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Heparin-Binding Epidermal Growth Factor-like growth factor eliminates constraints on activated Kras to promote rapid onset of pancreatic neoplasia

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Abstract

Pancreatic cancer remains one of the most deadly cancers with few treatment options at late stages and little information about how it develops through earlier stages. Activating mutation of the Kras gene has been implicated in, but is not sufficient for, tumorigenesis. In mouse models of pancreatic cancer, loss of tumor suppressor genes in conjunction with Kras mutation leads to gradual stochastic acquisition of neoplastic precursors and carcinomas, while many cells remain phenotypically unaltered in younger mice. Here, we demonstrate that two oncogenic events, mutation of Kras and production of the growth factor heparin-binding epidermal growth factor-like growth factor (HB-EGF), are sufficient for rapid and complete neoplastic transformation of the exocrine pancreas. We found that macrophages are the major source of HB-EGF production in pancreatic cancer tissue samples and that macrophages are present in high density and in close association with human pancreatic cancer lesions. In a mouse model, high macrophage density was observed at the earliest stages of neoplastic transformation. The consequence of elevated HB-EGF signaling was investigated without the confounding effects of other macrophage-produced factors via transgenic overexpression of the active form of HB-EGF. In this model, HB-EGF was sufficient to promote Kras-initiated tumorigenesis, inducing rapid and complete neoplastic transformation of the entire exocrine pancreas shortly after birth. HB-EGF overexpression and $Kras^{G12D}$ together, but neither alone, increased proliferation with increased CyclinD1 and

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decreased Cdkn2a/2d (p16/p19$^{Ink4A/Arf}$). These findings establish the importance of oncogenic synergy in cancer initiation and promotion and establish a molecular link between inflammation and the earliest stages of tumor induction.

Keywords

pancreatic ductal adenocarcinoma; inflammation; transdifferentiation; EGFR

Introduction

The early stages of pancreatic cancer are rarely seen in human patients, providing little information on how this deadly cancer originates. Development of robust models for the early stages of pancreatic cancer is needed to identify biomarkers for earlier diagnosis and to understand regulatory pathways for targeted therapies. In general, cancer is thought to arise from a series of genetic and epigenetic changes that confer oncogenic properties on a cell and its descendants. These changes typically include acquisition of oncogene expression and loss of tumor suppressor genes. Kras is one such protooncogene that when mutated can lead to pancreatic cancer. Kras mutations are found in 90–95% of pancreatic ductal adenocarcinomas (PDAC) (1, 2), the most common pancreatic cancer in humans (3). However, Kras mutations are also found in normal pancreas where they accumulate over a lifetime without leading to cancer in most instances (4). Thus, while Kras is a powerful oncogene, cells normally constrain its activity and other event(s) are required to release its oncogenic potential. Most cases of pancreatic cancer arise spontaneously suggesting somatic mutations and/or damage are required to promote Kras-initiated tumorigenesis. Damage in the form of chronic pancreatitis is a major risk factor, increasing the risk of pancreatic cancer by 4- to 19-fold (5).

The little we know about early stages of human pancreatic cancer comes from benign epithelial lesions that are often found in close proximity to PDAC lesions (6). Mouse models have supported the hypothesis that these lesions, called PanINs (Pancreatic Intraepithelial Neoplasms) in humans or mPanINs in mice, do indeed give rise to PDAC (7–9). Mice expressing mutant Kras in the pancreas, such as *Ptf1a*^{Cre}; *LSL-Kras*^{G12D} mice (8), gradually develop PanINs over weeks or months and progress to PDAC at a low frequency with long latency. Unlike some other tissues (10), presence of an activating Kras mutation does not immediately lead to hyperplasia or neoplasia. This suggests that 1) cells require a period of time for mutant Kras to induce neoplasia, 2) cells must acquire further mutations for mutant Kras to initiate tumorigenesis, or 3) some other event, perhaps in the cellular microenvironment, induces mutant Kras to initiate hyperplasia/neoplasia. In the Kras-driven mouse model, loss of various tumor suppressor genes led to earlier and more frequent development of pancreatic cancer (7, 9, 11–13) but precancerous lesions in these mice still did not arise immediately. We now report that cells of the microenvironment can provide signal(s) that allow mutant Kras to reach its full neoplastic potential. Extracellular signaling pathways often provide tumor-promoting effects, such as regulation of proliferation or survival. The epidermal growth factor receptor (EGFR) and many of its ligands (14–16) are overexpressed in PDAC, creating a constantly activated signaling pathway that may

contribute to tumor development (17). We show here that one EGFR ligand, heparin-binding epidermal growth factor-like growth factor (HB-EGF) is produced by macrophages that are abundant in both human and mouse cases of PDAC (14, 18–20). To understand the role of HB-EGF in tumor initiation, promotion, and progression without confounding effects of other factors produced by macrophages, we used a transgene to express the active form of HB-EGF, sHB-EGF, in pancreatic epithelium, thus abrogating the need for macrophage infiltration (21). Unlike results from tumor suppressor studies, we found that this growth factor in conjunction with Kras mutation is sufficient to induce pancreatic cancer precursors as soon as the pancreas reaches maturity in the early neonatal period.

Results

Macrophages are the primary source of HB-EGF in human pancreatitis and pancreatic cancer

Hbegf mRNA was detected in lysates from human pancreatic cancer tissue (14). However, by immunohistochemical staining of tissue sections from ten PDAC patients, we found that the predominant source of HB-EGF protein was not in tumor epithelium but in cells scattered through the stroma in all cases examined. Based on the morphology of HB-EGF-expressing cells, we performed co-immunofluorescence for HB-EGF and CD68, a marker of macrophages (Figure 1A–C). Virtually all cells expressing detectable levels of HB-EGF protein were positive for CD68, indicating that cancer-associated macrophages expressed HB-EGF. Macrophages present in chronic pancreatitis also expressed HB-EGF (Figure 1D–F). The number of macrophages present in human chronic pancreatitis and in pancreatic cancer tissues was elevated 5-to >20-fold (Figure 1G) consistent with findings in mouse models (19). Many PanINs and PDAC lesions had macrophages concentrated in close proximity to their basal surfaces (Figure 2A), effectively increasing the amount of HB-EGF produced near these lesions. The clustering of macrophages near ductal lesions was less pronounced in chronic pancreatitis (Figure 2B).

