A shifting adversary: control of pancreatic cancer transcriptomic subtypes

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Abstract

Cellular plasticity, the dynamic ability of cells to adopt distinct transcriptional states, plays a well-known role in the pancreas during the initiation of pancreatic ductal adenocarcinoma (PDA), the most common form of pancreatic cancer. It is now becoming clear that plasticity also plays an important role after the emergence of PDA. PDA is composed of two major transcriptional subtypes, classical and basal-like, with important biological differences. Recent work has indicated that individual tumors can be comprised of cells of each subtype, and that tumor subtype can change during the evolution of a tumor. This suggests that PDA cells can transit between transcriptional states, with important implications for disease progression. This review discusses what is currently known about inter-subtype plasticity and how this process is controlled.

Keywords: Pancreatic ductal adenocarcinoma, transcriptional subtypes, classical, basal-like, squamous, quasi-mesenchymal, cellular plasticity, transcription factors, tumor microenvironment

PANCREATIC CANCER INITIATION AND PROGRESSION

Pancreatic ductal adenocarcinoma (PDA) carries the bleakest prognosis of any common malignancy, with five-year survival rates hovering around 10%\(^1\). Because of difficulties in early detection, most PDA patients present at an advanced stage, precluding surgical resection. However, even among patients eligible for surgical resection, the five-year survival rate remains no higher than 20\(^1\), arguing that poor survival in
PDA patients is due in part to the intrinsically aggressive nature of the disease.

Potentially contributing to this clinical intractability is an emerging hallmark of pancreatic cancer, plasticity - the ability of tumor cells to adopt new identities in response to specific signals or through natural selection. A high degree of plasticity is already evident around the time of tumor initiation, when identity shifts can play a critical role in the emergence of pancreatic intraepithelial neoplasia (PanIN), the most common precursor to PDA\[^3\]. Approximately 90% of PDA tumors are driven by oncogenic KRAS mutations\[^4\], and despite the ductal histology of PDA tumors, acinar cells have proven to be more amenable to KRAS-mediated transformation than ductal cells in mouse models\[^5\]. Activation of mutant KRAS, combined with inflammation, converts acinar cells into duct-like cells through a process known as acinar-to-ductal metaplasia (ADM)\[^6\]-\[^8\], followed by the emergence of PanINs\[^9\]. The ease with which acinar cells are transformed is partly due to the fact that the duct-like cells that emerge during ADM harbor a proto-oncogenic transcriptional program that is locked into place by KRAS mutations\[^10\]. It is now clear that all major epithelial cell types of the pancreas (acinar, ductal, and endocrine) can give rise to PDA through a combination of KRAS activation, tumor suppressor gene inactivation, and inflammation\[^11\]-\[^14\].

While the plasticity exhibited by normal pancreatic cells in the face of oncogenic and inflammatory insults is relatively well understood, recent studies have suggested another form of plasticity that exists among cancer cells after the emergence of invasive carcinoma. For over a decade, it has been clear that PDA tumors can be separated into distinct transcriptomic subtypes\[^15\]. More recent evidence, discussed in detail in this review, argues that subtypes are not static; tumor cells can readily transit between these transcriptional states. This form of plasticity has important ramifications for tumor progression and patient outcomes.

**TRANSCRIPTIONAL SUBTYPES OF PANCREATIC CANCER**

Despite a relatively homogeneous landscape of driver mutations (i.e., KRAS mutations), pancreatic cancer exhibits important inter- and intra-tumoral transcriptomic heterogeneity. This heterogeneity has important implications for the biology of the disease.

