

An iPSC Line from Human Pancreatic Ductal Adenocarcinoma Undergoes Early to Invasive Stages of Pancreatic Cancer Progression

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SUMMARY

Pancreatic ductal adenocarcinoma (PDAC) carries a dismal prognosis and lacks a human cell model of early disease progression. When human PDAC cells are injected into immunodeficient mice, they generate advanced-stage cancer. We hypothesized that if human PDAC cells were converted to pluripotency and then allowed to differentiate back into pancreatic tissue, they might undergo early stages of cancer. Although most induced pluripotent stem cell (iPSC) lines were not of the expected cancer genotype, one PDAC line, 10–22 cells, when injected into immunodeficient mice, generated pancreatic intraepithelial neoplasia (PanIN) precursors to PDAC that progressed to the invasive stage. The PanIN-like cells secrete or release proteins from many genes that are known to be expressed in human pancreatic cancer progression and that predicted an HNF4 α network in intermediate-stage lesions. Thus, rare events allow iPSC technology to provide a live human cell model of early pancreatic cancer and insights into disease progression.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) carries a dismal prognosis, with less than a 5% survival rate (Hezel et al., 2006; Maitra and Hruban, 2008; Rustgi, 2006). Due to a paucity of bio-

markers for early-stage detection of the disease, PDAC is usually detected at advanced stages, with limited therapeutic options. Given the frequent occurrence of *KRAS* mutations in human PDAC, the prominent animal model of PDAC is based upon inducing a G12D mutant allele of *Kras* in the mouse pancreatic epithelium (Hingorani et al., 2003). The mice develop pancreatic intraepithelial neoplasias (PanINs) with prolonged latency and incomplete penetrance of PDAC. PDAC and related tumors develop much more rapidly when *Kras*G12D/+ mice also contain mutations of *Ink4a/Arf*, *Tgfbr2*, *p53*, or *PTEN* (Morris et al., 2010), although these mutations alone do not efficiently cause PDAC. In an effort to develop human models of early pancreatic cancer, PDAC cells have been grafted into immunodeficient mice either as tumor fragments (Rubio-Viqueira et al., 2006), dispersed cells (Kim et al., 2009), or cells sorted for cancer stem cell markers (Hermann et al., 2007; Ishizawa et al., 2010; Li et al., 2007). In these contexts, tumors rapidly arise that resemble the advanced PDAC stages from which the cells were derived and do not undergo the slow growing, early PanIN stages of PDAC (Ding et al., 2010). Presently, there is no dynamic, live human cellular model that undergoes the early stages of PDAC and disease progression.

Most of the secreted proteins from pancreatic cancers (Harsha et al., 2009) that could serve as biomarkers have been identified in advanced, invasive PDAC or cell lines thereof and thus may not represent markers for early stages of the disease. Markers have been sought for precancerous lesions, such as PanINs and intraductal papillary mucinous neoplasms (IPMNs) (Brat et al., 1998; Hruban et al., 2001), but the markers are typically intracellular or cell surface proteins (Harsha et al., 2009) and not known to be secreted or released proteins that would provide the best opportunity for diagnosis.

Although irreversible mutations in oncogenic and tumor suppressor genes promote human cancers, potentially reversible epigenetic changes also play a role (Esteller, 2007). Indeed, the cancer phenotype can be suppressed in certain medulloblastoma cells, RAS-induced melanoma cells, and embryonal carcinoma cells and renal tumor cells when they are reprogrammed to pluripotency by nuclear transfer (Blelloch et al., 2004; Hochedlinger et al., 2004; Li et al., 2003; McKinnell et al., 1969). More significantly, the resultant pluripotent cells can then differentiate into multiple early developmental cell types of the embryo. Such embryos die partly through organogenesis, presumably due to re-expression of the cancer phenotype. Still, it is remarkable that, in certain circumstances, the pluripotency network can suppress the cancer phenotype sufficiently to allow early tissue differentiation. Using iPSC technology (Takahashi and Yamanaka, 2006), cancer cell lines have been made into iPSCs (Carette et al., 2010; Miyoshi et al., 2010). However, no iPSC lines from solid primary human cancers have been reported.

Based on the above considerations, we hypothesized that creating iPSCs from an epithelial tumor would allow the cells to be propagated indefinitely in the pluripotent state and that, upon differentiation, a subset of the cells would undergo early developmental stages of the human cancer. This could provide a live cell human model for unprecedented experimental access to early stages of the disease. We therefore sought to reprogram epithelial cells from human PDAC, along with apparently normal, isogenic cells beyond the tumor margins and assess the reprogrammed cells' developmental potential. From a variety of initial PDAC samples, only once were we able to reprogram a cell from a recurrent, late-stage human pancreatic cancer to a near-pluripotent state. Yet the reprogrammed cells, when injected into immunodeficient mice, consistently generate PanIN lesions that can progress to invasive PDAC. We developed conditions for isolating the early-stage lesions, culturing the cells, and performing proteomic studies on proteins that are secreted or released and stable. We discovered known networks and a previously unappreciated network that we find to be associated with early to invasive stages of pancreatic cancer. These studies provide an example of where a rare iPSC line can be validated against known features of human cancer and provide potential biomarkers and insights into networks activated in early to intermediate stages of the disease.

RESULTS

Creating iPSC-like Lines from Human Pancreatic Ductal Adenocarcinoma

We obtained human pancreatic ductal adenocarcinoma samples immediately after resection (Table S1). Histologically normal pancreatic tissues at the margin of the specimens were used as controls. Epithelial cells were isolated and cultured in serum-free medium with cholera toxin, to impair the growth of fibroblasts. We performed two successive infections of the pancreatic cancer and margin cells with five lentiviruses separately encoding doxycycline-inducible mouse *Oct4*, *Sox2*, *Klf4*, and *c-Myc* and the rt-TA transactivator,

while isolating genomic DNA from the pancreatic specimen margin and cancer epithelial cells that had been cultured separately. Pyrosequencing analysis revealed *KRAS* mutant cells in seven of nine of the initial tumor samples (Tables S1 and S2). Embryonic stem cell (ESC)-like colonies began to arise after 7 days, with fewer colonies arising from cancer epithelial cells. ESC-like clones from cancer or margins started to differentiate and disappear 4 days after withdrawal of doxycycline. Thus, we maintained colonies in the presence of a low doxycycline concentration (50 ng/ml) and called the resulting lines "iPSC-like" because of their dependency upon doxycycline. We established iPSC-like lines from four of the nine tumor epithelial specimens and corresponding margin iPSC-like lines from three of the specimens (Figure 1A; Table S1).

We characterized the cells' pluripotency by RT-PCR, immunostaining, embryoid body formation, teratoma assays, karyotyping, and a subset by CpG methylation analysis. Most iPSC-like lines expressed endogenous pluripotency marker RNAs and protein (Figures 1B, 1C, and S1A–S1D). Teratoma assays in immunodeficient NSG mice and embryoid body assays revealed that the 10–12 pancreatic margin and 10–22 cancer iPSC-like lines from the tenth patient (Figures 1D, 1E, and S2A) and the 14–24 pancreatic margin and 14–27 cancer iPSC-like clones from the 14th patient (Figure S1E and S1F) could generate tissues of multiple germ layers, demonstrating pluripotency. The original tumor 10 was negative for *NANOG* expression and exhibited sporadic expression of *POU5F1/OCT4* (Figure S2B). Detailed pyrosequencing analysis of CpG methylation showed that the 10–22 iPSC-like line exhibited demethylation at nine of nine sites at the *NANOG* promoter, compared to the primary tumor and at three of six sites at the *OCT4* promoter; by comparison, the H1 huES line was similarly demethylated at *NANOG* and demethylated at four of six sites at *POU5F1* (Figure S2C). Thus, the iPSC-like lines exhibit diverse characteristics of reprogrammed pluripotent cells.

