2 and KLF4, either alone or in combination with each other, converted SCA1− cells into DP cells (Fig. 4 *C* and *D*) with low proliferative ability and suppressed tumorigenicity (Fig. S7*D*). Most surprisingly, overexpression of constitutively active AKT or activation of ERK and AKT caused

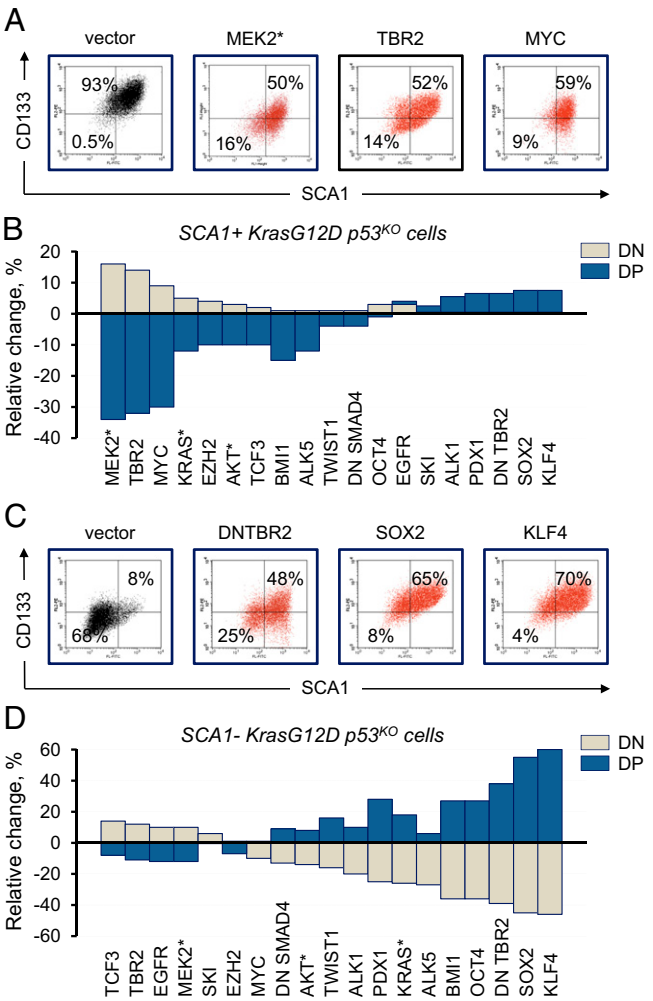

Fig. 4. Differentiation of tumorigenic pancreatic cells into quasi-normal cells with suppressed tumorigenicity. (*A* and *B*) Phenotypic conversion of SCA1+ KrasG12D $p53^{KO}$ clonal cell lines transduced with the indicated genes relative to vector-transduced controls. Asterisks indicate constitutively active mutants of Kras, AKT, and MEK2. (*C* and *D*) Phenotypic conversion of SCA1− KrasG12D $p53^{KO}$ clonal cell lines transduced with the indicated genes relative to vector-transduced controls.

by constitutively active KrasG12D failed to effectively convert SCA1+ cells into a DN-like state (Fig. 4*B*). These data support our previous findings that dedifferentiation precedes the acquisition of malignant properties (11), but indicate that dedifferentiation of pancreatic cells results from the deregulation of a large number of genes rather than just one or few genes.

Discussion

The ductal morphology of PDAC suggests that it derives from the ductal epithelium or from adult progenitor/stem cells capable of differentiating into duct-like cells. Surprisingly, convincing evidence that pancreatic tumors can arise from ductal cells is scarce (36, 37). The first mouse models of PDAC were generated by combining KrasG12D activation in embryonic pancreatic progenitors (using the PDX1 promoter) with homozygous deletion of p53 or CDKN2A (1, 2, 26). It was discovered that embryonic activation of KrasG12D in PDX1+ cells gives rise to widespread early neoplasms (1, 2, 38), whereas adult PDX1+ cells are considerably more resistant to KrasG12D-induced malignant transformation (39). More recent studies demonstrated that adult acinar, islet, and centroacinar cells (intercalated duct