Over the past decade, several studies developed classification systems based on gene expression profiling across tumors. The first such study, performed by Collisson \textit{et al.} used microarray data from microdissected PDA tumors, arriving at three distinct subtypes: classical, quasi-mesenchymal (QM-PDA), and exocrine-like\[^15\]. Mouse and human cell lines with gene expression profiles mirroring the classical and QM-PDA but not the exocrine-like tumors could be established, raising the possibility that the exocrine subtype reflected contamination from surrounding normal pancreatic tissue. Patients with QM-PDA tumors fared more poorly than those with classical tumors, the first indication that tumor transcriptional subtypes impact the biological properties of the cancer. Additionally, classical and QM-PDA cells differed in their response to chemotherapy drugs, while classical cells displayed a higher dependency on continued mutant KRAS expression.

The abundant stroma of PDA and the potential for contaminating normal tissue presents a confounding issue for the identification of cancer-specific gene expression signatures and the precise assignment of tumor subtypes. A second study refined these subtype definitions by employing a bioinformatic technique termed “digital microdissection” to untangle gene expression in the tumor epithelium from that in the stroma and surrounding normal tissue\[^16\]. Using this approach, these authors identified two subtypes, classical and basal-like. The exemplar genes defined by Moffitt \textit{et al.} in classical tumors corresponded well to the classical signature defined by Collisson \textit{et al.} confirming the existence of that subtype. Moffitt \textit{et al.} named the basal-like subtype due to the shared expression of keratins present in the basal subtype of other
tumor types\textsuperscript{[15,16]}. Basal-like tumors largely corresponded to the QM-PDA subtype defined in the earlier study, although these authors presented evidence that some of the mesenchymal gene expression in the Collison classifier was derived from the stroma. In addition to tumor-specific gene expression subtypes, these authors also identified two distinct stromal subtypes, normal or activated. The normal stromal subtype was enriched with gene expression characteristic of pancreatic stellate cells, while the activated subtype showed more diverse gene expression, mainly connected with macrophage-enriched genes. Importantly, both classical and basal-like tumor cell gene expression was found among both stromal subtypes, arguing against a causal connection between stromal and tumor gene expression.

A third study by Bailey \textit{et al.} combined gene expression profiling with a comprehensive genomic analysis (whole genome, exome and copy number analysis), all performed using bulk tumor samples with high (> 40\%) tumor cellularity\textsuperscript{[17]}. These authors identified four subtypes using RNA-seq: squamous, aberrantly differentiated exocrine, pancreatic progenitor, and immunogenic. The squamous and pancreatic progenitor subtypes corresponded well to the previously described QM-PDA/basal-like and classical subtypes, respectively. Not surprisingly, tumors of the squamous subtype were more frequently associated with adenosquamous histology, a long-observed subset of PDA tumors comprising up to 4\% of PDA\textsuperscript{[18,19]}, although not all tumors of the squamous subtype displayed this histology\textsuperscript{[17]}. Immunogenic tumors were characterized by increased B and T cell infiltration, a characteristic captured due to the inclusion of stroma in the samples used, while the tumor cell gene expression in the immunogenic subtype corresponded most closely to the classical subtype.

Subsequent studies have supported the conclusions of Moffitt \textit{et al.} arguing for the existence of two main gene expression states within the tumor epithelium: classical and basal-like\textsuperscript{[16]}. Examination of sample histology has suggested that the previously proposed exocrine/ADEX and immunogenic subtypes reflect normal or stromal contamination, respectively\textsuperscript{[20-22]}. Importantly, careful experimental microdissection validated the conclusions based on the “digital” microdissection performed by Moffitt \textit{et al.} Recent work has suggested further refinement of the classical/basal-like dichotomy, suggesting that the basal-like and classical categories can each be further broken down into A and B subtypes\textsuperscript{[16,22,23]}. Additionally, a set of “hybrid” tumors expressing genes of both subtypes were identified, suggesting the possibility that some tumors harbor a mixture of basal-like and classical cells, or that individual cells may simultaneously express both transcriptional programs. Single cell studies have indeed borne out both possibilities, identifying tumors containing individual cells that were fully basal-like or classical\textsuperscript{[22]}, and also a subset of tumor cells that indeed express genes of both programs simultaneously\textsuperscript{[24]}

