Challenges and Opportunities in Modeling Pancreatic Cancer

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The ability to faithfully model complex processes lies at the heart of experimental biology. Although a reductionist approach necessarily reduces this complexity, it is nevertheless required for untangling the contributions and interactions of the various system components. It has long been appreciated that cancer is a complex process that involves positive and negative interactions between tumor cells, normal host tissue, and the associated cells of the tumor microenvironment. However, accurate models for studying these complex interactions in vitro have remained elusive. We seek to generate models of mouse and human pancreatic cancer that are relevant to disease biology and useful for elucidating poorly understood facets of this deadly disease. The ability to model, manipulate, and predict the therapeutic response of an individual's disease outside their body represents the promise of precision medicine. Therefore, these models are patient-specific and allow the identification of new biomarkers and novel treatment modalities for rapid translation to the clinic. In this perspective we will discuss recent advances in modeling pancreatic cancer in vitro, the discoveries these models have enabled, and future challenges and opportunities awaiting further investigation.

Pancreatic ductal adenocarcinoma (PDA) is among the most highly lethal malignancies, with a median survival rate of $<8\%$ (Siegel et al. 2016). Several factors account for this poor outcome, including the advanced stage at which most patients are diagnosed and the minimal effectiveness of currently utilized therapeutic options (Ying et al. 2016). Although surgical intervention can be curative, the vast majority of patients ($>80\%$) present at diagnosis with metastases and are considered ineligible for surgery. In these patients, standard of care generally consists of chemotherapy, with minimal long-term benefit. The modest activity of systemic chemotherapy is thought to be a consequence of the highly desmoplastic, poorly vascularized nature of PDA, where up to 90% of the total tumor volume can consist of a dense extracellular matrix, fibroblasts, and cells of the immune system (Moir et al. 2015). Understanding the contribution of the individual components of the tumor microenvironment to tumor progression or control is imperative to developing more effective treatment options. Therefore, in vivo and in vitro models that accurately reflect this complexity are required for defining and manipulating the signals that mediate tumor–stromal interactions.

Human and mouse tumor-derived cell lines grown on tissue culture plastic in two dimensions (2D) have been a mainstay for understanding the genes and pathways critical for cancer cell growth and oncogenic processes such as cell migration. They are easily cultured and can be genetically manipulated, transplanted into mice for tumor growth and metastasis assays, and grown in large quantities for high-throughput chemical and genetic screens. However, several important drawbacks exist with this culture method. Importantly, normal, nontransformed human or mouse cells rarely proliferate under these conditions, hindering the ability to directly compare gene expression or the results of experimental manipulations between normal and tumor. When nontransformed cells can be cultured in a monolayer, this oftentimes results in the loss of cell polarity, a critical property required for epithelial cell function. It is also challenging to coculture distinct cell types in monolayers, hindering the ability to study tumor–stromal cell interactions. Finally, transplantation of 2D-cultured cancer cells into the mouse generally results in tumors that do not recapitulate the complex pathophysiology, microenvironment and therapeutic response of the primary tumor. Therefore, models that overcome these limitations are required.

Genetically engineered mouse models (GEMMs) of PDA have demonstrated the ability to accurately model disease progression, from the early stage pancreatic intraepithelial neoplasm (PanIN) stages through desmoplastic tumors with stroma characteristic of the human disease (Perez-Mancera et al. 2012). In particular, the "KC" mouse, harboring a mutant KRas allele ($KRas^{G12D}$), and the "KPC" mouse, harboring both mutant KRas and mutant p53 ($Trp53^{R172H}$), driven by *Pdx1-Cre* in the pancreas, have provided opportunities for preclinical studies with novel therapeutics, generating a rationale for

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clinical trials and changes in PDA clinical intervention (Hingorani et al. 2003, 2005). Although these mouse models are useful for understanding the genetic drivers of PDA and performing therapeutic trials, they are expensive and time-consuming to maintain. Furthermore, the inherent tumor microenvironmental complexity makes it difficult to untangle interactions between the epithelial-derived tumor cells, stromal fibroblasts, and immune components.

To address the deficiencies of both standard 2D cell culture and GEMMs, we developed an in vitro three-dimensional (3D) organoid model, allowing the growth and manipulation of normal, premalignant, and malignant pancreatic tissue from mouse and human (Boj et al. 2015). The organoids can be passaged indefinitely in semisolid matrices, stored as a frozen pellet, and resuscitated in a manner similar to traditional cell culture methods, genetically manipulated, grown in large quantities for therapeutic or genetic screens, and orthotopically transplanted into mice where they recapitulate the phenotype of the tissue from which they were derived. Importantly, the tumors derived from organoid transplants recapitulate many properties of human PDA, including desmoplasia and poor vascularization. This system provides several advantages over standard cell culture, including the ability to grow normal and malignant pancreatic ductal cells under identical conditions, allowing direct comparisons of differential gene expression, cell signaling networks, metabolism, and therapeutic response. We have also developed a coculture model whereby organoids can be grown alongside pancreatic stellate cells (PSCs, the resident fibroblast of the pancreas), allowing detailed investigations into the positive and negative interactions that occur between these cell types during tumorigenesis.