We screened all iPSC-like lines for mutations in *KRAS*, *CDKN2A*, and *BRAF*, which are common genetic alterations in pancreatic cancer (Moskaluk et al., 1997) (Tables S2 and S3). The 10–22 iPSC-like line, derived from the recurrent, invasive, and poorly differentiated PDAC of the tenth patient, harbors the same *KRAS* G12D mutation seen in the initial tumor epithelial population (Figure 1F; Tables S1 and S2). A total of 10–22 cells also possess a *CDKN2A* heterozygous deletion (Figure 1H; Table S2) and a comparative genomic hybridization (CGH) pattern with numerous chromosomal aberrations, more exaggerated than that of the primary cancer culture and a correspondingly aberrant karyotype (Figure 1I and S3). The exaggeration of the primary cancer CGH pattern in the 10–22 iPSC-like line is expected because the primary cancer culture contained some stromal cells and thus was contaminated by cells of a normal genotype. Specifically, we detected 23 gross chromosomal aberrations in the PDAC epithelial cell population of the tenth tumor and 20 were represented in the 10–22 line (Figures 1I and S3B). Decreased CGH signals in the primary tumor cells and the 10–22 line spanned *PTEN* and *DPC4 (SMAD4)*, consistent with observations of allelic loss of these loci in human

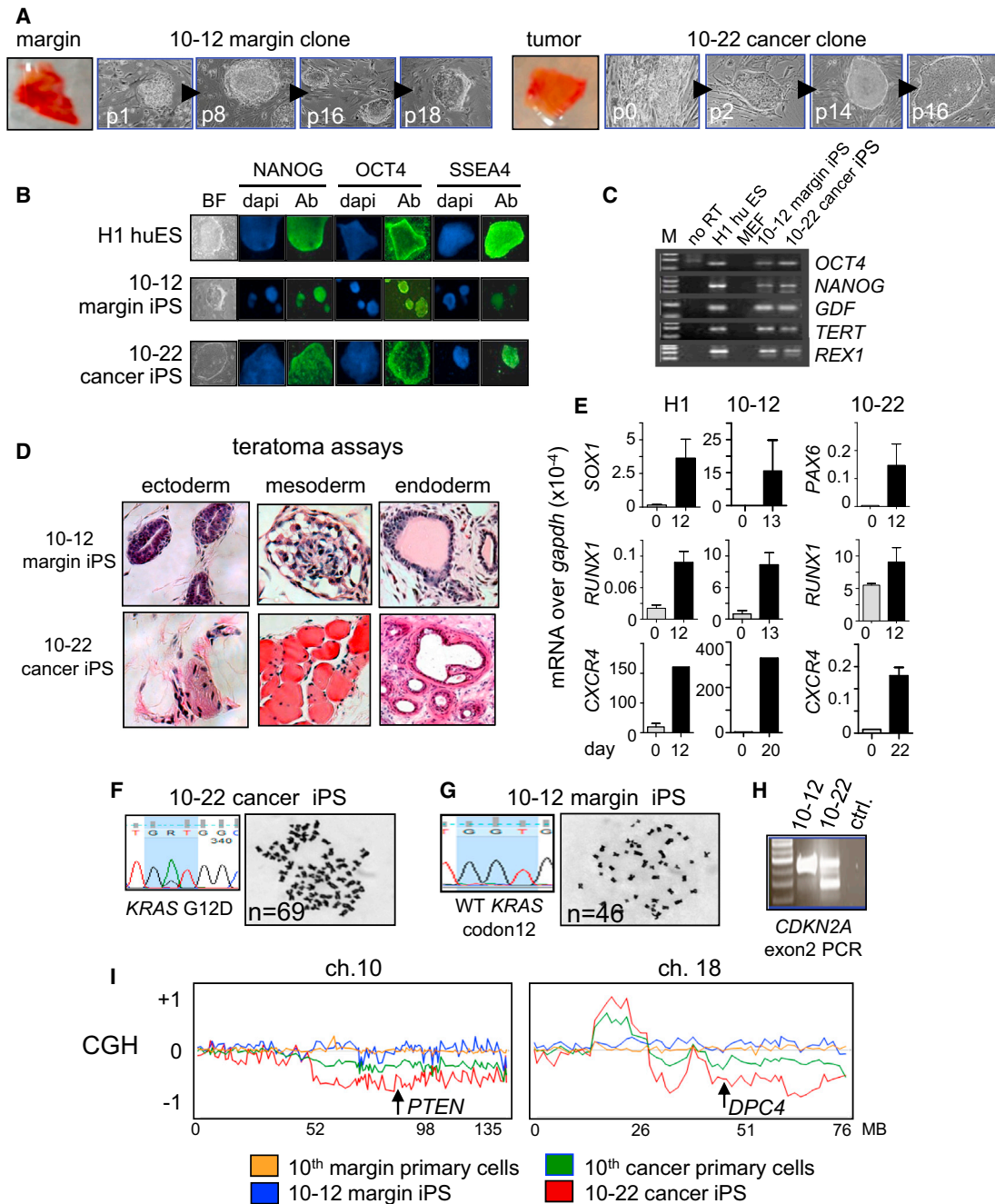


Figure 1. Establishing iPSC-like Lines from Patient-Matched Margin and Pancreatic Ductal Adenocarcinoma

(A) Cells from pancreatic cancer and margin tissues were reprogrammed and different passages of the 10–12 margin and 10–22 cancer iPSC-like clones from patient 10 are shown.

(B and C) Expression of pluripotency markers in 10–12 margin and 10–22 cancer iPSC-like lines by immunostaining (B) and RT-PCR (C).

(D) Three month teratomas in NSG mice from 10–12 margin and 10–22 cancer iPSC-like lines showed differentiation into tissues of all three germ layer lineages. See also Figure S2A.

(E) Expression of differentiation markers for endoderm (*CXCR4*), mesoderm (*RUNX1*), and ectoderm (*SOX1*, *PAX6*) relative to *GAPDH* in embryoid bodies from H1 huES cells and 10–12 margin and 10–22 cancer iPSC-like lines cultured for 12–14 days. Error bars represent mean \pm SD.

(F and G) DNA sequence tracks revealing *KRAS* mutation and karyotype showing subtetraploidy in the 10–22 cancer iPSC-like line (F) and wild-type *KRAS* and normal karyotype in the 10–12 margin iPSC-like line (G).

(H) PCR analysis of *CDKN2A* (*p16^{Ink4a}*) exon 2 reveals a heterozygous deletion in 10–22 cells.