cells located in the acinus) also have the potential to initiate invasive carcinoma, but each cellular context may require a different combination of genetic and/or environmental factors (3, 40, 41). Of primary importance, a phenotypic switch converting adult pancreatic acinar cells (the most numerous pancreatic cell type) to duct-like cells can lead to pancreatic intraepithelial neoplasia (PanIN) and eventually to PDAC, but only in conjunction with chemically induced pancreatitis (3, 4, 39, 42). In contrast, a population of nonislet PDX1+ cells from the adult pancreas was found to have heightened sensitivity to Kras activation in normal noninflammatory conditions, and was proposed to represent the cell of origin of PDAC (4). It is worth noting that PDX1 is widely expressed in early pancreatic progenitors, but in the adult pancreas its expression is largely restricted to insulin-producing β cells. Whether the population of nonislet PDX1+ cells represents remnants of embryonic progenitors, their descendants, or a separate stem/progenitor cell population remains to be established. These cells reportedly reside in the ductal and centroacinar compartments (29, 43), and make up ∼1% of the adult pancreas (44). More importantly, these PDX1+ cells are fully competent to form PanINs on activation of Kras alone and, in conjunction with p53 mutation or INK4A deletion, can develop into invasive and metastatic PDAC (2, 4, 38).

The purpose of this study was to determine which subsets of PDX1+ cells may be responsible for tumor growth. We show that endogenous expression of oncogenic KrasG12D induces expansion of PDX1+ cells and endows them with high propensity for metastatic dissemination while still at premalignant stages. We demonstrate that PDX1+ cells represent a heterogeneous population composed of cells corresponding to various stages of differentiation that can be discriminated on the basis of SCA1 and CD133 expression. We also demonstrate that the tumorigenic capacity of PDX1+ cells is limited and is progressively lost concomitantly with the acquisition of a mature CD133hi/SCA1hi phenotype. These data are consistent with the hypothesis that the adult pancreas harbors a dormant progenitor cell population that is activated under conditions of oncogenic stimulation and is capable of initiating cancer (4). Based on the results of our study, in combination with data from previous work (4), we propose that a drift of PDX1+ cells that have acquired Kras mutation toward ductal differentiation may provide the basis for pancreatic carcinogenesis, particularly in the conditions that do not actively promote inflammation.

The acquisition of metastatic properties by tumor cells is often considered a late event in neoplastic progression, although it has long been argued that certain genetic changes that are selected for the proliferative advantage they confer to primary tumor cells may also confer invasive and metastatic phenotypes (45, 46). Accumulating evidence suggests that some cell populations in early stage pancreatic tumors are predisposed to metastatic spread (47–49). Our results have several implications. First, we identified, isolated, and characterized a population of PDX1-expressing cells with CSC properties and an inherent capacity to metastasize. Second, our data suggest that seeding metastasis requires mutations (e.g., loss of WT Kras allele or MYC overexpression) beyond those observed in solid tumors. Third, metastases were too numerous and/or too large to be accounted for by random mutations occurring in vivo in the colonization-proficient cells. We surmised that cells of high metastatic capacity must preexist within precancerous KrasG12D $p53^{KO}$ cell lines before in vivo selection. Recent whole-genome sequencing of matched primary pancreatic tumors and metastases also revealed that both preexisting and further acquired mutations contribute to the mutational profiles of metastatic lesions (47, 48). The implication of these findings is that the primary carcinomas are mixtures of numerous subclones, each of which may have a different capacity to seed metastases. Moreover, pancreatic cancer metastasis may occur early in tumor evolution rather than late (47, 48). Computational models of pancreatic cancer also predict that all patients are likely to contain metastasis-competent cells at the time of diagnosis, even though the size of the primary tumor may be small (50). We are currently unable to explain how cells expressing "metastasis predisposition genes" (51) can arise at appreciable frequencies in the course of ex vivo expansion. Likewise, it is unclear if the epigenetic modifications that accompany the conversion of preneoplastic cells to a less mature state, as we have observed with cultured CD133+/lo KrasG12D $p53^{KO}$ cells, endow them with the capacity to spread, survive, and/or proliferate at distant sites. Whatever the underlying mechanisms, they need to be investigated and targeted with therapeutics.

