Notch mediates TGFα-induced changes in epithelial differentiation during pancreatic tumorigenesis

Yoshiharu Miyamoto,[1] Anirban Maitra,[1] Bidyut Ghosh,[1] Ulrich Zechner,[2] Pedram Argani,[1] Christine A. Iacobuzio-Donahue,[1] Virote Sriuranpong,[1] Tatsuya Iso,[3] Ingrid M. Meszoely,[4] Michael S. Wolfe,[5] Ralph H. Hruban,[1] Douglas W. Ball,[1] Roland M. Schmid,[2] and Steven D. Leach[1,*]

[1]Departments of Surgery, Oncology, and Pathology, The Sidney Kimmel Cancer Center at Johns Hopkins, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287
[2]Department of Medicine, University of Ulm, Ulm Germany
[3]Second Department of Internal Medicine, Gunma University School of Medicine, Gunma, Japan
[4]Department of Surgery, Fox-Chase Cancer Center, Philadelphia, Pennsylvania 19111
[5]Center for Neurologic Diseases, Harvard Medical School, Boston, Massachusetts 02115
*Correspondence: stleach@jhmi.edu

Summary

Notch signaling regulates cell fate decisions in a wide variety of adult and embryonic tissues. Here we show that Notch pathway components and Notch target genes are upregulated in invasive pancreatic cancer, as well as in pancreatic cancer precursors from both mouse and human. In mouse pancreas, ectopic Notch activation results in accumulation of nestin-positive precursor cells and expansion of metaplastic ductal epithelium, previously identified as a precursor lesion for pancreatic cancer. Notch is also activated as a direct consequence of EGF receptor activation in exocrine pancreas and is required for TGFα-induced changes in epithelial differentiation. These findings suggest that Notch mediates the tumor-initiating effects of TGFα by expanding a population of undifferentiated precursor cells.

Introduction

Pancreatic ductal adenocarcinoma represents the fifth leading cause of cancer death in Western populations and carries a five-year survival rate of less than 5% (Jemal et al., 2002). While precursors of pancreatic cancer are less well characterized than in many other human neoplasms, recent studies have identified pancreatic intraepithelial neoplasia (PanIN) as a premalignant lesion (Hruban et al., 1999). In transgenic mouse models, formation of PanIN-like lesions occurs in the context of a metaplasia/neoplasia sequence involving TGFα-induced acinar-to-ductal metaplasia. On both a histologic and a molecular level, metaplastic epithelium arising under the influence of TGFα has been identified as a direct precursor of malignant epithelium (Song et al., 1999; Wagner et al., 1998, 2001). Histologically, direct transitions between metaplastic and neoplastic epithelium have been demonstrated, with gradual progression frequently observed in immediately contiguous areas of metaplastic epithelium. It has also been demonstrated that, before the onset of dysplasia or cancer, metaplastic epithelium has already begun to progressively accumulate the molecular changes characteristic of invasive pancreatic cancer, including ras activation and accumulation of activated Erk, cyclin D, Cdk4, and nuclear p53 (Wagner et al., 2001). A similar metaplasia/neoplasia sequence has been observed in human pancreatic cancer (Parsa et al., 1985).

We have previously demonstrated that TGFα-induced acinar-to-ductal metaplasia involves expansion of undifferentiated cell populations resembling those found in embryonic pancreas (Song et al., 1999). During pancreatic development, differentiation of epithelial precursors is tightly regulated by Notch signaling, a highly conserved pathway known to regulate cell fate decisions in a variety of organisms (Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000). In this context, Notch appears to prevent cellular differentiation and maintain a population of undifferentiated precursor cells (Apelqvist et al., 1999; Jensen et al., 2000a, 2000b). We have therefore analyzed Notch pathway activation in normal human pancreas, pancreatic cancer precursors, and invasive pancreatic ductal adenocarcinoma. In addition, we have examined expression of Notch pathway components in premalignant epithelium and invasive pancreatic cancer generated by transgenic overexpression of TGFα in mouse pan-

SIGNIFICANCE

Pancreatic cancer is a highly lethal malignancy. Recent evidence has implicated overexpression of TGFα in the pathogenesis of this disease. In this report, we define Notch pathway activation as a direct consequence of EGF receptor signaling in exocrine pancreas and demonstrate that Notch is a requisite downstream mediator of TGFα-induced changes in epithelial differentiation. These studies define a novel role for Notch in regulating metaplastic conversion between epithelial cell types and suggest an important link between EGF receptor signaling and Notch pathway activation in the context of mammalian tumorigenesis. By implicating Notch signaling in the very earliest phases of pancreatic cancer initiation, the current results provide new strategies for early detection and chemoprevention.

creas. Finally, we have determined the effects of ectopic Notch activation in explant cultures of normal mouse pancreas, as well as the role of Notch in mediating TGFα-induced changes in epithelial differentiation. These studies demonstrate a requirement for Notch pathway activation in the earliest stages of pancreatic neoplasia and identify a requisite role for Notch in mediating metaplastic conversion between differentiated cell types. In addition, this report defines a novel direct link between EGF receptor signaling and Notch pathway activation in the context of mammalian tumorigenesis, providing new strategies for combinatorial chemoprevention.

Results

Expression of Notch pathway components in human pancreatic cancer

Based on the known role of Notch signaling in regulating epithelial differentiation in developing mouse pancreas (Apelqvist et al., 1999; Jensen et al., 2000a, 2000b), we examined expression of Notch target genes in human pancreatic cancer and pancreatic cancer precursors. cRNA prepared from either normal pancreas (n = 29) or pancreatic ductal adenocarcinoma (n = 26) was hybridized to the complete Affymetrix Human Genome U95 GeneChip® set (arrays U95 A-E). Within this set, 47 oligonucleotides covering 25 known genes were identified as components of the classical Notch signaling pathway, defined as either Notch receptors (e.g., Notch2, Notch3; fragments corresponding to Notch1 not present on these arrays), Notch ligands (e.g., delta, jagged1), Notch target genes (e.g., Hes1, Hey1), or factors known to regulate Notch pathway activity (e.g., presenilin-1, sel1L). Genes from each of these categories were highly expressed in human pancreatic cancer, with several demonstrating differential expression patterns in pancreatic cancer compared to normal pancreas (Figure 1). Transcripts corresponding to 25 fragments covering 17 Notch pathway components were assessed to be "present" in at least 50% of either tumor or normal samples. Among these 25 fragments, 11 fragments corresponding to 9 different Notch pathway components were significantly overexpressed in tumor tissue compared to normal pancreas (Table 1). Notch receptors and ligands significantly overexpressed in pancreatic cancer included Notch2, Notch3, Notch4, dlk1, and jag1. With the exception of Notch3, which exhibited 5.8-fold overexpression in cancer specimens, the degree of upregulated expression was moderate, typically in the range of 2- to 3-fold. In contrast, the human ortholog of *C. elegans* sel-1 (sel1L), a known inhibitor of Notch pathway activation, was downregulated in cancer versus normal, consistent with previous observations regarding downregulation of this gene in human pancreatic adenocarcinoma cell lines (Donoviel et al., 1998; Grant and Greenwald, 1997; Harada et al., 1999). Evidence that relative overexpression of Notch receptors and ligands resulted in functionally significant changes in Notch pathway activation was provided by associated upregulation of several known and candidate Notch target genes, including Hes1, Hes4, Hey1, and HeyL (Davis and Turner, 2001; Iso et al., 2001). Together, these data suggest activation of a Notch signaling "module" in human pancreatic adenocarcinoma. In contrast, expression profiling of Wnt pathway components revealed no similar tendency for modular upregulation of activating elements (Figure 1D); the Wnt pathway component with the highest level of overexpression in pancreatic cancer was secreted frizzled-related protein 2, a known inhibitor of Wnt signaling (Finch et al., 1997; Mayr et al., 1997).