Examining a mouse model of the premalignant stages of PDAC, the $Ptf1a^{Cre}$; $LSL\text{-}Kras^{G12D}$ genetic model in which mutant Kras is expressed throughout the pancreatic epithelium, we also found that macrophages were dramatically elevated as reported (19) and these macrophages also produced HB-EGF (Supplemental Figure S1) Looking at early stages of mPanIN formation in the mouse, dense clusters of macrophages could be found in areas of acinar-ductal metaplasia in which acinar cells appear to be undergoing transition to ductal cells, thought to be one of the earliest events associated with pancreatic neoplasia. This suggests that macrophages, perhaps through HB-EGF expression, may regulate the earliest steps in pancreatic cancer development.

$Kras^{G12D}$ and sHB-EGF induce complete neoplastic transformation of the pancreas

Macrophages produce many cytokines and growth factors. To isolate the effects of HB-EGF specifically, we used transgene expression to produce sHB-EGF in the pancreas without the necessity of macrophage infiltration (21). We then combined sHB-EGF overexpression with Kras mutation and looked for effects on tumor promotion and/or progression in Kras-initiated epithelium.

Mice with the three alleles needed for $Kras^{G12D}$ and sHB-EGF overexpression (henceforth $Kras^{G12D}$; sHB-EGF mice) were born at normal Mendelian frequency even though both genes were expressed beginning in embryonic development (8, 21). At 7 days of age, there was no significant difference in appearance or weight between $Kras^{G12D}$; sHB-EGF mice and normal mice (3.87+/−0.29 g vs. 4.05+/−0.34 g body weight, $p = 0.74$). By two weeks of age, however, most $Kras^{G12D}$; sHB-EGF pups appeared lethargic and were 33% smaller than normal littermates (4.86 +/−0.29 g vs. 6.67+/−0.23 g, p=0.005). Due to this weight loss, mice were euthanized no later than 2 weeks of age.

As reported previously, $Kras^{G12D}$ alone did not cause any pancreatic abnormalities until after birth (8) and only rare metaplasias by 14 days after birth (Supplemental Figure S2). sHB-EGF overexpression alone had little effect on pancreatic epithelium until after birth although an increase in pancreatic mesenchyme from embryonic stages through the postnatal period was observed as reported (21). Between postnatal days 7 and 14, overexpression of sHB-EGF alone led to a small number of lesions with the appearance of acinar-ductal metaplasia (Supplemental Figure S2) while the majority of the pancreatic epithelium was normal.

In striking contrast to either gene alone, the combination of $Kras^{G12D}$ and sHB-EGF together dramatically altered the composition of the pancreas shortly after birth (Figure 3). In the rodent pancreas, the exocrine pancreas first reaches maturity 1–2 days after birth (22). It was only after reaching this benchmark of maturity that synergy was observed between $Kras^{G12D}$ and sHB-EGF. One day after birth, $Kras^{G12D}$; sHB-EGF pancreas was similar to sHB-EGF alone with increased mesenchyme but with epithelium that was largely normal although with sporadic dilation of acinar and ductal lumena seen to a lesser extent in sHB-EGF mice (Supplemental Figure S3). However, by day 3 after birth, few structural acini remained in $Kras^{G12D}$; sHB-EGF mice and those remaining had reduced apical cytoplasm. Both ductal and acinar lumena were greatly dilated (Figure 3B and data not shown). By postnatal day 7 and continuing to day 14, no structural acini remained and the entire pancreatic epithelium consisted of abnormal ducts (Figure 3C, D), in striking contrast to sHB-EGF overexpression or $Kras^{G12D}$ expression alone (Figure 3E,F). Consistent with this ductal morphology, most cells expressed CK19, a marker of pancreatic ducts, and only a few cells expressed amylase, an acinar marker (Figure 4). Figure 4C showing the highest concentration of remaining acinar cells while other regions had fewer or no amylase-positive cells. Thus, in the presence of sustained HB-EGF release, $Kras^{G12D}$ was able to transform the entire exocrine pancreatic epithelium rapidly and completely.

Histologically, many ducts observed in $Kras^{G12D}$; sHB-EGF mice at postnatal day 7 were comparable to early PanIN lesions, with columnar morphology and mucin production, (Supplemental Figure S4A). By day 14, the percent of ducts containing mucin-positive cells increased to 93.1+/−0.7% (Supplemental Figure S4B). Normal pancreas controls never had mucinous ducts (data not shown). By day 14, most ductal lesions were PanIN-like with many having characteristics of more advanced mPanINs, including papillary architecture, loss of polarity, cellular atypia, and lumenal budding in ducts throughout each pancreas characteristic of PanIN3, which corresponds to carcinoma in situ (Figure 5).

Because some ductal lesions in Kras^{G12D}; sHB-EGF mice had characteristics of PanIN3 by postnatal day 14, we investigated whether they also progressed to invasive carcinoma. In three of four mice, we found evidence suggestive of early invasion characterized by single CK19+ cells surrounded by stroma and located near ductal lesions with early invasive morphology (Figure 6 and Supplemental Figure S5). These events, while rare, suggested that sustained signaling by sHB-EGF may allow oncogenic Kras to induce mPanIN lesions with the ability to become invasive PDAC.