One of the most robust findings in all subtyping studies has been the poor survival of patients with basal-like tumors in comparison with classical tumors. Several observations suggest more aggressive behavior by basal-like tumors. For example, patient-derived xenografts (PDX) of a basal-like phenotype grew considerably faster than PDX tumors of a classical phenotype\textsuperscript{[16]}. Until recently, most pancreatic tumor samples used for subtyping studies have been material obtained from surgically resected samples, skewing sample representation towards the ~15\% of relatively early-stage tumors that are eligible for surgical resection. Recently, the COMPASS trial has made available samples from late-stage patients not eligible for surgery, making their primary tumors and metastases available for subtyping. This allowed examination of subtype representation at various stages of tumor progression. This showed that basal-like cancers are more highly represented in tumors from Stage IV patients compared to those with resectable Stage I/II tumors (36\% vs. 14\%), suggesting that studies reliant only on resected tumors may underestimate the frequency of basal-like tumors\textsuperscript{[21]}. 

While the classical vs. basal-like dichotomy in PDA has become widely accepted by the field, additional questions remain to be answered. For one, the role of EMT in the acquisition of basal-like gene expression is unclear. Basal-like tumors usually show enrichment of markers of the epithelial-to-mesenchymal transition (EMT), although adenosquamous cancers that occur in the pancreas and is associated with the basal-like subtype\cite{25} are epithelial. PDA cell lines of epithelial and mesenchymal morphology can be obtained, although cell lines expressing the basal-like or squamous transcriptional program are often epithelial in morphology (unpublished observations). Are basal-like tumors simply more prone to undergo EMT in response to the correct signals, or is there a direct connection between EMT or EMT-promoting signals in driving cells towards basal-like gene expression? Second, while mouse models have shown an ability to recapitulate classical vs. basal-like gene expression, mice with pancreas-specific \textit{Cdkn2a} deletion combined with mutant KRAS activation develop sarcomatoid tumors that are not seen in human PDA\cite{26}. These tumors are completely devoid of epithelial features and have been suggested to be underrepresented in human surgical specimens due to their highly invasive nature, precluding their surgical resection\cite{27}. Do these tumors represent the extreme end of an EMT spectrum that includes basal-like tumors?

**DETERMINANTS OF TRANSCRIPTIONAL SUBTYPES**

What explains the tendency of a given tumor towards either of the two subtypes? One potential explanation is epigenetic, based on PDA cell of origin. This hinges on the fact that the introduction of appropriate genetic lesions in both ductal and acinar cells in mouse models can give rise to PDA\cite{28,29,30}. PDA derived from acinar cells is associated with PanIN lesions, both before and after progression to invasive carcinoma, while duct-derived carcinoma display far fewer attendant PanIN lesions\cite{31}. Importantly, duct-derived carcinoma cells show a tendency towards basal-like gene expression, while acinar-derived tumors more closely resemble the classical subtype identified in human cohorts\cite{32}. Definitively ascribing a cell of origin to advanced human malignancies is impossible, meaning the role of the cell of origin in human PDA subtypes will remain undetermined. That said, human ducts have been recently shown to harbor cells expressing the squamous lineage-determining transcription factor (TF) \textit{ΔNp63}, and displaying the full spectrum of basal markers in addition to the ducal marker KRT19\cite{33}. While mouse ducts did not contain basal cells, normal mouse ductal cells could be induced to express \textit{ΔNp63} under organoid growth conditions, suggesting ductal cells can easily transdifferentiate into a basal-like phenotype\cite{34}.

