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Resolving the HIF Paradox in Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is currently the third leading cause of cancer-related deaths and has a 5-year survival rate of less than 10%, far below the ~70% national average for all cancers. This poor prognosis is driven by an extreme resistance to nearly all known cancer treatments, which has long been attributed to hypoxia driven interactions between tumor cells and the supporting stromal microenvironment. The cellular response to hypoxia is driven by the transcription factors known as the hypoxia inducible factors (HIFs), which have been hypothesized to play a role in the pathobiology of PDAC as well as a potential therapeutic target based on years of cell culture data. Attempts to validate the oncogenic role of HIF in PDAC through rigorous spontaneous tumor models have paradoxically shown that the HIFs may act as a tumor suppressor in epithelial cells. Here, we seek to resolve this paradox by discussing the roles of HIFs both in cancer cells and the supporting microenvironment and place them into context of current model systems that could be used to interrogate these interactions. We suggest that HIF may exert its oncogenic influences by modulating the form and function of the stroma rather than direct effects on cancer cells.

Keywords

Stroma; Tumor microenvironment; Hypoxia; CAFs; TAMs

Introduction

Oxygen is a highly reactive element that plays a vital role in all eukaryotic life. As such, our cellular systems have evolved various mechanisms to adapt to changing oxygen levels. The atmosphere of Earth contains ~21% oxygen, however most cellular tissues only encounter oxygen levels of 3–13% [1,2]. To deal with the constant fluctuations in oxygen, [3] our cells have evolved a conserved program driven by the hypoxia inducible factor (HIF) family of transcription factors. In oxygenated environments, EGLN prolyl hydroxylases are responsible for hydroxylating the highly conserved two prolyl residues on HIF to create a

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binding site for the Von Hippel-Lindau (VHL) protein. The VHL E3 ubiquitin ligase complex then degrades HIF via the proteasome. During hypoxia, however, EGLN proteins no longer have sufficient oxygen to drive HIF hydroxylation, which results in the rapid stabilization of the HIF alpha subunit, which is then free to bind to HIF1β/ARNT. Together, this heterodimer binds to cognate hypoxia response elements (HREs) within enhancer elements to increase the transcription of its target genes. The ultimate results of activation of these transcriptional networks are a wide range of biological processes associated with cell survival in a low oxygen environment including embryogenesis, angiogenesis, and erythropoiesis. Additionally, hypoxic cells are known to shift energy production toward glycolysis and away from oxidative metabolism through the induction of mitophagy to reduce mitochondrial numbers [4–6]. The HIF program can even promote survival during tremendous stresses such as radiation damage [7].

The tumor microenvironment is a dynamic milieu consisting of not only the malignant tumor cells themselves, but also blood vessels, immune cells, fibroblast, adipocytes, and extracellular matrix [8]. Interestingly, the complex nature of interactions that shape the microenvironment can both promote [9,10] and antagonize [11] tumor growth. Despite the wide presence of endothelial cells, tumors often lose the normal tissue architecture that supports oxygenation, which leads to pockets of hypoxia throughout the tumor. Although adaptation to hypoxia is important to normal tissues, many have postulated that low oxygen levels also contribute to cancer growth, survival, and treatment resistance. Pancreatic ductal adenocarcinoma (PDAC) has one of the worst survival rates of all cancers driven by a poor response to most cancer treatments. We hypothesis that the hypoxic tumor microenvironment plays a large role in maintaining therapeutic resistance. Surprisingly HIF can play a tumor suppressive role in PDAC. Therefore, in this review, we seek to resolve this paradox by focusing on the current literature addressing the role of HIF in pancreatic cancer, as well as potential strategies to address gaps in knowledge. We believe that a better understanding of the role of hypoxia in PDAC will help guide important therapeutic approaches.

Hypoxia is Central to the Pathobiology of Pancreatic Cancer

PDAC is known for its desmoplastic stroma composed of activated stellate cells that express a large amount of extracellular matrix (ECM) and alpha-smooth muscle actin (αSMA) [12]. Traditionally, the desmoplastic stroma is known to increase intratumoral pressure [13–15] and acts as a barrier to impede the delivery of therapeutic agents such as chemotherapy and radiation as well as nutrients, and oxygen. These effects are seen readily on triphasic CT scans of pancreatic tumors, which have a pathognomonic feature of hypoenhancement relative to the nearby normal parenchyma [16]. Direct measurement of the oxygen tension within tumors have consistently shown that pancreatic cancer is among the most hypoxic of known tumors [1,17,18]. This intratumoral hypoxia leads to therapeutic resistance against chemotherapy and radiotherapy, which often depend on oxygen for full cytotoxic effects [19].