Genetic alterations of Kras, CDKN2A, p53, and SMAD4 are the most frequent events in pancreatic cancer (52, 53) and are considered to be founder mutations (47, 48). These alterations seem to arise sequentially (52), resulting in the development of increasingly aggressive cancer phenotypes. Recent evidence indicates that pancreatic cancers with high metastatic capacity may be represented by two genetic subtypes: p53 null and p53 mutant in association with SMAD4 loss (54). Overall, p53 mutations occur in 50–75% of pancreatic cancers (53, 54), of which ∼50% are null mutations (deletion, nonsense, or frame shift) that abolish gene expression (54). Our data predict that, apart from the abundant Kras and p53 mutations, deregulated MYC expression is an important determinant of metastatic predisposition. High MYC expression has been associated with pancreatic cancer (11, 48), and our current study underscores a major role for MYC in phenotypic switching and metastatic progression. We present evidence that it is the constitutive activation of the MAPK/ERK pathway and the subsequent stabilization of the MYC protein that are the two main driving forces behind the development of pancreatic cancer cells with stem-cell–like properties and high metastatic potential. The central role of this pathway was highlighted by the finding that pancreatic cells bearing KrasG12D mutation could be stimulated to differentiate into quasi-normal cells with suppressed tumorigenicity by the selective inhibition of the MAPK/ERK/MYC signaling cascade. In sum, our study underscores the molecular underpinnings of the conflict between neoplastic transformation and differentiation. In addition, our experiments provide proof of principle that the differentiation of pancreatic cells expressing oncogenic Ras can be driven by lineage-determining transcription factors. These methods may facilitate generation of pancreatic cells for in vitro disease modeling and future applications in cancer therapy.

Materials and Methods

All animal studies were approved by the Institutional Animal Care and Use Committee at Stony Brook University. We used previously described Lox-Stop-Lox (LSL) KrasG12D mice (10) and p53-null mice (55). Actively growing clumps of epithelial cells were obtained from pancreata of 4–6-wk-old LSL KrasG12D $p53^{KO}$ mice by collagenase and hyaluronidase treatment (11). Dispersed epithelial cells were purified by FACS by virtue of their EpCAM, CD24, and SCA1 expression. Nonsorted dispersed and sorted SCA1+ and SCA1− cells were cultured on gelatinized plates in CnT-17 media (CellnTec). The nonsorted cultured cells contained 78 ± 6% SCA1+ cells, which was not changed during prolonged passage. Subsequent analysis revealed that expression of PDX1 is equally as variable as that of SCA1 and CD133. Molecular, histological, and other analyses were performed as described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Alice Nemajerova (Stony Brook University) and Howard C. Crawford (Mayo Clinic Florida) for their invaluable help in histologic and immunohistologic analysis of tumor samples. This work was supported by US Public Health Service Grant CA42573 from the National Cancer Institute (to M.J.H.).