Our finding that sHB-EGF is a potent promoter of Kras^{G12D}-initiated pancreatic cancer prompted us to ask if sHB-EGF also promotes transformation of adult pancreatic cells. We isolated acinar-enriched pancreatic cells from two month old wildtype or Kras^{G12D} mice and cultured them in a three dimensional collagen matrix, with or without recombinant HB-EGF. After four days in culture, wildtype cells treated with vehicle maintained many cells of acinar morphology (Figure 7A) but transdifferentiated into ductal cysts with a flat cuboidal to simple squamous morphology in the presence of HB-EGF (Figure 7B (23)). Cells expressing Kras^{G12D} transdifferentiated into ductal cysts without HB-EGF treatment (Figure 7C). When Kras^{G12D}-expressing cells were treated with HB-EGF, cells also transdifferentiated into ductal cysts, but approx. one-third of cysts had altered cellular morphology with increased apical and/or basal cytoplasm compared to wildtype + HB-EGF or vehicle-treated Kras^{G12D} cells (Figure 7D). Kras^{G12D} cells, with or without HB-EGF treatment degraded and severely contracted the collagen matrix such that cultures could not be followed for longer periods of time (data not shown).

Mechanisms of sHB-EGF and Kras^{G12D} synergy

We found that sHB-EGF/Kras^{G12D} did not act via inducing an inflammatory response. On postnatal day 1, immediately prior to onset of the neoplastic phenotype, there were few CD3+ T cells or CD45R(B220)+ B cells in normal, Kras^{G12D}, sHB-EGF, or Kras^{G12D}; sHB-EGF pancreata with no apparent change with genotype (Supplemental Figure S6). Macrophages comprised ~5% of total pancreatic cells in normal day 1 pancreas and this percentage did not significantly change with Kras^{G12D} and/or sHB-EGF expression (Supplemental Figure S7A–D, H). However, macrophage numbers increased in Kras^{G12D}; sHB-EGF pancreata between days 3 and 7 consistent with a role of macrophages in later stages of tumorigenesis (Supplemental Figure S7E–G). In all genotypes, macrophages had lower levels of iNOS protein than did intestinal macrophages and high levels of Arginase I, suggesting they were alternatively activated macrophages (24) (Supplemental Figures S8 and S9 and data not shown). We also found that expression of sHB-EGF via this pancreas-specific Pdx1 promoter did not affect HB-EGF protein levels in macrophages (Supplemental Figure S10).

To understand the molecular events underlying the rapid and complete transformation of pancreatic exocrine epithelium by Kras^{G12D} and sHB-EGF, we examined the ability of these genes to induce cell cycle entry at postnatal day 1, immediately before the extensive transition from acinar to ductal structures. At this point, acinar cells have a low rate of proliferation while ducts and centroacinar cells are still rapidly proliferating. We measured the percent of acinar cells expressing cyclinD1 as a marker for transition from G_0/G_1 into

G_1/S phase of the cell cycle and phosphorylated histone H3 as a marker of M phase cells. Overexpression of sHB-EGF increased the number of cyclinD1-positive acinar cells two-fold while $Kras^{G12D}$ had no effect on cyclinD1 expression (Figure 8A). Although sHB-EGF alone induced cyclinD1 expression, it did not increase the number of acinar cells completing the cell cycle. The percent of mitotic acinar cells in sHB-EGF alone or $Kras^{G12D}$ alone pancreas was similar to that of normal mice. However, sHB-EGF and $Kras^{G12D}$ together doubled the number of acinar cells entering M phase and thus completing the cell cycle (Figure 8B and Supplemental Figure S11). This increase in cell cycle progression was likely due to a decrease in expression of two cell cycle inhibitors. In the background of sHB-EGF overexpression, $Kras^{G12D}$ decreased both Cdkn2a ($p16^{Ink4A}$) and Cdkn2d ($p19^{Arf}$) levels 2- to 3-fold while having little effect on expression of Cdkn1a ($p21^{Cip}$) or Cdkn1b ($p27^{Kip}$) (Figure 8C). While $Kras^{G12D}$ and sHB-EGF synergistically increased acinar proliferation, they had no effect on apoptosis as measured by immunohistochemistry for cleaved caspase 3 (data not shown).

Because HB-EGF and Kras are in the same signaling pathway, upstream and downstream of EGFR/ErbB4 receptors, respectively, the mechanism of their carcinogenic synergy was not apparent. Therefore, we used an unbiased analysis of multiple signaling components via antibody arrays that recognize different phosphorylated forms of proteins involved in signal transduction. Protein lysates were made from whole pancreas one day after birth, immediately before morphological changes were apparent. $Kras^{G12D}$ expression alone did not induce any phosphorylation changes greater than 2-fold compared to wildtype (Supplemental Table S1), consistent with a lack of phenotype from Kras mutation alone. Overexpression of sHB-EGF alone induced phosphorylation of many pathway components compared to wildtype. However, these data are difficult to interpret because sHB-EGF induces an increase in pancreatic mesenchyme, so some changes may be due to difference in cellular composition of the tissue. This was not a factor when comparing sHB-EGF alone to sHB-EGF plus $Kras^{G12D}$ because cellular composition is similar in these two genotypes. Comparing sHB-EGF alone to $Kras^{G12D}$; sHB-EGF, only four proteins had increased phosphorylation greater than 2-fold. These included p70 S6 kinase and one of its targets, the activation loop of GSK3α/β (Figure 7D). sHB-EGF alone produced high levels of phosphorylation of Erk1/2, Mek1/2 and AKT that was not altered by additional mutation of Kras (Figure 8D, top row).

To further investigate how sHB-EGF/$Kras^{G12D}$ synergy may occur, we analyzed whether sHB-EGF overexpression affected the level of Kras protein. Overexpression of mutant Kras via transgene was shown to be much more oncogenic than endogenous levels of $Kras^{G12D}$ expression (25). We found that sHB-EGF overexpression increased the amount of Kras protein in the pancreas, which could facilitate the rapid transformation observed (Figure 8E).