While these studies suggest that cell of origin may skew individual tumors towards one of the two major subtypes, additional data suggest that the transcriptional subtype is not predetermined, but is an evolving tumor phenotype. Importantly, there is evidence in at least one patient harboring a classical or hybrid primary tumor in which a metastasis that emerged later had switched to the basal subtype\cite{35}. Given the difficulty of obtaining tumor material at multiple stages of progression in individual patients, the data supporting the generality of this phenomenon are still limited, although several additional observations provide further support. An important recent study showed that basal and classical cells coexist within individual tumors, a phenomenon observed in 13/15 tumors examined\cite{36}, and which was recapitulated in organoid culture\cite{37}. This suggests that basal vs. classical tumor designations using bulk RNA-seq data reflect only tipping of the balance towards one or the other differentiation state. This implies that, given the correct circumstances, the balance could be tipped in the other direction.

What might drive tumor cell plasticity? One important contributor appears to be the tumor microenvironment (TME), which has recently been shown to determine the transcriptional phenotype of PDA cells\cite{38}. This conclusion is partly based on observations that \textit{in vitro} culture conditions played a role in determining transcriptional phenotype, often causing \textit{in vitro} models to deviate significantly from the tumors from which they were derived. Growth in tumor organoid conditions favored outgrowth of tumor
cells with classical gene expression, while adherent culture as cell lines tended to favor basal-like. Given the distinct media composition between the two culture systems, one possibility this suggested is that differential exposure to specific cytokines may underlie differences in transcriptional phenotype. For example, PDA organoid culture routinely calls for the inclusion of a TGF-β inhibitor in the culture medium. Adding TGF-β to the organoid culture medium was sufficient to shift the transcriptional profile back towards the basal expression state, suggesting that transcriptional states in vivo may be strongly influenced by microenvironment-derived TGF-β signaling.

In support of this idea, xenografted organoids displayed distinct transcriptional profiles depending on where they were implanted in the pancreas. To complement existing orthotopic xenograft models that involve an injection of cells into the pancreatic interstitial space, Tuveson and colleagues developed an intraductal organoid transplantation model. Interestingly, the tumors formed by individual organoid lines could differ in transcriptional subtype depending on the location of organoid implantation. Cancer cells implanted in the interstitial space of the pancreas were more prone to adopt a basal-like transcriptional profile than those implanted within pancreatic ducts. Tumors within the interstitial space frequently contained reactive desmoplastic stroma, while intraductal transplants were often only exposed to the normal murine ducts, suggesting that stromal-derived signals indeed play an important role in driving the adoption of the basal-like transcriptional phenotype. Importantly, depletion of macrophages from the PDA TME resulted in a switch away from the squamous/basal-like subtype in cancer cells, suggesting that macrophages may supply important signals enforcing that transcriptional phenotype. A recent study suggests this may occur through tumor necrosis factor (TNF)-α secretion. Additional work will be necessary to fully elucidate the function of stromal cell populations in driving classical versus basal-like differentiation.

Cell-autonomous mechanisms may also contribute to the evolution of PDA transcriptional phenotypes. Gains of mutant KRAS signaling have been implicated in PDA metastasis in mouse models, and cells derived from these models exhibit a transcriptional phenotype consistent with basal-like cells. Later work in human PDA confirmed a correlation between basal-like gene expression and gains in KRAS signaling. Basal-like tumors were significantly more likely than classical tumors to harbor genomic imbalances favoring the mutant allele of KRAS. Genomic imbalances usually involve loss of the wild-type KRAS allele coupled with genome duplication. In one patient, a classical primary tumor contained only a minor imbalance favoring the mutant KRAS allele (loss of the wild-type allele), while a basal-like metastasis that occurred later had gained copies of mutant KRAS. This data suggests that subtype switching, driven by amplification of KRAS signaling, can occur during pancreatic cancer progression. Direct evidence for such an idea has been provided in organoids engineered to express tamoxifen-inducible mutant KRAS. Activation of KRAS using this system was shown to push implanted tumors towards a more basal-like phenotype both histologically and transcriptionally. Given that mutations in KRAS initiate and drive all stages of pancreatic tumorigenesis, it will be of great interest to identify the mechanisms by which a quantitative change in KRAS output can contribute to such dramatic phenotypic and transcriptional differences during tumor progression.