1. Aguirre AJ, et al. (2003) Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes Dev* 17(24):3112–3126.
2. Bardeesy N, et al. (2006) Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. *Proc Natl Acad Sci USA* 103(15):5947–5952.
3. Guerra C, et al. (2007) Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer Cell* 11(3):291–302.
4. Gidekel Friedlander SY, et al. (2009) Context-dependent transformation of adult pancreatic cells by oncogenic K-Ras. *Cancer Cell* 16(5):379–389.
5. Collins MA, et al. (2012) Metastatic pancreatic cancer is dependent on oncogenic Kras in mice. *PLoS ONE* 7(12):e49707.
6. Reichert M, Rustgi AK (2011) Pancreatic ductal cells in development, regeneration, and neoplasia. *J Clin Invest* 121(12):4572–4578.
7. Jonkers YM, Ramaekers FC, Speel EJ (2007) Molecular alterations during insulinoma tumorigenesis. *Biochim Biophys Acta* 1775(2):313–332.
8. Guerra C, et al. (2011) Pancreatitis-induced inflammation contributes to pancreatic cancer by inhibiting oncogene-induced senescence. *Cancer Cell* 19(6):728–739.
9. Guerra C, et al. (2003) Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. *Cancer Cell* 4(2):111–120.
10. Tuveson DA, et al. (2004) Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell* 5(4):375–387.
11. Ischenko I, Zhi J, Moll UM, Nemajerova A, Petrenko O (2013) Direct reprogramming by oncogenic Ras and Myc. *Proc Natl Acad Sci USA* 110(10):3937–3942.
12. Zhang Z, et al. (2001) Wildtype Kras2 can inhibit lung carcinogenesis in mice. *Nat Genet* 29(1):25–33.
13. Fleming JB, Shen GL, Holloway SE, Davis M, Brekken RA (2005) Molecular consequences of silencing mutant K-ras in pancreatic cancer cells: Justification for K-ras-directed therapy. *Mol Cancer Res* 3(7):413–423.
14. Qiu W, et al. (2011) Disruption of p16 and activation of Kras in pancreas increase ductal adenocarcinoma formation and metastasis in vivo. *Oncotarget* 2(11):862–873.
15. Sarkisian CJ, et al. (2007) Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. *Nat Cell Biol* 9(5):493–505.
16. Marshall CJ (1995) Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80(2):179–185.
17. Parikh N, Shuck RL, Nguyen TA, Herron A, Donehower LA (2012) Mouse tissues that undergo neoplastic progression after K-Ras activation are distinguished by nuclear translocation of phospho-Erk1/2 and robust tumor suppressor responses. *Mol Cancer Res* 10(6):845–855.
18. Bazarov AV, et al. (2001) A modest reduction in c-myc expression has minimal effects on cell growth and apoptosis but dramatically reduces susceptibility to Ras and Raf transformation. *Cancer Res* 61(3):1178–1186.
19. Schleger C, Verbeke C, Hildenbrand R, Zentgraf H, Bleyl U (2002) c-MYC activation in primary and metastatic ductal adenocarcinoma of the pancreas: Incidence, mechanisms, and clinical significance. *Mod Pathol* 15(4):462–469.
20. Mahlamäki EH, et al. (2002) Frequent amplification of 8q24, 11q, 17q, and 20q-specific genes in pancreatic cancer. *Genes Chromosomes Cancer* 35(4):353–358.
21. Sears R, Leone G, DeGregori J, Nevins JR (1999) Ras enhances Myc protein stability. *Mol Cell* 3(2):169–179.
22. Ardito CM, et al. (2012) EGF receptor is required for KRAS-induced pancreatic tumorigenesis. *Cancer Cell* 22(3):304–317.
23. Navas C, et al. (2012) EGF receptor signaling is essential for k-ras oncogene-driven pancreatic ductal adenocarcinoma. *Cancer Cell* 22(3):318–330.
24. Jones S, et al. (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 321(5897):1801–1806.
25. Bardeesy N, et al. (2006) Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. *Genes Dev* 20(22): 3130–3146.
26. Ijichi H, et al. (2006) Aggressive pancreatic ductal adenocarcinoma in mice caused by pancreas-specific blockade of transforming growth factor-beta signaling in cooperation with active Kras expression. *Genes Dev* 20(22):3147–3160.
27. Kim CF, et al. (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 121(6):823–835.