Understanding the complex genetic underpinnings of PDA has been hampered by the low tumor cellularity and difficulty in obtaining samples from patients with advanced disease (Bailey et al. 2016). The organoid model overcomes these obstacles by allowing the selective culture and expansion of the epithelial-derived tumor cells, allowing deeper sequencing without stromal contamination. Furthermore, organoids can be generated from endoscopic ultrasound-guided fine-needle biopsies, including from those patients with inoperable, metastatic disease, allowing for the first time a large-scale analysis of advanced PDA. In this perspective, we will highlight recent discoveries in PDA biology made possible by the organoid model and propose new challenges and opportunities for studying this deadly disease.

ELUCIDATING THE ROLE OF NRF2 IN PDA PROGRESSION

Nearly all patients with PDA display an activating mutation in the *KRAS* oncogene, a small GTPase with a wide range of downstream effectors (Javle et al. 2016). Genetic ablation of oncogenic *KRas* in mouse models leads to tumor regression, demonstrating the importance of therapeutically targeting this protein in patients with PDA (Ying et al. 2012). However, effective drugs targeting KRas directly have yet to be developed. Therefore, much research has focused on understanding the downstream mechanisms by which oncogenic KRas promotes tumorigenesis. We previously showed that oncogenic KRas increased the expression of the redox regulator transcription factor Nrf2 (nuclear factor erythroid-derived 2-like 2, *Nfe2l2*/*Nrf2*), lowering intracellular reactive oxygen species (ROS) (DeNicola et al. 2011). Disruption of this pathway inhibited KRas-driven tumorigenesis, demonstrating that Nrf2-mediated ROS detoxification is a critical mediator of oncogenesis. However, as directly targeting transcription factors therapeutically has proved difficult, we endeavored to understand the mechanisms by which Nrf2 promoted tumorigenesis.

Accordingly, we utilized the organoid model system to determine the mechanism by which Nrf2 maintains PDA proliferation (Chio et al. 2016). Knockdown of Nrf2 in human tumor organoids reduced proliferation, whereas knockdown had little effect on normal human organoids. Mouse organoids harboring oncogenic KRas were able to form tumors upon orthotopic transplantation in athymic nude mice; in contrast, engraftment was impaired in mouse organoids lacking Nrf2. In concordance with previous results, activation of oncogenic KRas decreased levels of ROS, which was reversed upon knockdown of Nrf2. We hypothesized that Nrf2-regulated changes in ROS levels may alter patterns of protein cysteine oxidation, thereby modifying protein function and driving cancer cell proliferation. Therefore, we developed a proteomic platform to specifically detect reduced cysteine-containing peptides, independent of protein abundance differences. This method revealed that loss of Nrf2, in the context of oncogenic KRas and mutant p53 ("KP"), modified the oxidized cysteine content of proteins regulating mRNA translation. Organoids derived from KP mice displayed up-regulation of translation when compared with those derived from normal pancreata, and protein translation was attenuated in these organoids following Nrf2 loss. To determine if reversible oxidation of the translation machinery controlled translation activity, we expressed cysteine to aspartic acid oxidation mimics of several translational regulatory proteins and measured protein synthesis. Expression of one such mutant, of the elongation factor eEF2, decreased the rate of nascent protein synthesis as compared with wild-type eEF2, showing that eEF2 is a bona fide redox sensor and supporting the overall premise that Nrf2 protects PDA cells from oxidative stress to promote tumorigenesis.

In addition to the effects of Nrf2 on mediating translation through redox regulation, we observed that Nrf2-deficient cells displayed defects in cap-dependent translation, driven by changes in PI3K (phosphoinositide 3-kinase)/Akt/mTOR (mammalian target of rapamycin) signaling. Cells lacking Nrf2 had decreased EGFR (epidermal growth factor receptor) activation, leading to decreased Akt and Erk phosphorylation, known regulators of cap-dependent translation. The lack of EGFR activity resulted from decreased shedding of EGF by the metalloprotease Adam10. Adam10 was shown to contain a

reduced cysteine residue sensitive to changes in Nrf2 levels, and an Adam10 cysteine oxidation mimic reduced nascent protein synthesis in KP organoids. Therefore, we hypothesize that Nrf2 regulates Adam10 activity in a redox-dependent manner, mediating EGF autocrine signaling and cap-dependent translation in PDA.