In addition to KRAS gains, additional genetic correlates of tumor subtypes have been uncovered. Amplifications of the TF GATA6 and loss of SMAD4 are enriched in classical subtype tumors. GATA6 is an endodermal TF associated with normal pancreatic development, so its association with well-differentiated pancreatic tumors of the classical subtypes appears to fit. As discussed above, TGF-β signaling appears to play a role in the emergence of the basal-like phenotype, so a paucity of SMAD4 alterations in the basal-like subtype is consistent with those observations. In the basal-like subtype, loss of function of the
most common tumor suppressors \(TP53\) and \(CDKN2A\) were even more common than in classical tumors\(^{[23]}\). Given the sensitivity of these tumor suppressors to oncogene activation, this may be linked to \(KRAS\) gains observed in basal-like tumors. Squamous subtype tumors (a classification aligned with basal-like) also exhibit loss of the histone demethylase KDM6A\(^{[17,38]}\). Importantly, pancreas-specific knockout of \(Kdm6a\) in a \(KRAS^{G12D}\)-driven mouse model of PDA leads to the formation of squamous-like PDA, suggesting a causal relationship between KDM6A alterations and tumor subtype\(^{[38]}\).

In summary, evidence has been presented suggesting that cell of origin, microenvironment, and genetic alterations all play a role in determining PDA transcriptional subtypes. Cases in which, within the same patient, tumor progression is accompanied by gains in KRAS signaling and acquisition of basal-like properties would argue against a role for the cell of origin in determining subtype in that patient, though it does not rule out a role for the cell of origin in globally influencing PDA transcriptional phenotypes. Studies in mice or humans that follow the evolution of transcriptional subtypes throughout tumor progression should provide an idea about the generality of classical-to-basal transitions during tumor evolution.

TRANSCRIPTIONAL CONTROL OF TUMOR SUBTYPES

While the mechanisms that might allow the interconversion of PDA subtypes demand more study, several TFs have been implicated in the maintenance of each subtype. With some important exceptions, the TFs that control the two subtypes are generally lineage-specific TFs that control transcriptional enhancers specifying each differentiation state. According to the study of these TFs and their target genes, it has become clear that while they cooperate to maintain subtype identity, many of these TFs control distinct aspects of the biological properties of each subtype. Therefore, these key TFs provide important insights into the specific biology of each PDA subtype.

**TF determinants of classical PDA**

As discussed above, frequent GATA6 amplifications have been identified in PDA\(^{[39,40]}\), suggesting that it functions as a PDA lineage survival oncogene\(^{[41]}\). The fact that these amplifications preferentially occur in classical PDA makes it an obvious candidate for determining the classical state\(^{[23]}\). Indeed, the expression of GATA6 is a robust discriminator between basal and classical PDA\(^{[25,42]}\). Like lineage survival oncogenes in other tissues, GATA6 inhibits EMT in PDA cell lines, likely through direct transcriptional control of E-cadherin and activation of other epithelial-promoting TFs such as FOXA1 and FOXA2\(^{[43]}\). Later studies suggested that GATA6 stands as a guardian against the acquisition of basal-like characteristics\(^{[43,44]}\). Pancreas-specific deletion of GATA6 upon tumor initiation by mutant KRAS accelerated tumor formation but failed to result in tumors of squamous histology\(^{[46]}\). On the contrary, deletion of GATA6 in established PanIN lesions resulted in invasive tumors with increased squamous differentiation, determined by the expression of basal markers KRT5 and KRT14\(^{[45]}\). The tumors resulting from “late” GATA6 knockout were not only more proliferative and chemoresistant, but also displayed evidence of increased ability to evade adaptive immunity, most notably through downregulation of class I MHC molecules. Consistent with this, human tumors with low GATA6 expression exhibited significantly fewer infiltrating CD8+ T cells than high GATA6 tumors. These results suggest that transcriptional subtypes may dictate PDA immune phenotype, with implications for immunotherapy that require follow-up.