Discussion

HB-EGF signaling promotes Kras-driven oncogenesis

We have shown that two genes in combination, but neither alone, are sufficient to initiate and promote the early stages of pancreatic tumorigenesis in the mouse. Transformation occurred rapidly and completely within days after cells reached functional maturity. This

striking phenotype reflects results typically seen in cultured cells. Early studies in chicken and rodent cells demonstrated that two oncogenes such as v-ErbA and v-ErbB (a viral EGFR homolog) (26) or v-Hras (a viral Ras homolog) and c-myc (27) were necessary and sufficient to transform cells. However, when these and other oncogenes were tested in mice, they were not sufficient to induce immediate and widespread tumors. Rather, tumors arose stochastically as mice aged, indicating that further somatic events were required for tumorigenesis in vivo (27, 28). Our results suggest that some combinations of specific oncogenes are sufficient to induce transformation in specific tissues such as the pancreas. Individually, either Kras mutation or overexpression of sHB-EGF led to gradual accumulation of benign lesions and, with long latency, PDAC in $Kras^{G12D}$ mice (8, 21) and acinar carcinomas in approx. 15% of sHB-EGF mice over a year in age (data not shown), suggesting that either alone can initiate but not promote tumorigenesis. Together, sHB-EGF was a potent promoter of $Kras^{G12D}$-initiated PanIN formation such that the entire exocrine pancreas adopted a transformed phenotype. Several downstream effectors were associated with KrasG12D/sHB-EGF synergy: elevation of Kras protein level; phosphorylation of p70S6K and one if its targets; and downregulation of Cdkn2a/2d ($p16/p19^{Ink4A/Arf}$).

Numerous tumor suppressors including $p16/p19^{Ink4A/Arf}$, tp53, Smad4/DPP4, and TGFβ Receptor type II, have been examined in the context of $Kras^{G12D}$ expression in mouse models (7, 9, 12, 13, 29). Even though these genes were mutated embryonically, young mice retained a phenotypically normal pancreas and tumors only arose stochastically as mice aged. However, once formed, these tumors progressed much more rapidly than tumors arising from Kras mutation alone. These studies suggest either that pancreatic cells of young mice are not competent to respond to tumor inducers, that pancreatic cells are intrinsically capable of responding but require a long period of time for phenotypic changes, or that other intrinsic (e.g., mutations) or extrinsic (e.g. signals from the microenvironment) events are required for tumor induction. Our work demonstrates that pancreatic cells of young mice are competent to respond to at least some inducers of tumorigenesis and that this response can occur in a matter of days rather than weeks or months. We suggest that pancreatic cancer varies from the classic paradigm of tumorigenesis seen in colon cancer, in which loss of tumor suppressor activity is a necessary initiating event (30). Rather, in the pancreas, two oncogenic events, such as mutation of Kras and activity of an EGFR ligand, may be required to initiate and promote cancer precursors, with loss of tumor suppressor activity functioning in tumor progression. Inflammation has been shown to promote $Kras^{G12D}$-initiated tumorigenesis (31, 32). Our results suggest a major mechanism of inflammation-induced tumor promotion is elevated production of secreted HB-EGF by infiltrating macrophages.

While the complete transformation of the exocrine pancreas clearly indicates that $Kras^{G12D}$ and sHB-EGF are sufficient to initiate and promote tumor formation, progression to more advanced stages, e.g., PanIN3, happened stochastically; PanIN3-like lesions were observed in each day 14 mouse pancreas, but the majority of lesions in each pancreas remained as earlier precursors with an average of one area per mouse displaying early invasive phenotype. The random nature of progression suggests that either a subset of cells are competent to develop into PanIN3 or these two genes only regulate initiation and promotion but other events are required for progression. The ability of $Kras^{G12D}$ and sHB-EGF to induce tumorigenesis as soon as exocrine cells matured resulted in induction during the

neonatal period when some tumor suppressor genes may be expressed at lower levels than in adult tissue. However, high grade PanINs also arose rapidly in adult mice when pancreatitis was induced in adult $Kras^{G12D}$ mice (31). Pancreatitis involves the influx of multiple inflammatory cells including macrophages and is thus consistent with our results showing both promotion and rapid progression resulting from Kras mutation and a source of HB-EGF. Future studies, in which Kras mutation and sHB-EGF overexpression are induced in adult tissue will determine whether progression is as rapid in the adult pancreas as in the neonatal pancreas.

Macrophage expression of HB-EGF links chronic pancreatitis and PDAC

We found that in pancreatic cancer tissue, macrophages were the predominant source of HB-EGF production. HB-EGF protein was initially identified in media from the macrophage-like cell line U-937 (33). Recently, Mosser and colleagues (34) reported that type II macrophages, which have similarities to tumor associated macrophages, had an eight-fold elevation of Hbegf mRNA expression compared to classically activated macrophages. In breast cancer, macrophages produce multiple EGFR ligands including HB-EGF (35). Thus, macrophages may be a potent source of EGFR activation in multiple cancers. While macrophages may play multiple roles in cancer induction, progression and maintenance, we found an elevated macrophage presence during the earliest epithelial changes associated with tumor induction, the conversion of acini into ductal lesions (acinar-ductal metaplasia). Acinar-ductal metaplasia also occurs in chronic pancreatitis, another disease with an increased presence of HB-EGF-expressing macrophages. We have shown previously that EGFR ligands induce adult acinar cells to alter their identity, or transdifferentiate, into ductal cells within three days of exposure to an EGFR ligand (23, 36). We propose that macrophage infiltration produces HB-EGF (possibly acting in concert with other cytokines) that leads only to acinar-ductal metaplasia in the absence of Kras mutation. When macrophage populations are cleared, e.g., after tissue repair, the epithelium may then return to normal. However, if the affected epithelium contains an activating Kras mutation, HB-EGF instead can convert pancreatic epithelium to PanINs that are then self-sustaining (32) and capable of progressing to PDAC following further genetic alterations. PanIN1-like lesions are often seen in the pancreas of older people and frequently contain Kras mutations (4). Our work suggests that these early PanINs may have arisen from an earlier coincidence of Kras mutation and an inflammatory response.