We further evaluated Notch pathway activation in human pancreatic cancer using real-time quantitative RT-PCR analysis of ten additional infiltrating ductal adenocarcinomas of the pancreas and five specimens of normal pancreas. This analysis confirmed upregulated expression of Notch ligands and receptors in bulk tumor material. Relative expression values for these components were similar to the values derived from microarray analysis, with 3.4-fold overexpression of Notch2, 5.1-fold overexpression of Notch3, and 4.2-fold induction of jag1 observed in tumor samples (Figure 1E). With respect to Notch target genes (Hes1, Hes4, Hey1, and HeyL) assessed by real-time RT-PCR, 9 of 10 cancers expressed at least one target gene at a level >2-fold over normal, and 4 of 10 cancers expressed one target gene at a level >3-fold over normal, confirming Notch pathway activation in a majority of human pancreatic cancers. In order to confirm this observation at the protein level, we analyzed Hes1 by Western blot analysis of ten human pancreatic ductal adenocarcinoma cell lines. We detected significant levels of Hes1 protein in 6/10 pancreatic cancer cell lines, with high levels noted in four and lower levels in two (see Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/3/6/565/DC1). Hes1 protein levels in the four high-expressing pancreatic cancer cell lines were similar to those observed in association with previously documented Notch pathway activation in non-small cell lung cancer lines (Chen et al., 1997).

Further validation of upregulated Notch signaling in human pancreatic cancer was obtained by immunohistochemical analysis involving 50 specimens of resected human pancreas. Among these specimens, 34 were contained on tissue microarrays, allowing for simultaneous staining of multiple specimens. Immunohistochemical staining was performed using polyclonal antibodies recognizing Notch1, Notch2, Notch3, Notch4, and jagged2, while jagged1 expression was evaluated by in situ hybridization. For each of these Notch pathway components, little-to-no expression was observed in normal pancreatic ductal epithelium (Figures 2A and 2C), and moderate-to-high level expression (defined as a score of ≥2 on a scale of 1–3) was never observed. In contrast, upregulated expression of Notch receptors and ligands was frequently observed in resected pancreatic cancer as well as PanIN lesions (Figures 2B and 2D–2G). Among the four Notch receptors, moderate-to-high levels of detectable protein were observed as follows: Notch1, 16 of 34 specimens; Notch2, 16 of 34; Notch3, 12 of 33; and Notch4, 15 of 33. Expression of different Notch family members tended to be concordant within individual tumor specimens (see Supplemental Table S1 on the *Cancer Cell* website). Among all examined specimens, 21 of 34 (62%) showed moderate-to-high level expression of at least one Notch protein. Similarly, tumor sections frequently contained moderate-to-high levels of jagged2 protein (11 of 33 by immunohistochemistry) and jagged1 RNA (21/33 scored as positive by in situ hybridization). As with the four Notch receptors, jagged1 and jagged2 expression tended to be concordant within individual tumor specimens; among the 12 tumors lacking jagged1, only one expressed jagged2 protein at moderate-to-high levels.

These data confirm upregulated expression of Notch receptors and ligands in human pancreatic cancer. However, upregulated expression of receptors and ligands does not necessarily imply functional pathway activation. In order to specifically de-

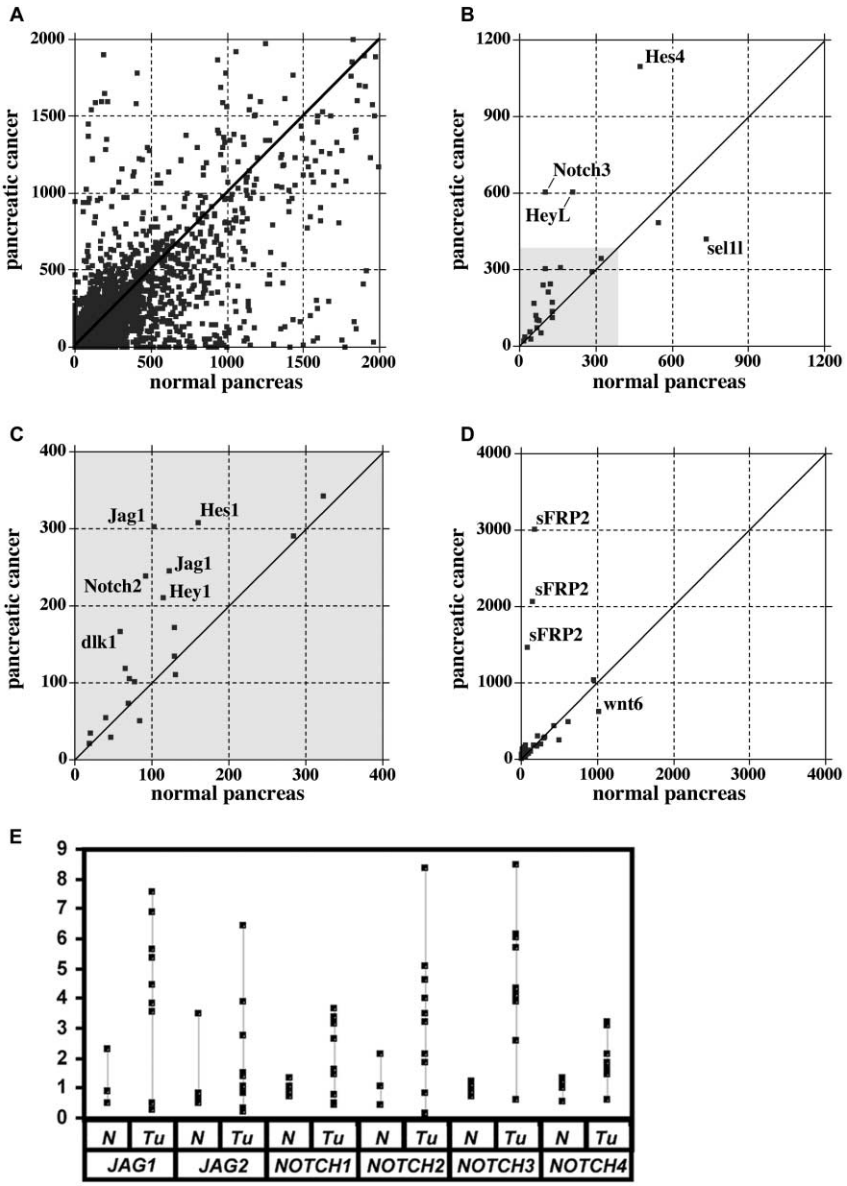

Figure 1. Modular upregulation of Notch pathway components in pancreatic ductal adenocarcinoma

A: Normalized hybridization intensities for 8,863 gene fragments included on Affymetrix U95A GeneChip. Data represent mean values from 26 pancreatic ductal cancers and 29 specimens of normal pancreas. Values distribute symmetrically about line of unity, with multiple fragments either overexpressed or underexpressed in cancer versus normal.

B: Expression values for 25 gene fragments representing 17 different Notch pathway components; fragments corresponding to Notch1 are not present on these arrays. Note moderate overexpression of Notch3, Hes4, and HeyL, as well as relative underexpression of sel-1L.

C: Expanded view of shaded area in panel B allowing examination of expression values for Notch pathway components with lower hybridization intensities. Note moderate overexpression of Notch2, jag1, dlk1, Hes1, and Hey1.

D: Expression values for 40 gene fragments representing 28 different Wnt pathway components. Gene fragments with highest degree of overexpression in cancer versus normal correspond to sFRP2 (secreted frizzled-related protein 2), a known inhibitor of Wnt signaling.