(I) Comparative genomic hybridization showing normal profiles for the tenth primary margin cells (orange) and the 10–12 margin iPSC-like line (blue). Gross chromosomal rearrangements are evident in the tenth primary cancer cell culture (green), which was mixed with normal stromal cells; the rearrangements are

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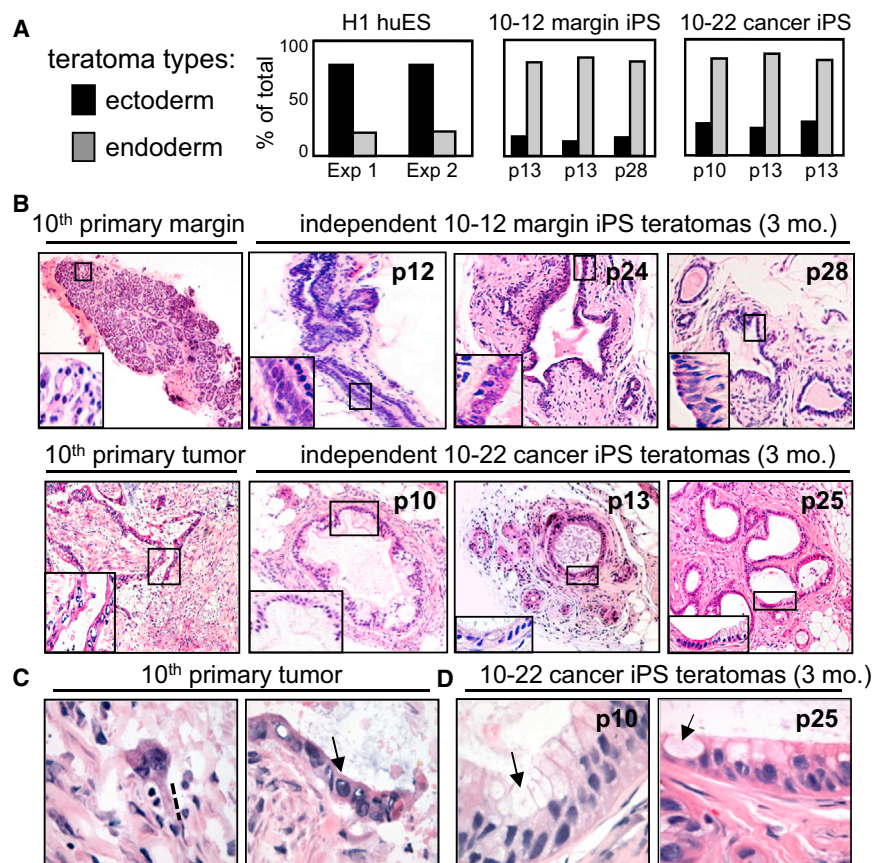


Figure 2. The 10-22 iPSC-like Cells Preferentially Generate Ductal Structures in Teratomas

(A) Summaries of teratoma tissue types arising in 3 months in immunodeficient mice based on the number of histologic structures seen. Teratomas from huES cells are mostly ectodermal in appearance, whereas teratomas from 10–12 and 10–22 iPSC-like lines are mostly endodermal/ductal.

(B) Hematoxylin and eosin staining of the margin and tumor tissue of patient 10 and of teratomas formed by 10–12 margin and 10–22 cancer iPSC-like lines after 3 months. The latter lines create tubular, duct-like structures independent of passage number. Notably, the 10–22 cancer teratomas show a much a higher degree of differentiation than the primary tumor.

(C) The primary tumor shows an invasive phenotype (dotted line) and high nuclear to cytoplasmic ratio (arrow).

(D) 10–22 tumor iPSC teratomas at 3 months show a low nuclear to cytoplasmic ratio, intracellular mucins (arrows), and a more differentiated phenotype.

See also Tables S1, S2, and S3.

10-22 iPSC-like Cells from Advanced Human PDAC, upon Differentiation, Undergo the Early Stages of Pancreatic Cancer

Despite being derived from cancer, the 10–22 cancer iPSC-like line as well as its

pancreatic cancers (Hahn et al., 1995, 1996). By contrast, the isogenic 10–12 pancreatic margin iPSC-like line had wild-type *KRAS*, a flat CGH chromosomal profile, similar to the parental margin cell culture and a normal karyotype (Figures 1G–1I and S3; Table S2). We conclude that the 10–12 and 10–22 iPSC-like lines are derived from pancreatic margin and cancer epithelial cells, respectively, with the 10–22 line harboring the marked genomic rearrangements seen in the initial advanced tumor epithelial population.

The iPSC-like lines from the 14th patient (Figure S1A and S1B), with moderately differentiated PDAC containing scattered 1 mm foci, showed point mutations in *CDKN2A* and *BRAF* but a wild-type *KRAS*, as with the parental epithelial culture (Tables S1 and S2), and flat CGH profiles (data not shown). A pair of margin- and cancer-derived iPSC-like lines from the 19th patient (Figures S1C and S1D) also had somatic mutations in *CDKN2A*, with wild-type *KRAS* and *BRAF* (Table S2). Because the 10–22 cancer iPSC-like line from the tenth patient contained the *KRAS* mutant background, typical of PDAC, we performed a detailed study of that line and its isogenic 10–12, *KRAS* wild-type margin iPSC-like clone.

companion 10–12 margin line gave rise to much smaller subcutaneous teratomas, at 3 months, compared to those seen from the control H1 human ESCs. More striking, there was a much higher proportion of endodermal, Dolichos biflorus agglutinin (DBA) lectin-positive ductal structures in teratomas from the 10–12 and 10–22 iPSC-like lines (Figures 2A and S4A), whereas H1 cells generated mostly neuronal lineages, as seen previously (Bock et al., 2011). These results are in concordance with reports on the propensity of other iPSC lines to differentiate into the lineage from which they were derived (Bar-Nur et al., 2011; Kim et al., 2011).

We next compared the endodermal teratomas arising from the margin (10–12) and tumor (10–22) iPSC lines with the original tumor of the tenth patient. The original tumor exhibited many areas of poorly differentiated foci and infiltration, although occasionally showed a more organized epithelium, but no PanINs (Figures 2B, 2C and S4B). The tumor epithelium exhibited irregularly shaped and hyperchromatic nuclei and a high nuclear to cytoplasmic ratio (Figure 2C, arrow), along with cytoplasmic protrusions indicative of an invasive phenotype (dotted line in Figure 2C). By contrast, teratoma ductal tissues at 3 months from

evident more clearly in the 10–22 cancer iPSC-like line (red) (see also Figure S3B), demonstrating that the 10–22 line is clonally derived representative of the initial tumor epithelial cell population. The locations of *PTEN* and *DPC4* (*SMAD4*) loci are shown in regions of chromosome loss in the tenth cancer and 10–22 cells.

See also Tables S1, S2, and S3.