Synergy between EGFR ligands and activated Ras

Kras is a downstream effector of HB-EGF signaling yet this study demonstrates that HB-EGF is not redundant with activated Kras, but rather is synergistic for the induction of pancreatic cancer. A similar result was obtained in rat intestinal epithelial cells in culture. While transfection with activated Hras could transform those cells, this transformation was blocked by inhibiting EGFR activity (37, 38). Recently, a small molecule inhibitor of EGFR activity or deletion of the Egfr gene was found to decrease the number of mPanINs that formed in $Ptf1a^{Cre}$; $LSL\text{-}Kras^{G12D}$ mice (39–41). This requirement of EGFR activity for mPanIN development supports our finding that an EGFR ligand, HB-EGF, is involved in the earliest steps of promotion of Kras-initiated pancreatic tumorigenesis.

Multiple signaling pathways are activated downstream of the EGFR family of receptors and Ras proteins have only been implicated in some of them. While Ras proteins are upstream of the MAPK pathway and can facilitate PI3 Kinase/AKT activation, we did not find any synergistic activity of $Kras^{G12D}$ and HB-EGF in those pathways. However, $Kras^{G12D}$; sHB-EGF mice had increased phosphorylated p70 S6 kinase and its target, the activation loop of GSK3α/β. p70 S6 kinase is downstream of the mTOR complex which can be activated by multiple pathways. While AKT is one of the best understood activators of mTORC1, our data suggest that another pathway is synergistically activated by HB-EGF and $Kras^{G12D}$ such as the phospholipase/phosphatidic acid and/or Rheb pathways (42). AMP-dependent protein kinase (Ampk), a negative regulator of mTORC1, also had increased phosphorylation and may represent a negative feedback response. Hck, a Src family member, is found predominantly in inflammatory cells and may therefore not represent an epithelial regulatory event. However, this elevation did not coincide with any apparent increase in macrophages, T cells or B cells.

It was reported that overexpression of TGFα in acinar cells accelerated tumor progression in $Kras^{G12D}$ mice (43), but with very different kinetics and phenotypes to those shown here for sHB-EGF overexpression. The increased oncogenicity of sHB-EGF may result from production of constitutively secreted rather than transmembrane growth factor. Other factors that may have influenced different effects of HB-EGF and TGFα are activation of the ErbB4 receptor to which HB-EGF but not TGFα binds (44), and the use of different promoters affecting the level, timing and cell specificity of protein production.

Methods

Mice

All experiments were done with the approval of the Vanderbilt Institutional Care and Use Committee. *Pdx1-sHBEGF* mice (21), $Ptf1a^{Cre}$ mice (45) and $LSL\text{-}Kras^{G12D}$ ($Kras^{tm4Tmj}$) mice (8) were described previously. All alleles were bred for at least 10 generations into the C57BL6/J background. The morning that pups were first present in a cage was defined as day 0. Sample sizes are listed in Supplemental Table S2.

Histology

Mouse tissues were fixed in fresh 4% paraformaldehyde, 4–5 h, 4°C, washed and stored in 70% ethanol until embedding. Tissues were serially dehydrated, paraffin-embedded, and sectioned at 5 μm depth onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). Immunolabeling was done using Vectastain Elite ABC kits (Vector Labs, Burlingame, CA) with the following antibodies: goat anti-HB-EGF (R&D Systems, Minneapolis, MN), rat anti-CK19 (Troma III, Developmental Studies Hybridoma Bank, Iowa City, IA), goat anti-amylase (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-histone H3 (Millipore, Billerica, MA), rabbit anti-Ki67 (Abcam), rabbit anti-cyclin D1 (Dako, Glostrup, Denmark), Rat anti-F4/80 (AbD Serotec, Raleigh, NC), rabbit anti-cleaved caspase 3 (Cell Signaling Technology, Boston, MA), rabbit anti-iNOS (Abcam, Cambridge, MA), rabbit anti-Arginase I (Abgent, San Diego, CA). Peroxidase activity was detected with 3,3'-Diaminobenzidine (Invitrogen, Carlsbad, CA) and cells were counterstained with

hematoxylin (Fisher Scientific). Mucin was detected by staining with Periodic acid-Schiff reagent (PAS) according to manufacturer's specifications (Sigma, St. Louis, MO).

Deidentified human pancreatic tissues, three normal, five chronic pancreatitis and ten PDAC, were obtained from the Vanderbilt Ingram Cancer Center Tissue Acquisition and Pathology Core and labeled with mouse anti-CD68 (Dako) and goat anti-HB-EGF (R&D Systems, Minneapolis, MN) antibodies using the TSA-Plus kit (Perkin Elmer LAS, Boston, MA) for detection of CD68 as per manufacturer's protocol and fluorophore-conjugated anti-goat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for detection of HB-EGF. Nuclei were counterstained with Toto3 (Molecular Probes, Eugene, OR).