E: Real-time RT-PCR analysis demonstrating relative expression levels of Notch receptors and ligands in ten additional resected cancers and five specimens of normal pancreas. N, normal pancreas; Tu, tumor.

termine the functional status of Notch signaling in normal and neoplastic human pancreas, immunohistochemical analysis of Hes1 protein was performed on a total of 33 resected specimens, of which 17 were contained within tissue microarrays. Examination of nonpancreatic tissues revealed high levels of nuclear Hes1 protein in basal epithelial cells of normal prostate and in crypt cells of normal colon (data not shown), consistent with Notch pathway activation in known epithelial precursor populations. Hes1 protein expression in normal human pancreas was infrequent and largely confined to centroacinar cells and terminal ductal epithelium (Figure 2H), consistent with a possible precursor function for these cell populations (Gasslander et al., 1992; Hayashi et al., 1999). Normal interlobular ductal epithelium showed occasional low-level Hes1 expression, typically involving only a very small number of epithelial cells (Figure 2I). Hes1 protein expression was significantly expanded in metaplastic ductal epithelium as well as in PanIN lesions and invasive pancreatic cancer (Figure 2J–2L). Compared to normal interlobular ductal epithelium, there was a 15-fold increase in frequency of Hes1-positive cells in metaplastic duct lesions, a 5-fold increase in PanIN lesions, and a >7-fold increase in epithelial cells of infiltrating ductal adenocarcinoma ($p < 0.01$ for metaplastic epithelium versus normal, $p = 0.06$ for PanIN versus normal, $p < 0.01$ for cancer versus normal).

Ectopic Notch pathway activation in normal pancreas

The frequent expression of Hes1 in metaplastic duct lesions and PanIN epithelium suggested that Notch pathway activation might occur as an early event in pancreatic tumorigenesis. We therefore examined the effects of ectopic Notch activation in normal pancreatic tissue using an explant culture system. Collagen-digested mouse pancreas was depleted of stromal elements, large ductal structures, and pancreatic islets, resulting in epithelial explants predominantly comprised of acinar cells,

Table 1. Expression values for 25 cDNA fragments covering 17 known Notch pathway components in pancreatic cancer and normal pancreas

Fragment name	Known gene name	Fold difference (cancer versus normal)	t test
52819_at	delta-like 4 homolog (*Drosophila*)	0.9	NS
32648_at	delta-like homolog (*Drosophila*)	2.4	0.01
37393_at	hairy (*Drosophila*)-homolog (Hes1)	1.9	0.0001
44783_s_at	hairy/enhancer-of-split related with YRPW motif 1	1.8	0.001
46813_at	hairy/enhancer-of-split related with YRPW motif 2	1.4	NS
79699_at	hairy/enhancer-of-split related with YRPW motif 2	1.1	NS
43489_at	hairy/enhancer-of-split related with YRPW motif-like	2.9	0.0001
54852_at	hairy/enhancer-of-split related with YRPW motif-like	1.8	0.03
77244_at	Hes4	2.3	0.0001
35414_s_at	jagged1 (Alagille syndrome)	2.0	0.0001
33178_at	jagged1 (Alagille syndrome)	1.1	NS
74827_s_at	jagged1 (Alagille syndrome)	3.0	0.0003
32137_at	jagged2	1.3	NS
48486_at	Notch (*Drosophila*) homolog 2	2.6	0.0006
38750_at	Notch (*Drosophila*) homolog 3	5.8	0.0001
39048_at	Notch (*Drosophila*) homolog 4	1.7	0.002
37693_at	numb (*Drosophila*) homolog	1.0	NS
641_at	presenilin 1 (Alzheimer disease 3)	1.0	NS
642_s_at	presenilin 1 (Alzheimer disease 3)	1.1	NS
40689_at	sel-1 (suppressor of lin-12, *C. elegans*)-like	0.6	NS
65740_at	sel-1 (suppressor of lin-12, *C. elegans*)-like	0.6	0.03
76761_at	sel-1 (suppressor of lin-12, *C. elegans*)-like	0.6	NS
41489_at	transducin-like enhancer of split 1, homolog of *Drosophila* E(sp1)	1.3	NS
56754_at	transducin-like enhancer of split 1, homolog of *Drosophila* E(sp1)	1.4	NS
40837_at	transducin-like enhancer of split 2, homolog of *Drosophila* E(sp1)	0.8	NS

Fragments corresponding to Notch1 are not present on utilized arrays.

centroacinar cells, and associated terminal ductal epithelium. Epithelial explants were infected with previously characterized adenoviral vectors encoding either GFP (Ad-GFP) or GFP plus the activated intracellular domain of either Notch1 (Ad-GFP/Notch1-IC) or Notch2 (Ad-GFP/Notch2-IC) (Sriuranpong et al., 2001). In addition, direct expression of Notch target genes was accomplished using adenoviral vectors encoding GFP plus either Hes1 (Ad-GFP/Hes1), Hey1 (Ad-GFP/Hey1), or Hey2 (Ad-GFP/Hey2). High-efficiency gene delivery was confirmed by GFP visualization as well as by RT-PCR, confirming successful expression of Notch1-IC and/or induced target genes at 2 and 4 days following infection (Figures 3A–3C). As shown in Figure 3C, adenoviral delivery of Notch1-IC resulted in upregulated Hes1 expression at levels similar to those achieved following Ad-GFP/Hes1 infection, while Hey1 and Hey2 were induced at lower levels. Previous studies have suggested significant cell type specificity in the relative responsiveness of Hes1, Hey1, and Hey2 to induction by Notch (Iso et al., 2001, 2002); these data define each of these as Notch-responsive elements in mouse pancreas.

During the five day culture period, Ad-GFP-infected epithelium behaved in a manner identical to that observed for uninfected control epithelium, with a predominant acinar cell population persisting until loss of explant viability (Figure 3D). In contrast, explants infected with either Ad-GFP/Notch1-IC or Ad-GFP/Notch2-IC underwent metaplastic conversion from an acinar cell-predominant epithelium to a ductal cell-predominant epithelium. This process involved conversion from acinar to ductal morphology (Figures 3E and 3F), gradual loss of acinar cell-specific gene expression (Figures 3G–3I), and acquired expression of ductal markers including carbonic anhydrase (data not shown) and cytokeratin-20 (Figures 3J–3L). Notch-mediated acinar-to-ductal metaplasia appeared to proceed through an undifferentiated intermediate characterized by upregulated expression of nestin (Figures 3M–3O), an intermediate filament expressed by precursor cell types in pancreas and CNS (Kawaguchi et al., 2001; Messam et al., 2000; Zulewski et al., 2001). These data suggest that Notch activation results in expansion of an undifferentiated precursor population in exocrine pancreas. In this regard, the effects of ectopic Notch activation appeared identical to those previously reported for TGFα (Song et al., 1999; Wagner et al., 2002). Adenovirus-mediated overexpression of several known Notch target genes, including Hes1, Hey1, and Hey2, failed to recapitulate the effects of activated Notch (data not shown), suggesting a requirement for combinations of target genes and/or additional downstream effectors.