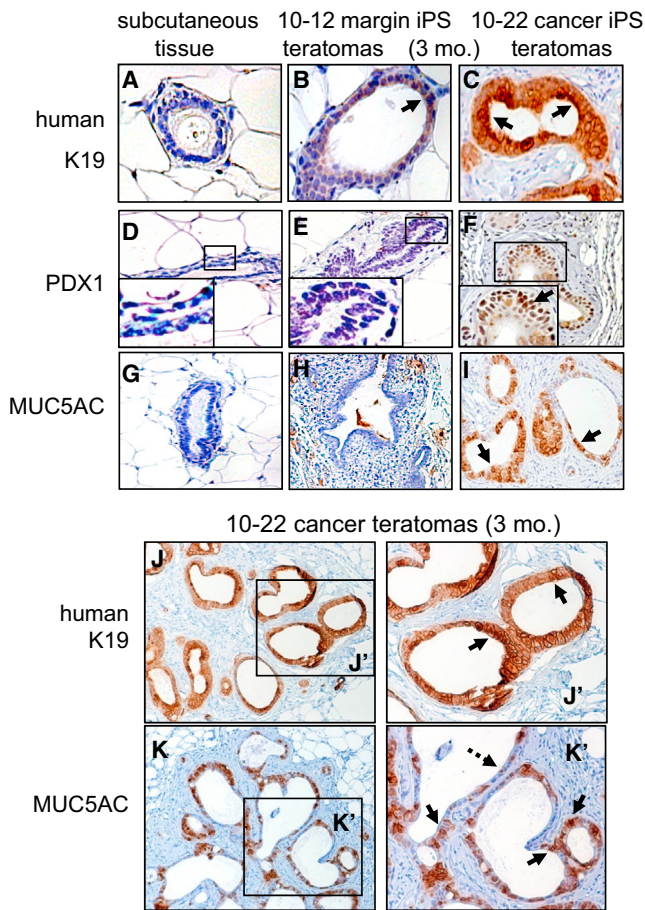


Figure 3. Teratomas at 3 Months from 10–22 Cancer iPSC-like Cells Exhibit PanIN-like Structures and Marker Expression

(A–C) No K19 staining in mouse subcutaneous tissue (negative control), weak K19 staining of 10–12 margin iPSC-like teratomas at 3 months, and strong K19 staining of 10–22 cancer iPSC-like teratomas at 3 months.

(D–I) Nuclear staining of PDX1 (arrow) and cytoplasmic staining of MUC5AC (arrow) only in teratomas of 10–22 cancer iPSC-like teratomas at 3 months.

(J and K) Higher magnification shows uniform K19 staining (J and J', arrow) and heterogeneous MUC5AC staining (K and K', arrow) of teratomas from 10–22 cancer iPSC-like cells.

See also Tables S1, S2, and S3.

the 10–22 cancer iPSC-like line had a high level of architectural organization, with abundant gland formation and a more differentiated cytology compared to the primary tumor (Figure 2B). This included an increased cytoplasm and smaller, less hyperchromatic nuclei with even nuclear membrane contours, a decreased nuclear to cytoplasmic ratio and increased cell polarity, indicative of PanIN stages. Abundant mucin was present in the apical aspect of the cells (arrows in Figure 2D). There was no evidence of acinar differentiation or neuroendocrine neoplasia. PanINs are graded by the extent of dysmorphic structures compared to normal ducts (Hruban et al., 2001) and we observed a range of PanIN1-, PanIN2-, and PanIN3-like structures in the histology of the endodermal teratomas from the 10–22 iPSC-like line at 3 months; although predominantly structures resembling the PanIN2 and PanIN3 stages (Figures S4C–

S4G). Taken together, these findings suggested that the 10–22 cancer iPSC-like line generates ductal structures that resemble PanIN (Maitra and Hruban, 2008).

PanIN-like teratomas formed in nine of ten teratoma experiments at 3 months, regardless of the passage number of the 10–22 cells. By contrast, the 10–12 pancreatic margin iPSC-like line did not generate PanIN-like structures in teratomas ($n = 4$) (Figure 2B). Also, iPSC-like lines from the 14th and 19th tumors, containing predisposing mutations, but not of *KRAS*, did not generate PanIN-like lesions ($n = 5$, data not shown) and therefore were not studied further.

PCR of DNA obtained by laser capture microdissection showed that the stromal cells surrounding the ductal epithelium in the PanIN-like teratomas of the 10–22 line contained the rt-TA lentiviral DNA and thus were derived from the starting 10–22 cells (Figure S4H). This was confirmed by detecting rt-TA expression in the PanIN-like epithelium as well as throughout the local stroma, but not in the distal stromal portions of subcutaneous tissue (Figure S4I). Recently, stromal-like cells surrounding PDAC have been found to be derived by EMT from the pancreatic cancer epithelium in a mouse model (Rhim et al., 2012). However, the study observed very few EMT-derived stromal cells at the PanIN stages. Taken together, it seems that most but perhaps not all of the human stromal cells in the PanIN regions of the teratomas were derived by local codifferentiation of the pluripotent 10–22 cells into epithelial and mesenchymal tissues.

Characterizing the ductal structures in teratomas of the 10–22 cancer iPSC-like line in further detail, we found that they expressed keratin 19 (K19) as well as nuclear PDX1, the pancreatic determination factor, and nuclear SOX9 (Figures 3A–3F, 3J, and S5A–S5E). PDX1 is expressed at very low levels in adult pancreatic duct cells, it is not expressed in adult exocrine cells, and it is upregulated in pancreatitis, PanIN, and PDAC (Miyatsuka et al., 2006). SOX9 is a coordinate effector, with mutant *Kras*, of precursor pancreatic lesions in a mouse model (Kopp et al., 2012). Teratomas from the 10–12 pancreatic margin iPSC-like cells did not express the gastric mucin MUC5AC, whereas the PanIN-like structures of the teratomas from the 10–22 cells expressed abundant MUC5AC (Figures 3G–3I, 3K, brown staining, and S5A), as observed in PanIN lesions and well-differentiated PDAC (Kim et al., 2002). Considering the histology and CGH genomic profile of the parental pancreatic cancer tissue from which the 10–22 iPSC-like line was derived, the data indicate that the 10–22 iPSC-like line from poorly differentiated, late-stage PDAC can differentiate into PanIN lesions associated with the early stage of the disease.

The 10–22 iPSC-like Line PDAC Progresses to the Invasive Stage of Human Pancreatic Cancer

To assess whether PanIN-like structures from the 10–22 line could progress to later stages of PDAC, we investigated teratomas grown for 6–9 months in NSG mice. By 9 months, two solid, palpable tumors arose in each of two injected mice (3–6 mm diameter), all with a genotype characteristic of 10–22 cells (Figure S5F). Although palpable tumors were not evident at 6 months, histological analysis showed highly glandular structures with nuclear heterotypia and hypochromia at both 6 and