However, the lack of chemical oxygen also provokes a biological response through the stabilization of the HIF proteins. As reviewed elsewhere, the EGLN family of prolyl

hydroxylases are inhibited in low oxygen, preventing hydroxylation of the HIF alpha subunit and its subsequent degradation by proteasomes [20]. HIF1α and HIF2α are the two major isoforms in mammalian cells and are the primary drivers of hypoxia response. HIF1 is expressed ubiquitously and responds rapidly to changes in oxygen levels [21]. HIF2, on the other hand is expressed in select cell types including vascular endothelial, lung type II pneumocytes, liver parenchyma, interstitial cells in the kidney, and stem cells [22–27] and may be stabilized at higher concentrations of oxygen [7]. When HIF is expressed at high levels in tumors, many cancer cells become more resistant to chemotherapy [28,29] and radiation [30–32] and may exhibit enhanced proliferation and migration [33–36].

The role of HIF in pancreatic cancer remains unclear. The specific deletion of either HIF1α [37] or HIF2α in the pancreatic epithelia accelerated PDAC formation [38], suggesting a potential tumor suppressive role. Moreover the direct overexpression of HIF2 suppressed PDAC growth and improved survival [39]. It is important to note that the previously mentioned studies involved manipulation of HIF expression in only the epithelium. This is in contrast to the physiological response of HIFs which would occur in most if not all cell types in the tumor. The tumor suppressive role of HIF is not isolated to PDAC, but is further supported by preclinical models of lung cancer [40–42] and sarcoma [43] which also exhibited a growth suppressive phenotype for HIF. We note, however, that one tumor where HIF is oncogenic is clear cell renal carcinoma (ccRCC), where HIF2 expression enabled by VHL-loss drives changes in growth and fatty acid metabolism [44,45]. Specific inhibitors to HIF2α are now progressing through clinical trials to treat ccRCC [46,47], but whether HIF2 inhibition might be beneficial in other cancers is not known but is an area of active investigation.

Resolving the Paradox HIF in PDAC Microenvironment

The tumor suppressive role of HIF in preclinical pancreatic cancer models is at odds with studies correlating the poor outcomes in PDAC with its hypoxic nature. Although several possible explanations exist, including heterogeneity of hypoxia within the tumor [17,18,48] and physiologic differences between mouse and man [49], perhaps the most striking possibility may be that previous studies have not accounted for the direct actions of HIF signaling within the tumor microenvironment. The tumor microenvironment is now appreciated to play as critical a role in oncogenesis as the individual driver mutations [9,10]. Unfortunately, most studies have focused exclusively on HIF within cancer cells and have ignored the possibility that HIF might be acting through stromal components, like the cancer associated fibroblasts or immune cells.

A pancreatic tumor contains only a small fraction of adenocarcinoma cells [50]. The major cellular component of PDAC is actually the cancer-associated fibroblast (CAF), which may account for up to 80% of the tumor volume [51,52]. There is significant debate about the roles and subtypes of CAFs in pancreatic cancer. Recent work using single cell methodology has revealed that many different subtypes of fibroblasts promote tumor progression [53], some which express inflammatory cytokines [54] and some that provide metabolic support through delivery of non-essential amino acids [55] or lipids [56]. Hypoxia metabolically reprograms CAFs to take on a more catabolic phenotype [57] and promotes the secretion of

pro and anti-angiogenic factors [58]. In regards to secretion, it is noteworthy that hypoxia induces lipid remodeling [59] and free cholesterol accumulation [60]. The importance of this is highlighted by recent work linking levels of cholesterol in the tumor microenvironment with antitumor activity [61]. Therefore, we speculate that the hypoxic tumor microenvironment induces the excretion of critical lipids from CAFs which contributes to therapeutic resistance by attenuating the host antitumor immune response.

Besides cancer cells and CAFs, pancreatic tumors possess microvessels lined by endothelial cells, which grow in response to vascular endothelial growth factor (VEGF), a known HIF target. HIF1α and HIF2α may have complementary functions in physiological and pathological angiogenesis. For instance, the loss of HIF1α in endothelial cells inhibits blood vessel growth and tumor size by decreasing VEGF expression [62], whereas endothelial HIF2α null mice have reduced capacity for metastases [63,64].