Transgenic overexpression of TGFα activates Notch signaling in exocrine pancreas

Based on the striking similarity between Notch-mediated acinar-to-ductal metaplasia and events induced by TGFα, we next determined the role of Notch signaling as a possible mediator of EGF receptor activation in mouse pancreas, using both in vivo and in vitro systems. Transgenic overexpression of TGFα driven by either an elastase-1 promoter (Ela-TGFα) or a zinc-inducible metallothionein promoter (MT-TGFα) results in initiation of a metaplasia/neoplasia sequence characterized by dramatic changes in epithelial differentiation including loss of acinar cell mass, expansion of Pdx1-positive precursors, acinar-to-ductal metaplasia, and formation of PanIN-like lesions. When placed on a $p53^{+/-}$ background, these precursors show accelerated progression to invasive pancreatic cancer (Jhappan et al., 1990; Sandgren et al., 1990; Song et al., 1999; Wagner et al., 1998, 2001). We therefore evaluated expression of Notch path-

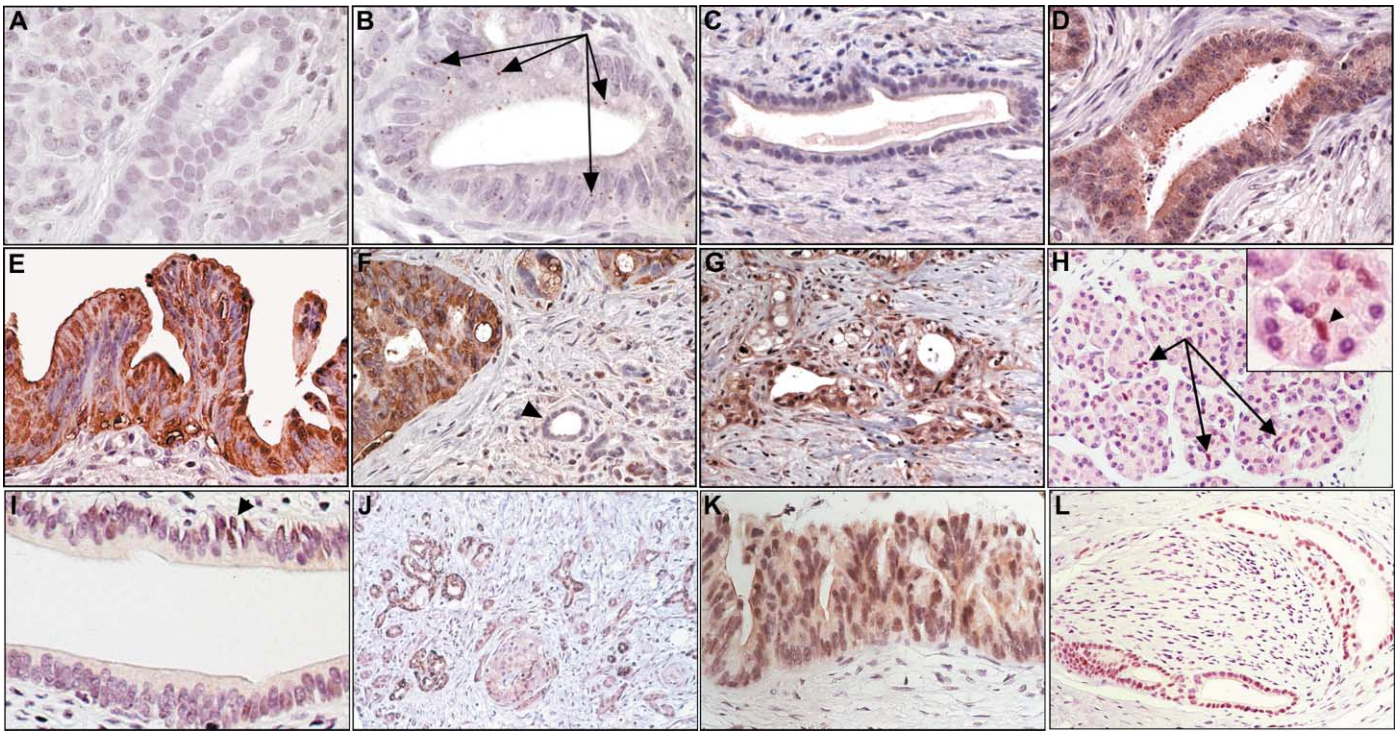

Figure 2. Expression of Notch pathway components in invasive pancreatic cancer and pancreatic cancer precursors

A and **B:** (400×), Detection of jagged1 transcripts by in situ hybridization in normal intralobular ductal epithelium (**A**) and invasive pancreatic cancer (**B**). Arrows in (**B**) indicate punctate staining indicative of jagged1 expression.
C and **D:** (400×), Immunohistochemical detection of Jagged2 in normal interlobular ductal epithelium (**C**) and invasive pancreatic cancer (**D**).
E: Immunohistochemical detection of Notch1 protein in PanIN2 epithelium (400×).
F and **G:** Immunohistochemical detection of upregulated Notch1 (**F**) and Notch2 (**G**) expression in invasive pancreatic cancer. Arrow in (**F**) indicates absence of Notch1 protein in adjacent normal ductal epithelium.
H–L: Immunohistochemical detection of nuclear Hes1 protein in human pancreatic tissue. **H:** Normal exocrine pancreas (100×); arrows indicate Hes1-positive centroacinar cells. Inset (400×) shows single acinar unit with Hes1-positive centroacinar cell (arrowhead). **I:** Normal interlobular pancreatic ductal epithelium (400×); arrow indicates low-frequency Hes1-positive duct cells. **J:** Hes1-positive metaplastic ductal epithelium in area of chronic pancreatitis (20×). **K:** High-level Hes1 expression in PanIN 2 epithelium (400×). **L:** Nuclear Hes1 immunoreactivity in invasive pancreatic ductal adenocarcinoma with perineural infiltration (100×).

way components in pancreatic tissue from both Ela-TGFα and MT-TGFα transgenic mice at different steps along this metaplasia/neoplasia progression, using both real-time quantitative PCR and immunohistochemistry. Pancreatic tissue from 24-week Ela-TGFα mice frequently demonstrated upregulated expression of Notch ligands, receptors, and target genes relative to normal mouse pancreas, as assessed by real-time RT-PCR (Figure 4A). These elevated expression levels were generally maintained without additional increases in pancreatic tumor material from Ela-TGFα;p53$^{+/-}$ mice. Expression levels of certain genes (e.g., Hes1) returned to normal in tumor material, suggesting a role in early precursors but not necessarily in invasive cancer. Further evaluation of Notch activity in premalignant epithelium from TGFα transgenic pancreas was performed using immunohistochemistry for Notch1 and Hes1. As in the case of normal human pancreas, pancreatic tissue from nontransgenic littermates demonstrated little-to-no expression of either of these proteins (Figures 4B and 4D). In contrast, metaplastic duct lesions and low-grade PanIN lesions demonstrated frequent expression of Notch1 (Figure 4C). Similarly, a striking pattern of Hes1 protein expression was observed, with high-level nuclear expression observed in TGFα-induced premalignant metaplastic epithelium (Figures 4E and 4G) and in centroacinar cells (Figure 4F). Similar to the pattern previously reported for Pdx1 expression in these lesions (Song et al., 1999), abrupt transitions were observed between Hes1-positive metaplastic epithelium and adjacent Hes1-negative acinar cells (see arrowheads in Figure 4G). These results demonstrate that transgenic overexpression of TGFα in mouse pancreas results in Notch pathway activation in pancreatic cancer precursors. In addition, the documentation of both Hes1 and Pdx1 expression in premalignant metaplastic epithelium further emphasizes the similarity between this epithelium and the undifferentiated epithelium of the developing pancreas.