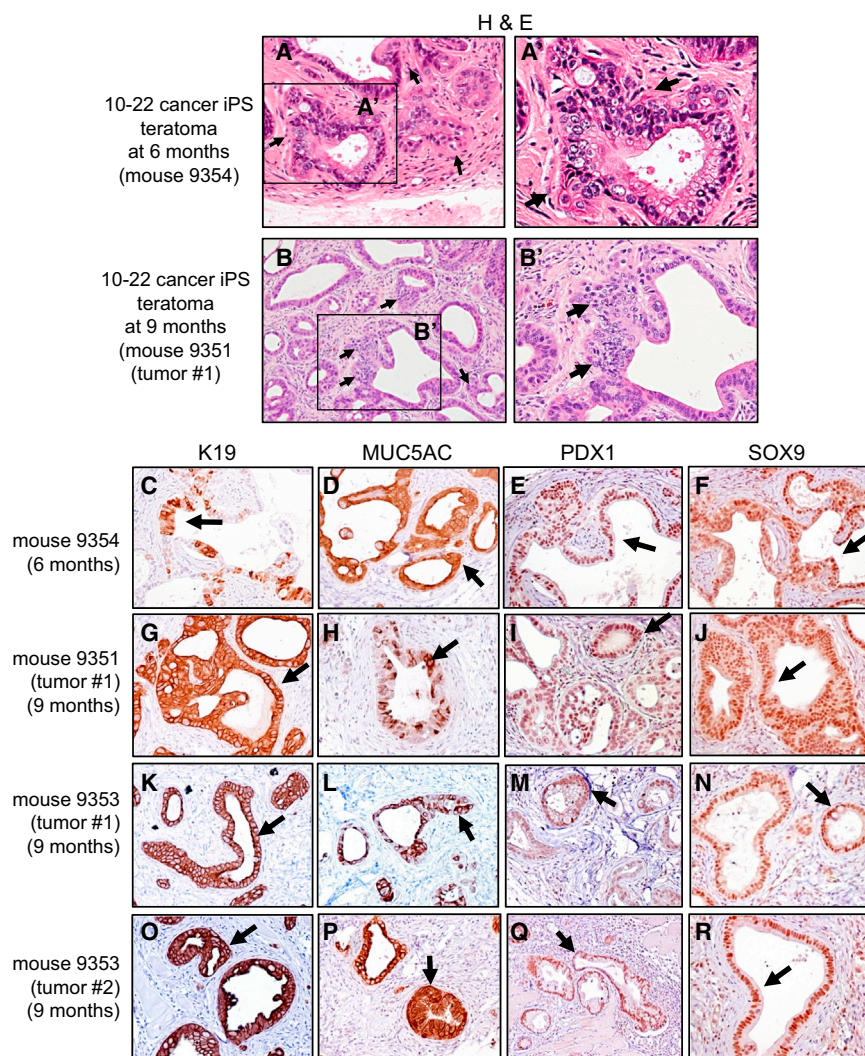


Figure 4. Teratomas at 6 and 9 Months from 10-22 Cancer iPSC-like Cells Exhibit Invasive Stages of Pancreatic Cancer

(A and B) Hematoxylin and eosin staining of 10-22 teratomas in NSG mice at 6 months (A and A') and 9 months (B and B'). Arrows indicate epithelial cells with a more dysmorphic phenotype than at 3 months; nuclei are heterotypic and epithelia with hypochromic nuclei are invading the stroma (A and B, arrows).

(C-R) Immunohistochemistry showing epithelial cells positive for K19, MUC5AC, PDX1, and SOX9 in tumors arising at 6 and 9 months in NSG mice injected with 10-22 cells. See Figure S5F for genetic confirmation of 10-22 cells in the tumors. See also Tables S1, S2, and S3.

10-22 line genotype, which was absent from contralateral control explants (Figure S6B). The organoids from the 10-22 cancer iPSC-like cells retained the expression of human K19 and MUC5AC (Figures 5A-5C, sections).

Given the poor prognosis of PDAC, we used the organoid system to identify biomarkers and pathways that could permit early detection and facilitate disease monitoring after therapy. We used nanoLC/MS/MS to examine the proteins that were secreted or released from explants of three independent teratomas that were cultured for 6 days (Figure 5D, left). The data were compared to proteins secreted or released from explants of contralateral control tissue cultured similarly and from the parent 10-22 iPSC-like line cultured in the undifferentiated state. We then selected peptides with

9 months (Figures 4A and 4B). The epithelial cells were positive for K19, MUC5AC, PDX1, and SOX9 (Figures 4C-4R, arrows). Notably, we observed structures indicative of a locally invasive phenotype (Figures 4A and 4B, arrows). We conclude that the PanIN-like stage of teratomas from the 10-22 iPSC-like line is succeeded in vivo by the invasive stage of PDAC, indicating that the 10-22 iPSC-like model undergoes a spectrum of pancreatic carcinogenesis.

Cultured Organoids of PanIN-like Cells from 10-22 Line Teratomas Secrete or Release Proteins Indicative of Early-Stage Pancreatic Cancer

We sought to generate a system where the PanIN-like structures occurring within teratomas from the 10-22 cells could be studied as a live, in vitro model of early-stage human pancreatic cancer. Accordingly, we harvested tissues from teratomas 3 months after injection, along with contralateral control tissue and established conditions where the tissues were embedded separately into Matrigel and cultured in vitro (Figure S6A). PCR analysis of DNA from the resulting sphere-like organoids confirmed the

perfect matches to human peptides and that were specific to the human PanIN-like teratoma explants (Figure 5D, right). Of the 25 proteins that were secreted or released from all three 10-22 teratoma explants (Figure 5D, right; Table S4), eight were previously reported to be expressed at the RNA or protein levels within PanIN, IPMN, and/or PDAC, whereas the remaining proteins have not been reported (Harsha et al., 2009) (Table S5). Of the 82 proteins that were identified from the intersection of pairs of 10-22 teratoma explants (Figure 5D, right; Tables S6, S7, and S8), 15 proteins have been previously reported in PanIN/IPMN/PDAC (Harsha et al., 2009) (Table S5). Although certain mRNAs were previously identified in PanIN, such as PGAM-M and VWF (Buchholz et al., 2005), we have determined that the corresponding proteins are secreted or released from cells and remain stable in the medium.

Of the total 107 proteins secreted or released from at least two 10-22 teratoma explants, Ingenuity pathway analysis revealed that 42 fall into interconnected TGF β 1 and integrin signaling networks (Figure 5E, for 38 proteins). These pathways have been previously reported in PanIN, IPMN, and PDAC (Bardeesy

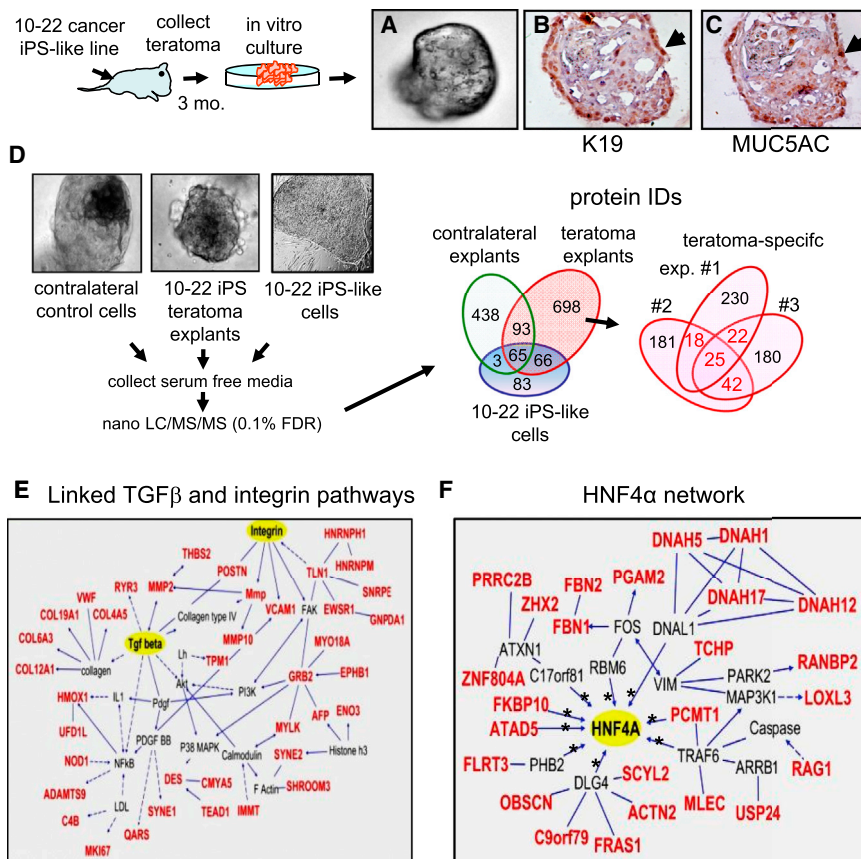


Figure 5. Proteins Secreted and HNF4α Regulatory Network within Teratoma Explants of 10-22 iPSC-like Cells at 3 Months

Scheme for in vitro explants of 3 month teratomas from 10-22 cancer iPSC-like lines.