Ex vivo culture of primary cells

Pancreata from wildtype or $Kras^{G12D}$ mice were isolated, dispersed by partial collagenase digestion, and placed into three-dimensional collagen matrix as described but with 1% fetal bovine serum to reduce effects of other growth factors (23). Cultures were treated on days 0, 1 and 3 with 100 ng/ml recombinant HB-EGF (R&D Systems), fixed with 4% paraformaldehyde on day 4, paraffin embedded, and processed as described above.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from day 1 pancreas from at least 3 mice of each genotype (RNeasy Mini Kit, Qiagen, Valencia, CA). cDNA was synthesized from 1.5 ng total RNA (+ reverse transcriptase, +RT, reactions) or 0.5 ng (− RT reactions) using the Transcriptor Universal kit (Roche Applied Sciences, Indianapolis, IN). qPCR was performed on 1/15th of resulting cDNA per reaction (or equivalent volume –RT mix) using Express SYBR Green ER Supermix (Invitrogen), in a LightCycler 480 (Roche) using primers: Cdkn1a: 5'TGCTCAGACCTGTGAAGACA3' (F)/5'CTTCCAGTCCACTGAGCTGT3' (R); Cdkn1b: 5'AGCGTTTCTTCATTGCCTGT3' (F)/5'CACAAAACATGCCACTTTGG3' (R); Cdkn2d: 5'TCCATTGAAGAAGGGAGTGG3' (F)/5'CACCAAAAGGGGTGAGAAAA3' (R); βActin: 5'AAGAGCTATGAGCTGCCTGA3' (F) /5'TACGGATGTCAACGTCACAC (R) (Real Time Primers, Elkins Park, PA); Cdkn2a: 5'CGTACCCCGATTCAGGTG3' (F)/ 5'ACCAGCGTGTCCAGGAAG3' (R); (Sigma).

Immunoblotting

Total neonatal pancreas was lysed and analyzed by western blotting as described (46) using 20 μg lysate separated on 10% polyacrylamide gels. Blots were probed with mouse anti-Kras (Santa Cruz Biotechnology) and mouse anti-β actin (Sigma Aldrich, St. Louis, MO).

Phospho-kinase antibody array

Phosphorylation patterns of signaling molecules were determined using the Proteome Profiler Phospho-Kinase Array Kit (R&D Systems) using whole postnatal day 1 pancreata per manufacturer's protocol. Intensities were determined with ImageJ software (NIH).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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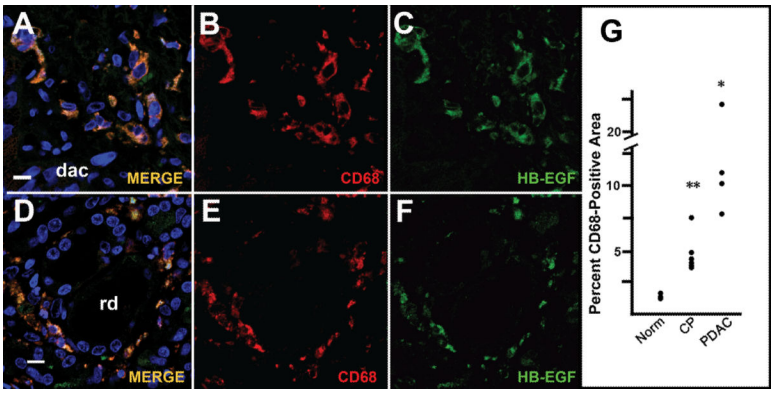

Figure 1. Macrophages are an endogenous source of HB-EGF that is enriched in human chronic pancreatitis and pancreatic cancer

PDAC (A–C) and chronic pancreatitis (D–F) tissues contain an abundance of macrophages labeled with CD68 (red) that are co-labeled with HB-EGF (green). Left images show merge (yellow) of panels to right. Dac, edge of ductal adenocarcinoma; rd, reactive duct; blue, nuclei. G. Quantification of the percent area of CD68 staining in fibrotic regions from chronic pancreatitis patients (CP) and from PDAC patients shows a significant enrichment of macrophages compared to normal human pancreas (Norm). Single asterisk, $p < 0.05$; two asterisks, $p < 0.01$.

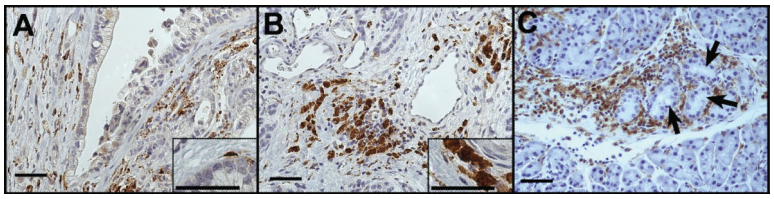

Figure 2. Macrophages are found in close proximity to neoplastic lesions

A. CD68-positive (brown cells) are found at high density within a few cell diameters of a human PDAC lesion. Inset, occasional CD68-positive macrophages are found immediately juxtaposed to PDAC cells. B. CD68-positive macrophages are also found at high density in human chronic pancreatitis but with less obvious proximity to ductal lesions. Inset, higher magnification of CD68-positive cells. C. In the Ptf1a^{Cre}; LSL-Kras^{G12D} mouse without sHB-EGF overexpression, acinar-ductal metaplasias are observed in regions of F4/80-positive macrophage infiltration. Arrows, acini losing apical cytoplasm and forming lumena, characteristics of acinar-ductal metaplasia.

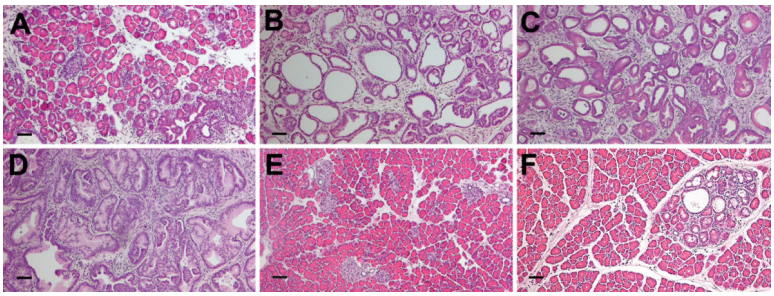

Figure 3. PanIN-like lesions developed within the first two weeks after birth when sHB-EGF was overexpressed in conjunction with Kras activation

$Kras^{G12D}$; sHB-EGF mice (A–D), $Kras^{G12D}$ alone (E) and sHB-EGF alone (F) were analyzed at 1 day (A), 3 days (B), 7 days (C) and 14 days (D–F) after birth by hematoxylin (blue) and eosin (pink) staining. A. In $Kras^{G12D}$; sHB-EGF mice on postnatal day 1, some acinar and ductal lumena were slightly dilated. By postnatal day 3, large cystic ducts with columnar cell morphology were apparent with few acini remaining. By day 7, no structural acini remained and cells within ducts became columnar with occasional papillary architecture and lumenal budding observed. By 14 days, these structural alterations and other cellular changes were consistent throughout each $Kras^{G12D}$; sHB-EGF pancreas while no or few alterations were observed with $Kras^{G12D}$ or HB-EGF alone. Size bars, 50 μm.