TGFα-induced Notch activation is required for initiation of the metaplasia/neoplasia sequence

In order to clarify the functional significance of TGFα-induced Notch activation in exocrine pancreas, we further evaluated the relationship between these signaling pathways in epithelial explants. We have recently demonstrated that explant cultures of pancreatic tissue from transgenic mice overexpressing TGFα spontaneously initiate the metaplasia/neoplasia sequence, and that this effect can be induced in explant cultures of normal

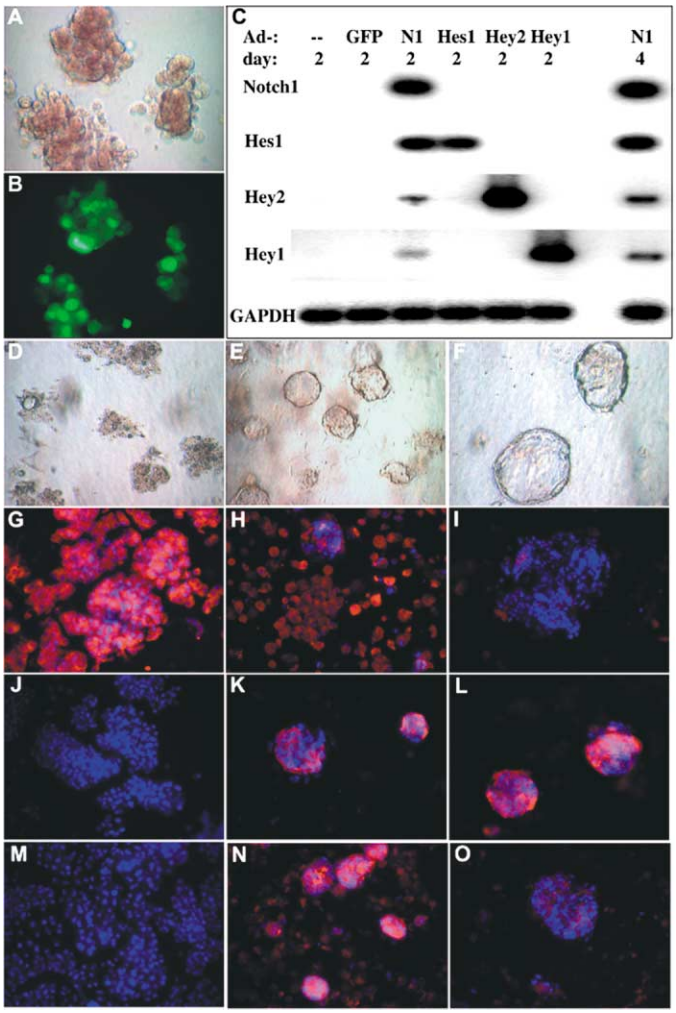

Figure 3. Acinar-to-ductal metaplasia induced by ectopic Notch activation in pancreatic explants

A and **B:** Corresponding phase-contrast (**A**) and fluorescent (**B**) images demonstrating high-efficiency expression of GFP in pancreatic explants 24 hr after infection with Ad-GFP (400×). Similar levels of GFP expression were obtained for all adenoviral vectors.

C: RT-PCR analysis of Notch1 and Notch target gene expression at 2 and 4 days following infection with Ad-GFP, Ad-GFP/Notch1-IC (N1), Ad-GFP/Hes1, Ad-GFP/Hey1, and Ad-GFP/Hey2.

D: Low-frequency of acinar-to-ductal metaplasia in pancreatic explants 5 days after infection with Ad-GFP (20×).

E (20×) and **F** (100×): High-frequency acinar-to-ductal metaplasia in pancreatic explant 5 days after infection with Ad-GFP/Notch1-IC.

G–O: (100×), Immunofluorescent analysis of amylase (**G–I**), cytokeratin 20 (**J–L**), and nestin (**M–O**) expression on day 0 (**G**, **J**, and **M**), day 3 (**H**, **K**, and **N**), and day 5 (**I**, **L**, and **O**) following infection of pancreatic explants with Ad-GFP/Notch1-IC.

pancreas by treatment with soluble recombinant TGFα (Wagner et al., 2002). We therefore treated explant cultures of normal mouse pancreas with and without TGFα and evaluated expression of endogenous Notch target genes by semiquantitative RT-PCR (Figure 5A). Prior to the onset of TGFα-induced acinar-to-ductal metaplasia, transcripts for Hes1 and Hey1 were minimally detectable following 25 cycles of PCR amplification. Increased expression of these Notch target genes was never observed in untreated explant cultures over a five day time course. TGFα-treated explants demonstrated accumulation of transcripts for both Hes1 and Hey1, coincident with initiation and progression of acinar-to-ductal metaplasia. In contrast, no activation of Hey2 was observed during this process (data not shown). These data suggest that TGFα activates Notch signaling during initiation of the metaplasia/neoplasia sequence in vitro, similar to the in vivo activation observed in TGFα transgenic mice.

Further evidence of Notch pathway activation during TGFα-induced acinar-to-ductal metaplasia was provided by examining the acetylation status of histone proteins associated with *cis*-acting elements in the Hes1 promoter. In the absence of Notch-IC, RBP-Jκ functions as a transcriptional repressor in complex with SMRT and the histone deacetylase, HDAC-1. In this setting, histone proteins associated with RBP-Jκ-regulated transcriptional units are characterized by low acetylation levels. Transcriptional activation of Notch target genes involves disruption of the RBP-Jκ/SMRT/HDAC-1 corepressor complex in favor of a complex involving RBP-Jκ and Notch-IC, resulting in histone acetylation and conversion of RBP-Jκ from a transcriptional repressor to a transcriptional activator (Kao et al., 1998). We therefore examined the acetylation status of histone proteins associated with *cis*-acting elements in the Hes1 promoter using chromatin immunoprecipitation (ChIP) assays. Following cross-linking and immunoprecipitation of cellular lysates using antibodies recognizing either acetylated histone H3 or acetylated histone H4, we utilized semiquantitative PCR to determine the amount of associated genomic DNA corresponding to a 545 bp element spanning an RBP-Jκ binding site in the Hes1 promoter. Initial assay validation confirmed a Notch-dependent increase in Hes1 promoter-associated acetylated histone H3 (but not histone H4) in cultured pancreatic acinar cells (Figure 5B). An identical increase in Hes1 promoter-associated acetylated histone H3 was achieved by treatment of pancreatic acinar cells with trichostatin A (TSA), an inhibitor of HDAC previously shown to potentiate expression of Notch target genes in *Xenopus* animal caps (Kao et al., 1998). In cellular lysates prepared from explant cultures of mouse pancreas treated with and without TGFα, a consistent 4-fold increase in Hes1 promoter-associated acetylated histone H3 was observed following treatment with TGFα (Figure 5C). These data demonstrate that TGFα-induced acinar-to-ductal metaplasia is associated with changes in Hes1 promoter-associated histone H3 acetylation identical to those induced by Notch or by pharmacologic inhibition of HDAC.

To further determine whether TGFα-induced upregulation of Hes1 and Hey1 was mediated by classical Notch pathway activation, we utilized pharmacologic inhibitors of γ-secretase, an intramembrane protease known to be required for activating cleavage of Notch receptors following ligand binding (Berezovska et al., 2000; De Strooper et al., 1999). Compound 1, a previously characterized peptidomimetic inhibitor of γ-secretase (Wolfe et al., 1999), eliminated induction of both Hes1 and Hey1 following treatment of pancreatic explants with TGFα (Figure 5D). Similarly, this compound effectively downregulated Hes1 protein levels in BXPC3 human pancreatic cancer cells (data not shown). In both BXPC3 cells and mouse pancreatic explants, the IC_{50} for downregulation of Hes1 expression by compound 1 was ~30 μM, consistent with previous reports regarding inhibition of Notch1-IC generation in CHO cells (Berezovska et al., 2000). These findings suggest that Hes1 expression in human pancreatic cancer cell lines and TGFα-induced