(A–C) Whole mount view of teratoma explant from 10-22 iPSC-like cells (A), along with the explant sectioned and stained for K19 (B), and MUC5AC (C); arrows indicate positive (brown) domains.

(D) Proteomic analysis of conditioned media of 3 independent teratoma explants (7761, 9223, and 9225) from 10-22 cancer iPSC-like cells. Conditioned media was analyzed by nanoLC/MS/MS and protein candidates were identified by a Sequest search in the International Protein Index database. Using UniProtKB TrEMBL (downloaded in October, 2011), we included peptides in our list that either consists of uniquely human sequences or sequences that are common to humans and mice. Mouse-specific sequences were excluded. Protein identifications of conditioned media from teratoma explants were compared to those in conditioned media from explants of contralateral control tissue and of media from undifferentiated 10-22 cells (left Venn diagram). The total 698 secreted or released proteins specific to the teratomas are indicated in the three different experiments (right Venn diagram). Proteins secreted from at least two teratoma explants (red numbers) are summarized in Table S4 and were used for pathway analysis.

(E) Numerous proteins fall into linked pathways for TGFβ and integrin signaling. Lines and arrows connecting molecules indicate direct interactions and dashed lines and arrows indicate indirect interactions.

(F) All proteins in red are secreted or released specifically from the 10-22 teratoma explants and fall within a network controlled by the transcription factor HNF4α (Table S4). Asterisks denote proteins whose genes are directly bound by HNF4α. See also Tables S5, S6, S7, S8, and S9.

et al., 2006; Jones et al., 2008). In summary, we discovered numerous proteins that are secreted or released from the 10-22 live cell model of early-stage human pancreatic cancer, as well as evidence of well-documented pathways involved in early cancer progression.

HNF4α Network Activated in Early- to Intermediate-Stage, but Not Late-Stage, Pancreatic Cancer

Further Ingenuity analysis revealed that another 25 of the secreted or released proteins were in a network that we observed to be centered on the transcription factor HNF4α, including numerous direct gene targets (Odom et al., 2004) (Figure 5F; Table S9). Nine of the proteins in the HNF4α network were also secreted into the plasma during the PanIN stage of a mouse PDAC model (Table S9) (Taguchi et al., 2011). Although HNF4α has not been reported in the development or progression of PDAC, by searching databases, we noted the amplification of the *HNF4α* locus (Maser et al., 2007) and the upregulation of *HNF4α* mRNA (Iacobuzio-Donahue et al., 2003; Logsdon et al., 2003) in human PDAC. Although some reports show HNF4α in pancreatic acinar cells as well as islets in adult mice (Gupta et al., 2007), we found HNF4α to be predominantly expressed in islets, with very low or no expression elsewhere in the normal

pancreas (Figure S6C). Strikingly, although HNF4α was not expressed in normal human or mouse pancreatic ducts (Figures 6A and S6C), it was expressed in the nucleus of 10-22 teratoma PanIN-like cells at 3 months and invasive-stage cells at 9 months (Figures 6B, 6C, and S6D). HNF4α was detected cytoplasmically in moderately differentiated domains of the original tumor of the tenth patient, from which 10-22 cells were derived, but not in undifferentiated portions of the tumor (Figure 6D).

To more quantitatively assess HNF4α expression at different stages of human pancreatic cancer, we used a tissue microarray to assess multiple samples of human PanINs at different stages as well as samples of pancreatic cancer. HNF4α was barely detected in the nuclei of normal pancreatic ducts and very weakly in the samples of PanIN-1 cells, but exhibited a statistically significant increase in nuclear expression in the samples of PanIN-2 ($p < 0.05$) and stronger and more uniform expression in PanIN-3 epithelia ($p < 0.05$) (Figures 6E–6G, and 6J; Table S10). HNF4α was most frequently detected in well-differentiated mucinous sections of PDAC ($p < 0.01$) and barely or not detectable in undifferentiated or poorly differentiated epithelial structures of PDAC (Figures 6H–6J), as seen in the original patient 10 tumor (Figure 6D). We also note that in the human protein atlas (Uhlen et al., 2010), HNF4α appeared positive

in well-differentiated epithelial structures of PDAC but either cytoplasmic or not expressed in poorly differentiated or undifferentiated epithelial structures of PDAC and not expressed in metastatic PDAC.

We assessed HNF4 α in a mouse model of PDAC arising in a *Kras*^{G12D};*p53*^{L/+}; *Pdx1-Cre*; *Rosa*^{LSL-YFP} background (Rhim et al., 2012). As in humans, HNF4 α was sporadically expressed at the PanIN-1 stage (Figure 6K), it was expressed in most nuclei of PanIN-2 (Figure 6L) and PanIN-3 lesions (Figure 6M) and observed in nuclei of the more differentiated portions of the murine tumors but not in the undifferentiated portions (Figures 6N and 6O, white arrows). In conclusion, we validated the ability of the 10–22 iPSC-like cells to undergo early stages of human pancreatic cancer by their morphology, histology, secreted, or released proteins, and using them to discover a previously unappreciated network associated with early to invasive-stage pathology in human clinical samples and a mouse model of the disease.

DISCUSSION

There has been an absence of live human cell models of PDAC progression and consequently little information about proteins that could serve as released biomarkers and pathway indicators for early stages of the disease. As noted in the Introduction, when human PDAC or pancreatic cancer stem cells are grafted into immunodeficient mice, tumors rapidly arise that resemble the advanced PDAC stages from which the cells were derived and they do not undergo the slow growing phenotype of PDAC precursors. We hypothesized that, based on the ability of certain cancer cells to be reprogrammed to pluripotency by nuclear transfer and then to undergo early mammalian development, pluripotent stem cell lines from human pancreatic tumors might have the capacity to progress through early developmental stages of the cancer. This would provide an opportunity for discovering intrinsic processes and secreted protein biomarkers of live, early-stage human cells for a devastating cancer. Indeed we showed that a rare, single pancreatic cancer iPSC-like line, 10–22 cells, can provide insights into human cancer progression.