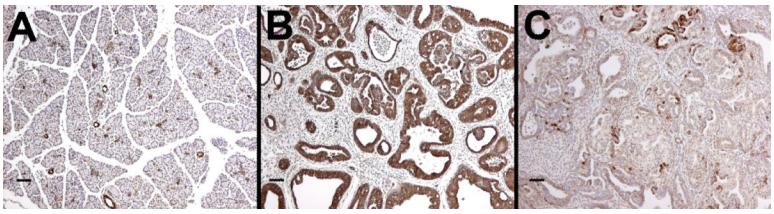

Figure 4. Overexpression of sHB-EGF in conjunction with activated Kras resulted in acinar-ductal metaplasia of most exocrine cells

A. Normal pancreas at postnatal day 14 was comprised largely of acinar cells with a smaller number of duct cells as visualized by CK19 immunolabelling (brown). B. By day 14 in $Kras^{G12D}$; sHB-EGF mice, CK19 labeled the majority of epithelial cells in the pancreas. C. Immunolabeling for amylase (brown) revealed that no acinar structures remained in $Kras^{G12D}$; sHB-EGF mice and only rare amylase-positive acinar cells were found within ductal structures. Many fields of view completely lacked amylase-positive cells (data not shown). Size bars, 50 μm.

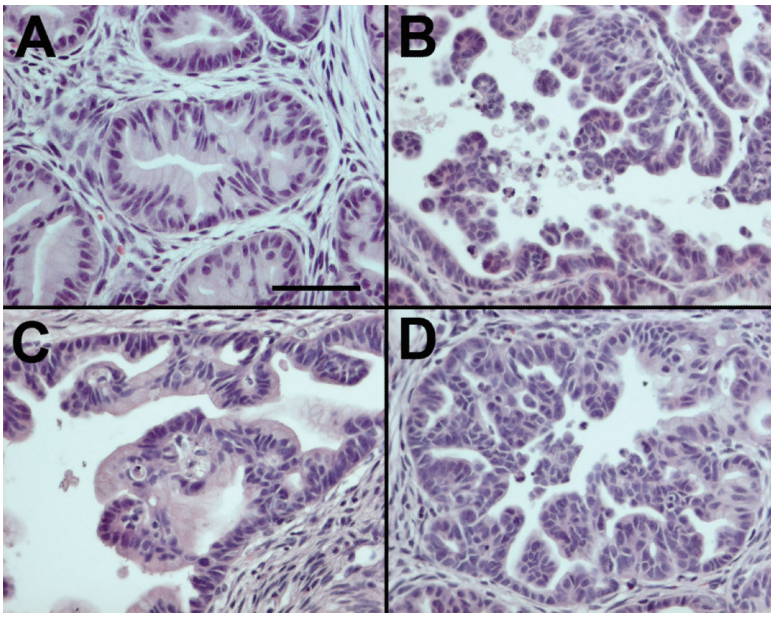

Figure 5. sHB-EGF and Kras^{G12D} together induced PanIN-like lesions

Kras^{G12D}; sHB-EGF mice were examined at day 14 for histology of lesions. A. A lesion showing loss of polarity with apically localized nuclei. B. A lesion showing luminal budding of epithelial cells. C. Cribriform architecture with loss of polarity and nuclear atypia are similar to PanIN3 or carcinoma in situ. D. complex papillary architecture in conjunction with loss of polarity and nuclear atypia are also indicative of PanIN3-like lesions. Size bars, 50 μm.

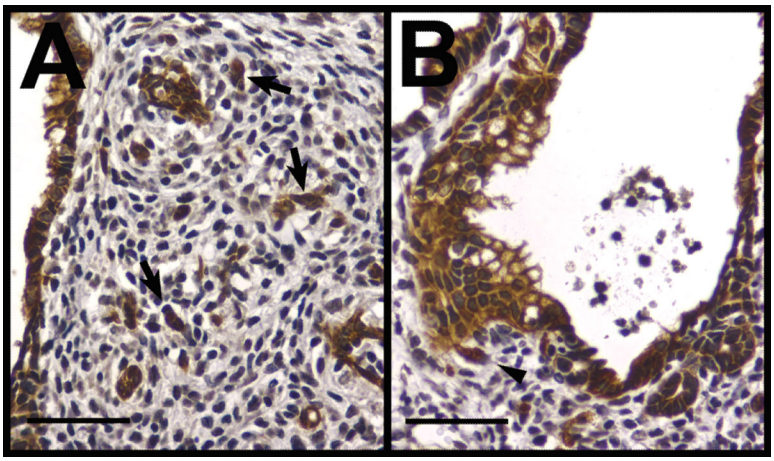

Figure 6. sHB-EGF and Kras^{G12D} expression allowed progression to invasive ductal adenocarcinoma

Tissue sections from 2 week old Kras^{G12D}; sHB-EGF mice were stained for ductal marker CK19 to identify epithelial cells with invading morphology. A. Individual ductal cells present in stroma (arrows). B. Ductal lesion exhibiting invasive morphology with a cell appearing to detach (arrowhead). Size bars, 50 μm.