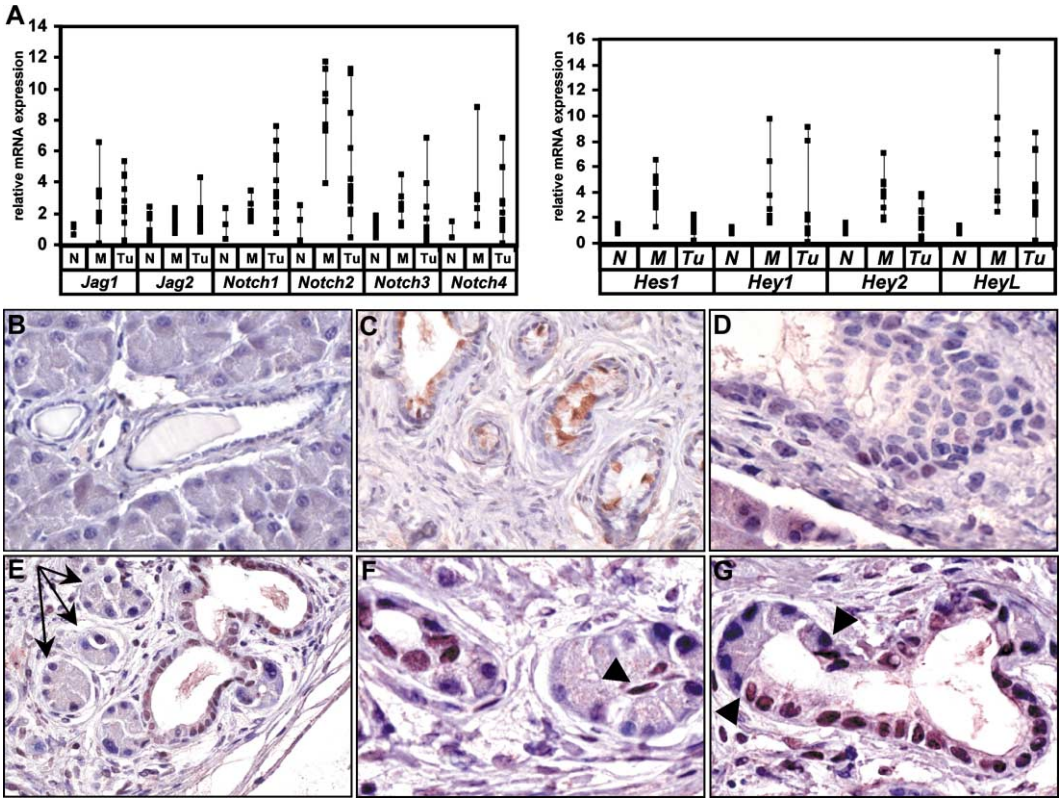

Figure 4. Transgenic overexpression of TGFα results in Notch pathway activation in premalignant pancreatic epithelium and malignant pancreatic cancer

A: Real-time RT-PCR analysis demonstrating relative expression of Notch receptors, ligands, and target genes in normal mouse pancreas (N), metaplastic Ela-TGFα mouse pancreas (M), and invasive tumors from Ela-TGFα;p53$^{+/-}$ mouse pancreas (Tu).
B: Absence of immunohistochemically detectable Notch1 protein in normal pancreatic ductal epithelium from nontransgenic littermate control (100×).
C: Upregulated Notch1 protein in premalignant metaplastic epithelium from MT-TGFα transgenic mouse pancreas (100×).
D: Absence of immunohistochemically detectable Hes1 protein in normal pancreatic ductal epithelium from nontransgenic littermate control (400×).
E: Detection of nuclear Hes1 protein in premalignant metaplastic epithelium from MT-TGFα mouse pancreas. Arrows indicate absence of Hes1 protein in adjacent normal acini (100×).
F: Nuclear Hes1 protein in metaplastic epithelium as well as centroacinar cells (arrow) from MT-TGFα mouse pancreas (400×).
G: Abrupt transition (arrows) between Hes1-positive metaplastic epithelium and Hes1-negative acinar cells (400×).

Hes1 expression in mouse pancreatic explants are both mediated by classical γ-secretase-dependent Notch pathway activation.

To determine whether Notch pathway activation was required for TGFα-mediated changes in epithelial differentiation, we examined the effects of compound 1 and three additional peptidomimetic inhibitors of γ-secretase on TGFα-induced acinar-to-ductal metaplasia. In explant cultures of normal mouse pancreas, compound 1 effectively prevented acinar-to-ductal metaplasia at concentrations identical to those required for downregulation of TGFα-induced Hes1 activation (Figure 6). In addition, this compound effectively inhibited spontaneous initiation of the metaplasia/neoplasia sequence in explant cultures of MT-TGFα pancreas. Dose-dependent effects were also observed for compound 11, WPE-III-31C, and DAPT. In contrast, a stereoisomer of compound 11 containing a D-valine residue had no effect, minimizing the possibility that the observed effects of γ-secretase inhibition in this system were mediated through nonspecific cellular toxicity. Further confirmation that the effect of γ-secretase inhibition was specifically mediated by a disruption in Notch signaling was achieved using Ad-GPF/Notch1-IC, encoding a "pre-cleaved" Notch1 intracellular domain not requiring γ-secretase-mediated proteolytic activation. Ad-GFP/Notch1-IC remained fully capable of "rescuing" acinar-to-ductal metaplasia in compound 1-treated explants, even at compound 1 concentrations associated with effective inhibition of the TGFα effect (Figure 7). These findings confirm that the effects of γ-secretase inhibition in this system were specifically due to a deficit in generation of activated Notch. Thus, TGFα not only induces γ-secretase-dependent Notch activation in pancreatic epithelial explants, but this activation of Notch is also required for TGFα-induced initiation of pancreatic epithelial metaplasia. Further insights regarding functional interactions between Notch and EGF receptor signaling in the induction of pancreatic epithelial metaplasia were provided by evaluating the effects of AG1478, a specific inhibitor of EGF receptor tyrosine kinase activity. At low micromolar concentrations, AG1478 completely abolished induction of acinar-to-ductal metaplasia by TGFα, but had little-to-no effect on changes in epithelial differentiation induced by Ad-GFP/Notch1-IC (Figure 7). Notch pathway activation is therefore capable of bypassing a requirement for EGF receptor signaling in the induction of

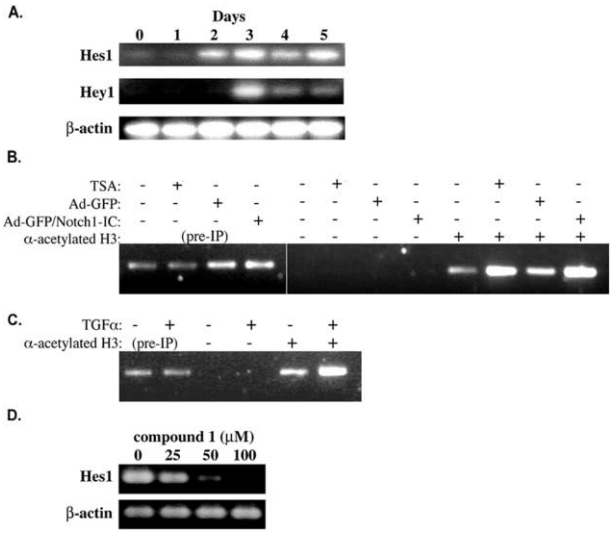

Figure 5. TGFα induces Notch pathway activation during acinar-to-ductal metaplasia in explant cultures of normal mouse pancreas

A: Semiquantitative RT-PCR analysis demonstrating activation of endogenous Notch target genes during TGFα-induced acinar-to-ductal metaplasia.
B: Pre-IP material and ChIP assay for Hes1 promoter-associated acetylated histone H3 following treatment of 266-6 cells with either TSA or Ad-GFP/Notch1-IC. Pre-IP material demonstrates equal amounts of input Hes1 genomic DNA in TSA versus control and Ad-GFP/Notch1-IC versus Ad-GFP conditions. Both TSA and Ad-GFP/Notch1-IC increase Hes1 promoter-associated acetylated histone H3.
C: Pre-IP material and ChIP assay for Hes1 promoter-associated acetylated histone H3 following treatment of mouse pancreatic explants with and without TGFα. TGFα-treated explants demonstrate an increase in Hes1 promoter-associated acetylated histone H3.
D: Semiquantitative RT-PCR analysis demonstrating effective inhibition of TGFα-mediated Hes1 activation by increasing concentrations of compound 1.

acinar-to-ductal metaplasia. Together with documented Notch pathway activation following transgenic overexpression of TGFα, these results demonstrate that Notch pathway activation represents a required downstream mediator of EGF receptor signaling in exocrine pancreas.