How does the ectopic expression of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* and a pluripotent-like state suppress the cancer phenotype? Apparently the pluripotency epigenetic environment can dominate over certain oncogenic states (Esteller, 2007). In nuclear transfer studies, only certain cancer cells are amenable to reprogramming (Blelloch et al., 2004; Hochedlinger et al., 2004; Li et al., 2003) and, similarly, we only obtained one iPSC-like line from pancreatic cancer harboring a *KRAS* mutation, the predominant driver of PDAC. Although the *KRAS* mutation induces MAPK signaling, which can trigger mouse ESCs to differentiate (Kunath et al., 2007), in human ESCs, MAPK signaling can promote self-renewal (Eiselleova et al., 2009). Oncogenic *RAS* induces cellular senescence by the accumulation of p53 or CDKN2A (Serrano et al., 1997) and the expression of the four reprogramming factors also triggers senescence by inducing p53 and CDKN2A, thereby impairing reprogramming (Banito et al., 2009). Only patient 10 in our study had a deletion in exon2 of *CDKN2A*, possibly explaining how the 10–22 cells

could escape a senescent phenotype. Additional mutations could have arisen in the 10–22 cells that made the cells particularly amenable to iPSC formation. Further work will determine what kind of mutations predict whether a cancer cell can be reprogrammed to pluripotency.

How does release from pluripotency allow the cancer genome to be expressed in a stage-specific fashion, as opposed to undergoing an immediate regression to the late-stage phenotype? Although the answers to these questions are not in hand, release from pluripotency is normally accompanied by the development of germ layer cells and then specialized tissues, which may continue to dominate, epigenetically, over the resident cancer genome (Blelloch et al., 2004; Hochedlinger et al., 2004; Li et al., 2003). The 10–22 cells from PDAC generated diverse tissue types in teratomas as well as pancreatic ductal tissue that exhibited PanIN lesions and later progression. The apparent preference for pluripotent cells to regenerate the cancer type from which they were derived reflects the tendency of iPSC lines in general to preferentially differentiate into their lineages of origin (Bar-Nur et al., 2011; Kim et al., 2011).

Several lines of evidence indicate that the 10–22 iPSC-like line is derived from PDAC. First, the pathology of the original, recurrent tumor was that of PDAC and the CGH profile of the bulk population of cultured cells, which had a highly disrupted genome, was represented in the CGH profile of the 10–22 iPSC-like line (Figures 1I and S3B). Although the tumor harbored pockets of more differentiated epithelial cells amidst a vast majority of undifferentiated cells, and therefore it is not certain which type of cell was immortalized in the 10–22 line, the 10–22 cells' disrupted genome does reflect that of a typical epithelial cell in the recurrent tumor. Second, the PanIN-like structures from the 10–22 cells' teratomas expressed SOX9 (Figures S5B–S5E), which is required for early, *Kras*^{G12D}-dependent pancreatic precursor lesions in a mouse model (Kopp et al., 2012), as well as PDX1, a definitive pancreatic cancer epithelial marker. Thus the ductal lesions from the 10–22 cells are of a pancreatic type. Third, teratomas at 9 months from 10–22 cells progressed to the histology and locally invasive characteristics of later-stage PDAC (Figure 4). Thus, the 10–22 line was not from an early-stage cell that would solely undergo an early-stage phenotype. Taken together, the evidence indicates that the 10–22 iPSC-like line is from PDAC cells in the original tumor and that, upon redifferentiation in teratomas, it undergoes progression of the disease. This is unlike other human PDAC lines, which exhibit late stages of cancer (Lieber et al., 1975; Yunis et al., 1977).

Could the 10–22 iPSC-like line be derived from cancer stem cells within the original tumor? Pluripotency genes such as *NANOG* are expressed in sphere cultures of pancreatic cancer stem cells (CSCs), suggesting that such cells might be more susceptible to reprogramming (Lonardo et al., 2011). However, CD133+CXCR4+ pancreatic CSCs are not enriched and the expression of pluripotency genes is not observed in the adherent culture conditions we used to derive the 10–22 cells (Hermann et al., 2007). Also, the *OCT4* and *NANOG* pluripotency genes were highly methylated in the parental tumor 10 epithelium cultures, in contrast to the 10–22 cell line and the huES H1 control, and *NANOG* itself was not expressed in the primary tumor,

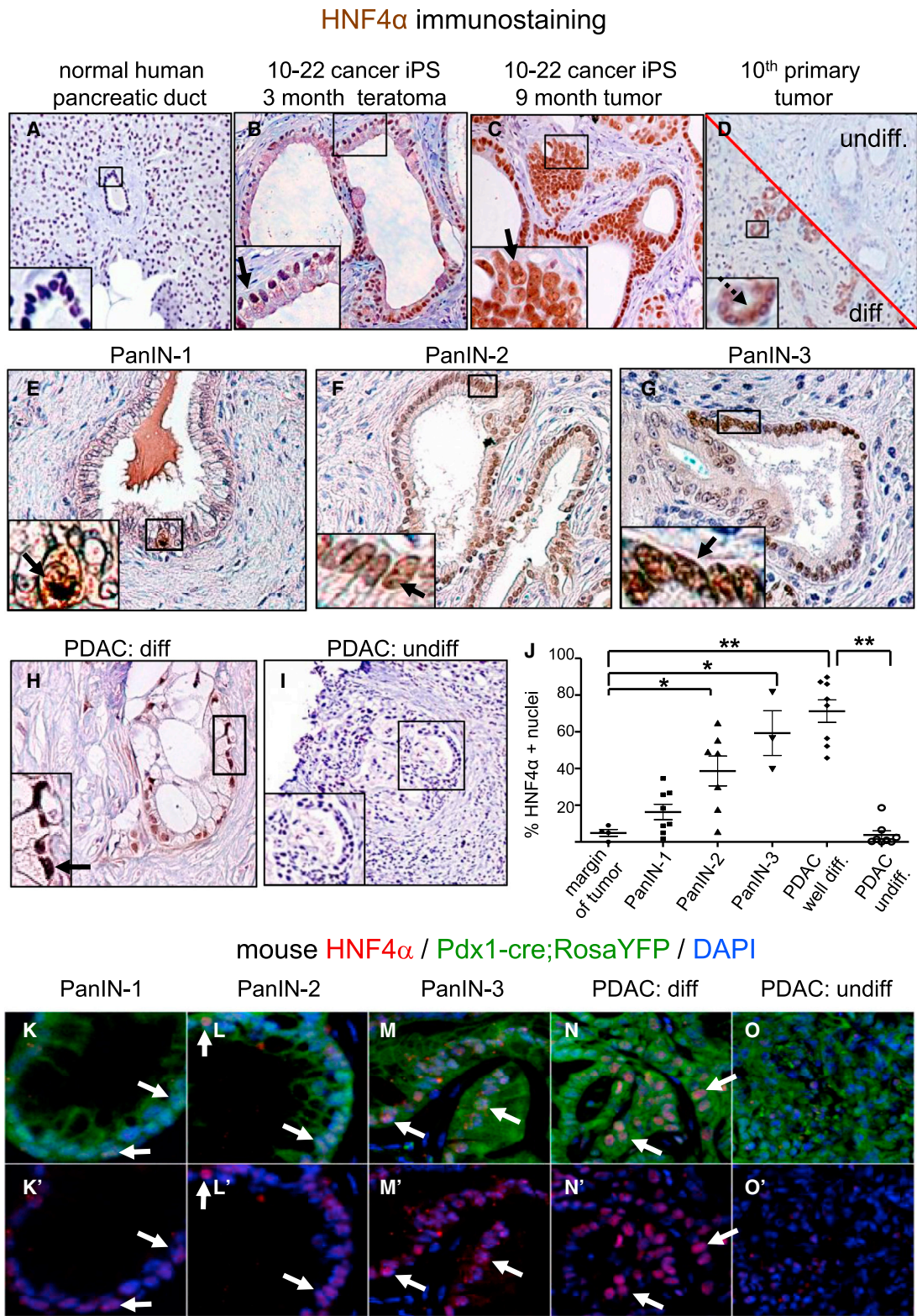


Figure 6. Activation of HNF4 α in PanIN Cells and Well-Differentiated Early Pancreatic Cancers in Human Clinical Samples and a Mouse Model of Human PDAC