As Akt is a critical regulator of KRas signaling and tumor proliferation, Akt inhibitors are under clinical investigation in pancreatic cancer (Akinleye et al. 2015) (Clinical Trials Identifier: NCT01783171). However, KRas mutant cells overcome Akt inhibition through reactivation of EGFR, in a process that requires autocrine EGF signaling (Mendoza et al. 2011). Because this autocrine EGF signaling is disrupted in tumor cells lacking Nrf2, we hypothesized that Akt inhibition would synergize with the pro-exidant buthionine sulfoximine (BSO), an inhibitor of intracellular glutathione (GSH) synthesis. KRas mutant organoids treated with both inhibitors failed to reactivate EGFR, and the combination was synergistic in reducing proliferation in organoids and human PDA cells. Finally, the combination of Akt inhibiton and BSO treatment was more effective than either drug alone in reducing tumor growth in KPC mice. Therefore, we propose that modulating redox regulation in combination with Akt inhibition should be considered for clinical translation (Chio et al. 2016).

UNTANGLING THE COMPLEXITY OF THE TUMOR MICROENVIRONMENT

The complex role of the tumor microenvironment as a regulator of PDA pathogenesis is an area of intense investigation. Cancer-associated fibroblasts (CAFs) are responsible for secretion of extracellular matrix (ECM) and production of tumor-promoting growth factors, resulting in tumor cell growth, decreased drug delivery, and drug resistance (Bachem et al. 2005; Hwang et al. 2008; Straussman et al. 2012; Jacobetz et al. 2013). Therefore, disruption of the ECM, either through enzymatic digestion or targeting CAFs directly, has been carried out in both mouse models and human clinical trials (Froeling et al. 2011; Olive et al. 2009; Hingorani et al. 2016). However, depletion of smooth muscle actin (αSMA)-expressing CAFs (Ozdemir et al. 2014) and long-term pharmacological inhibition of the CAFs that respond to sonic hedgehog ligand (Lee et al. 2014; Rhim et al. 2014) reduced survival in mice and produced highly undifferentiated PDA tumors. These divergent results may reflect the presence of CAF heterogeneity, as evidenced by the expression of differing patterns of fibroblast markers in vivo (Öhlund et al. 2014). Therefore, identifying these different CAF subpopulations, and probing their effects on tumorigenesis, is required for developing more specific CAF-targeted therapeutics.

To address the role of CAFs in PDA, we developed a coculture model allowing direct interactions between organoids and pancreatic stellate cells (PSCs), the resident fibroblast of the pancreas, which can be activated to form CAFs. Upon coculture in Matrigel with tumor-derived organoids, the PSCs acquired an activated phenotype, including cellular extensions in close contact with organoids, and deposited a collagen I–containing ECM organized into collagen fibrils. Coculture enhanced the proliferative rate of both PSCs and organoids, as well as allowed indefinite culture of organoids in growth factor–reduced media. Therefore, this model accurately reflects the desmoplastic stroma of PDA and provides opportunities to study the reciprocal interactions driving cell proliferation of each cell type. Furthermore, CAF heterogeneity was observed in the coculture model, as PSCs expressing high levels of αSMA were found exclusively in contact with organoids (termed myCAFs for myofibroblastic CAFs), whereas PSCs not in direct contact with organoids were αSMA^{low} and secreted high levels of the inflammatory cytokine interleukin 6 (IL6) (termed iCAFs for inflammatory CAFs). These observations were extended to mouse and human PDA, where SMA expression was restricted to fibroblasts in direct proximity to neoplastic cells, whereas fibroblasts expressing IL6 were found far from the neoplastic cells and surrounded by stroma. Although contact between organoids and PSCs was required for myCAF formation, spatial separation of organoids and PCSs through transwell culture increased iCAF formation exclusively. Although these two PSC populations are mutually exclusive in terms of gene expression profiles and secretory activity, they can switch phenotype in response to organoid contact or secreted factors (Öhlund et al. 2017). Therefore, the PDA stroma is more complex than previously appreciated and may inform our understanding of previous experiments disrupting CAF activity and the resultant effects on tumor progression. Importantly, this organoid-PSC coculture model should continue to provide fundamental insights into the molecular and cellular details of the PDA tumor microenvironment and guide us toward new and more fruitful therapeutic avenues for stromal targeting.