28. Haigis KM, et al. (2008) Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. *Nat Genet* 40(5): 600–608.
29. Rovira M, et al. (2010) Isolation and characterization of centroacinar/terminal ductal progenitor cells in adult mouse pancreas. *Proc Natl Acad Sci USA* 107(1):75–80.
30. Li C, et al. (2007) Identification of pancreatic cancer stem cells. *Cancer Res* 67(3): 1030–1037.
31. Hermann PC, et al. (2007) Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 1(3): 313–323.
32. Seaberg RM, et al. (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nat Biotechnol* 22(9): 1115–1124.
33. Immervoll H, Hoem D, Sakariassen PO, Steffensen OJ, Molven A (2008) Expression of the "stem cell marker" CD133 in pancreas and pancreatic ductal adenocarcinomas. *BMC Cancer* 8:48.
34. Lardon J, Corbeil D, Huttner WB, Ling Z, Bouwens L (2008) Stem cell marker prominin-1/AC133 is expressed in duct cells of the adult human pancreas. *Pancreas* 36(1):e1–e6.
35. Delmore JE, et al. (2011) BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 146(6):904–917.
36. Brembeck FH, et al. (2003) The mutant K-ras oncogene causes pancreatic periductal lymphocytic infiltration and gastric mucous neck cell hyperplasia in transgenic mice. *Cancer Res* 63(9):2005–2009.
37. Ray KC, et al. (2011) Epithelial tissues have varying degrees of susceptibility to Kras (G12D)-initiated tumorigenesis in a mouse model. *PLoS ONE* 6(2):e16786.
38. Hingorani SR, et al. (2005) Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* 7(5):469–483.
39. Habbe N, et al. (2008) Spontaneous induction of murine pancreatic intraepithelial neoplasia (mPanIN) by acinar cell targeting of oncogenic Kras in adult mice. *Proc Natl Acad Sci USA* 105(48):18913–18918.
40. Stanger BZ, et al. (2005) Pten constrains centroacinar cell expansion and malignant transformation in the pancreas. *Cancer Cell* 8(3):185–195.
41. Kopp JL, et al. (2012) Identification of Sox9-dependent acinar-to-ductal reprogramming as the principal mechanism for initiation of pancreatic ductal adenocarcinoma. *Cancer Cell* 22(6):737–750.
42. De La O JP, et al. (2008) Notch and Kras reprogram pancreatic acinar cells to ductal intraepithelial neoplasia. *Proc Natl Acad Sci USA* 105(48):18907–18912.
43. Huch M, et al. (2013) Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. *EMBO J* 32(20):2708–2721.
44. Jin L, et al. (2013) Colony-forming cells in the adult mouse pancreas are expandable in Matrigel and form endocrine/acinar colonies in laminin hydrogel. *Proc Natl Acad Sci USA* 110(10):3907–3912.
45. Bernards R, Weinberg RA (2002) A progression puzzle. *Nature* 418(6900):823.
46. Hynes RO (2003) Metastatic potential: Generic predisposition of the primary tumor or rare, metastatic variants-or both? *Cell* 113(7):821–823.
47. Yachida S, et al. (2010) Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 467(7319):1114–1117.
48. Campbell PJ, et al. (2010) The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature* 467(7319):1109–1113.
49. Rhim AD, et al. (2012) EMT and dissemination precede pancreatic tumor formation. *Cell* 148(1-2):349–361.
50. Haeno H, et al. (2012) Computational modeling of pancreatic cancer reveals kinetics of metastasis suggesting optimum treatment strategies. *Cell* 148(1-2):362–375.
51. Nguyen DX, Bos PD, Massagué J (2009) Metastasis: From dissemination to organ-specific colonization. *Nat Rev Cancer* 9(4):274–284.
52. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA (2006) Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev* 20(10):1218–1249.
53. Maitra A, Hruban RH (2008) Pancreatic cancer. *Annu Rev Pathol* 3:157–188.
54. Yachida S, et al. (2012) Clinical significance of the genetic landscape of pancreatic cancer and implications for identification of potential long-term survivors. *Clin Cancer Res* 18(22):6339–6347.
55. Jacks T, et al. (1994) Tumor spectrum analysis in p53-mutant mice. *Curr Biol* 4(1):1–7.