(A–D) Immunohistochemistry for HNF4 α showing the absence of staining in the main human pancreatic mass and ducts (A), strong nuclear staining in PanIN-like structures in a 3 month teratoma (B, arrow) and in invasive cells in a 9 month tumor (C, arrow) generated from NSG mice injected with 10–22 cells, and mostly

(legend continued on next page)

although OCT4 was expressed sporadically (Figures S2B and S2C). Taken together, it seems unlikely that 10–22 cells were derived from pancreatic CSCs. In addition, pancreatic CSCs (Hermann et al., 2007; Ishizawa et al., 2010; Li et al., 2007) rapidly generate aggressive tumors that represent the primary tumors; whereas the 10–22 cells generate slow growing PanINs (Figures 3 and S4). Finally, tumors generated with pancreatic CSCs give rise to both cytokeratin negative and positive cells in the resultant tumors, in contrast to the homogenous K19 positive staining in PanIN-like ducts at 3 month teratomas (Figures 3 and S2A). Thus, 10–22 cells appear not to exhibit properties of pancreatic cancer stem cells.

The proteins released or secreted from the PanIN-like teratomas fell into three major networks, including interconnected networks for TGF β and integrin signaling that suppress PDAC progression (Hezel et al., 2012). We also report the activation of an HNF4 α network distinctive for the late PanIN stages. HNF4 α is not or barely expressed in normal pancreatic ductal cells, poorly expressed in the PanIN1 stage, but is activated in PanIN2 and PanIN3 stages, invasive stages, and in early well-differentiated human pancreatic cancer. HNF4 α levels then decrease markedly in advanced or undifferentiated PDAC. We found that these expression states also occur in a mouse model of PDAC progression. Dynamics in HNF4 α expression affect the oncogenic transformation of liver cells (Hatziaepostolou et al., 2011). It remains to be determined whether the expression of HNF4 α and its target genes is a cause or consequence of pancreatic cancer progression. Yet considering that pancreatic cancer is typically discovered in advanced or metastatic stages, activation of HNF4 α and the release or secretion of proteins from the factor's target genes specifically in the late PanIN stages should provide useful diagnostics.

Of 107 proteins reproducibly released or secreted from PanIN-like cells derived from the 10–22 line, a total of 68 proteins overlap with genes, proteins, and networks expressed in human PanIN and PDAC, further validating the origin of the cells. In addition, we show that such proteins are released from the cells and stable, thus serving as potential biomarkers of early PDAC. A subset of the secreted proteins could be from locally activated stromal cells in the explants. We suggest that the combined detection of released proteins that are the products of the HNF4 α , TGF β , and integrin networks within PanIN and invasive PDAC cells may provide the best means for noninvasively detecting the progression of pancreatic cancer in humans.

Based on our extensive efforts with diverse initial PDAC samples (see Tables S1 and S2), iPSCs arising from epithelial

cells of solid tumors appear to be rare events, possibly involving secondary changes that allow iPSC formation. It is therefore unclear whether the iPSC approach will work with other solid tumor samples. Despite these caveats, the 10–22 cells behaved in a highly consistent fashion, and this study revealed information about human PDAC. It is hoped that a better understanding of how to create iPSCs from human epithelial cancers will provide opportunities in the future with other types of solid tumors.

EXPERIMENTAL PROCEDURES

Tissue (1–2 cc) was taken from the center of pancreatic ductal adenocarcinoma samples and sample margin furthest from the cancers, under the patient's informed consent under IRB 09-801 at the Fox Chase Cancer Center. Tissue was dissociated and cells were cultured in defined K-SFM supplemented with EGF, cholera toxin, and bovine pituitary extract on dishes coated with collagen at 37°C for 3–4 days. The resultant epithelial cells were infected twice with TetO mouse Oct4, Sox2, Klf4, and c-Myc and PWPT-rtTA lentiviruses for 72 hr. Approximately 10⁵ cells surviving per starting tumor or margin tissue were detached, resuspended in ESC media supplemented with Y27632, and plated onto irradiated mouse embryonic fibroblasts (MEFs). ESC-like colonies were picked 12–36 days postinfection and passaged mechanically every 5–7 days onto irradiated MEFs, with daily feedings. The iPSC-like lines were characterized by RT-PCR and immunostaining for pluripotency markers, embryoid body and teratoma assays for pluripotency, and CGH, karyotyping, PCR for *KRAS*, *CDKN2A*, and *BRAF* mutations, and pyrosequencing for *KRAS* mutations. Female 4–6-week-old NOD-SCID-IL2R γ c null (NSG) mice (Shultz et al., 2005) were used for subcutaneous injection of the iPSC-like lines. The injection area was isolated after 3–9 months and analyzed by histology or dissociated in liberase T-flex (1.3 W/ml), embedded into Matrigel, and cultured in SFM. Conditioned media was collected and proteins were processed for analysis by nanoLC/MS/MS. See the Extended Experimental Procedures for details.

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the CGH data reported in this paper is GSE47985.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and ten tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.05.036>.

LICENSING INFORMATION

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cytoplasmic staining in small, moderately differentiated ducts (D, image section below the diagonal, brown, dashed arrow) and an absence of staining in undifferentiated ducts (D, image section above the diagonal) of the tenth patient's pancreatic tumor epithelium.

(E–I) HNF4 α is weakly labeled in human PanIN-1 cells (E, arrow, n = 8), strongly in PanIN2 (F, arrow, n = 7), PanIN3 (G, arrow, n = 3), and mucinous well-differentiated human PDAC (H, arrow, n = 8) but decreased or not in undifferentiated/or poorly differentiated human PDAC (I, n = 8).

(J) Percentage of HNF4 α positive nuclei to total nuclei counted (see Table S4) in multiple PanIN and PDAC samples on human tissue microarrays. Error bars represent SEM. The differences in HNF4 α expression is statistically significant between margin and PanIN-2 and PanIN-3 stages (*p < 0.05); margin and well-differentiated PDAC (**p = 5.46E-06); well-differentiated PDAC and poorly/undifferentiated PDAC (**p = 3.05E-06), as per two-tailed Student's t tests.

(K–O) In a mouse model of PDAC, HNF4 α is expressed weakly at the PanIN1 (K and K'), strongly at the PanIN2-3 stages (L, L', M, and M') and in differentiated portions of tumors (PDAC) (N and N'), and weakly or not expressed in undifferentiated portions of the same tumor (O and O').

See also Table S10.

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