CHALLENGES AND OPPORTUNITIES IN PDA RESEARCH

The development of accurate models presents exciting opportunities for making impactful discoveries. The pancreatic organoids have provided insight into the role of Nrf2 in PDA progression and the complexity of the tumor microenvironment, generating hypotheses for new therapeutic interventions. We have adapted the human organoid model as a screening platform for therapeutic discovery, allowing the unbiased identification of drugs or drug combinations that are efficacious in PDA. We have also utilized both mouse and human organoids to identify and validate new tumor-specific biomarkers. Finally, we envision using organoids to study poorly understood facets of cancer cell biology, including the role of cell polarity, noncoding mutations, and perineural invasion.

Many patients diagnosed with PDA are ineligible for surgery because of the presence of advanced metastatic disease. As surgical samples provide the raw material for large-scale sequencing efforts, interrogating the genetics

of late-stage, metastatic PDA has been technically challenging. Furthermore, the low cellularity of PDA requires deep sequencing to ensure detection of even the most common mutations. The organoid model overcomes these challenges in several ways. First, we are able to successfully culture organoids with high reliability (>80%) from endoscopic ultrasound-guided fine-needle biopsies, a diagnostic procedure performed on patients with advanced disease. By growing these samples as organoids, we are able to selectively culture the epithelial component, allowing high-resolution DNA and RNA sequencing at lower coverage. In parallel, we have developed a high-throughput, 384-well-based therapeutics platform to determine the sensitivity of an individual patient's organoids to standard of care chemotherapy, FDA-approved investigational agents, or drugs targeting proteins of interest. The primary goal of such an approach is to identify the most active drug or combination of drugs that can be applied directly to the patient. Combining the genotype, gene expression, and therapeutic sensitivity information, we hope to develop algorithms for rapidly choosing the optimal course of action for future patients. This approach to precision medicine necessitates the generation and characterization of a large-scale, living biobank of PDA samples, an undertaking currently in progress.

Among the reasons that PDA is often diagnosed at a late stage is the absence of specific biomarkers of disease. The existing PDA biomarker, the carbohydrate antigen CA-19-9, cannot be used as a screening tool because of high false-positive and false-negative rates (Swords et al. 2016). Furthermore, CA-19-9 can be elevated in the absence of cancer, including in patients with pancreatitis. Current approaches to identifying novel biomarkers are hampered by their inability to compare cell surface markers or secreted factors from neoplastic cells versus those from the normal ductal epithelium. The organoid system overcomes this "lack of the best control" complication by allowing direct comparison between panels of normal or tumor organoids from individual patients. Accordingly, we have used mass spectrometric approaches to identify differentially modified protein carriers of CA-19-9 that may improve the specificity of this biomarker, and our preliminary results are encouraging. The ability to culture and manipulate normal human pancreatic ductal cells has been critical to these endeavors.

Finally, another hallmark of PDA is perineural invasion (PNI), the process by which tumor cells migrate along the surface of nerves and invade the neuronal sheath (Amit et al. 2016). The presence of PNI correlates with poor prognosis and is associated with extreme pain. Intriguingly, genes regulating axon guidance are frequently mutated in PDA, providing a possible cell-intrinsic mechanism for this process (Biankin et al. 2012). It has been well-established that tumor cells can promote neural sprouting and Schwann cell recruitment (Demir et al. 2016), and that neuron and Schwann cell–derived signals can regulate tumor cell invasion (Liang et al. 2016). However, models faithfully recapitulating neuronal–epithelial interactions are required for understanding the mechanisms driving PNI and for screening compounds that can inhibit this process. Existing models for neural invasion rely on cancer cells grown in 2D or cell suspension or utilize nonphysiological neural tissue. Therefore, we propose to develop a coculture model containing tumor organoids and nerves from the celiac ganglia that innervate the pancreas. Such a model would allow interrogation of the signals regulating PNI in PDA and directly test the hypothesis that the axon guidance pathway mediates this process.

CONCLUSION

PDA is a highly lethal malignancy, with numerous outstanding challenges standing in the way of improved outcomes for patients. First, more specific biomarkers are needed for detecting disease at an early stage where surgical intervention is possible. Second, a deeper understanding of the complexities in the PDA tumor microenvironment is required if we hope to target only those interactions driving tumor progression. Third, we must overcome the difficulties in drug delivery, stemming from an abnormal vasculature, as well as determine optimal drug combinations and dosing strategies. Fourth, we need to develop a means to effectively target the oncogene KRas, either directly, or indirectly, or through disruption of downstream signaling pathways, or synthetic lethal interactions. Finally, it is imperative to identify additional molecular events that promote PDA progression and metastasis, as these logically serve as ideal therapeutic targets. We have sought to address these challenges through the development and utilization of in vivo and in vitro models that reflect the complex genetic and pathophysiological characteristics of human PDA. These models present new opportunities for a deep molecular understanding of PDA and rapid translation of findings into the clinic for patient benefit.

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