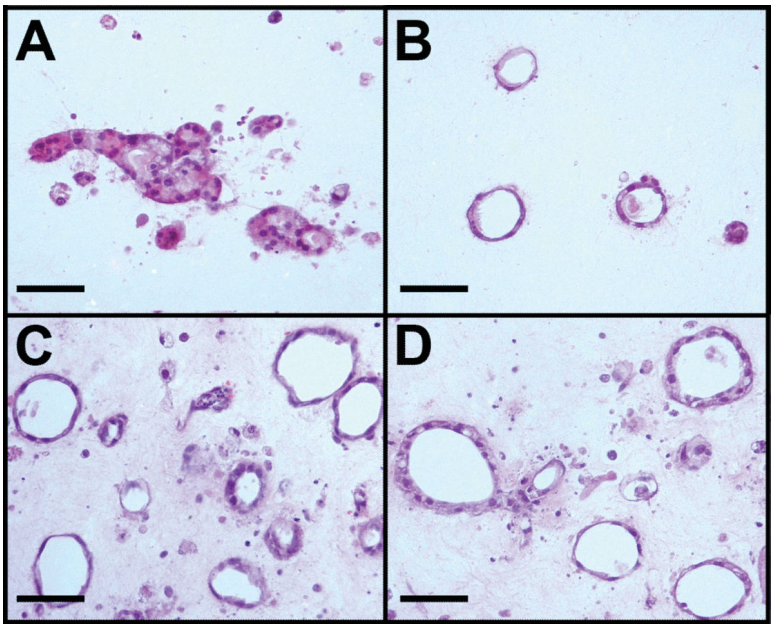

Figure 7. Acinar cells give rise to ductal cysts in response to HB-EGF or Kras mutation

Acinar-enriched pancreatic cells were isolated by collagenase digestion, embedded in collagen matrix, and treated with recombinant HB-EGF or PBS vehicle on days 0, 1 and 3. Cells were then analyzed on day 4. A. Without HB-EGF, many wildtype cells maintained acinar identity as indicated by high eosin uptake (dark pink). B. Wildtype cells all transdifferentiated into ductal cysts in the presence of HB-EGF. C. Even without HB-EGF and in the presence of only 1% fetal bovine serum, all acinar cells expressing $Kras^{G12D}$ transdifferentiated into ductal cysts. Similarly to wildtype cells treated with HB-EGF, cells were flat cuboidal or simple squamous in morphology. D. In the presence of HB-EGF, $Kras^{G12D}$-expressing cells also became ductal cysts but exhibited a higher cytoplasm/nucleus ratio with nuclei that were located basally, apically or centrally.

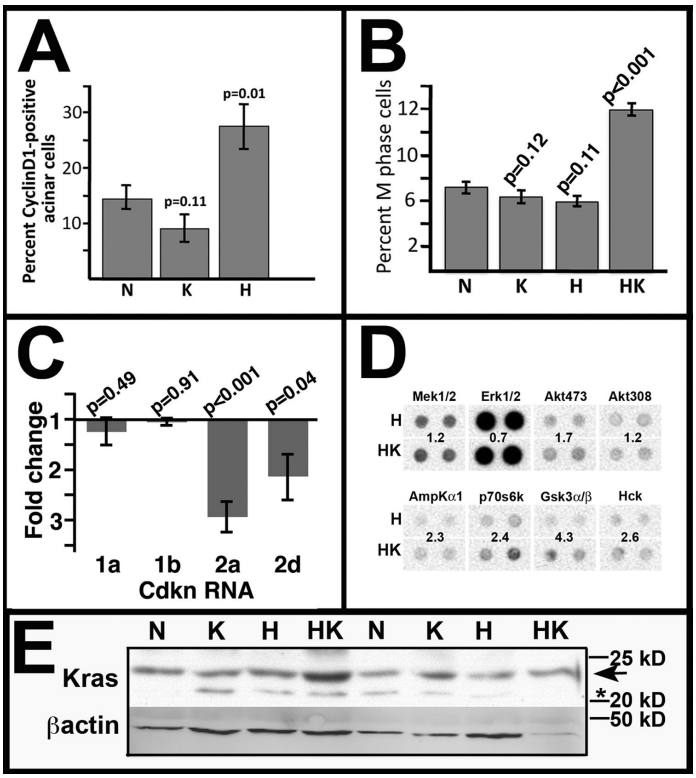

Figure 8. Synergy between sHB-EGF and Kras^{G12D} occurred at the level of proliferation, protein phosphorylation, and Kras expression

Pancreas samples were compared at postnatal day 1, immediately prior to morphological change. P values are relative to normal controls. A. Percent of cyclinD1-positive acinar cells according to genotype. B. Percent of mitotic acinar cells as measured by immunolabeling for phospho-histone H3. C. Quantitative comparison of RNA from sHB-EGF alone or from Kras^{G12D}; sHB-EGF mice revealed little change in Cdkn1a or Cdkn1b but a 2.8-fold decrease in Cdkn2a and a 2.1-fold decrease in Cdkn2d when Kras^{G12D} was combined with sHB-EGF overexpression. D. Comparison of phosphorylation levels in sHB-EGF mice and Kras^{G12D}; sHB-EGF mice. Numbers indicate fold difference of intensities between genotypes. Intensities for Erk1/2 were quantified on a lighter exposure. Specific phosphorylation sites detected were: Mek1/2, S218/S222, S222/S226; Erk1/2,T202/Y204, T185/Y187; Akt, S473, T308; Ampk1a,T174; p70S6K, T229; Gsk3α/β, S21/S9; Hck, Y411. E. Total protein lysates were analyzed for Kras protein levels. While Kras^{G12D} expression alone or sHB-EGF overexpression alone had no effect on total Kras levels, together they increased the amount of Kras protein relative to β actin. N, normal pancreas; K, Kras^{G12D} alone; H, sHB-EGF alone; HK, both Kras^{G12D} and sHB-EGF expression; arrow, expected Kras band size; asterisk, location of nonspecific band. Protein size standards are indicated on right.