GATA6 is a key endodermal TF that plays a crucial role in pancreatic development\(^{[46-49]}\), and it cooperates with additional endodermal TFs to maintain the classical subtype. In addition to FOXA1/A2, GATA6 cooperates with HNF4A and HNF1A, both of which are also developmentally important endodermal TFs whose loci are frequently methylated and silenced in squamous/basal-like PDA\(^{[17,45]}\). HNF4A was also shown to directly contribute to the expression of genes associated with the classical phenotype, and like Gata6,
deletion of Hnf4a in a mouse model of PDA accelerated tumorigenesis\cite{49}. Basal-like and classical PDA are metabolically distinct, with basal-like tumors displaying a higher degree of glycolysis and classical tumors exhibiting a more lipogenic phenotype\cite{50}. HNF4A depletion in PDA cells promoted increased glycolysis, presumably mediated by increased expression of key enzymes in the glycolytic pathway\cite{44}. It is unclear whether HNF4A directly regulates the expression of glycolytic enzymes, or whether the metabolic phenotype upon HNF4A knockdown is the indirect result of activation of oncogenic pathways such as PI3K, which were also activated by HNF4A depletion. In any event, despite their shared role in maintaining classical gene expression, GATA6 and HNF4A appear to control distinct sets of genes, and the glycolytic phenotype upon depletion was specific to HNF4A\cite{44}. Like HNF4A and GATA6, HNF1A is another endodermal TF whose expression is associated with the classical subtype of PDA. As with the other TFs associated with the classical subtype, pancreas-specific KO of Hnf1a coupled with mutant KRAS activation accelerated tumorigenesis, and the emergence of tumors with sarcomatoid features\cite{51}. The same study also showed that HNF1A cooperates with KDM6A to promote acinar cell differentiation, consistent with the fact that knockout of either factor in acinar cells results in widespread ADM. Contrary to these results in autochthonous mouse models, HNF1A has also been shown in some contexts to promote tumorigenesis.

Using primary human PDA cell lines, HNF1A was found to be a pro-tumor factor expressed more highly in an EPCAM\textsuperscript{high}/CD44\textsuperscript{high} population enriched with tumor-forming ability\cite{52}. These authors went on to show that in the setting of human cell lines, HNF1A depletion inhibits tumor formation. It is currently unclear what determines whether HNF1A plays a pro- or anti-tumor role in PDA cells.

Another series of studies performed in human cell lines aimed at examining the transcriptional determinants of PDA tumor grade, which the authors noted is closely linked with classical (low grade) and basal-like (high grade) subtypes\cite{43,44}. The authors first assembled a collection of cell lines that represented low- and high-grade tumors, and performed epigenomic profiling, identifying sets of enhancers marked by H3K27 acetylation specific to each grade. This approach was combined with RNA-seq to identify TFs differentially expressed in cell lines of each grade. The most strongly enriched motif in enhancers specific to the low-grade cells was that of the ETS-domain family of TFs, consistent with the fact that family member ELF3 was the second most highly expressed TF in low-grade compared to high grade. KLF5 was the most highly expressed TF in low-grade versus high-grade tumors, and despite its motif not showing enrichment in low-grade enhancers, the authors demonstrated that KLF5 is an important determinant of epithelial differentiation in low-grade tumor cell lines\cite{50}. Unlike other TFs linked with the classical subtype, KLF5 knockout in mouse models completely abolished tumorigenesis, apparently by preventing acinar-to-ductal metaplasia, an essential first step in tumorigenesis initiated in acinar cells\cite{18,53}.