Discussion

The current results demonstrate activation of Notch signaling as an extremely early event in pancreatic tumorigenesis and further suggest that Notch pathway activation is a required downstream mediator of EGF receptor activity in the pathogenesis of this disease. As a surrogate marker of Notch activity, Hes1 protein was identified not only in invasive pancreatic ductal adenocarcinomas, but also in metaplastic and neoplastic pancreatic cancer precursors in both mouse and human. The association of Notch with early events in a metaplasia/neoplasia sequence is further supported by the finding that ectopic Notch activation induces acinar-to-ductal metaplasia in explant cultures of normal mouse pancreas, and that Notch is required for mediation of TGFα-induced changes in epithelial differentiation. Future study will determine whether these effects are unique to pancreas, or whether Notch signaling represents a general mechanism for initiating metaplastic conversion between epithelial cell types during mammalian tumorigenesis.

In developing pancreas and other foregut derivatives, Notch signaling inhibits early commitment of epithelial progenitors to an endocrine lineage, apparently by suppressing expression of ngn3 and neuroD/β2 (Apelqvist et al., 1999; Jensen et al., 2000a, 2000b). Targeted deletion of Notch pathway components results in precocious endocrine differentiation, depletion of epithelial progenitors, and failure to differentiate normal exocrine lineages. Although these previous studies demonstrate that Notch pathway activation is required to reserve a population of exocrine precursors during early pancreatic development, our data suggest that Notch signaling is silenced in mature exocrine pancreas and that ectopic reactivation of Notch results in loss of acinar cell differentiation. As in other cellular contexts, Notch target genes may actively repress expression of lineage-specifying transcription factors in exocrine pancreas. In this regard, our demonstration of nestin-positive intermediates during Notch-induced acinar-to-ductal metaplasia suggests that Hes1 and/or Hey1 may actively repress expression of transcription factors required for maintenance of acinar cell differentiation, resulting in expansion of a dedifferentiated nestin-positive population and initiation of the metaplasia/neoplasia sequence. While the role of nestin-positive epithelial precursors in developing pancreas remains controversial (Hunziker and Stein, 2000; Lechner et al., 2002; Selander and Edlund, 2002; Zulewski et al., 2001), our finding of Notch-induced expansion of nestin-positive intermediates is consistent with previous reports demonstrating an association between Notch signaling and maintenance of nestin-positive precursors in developing mouse brain (Irvin et al., 2001; Lutolf et al., 2002).

Based on the requirement for Notch pathway activation in mediating the effects of TGFα, the current data demonstrate a direct relationship between EGF receptor activation and Notch signaling in the generation of pancreatic cancer precursors. There is significant precedent for crosstalk between EGF and Notch signaling in a number of developmental systems, with both cooperative and antagonistic interactions previously reported (Price et al., 1997; zur Lage and Jarman, 1999). However, interactions between Notch and EGF receptor signaling have not been previously reported in the context of mammalian neoplasia. Based on the role of ras in mediating certain aspects of EGF receptor signaling, it is interesting to consider interactions between ras signaling and Notch pathway activation during pancreatic tumorigenesis. Reciprocal interactions between ras and Notch have been described during vulval development in *C. elegans* (Berset et al., 2001) and during retinal, mesodermal, and peripheral nervous system development in *Drosophila* (Carmena et al., 2002; Culi et al., 2001; Tomlinson and Struhl, 2001). In mammalian cells, activated forms of both Notch1 and Notch2 are capable of substituting for activated ras in cooperative transformation assays (Capobianco et al., 1997), and interactions between ras signaling and Notch are required for maintenance of the malignant phenotype induced by activated Notch4 expression in mouse mammary epithelium (Fitzgerald et al., 2000). Additional evidence suggests that Notch is required for initiation and maintenance of the malignant phenotype in ras-transformed cells and that oncogenic ras is capable of activating Notch signaling, apparently by increasing expression of Notch1, Delta1, and presenilin-1 (Ruiz-Hidalgo et al., 1999; Weijzen et al., 2002). Based upon previously documented ras pathway activation in pancreatic tissue from transgenic mice overexpressing TGFα (Wagner et al., 2001), as well as the high rate of

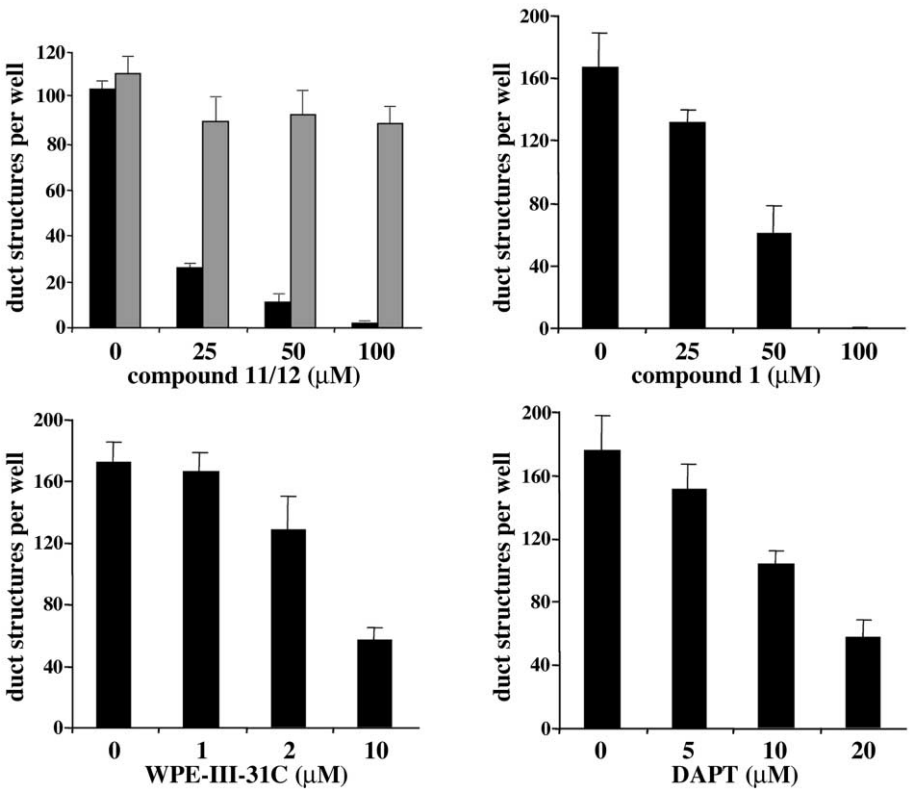

Figure 6. Requirement for Notch signaling in TGFα-mediated acinar-to-ductal metaplasia

Graphs demonstrate effect of four different γ-secretase inhibitors (compound 11, compound 1, WPE-III-31C, and DAPT) as well as inactive stereoisomer (compound 12) on TGFα-induced acinar-to-ductal metaplasia. Black bars indicate effect of active inhibitors, gray bars indicate effect of compound 12.