The emergence of immunotherapy to stimulate a patient's immune system to destroy a solid tumor has transformed modern cancer care [65]. The ability for T-effector lymphocytes to infiltrate and recognize a tumor are critical for immunotherapy to work [66,67]. Even if an active CD8+ lymphocyte can enter a tumor, there may not be sufficient antigen quantity or quality to induce an immune response [68–70]. Moreover, nearby cells can reduce the activity of effector lymphocytes, such as immunosuppressive regulatory T (Treg) cells [71] and tumor-associated macrophages (TAMs) [72]. Interestingly, high levels of HIF2α was observed in some populations of TAMs, even in tumors that lack expression of HIF2 [73].

The function of these immune effectors in hypoxic tumors appears to be context dependent. For instance, hypoxia both limited cytotoxic T-cell infiltration [74] and enhances Treg infiltration [75] but may also inhibit Treg differentiation [76]. Stabilized HIF expression by knockout of the EGLN proteins was recently shown to reduce tumor colonization in the lung [77]. In macrophage populations, however, HIF1 enhances PD-L1 expression [78] while HIF2α knockout reduces TAM infiltration in different carcinoma models, which is associated with reduced tumor cell proliferation and progression [79].

Modeling the Hypoxic Interplay Between Cancer Cells and Stroma

One common method to study stromal effects are *ex vivo* systems using co-culture. These systems are relatively easy to use and have provided the framework for much of our current understanding of tumor-stroma crosstalk [80,81]. There are several ways to preserve and culture stromal tissue *ex vivo*, each having its own advantages and disadvantages. Enzymatic and/or mechanical methods can be used to dissociate stromal samples, which can subsequently be cultured in either a 2D monolayer or in a 3D spheroid culture [82]. 3D spheroid models of hypoxia exhibit distinct signaling patterns and drug response that can help better predict responses *in vivo* [83–85]. The organotypic matrix invasion assay is another mixed cell 3D model which generates a reproducible hypoxia gradient that is compatible with various immunohistochemical and fluorescence techniques [17], including the commonly used hypoxia marker pimonidazole [86]. To further resemble the *in vivo* condition as closely as possible, dissected samples can alternatively be implanted in immunodeficient mice to generate patient-derived xenograft models [82]. In order to keep

tissue architecture intact, organotypic tissue slices can also be generated by careful sectioning of the specimen [82]. Additionally, in vitro “tumor/organ-on-a-chip” models rely on complex fluidics and engineered scaffolds [87,88] in order to replicate some of the complex interactions that occur within the tumor-stromal environment. The advantage of these microfluidic devices is that they are amendable to advanced microscopy techniques [88].

Models that utilize immunocompetent mice exploit tumors that are syngeneic to a particular host, such as KPC or LLC cells derived from C57BL/6 mice [89] or 4T1 cells derived from BALB/c strains. KPC mice contain a conditionally activated *KRas* allele (G12D) and a heterozygous *Trp53* loss-of-function allele, driven by a pancreas- specific Cre (Ptf1a-Cre) [90]. To study the effects of the stroma, germline mutations can be induced to create null or hypomorphic expression of a particular protein in every tissue of the mouse. This is possible when the gene of interest does not cause embryonic or perinatal lethality, but unfortunately, the major genes in the hypoxia response—HIF1 [91], HIF2 [92] and EGLN1 [93] do not develop to adulthood, which limits this approach.

These limitations may be overcome with the use of a dual recombinase system that enables the independent manipulation of the tumor genetics and the stroma. We and others [94] have developed a system that that initiates pancreatic tumorigenesis in a Flp-dependent fashion using a Pdx1-Flp along with a coordinated activation of Kras (G12D) oncogene (FSF-Kras) and a p53 allele ($Trp53^{FRT/FRT}$) in a Flp-dependent fashion. We can also breed in floxed alleles of the EGLN proteins, HIF proteins, or other key regulators in order to knock out these genes in specific cell types without also deleting these same genes in the tumor. This allows complete spatial and temporal control of gene deletion in the stroma or tumor tissues. We believe that his powerful system could be used to interrogate each stromal component in a definitive fashion (Figure 1).