While most of the classical subtype-linked TFs described so far have been endoderm-derived lineage regulators, another low-grade-specific TF identified in these studies is interferon regulatory factor 1 (IRF1), a broadly expressed immune regulatory TF important for the interferon response\cite{56}. IRF1 is activated by KLF5 and ELF3, explaining its expression in low-grade tumor cells. While IRF1 was not shown to play a role in maintaining classical subtype identity, its expression in low-grade tumor cells resulted in greater interferon responsiveness, resulting in higher expression of antigen presentation and processing genes, echoing observations made in experiments targeting GATA6\cite{45,56}. Contrary to classical tumors, basal-like tumors upregulate ZBED2, a zinc finger protein that binds to the promoters of IFN-regulated genes and antagonizes their activation by IRF1\cite{76}. These observations regarding differential IFN responsiveness of the two subtypes appear consistent with the observation that basal-like PDA tumors are depleted of infiltrating T cells relative to other subtypes\cite{42}, and again suggest that tumor subtype may play a role in determining sensitivity to immunotherapy.
Classical PDA cells are highly secretory, producing not only mucins but also large quantities of gastrokines such as GKN2, GKN3, TFF1, and TFF3. Mucin and gastrokine production are also associated with PanIN lesions, an observation that suggests classical PDA and PanIN are closely related entities. The high-level production of secreted proteins in these cells makes them potentially sensitive to ER stress. Recent work suggests that the secretory phenotype of classical PDA is ameliorated by the classical-specific TF MYRF. MYRF is an ER membrane-associated factor that undergoes autocatalytic cleavage to release an N-terminal fragment that translocates to the nucleus to regulate transcription. Depletion of MYRF resulted in activation of the transcriptional hallmarks of the unfolded protein response, together with a massive disruption in ER structure. The mechanisms by which MYRF maintains proper ER function appear to be multiple. On the one hand, MYRF knockout led to increased production of mRNAs encoding secreted factors that are normally highly expressed in classical PDA, such as CEACAM5 and CEACAM6. While this may seem counterintuitive given that MYRF and the secreted factors are co-expressed in classical tumors, these authors demonstrated that MYRF binds directly to secreted factor loci and attenuates their expression, presumably avoiding ER stress that might accompany their overproduction. When MYRF was introduced into basal-like PDA cells, ER function was improved, which partially occurred using a version of MYRF lacking its DNA binding domain, suggesting that the membrane-bound form that remains in the ER may also have effects on ER function that do not involve transcription. Another classical-linked TF that may contribute to ER homeostasis is SPDEF. SPDEF is an ETS-family TF expressed in secretory Goblet and Paneth cells in the intestine. SPDEF is preferentially expressed in classical PDA cells and promotes the expression of ER-resident AGR2 and ERN2, two proteins that contribute to ER homeostasis. SPDEF knockout in classical but not basal-like cell lines restrained tumorigenesis, supporting the idea that classical PDA lines are particularly dependent on pathways that maintain ER function.

**Classical TFs as tumor suppressors and dependencies**

We have seen that TFs that enforce the classical state are often not only dispensable for tumor development but even serve as an impediment to carcinogenesis. The fact that TFs linked to maintenance of the classical state restrain tumorigenesis appears consistent with the better overall survival of patients with classical compared to basal-like tumors and indicates that PDA subtypes can readily interconvert in response to experimental TF manipulation. However, the enrichment of GATA6 amplifications in classical tumors implies that at certain points in the development of these tumors, the high-level expression of GATA6 provides a selective advantage. In that light, experiments showing that depletion of GATA6 can result in a smooth transition to a basal-like phenotype are somewhat surprising. In fact, classical PDA cells do not always readily abandon the classical state for basal-like state. KLF5 is repressed during a transition from classical to basal-like PDA, which is consistent with the fact that KLF5 is repressed by SMAD TFs downstream of TGF-β during the EMT. EMT in classical tumor cells can result in cell death and tumor suppression, and experimental depletion of KLF5 can result in the same. In the case of KLF5, dispensability in classical cells stems from its collaboration with another TF, SOX4. SOX4 normally plays a tumorigenic role in classical PDA, and binds the genome with a high degree of overlap with KLF5. When KLF5 is depleted through TGF-β repression during EMT, SOX4 becomes a driver of apoptosis. Importantly, activation of certain signaling pathways such as AKT/Pi3K can smoothen the transition by preventing cell death. In this way, under some conditions, the EMT serves as a bottleneck for classical PDA cells, but the bottleneck can be "widened" by specific signaling pathways. Successful transit through this bottleneck may yield tumor cells of the basal-like subtype.