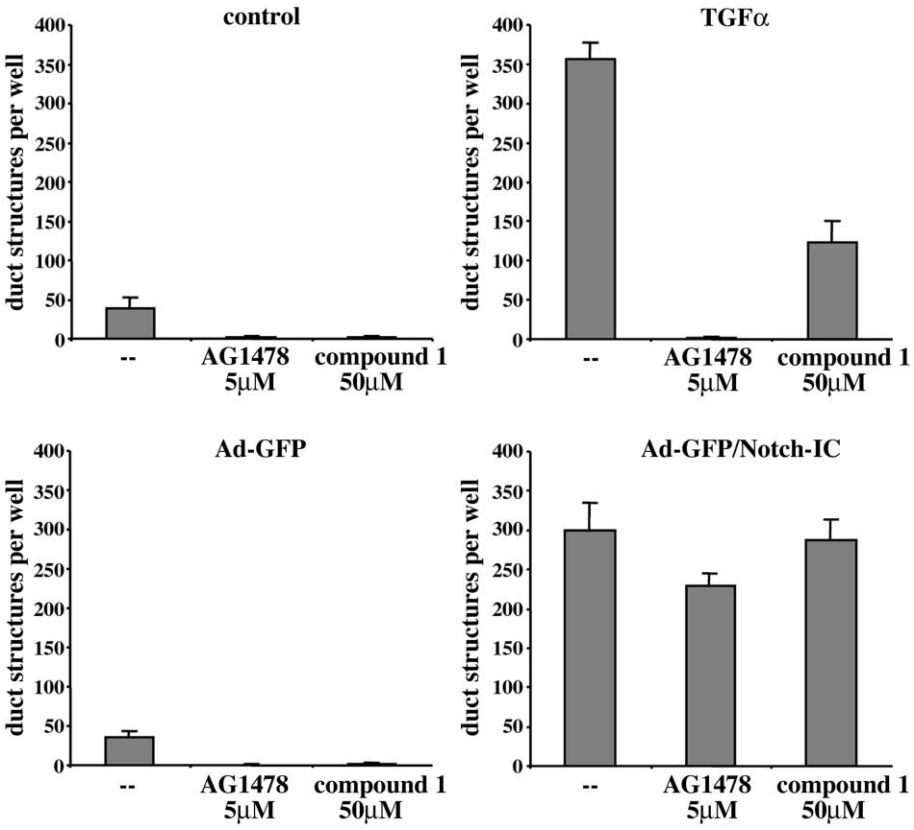

Figure 7. Functional interactions between EGF and Notch signaling during TGFα-mediated acinar-to-ductal metaplasia

The EGF receptor tyrosine kinase inhibitor AG1478 eliminates both basal and TGFα-induced acinar-to-ductal metaplasia, but has minimal effect on acinar-to-ductal metaplasia induced by Ad-GFP/Notch1-IC. The γ-secretase inhibitor compound 1 effectively prevents TGFα-induced acinar-to-ductal metaplasia, but has no effect on acinar-to-ductal metaplasia induced by Ad-GFP/Notch1-IC.

activating ras mutations observed in human pancreatic cancer (Hruban et al., 1999), this relationship may contribute to the upregulated expression of Notch receptors and ligands observed in the current study.

In combination with the recent identification of acinar-to-ductal metaplasia and PanIN formation as precursor lesions for pancreatic ductal adenocarcinoma (Hruban et al., 1999; Wagner et al., 1998, 2001), the current data provide an opportunity to generate a unifying model for pancreatic tumorigenesis. We propose that augmented EGF receptor activity represents an initiating event (Korc, 1998; Wagner et al., 2001), resulting in Notch pathway activation in exocrine tissue. Notch activation induces dedifferentiation of mature exocrine cells and expansion of metaplastic ductal epithelium sharing features with embryonic pancreas (Song et al., 1999). These Notch-mediated changes in epithelial differentiation promote formation of early PanIN lesions, arising in either metaplastic or preexisting ductal epithelium. Once formed, PanIN lesions undergo progressive accumulation of well-characterized genetic changes (Moskaluk et al., 1997; Wilentz et al., 2000), ultimately resulting in invasive ductal cancer. In addition to altering epithelial differentiation to promote generation of precursor lesions, Notch may also provide an ongoing growth advantage to malignant epithelium, reflected by maintenance of an active Notch pathway in invasive pancreatic cancer. In this regard, Notch signaling has previously been associated with activation of NF-kappaB (Bellavia et al., 2000; Cheng et al., 2001; Nickoloff et al., 2002; Oswald et al., 1998) and enhanced expression of bcl-2 and cyclin D1 (Deftos and Bevan, 2000; Ronchini and Capobianco, 2001), factors which potentially contribute to the malignant phenotype of human pancreatic cancer (Arlt et al., 2001; Gansauge et al., 1997; Hu et al., 1999).

Experimental procedures

For detailed procedures, See Supplemental Data online at http://www.cancercell.org/cgi/content/full/3/6/565/DC1.

Expression profiling in normal and malignant pancreas

Total RNA was extracted from bulk normal pancreas (n = 29) and bulk resected pancreatic ductal adenocarcinoma (n = 26). cRNA was hybridized to the complete Affymetrix Human Genome U95 GeneChip® set, and data analysis was performed using Gene Logic Inc. BioExpress™ analysis software (Iacobuzio-Donahue et al., 2002a). Relative cDNA levels were also determined in ten additional cancers and five samples of normal pancreas using real-time PCR (TaqMan, PE Applied Biosystems) as previously described (Wagner et al., 2001).

Immunohistochemistry and in situ hybridization

Immunolabeling was performed using both traditional formalin-fixed paraffin blocks as well as tissue microarrays containing samples of formalin-fixed nonpancreatic tissue, normal pancreas, chronic pancreatitis, PanIN, and invasive pancreatic cancer. Fifty total human pancreatic cancer specimens were utilized, of which 34 were included on tissue microarrays. All staining was conducted simultaneously with negative control slides utilizing secondary antibody only. In the case of Hes1, specificity was additionally confirmed by preincubation of primary antibody with synthetic Hes1 peptide. In the case of jagged1, expression was assessed by in situ hybridization using previously described techniques (Iacobuzio-Donahue et al., 2002b).

Transgenic mouse lines

The Ela-TGFα and MT-TGFα mouse lines were initially generated and provided by Dr. Eric Sandgren (University of Wisconsin). Breeding, genotyping, and transgene induction were performed as previously described (Song et al., 1999; Wagner et al., 2001).

Ectopic Notch pathway activation in pancreatic explants

Explant cultures of collagenase-digested mouse pancreas were prepared as previously described (Wagner et al., 2002) and infected with previously characterized recombinant adenoviral vectors encoding either GFP alone or GFP with Notch1-IC, Notch2-IC, or Hes1 (Sriuranpong et al., 2001). In addition, adenoviral vectors encoding N-terminal Flag epitope-tagged Hey1 and Hey2 were generated in a pShuttle backbone using previously established techniques (He et al., 1998). All infections were performed at an MOI of 20:1 for 1 hr at 37°C. Confirmation of successful viral transduction was accomplished by examination of high-efficiency GFP expression as well as RT-PCR for encoded cDNA's and Notch target genes.

Evaluation of endogenous Notch pathway activation in TGFα-treated explants

Acinar-to-ductal metaplasia was induced in explant cultures of normal mouse pancreas by daily treatment with recombinant human TGFα (50 ng/ml; R&D Systems). Endogenous Notch pathway activation was determined by semiquantitative RT-PCR for Hes1, Hey1, and Hey2. To evaluate TGFα-induced changes in histone acetylation in association with Hes1 promoter elements, chromatin immunoprecipitation (ChIP) assays were performed using Acetyl-Histone H3 and Acetyl-Histone H4 Immunoprecipitation Assay Kits (Upstate Biotechnology) according to manufacturer's recommendations. Hes1 promoter sequences in complex with acetylated histones were amplified with PCR primers chosen to specifically amplify genomic DNA flanking a highly conserved RBP-Jκ binding site in the Hes1 promoter.

Notch pathway inhibition

Endogenous Notch pathway inhibition was achieved using the following peptidomimetic inhibitors of γ-secretase: compound 1 (DFK-167; Enzyme Systems Products, Livermore, California), compound 11 (Wolfe et al., 1999), WPE-III-31C (Esler et al., 2002), and DAPT (Dovey et al., 2001). Control explants were treated with either DMSO vehicle or with compound 12, an inactive stereoisomer of compound 11 (Wolfe et al., 1999). Compounds 11 and 12, WPE-III-31C, and DAPT were synthesized as previously described and assessed for purity by 1H NMR (300 MHz), mass spectrometry (MALDI-TOF), and elemental analysis (Desert Analytics, Tucson, Arizona).

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