Conclusion and Future Directions

The function of HIF on the interplay between tumor and its stroma may be a key to unraveling the complex effects of hypoxia on cancer progression and treatment resistance. These data are of critical importance not only from a basic biology standpoint, but also because we now have the capability to target the function of the HIF transcription factors. For instance, the small molecule inhibitor PT2399 specifically binds to the HIF2α PAS B domain and causes tumor regression in ccRCC [47], which is depends on oncogenic expression of HIF2. It is unknown whether PT2399 or its derivatives may be useful in other cancer types that do not have canonical dependencies on HIF2. Similarly, the established tumor suppressive effects of epithelial HIF in PDAC suggest that selective targeting of stroma, or even specific stromal cell types is required for small molecule inhibition of HIF to induce tumor regression. Moreover, there still leaves the question of if HIF1 expression in CAFs have an effect on stromal formation or treatment resistance. The answers to these questions will provide essential insight into the role of HIF within the tumor microenvironment and should be fully understood before pursuing other HIF antagonists for the clinic.

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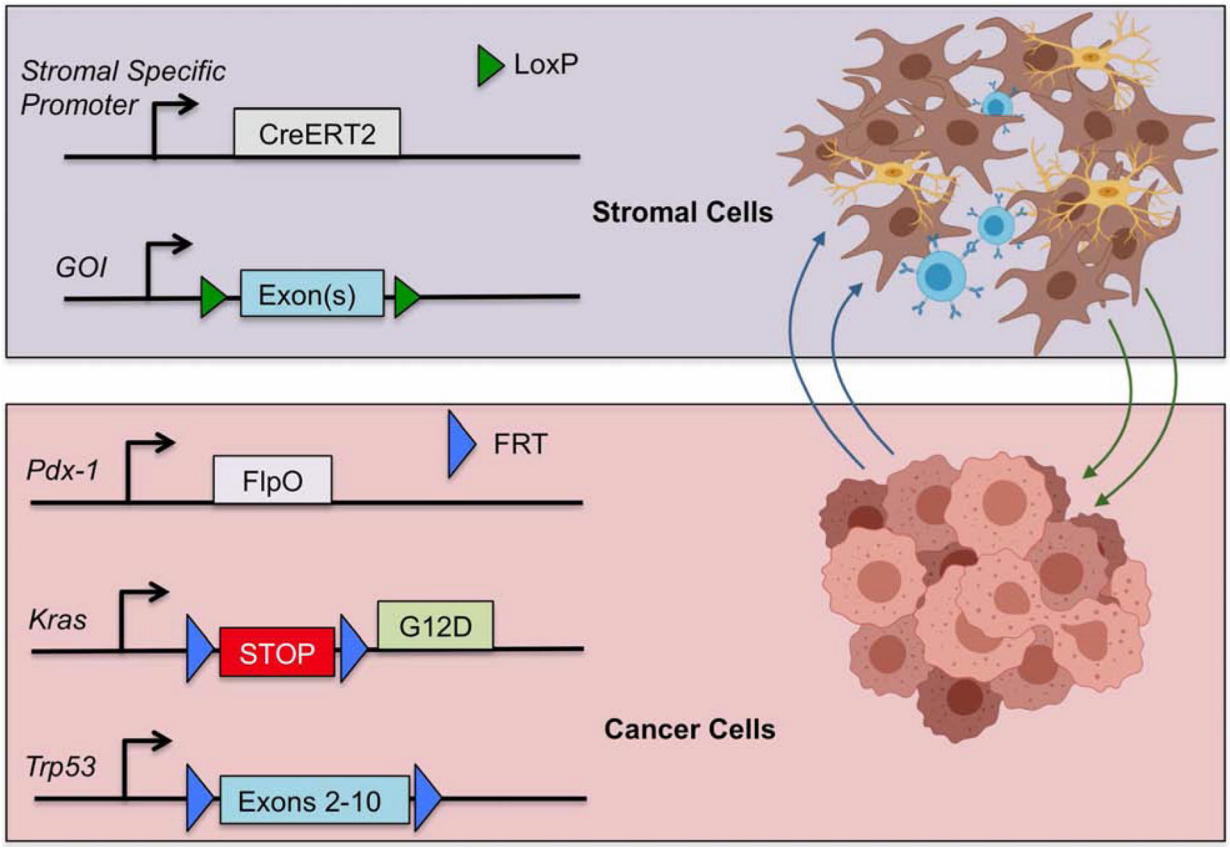

Figure 1. Dual recombinase system for pancreatic cancer.

Flp recombinase directs tumorigenesis in the target tissue by a tissue-specific Flp (in this case, Pdx1-FlpO), which activates *Kras* and knocks out *Trp53*. This leaves stromal components available to be regulated by the Cre/lox system where a gene of interest (GOI) can be manipulated as desired with a tissue-specific Cre.