**TF determinants of basal-like PDA**

Fewer TFs have been implicated in maintenance of the basal-like state. Given the histological and transcriptomic resemblance of basal-like PDA to squamous cell carcinoma, it is unsurprising that a key squamous lineage regulator, TP63, plays an important role in determining the basal-like state. TP63 is a
member of the p53 family\cite{71}, and it functions as a lineage determinant in the development of the normal squamous lineage\cite{72}. An isoform encoded by the TP63 gene lacking a portion of the full-length N-terminus (ΔNp63) is also an indispensable part of the transcriptional program of squamous cell carcinomas derived from skin, lung, esophagus, and head and neck\cite{73-75}. While it is not expressed in the normal pancreas, expression of the ΔNp63 isoform is abundant in tumors of the basal-like subtype\cite{17}. In PDA with squamous characteristics that form upon KDM6A knockout in the pancreas, an enhancer at the Trp63 locus activates ΔNp63 expression\cite{38}.

Functional experiments with ΔNp63 have now made it clear that it is an important master regulator of the basal-like subtype. Using loss- and gain-of-function experiments in human cell lines, it was demonstrated that ΔNp63 expression was necessary and sufficient to confer squamous-like gene expression on PDA cells\cite{76,77}. Activation of the squamous program by ΔNp63 overexpression in classical cells conferred the cells with increased migratory capacity in vitro and enhanced growth in vivo, consistent with the worse prognosis associated with basal-like tumors\cite{76}.

In addition to ΔNp63, the Hedgehog (Hh) signaling TF GLI2 also contributes to the basal-like state\cite{78}. The activation of this TF in PDA cells appears independent of Hh ligands and correlates strongly with EMT-high basal-like characteristics in PDA cell lines. As expected of a subtype determinant, GLI2 overexpression was sufficient to confer basal-like properties on classical cell lines, while its depletion shifted basal-like cells towards classical attributes. The enforcement of the basal-like state by GLI2 was at least partly dependent on one of its target genes, the inflammatory secreted factor osteopontin (OPN). Depletion of OPN reversed the basal-like phenotype of PDA cells, while supplying exogenous OPN could confer basal-like properties on others\cite{49}. This suggests that cancer cells that adopt a basal-like phenotype may induce others to follow suit through secreted signals such as OPN.

**PERSPECTIVE**

The importance of PDA transcriptional subtypes to the biology of the disease and the outcome of patients has gained recognition over the past several years, and it remains a highly active area of investigation. The distinct biology of the two subtypes, highlighted by work uncovering distinct gene expression programs governed by TFs associated with each state, suggests that each subtype will have distinct dependencies. Based on the work discussed above, these dependencies could be metabolic, signaling, or immune-related, and it appears likely that additional specific dependencies will be revealed for each subtype.

While subtype-specific dependencies are of interest, it will be even more important to find shared requirements that unite all PDA cells. KRAS signaling appears to represent a unifying PDA dependency (at least in 90% of PDA tumors with KRAS mutations), although some details remain to be cleared up about the role of KRAS in each subtype. As discussed, gains in KRAS signaling are associated with a tendency towards a basal-like phenotype, although it is unclear what, if anything, connects gains in KRAS signaling with the more aggressive subtype. In apparent discord with that data, evidence has also been presented indicating that basal-like cells are more resistant to KRAS depletion\cite{78}. Deciphering the role of KRAS in shaping and then maintaining PDA subtypes will be of great interest to future studies.

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