Inhibition of γ-Secretase Activity Inhibits Tumor Progression in a Mouse Model of Pancreatic Ductal Adenocarcinoma

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Abstract

Background & Aims—The Notch signaling pathway is required for the expansion of undifferentiated pancreatic progenitor cells during embryonic development and has been implicated in the progression of pancreatic ductal adenocarcinoma (PDAC). The interaction of Notch ligands with their receptors promotes a γ-secretase-dependent cleavage of the Notch receptor and release of the Notch intracellular domain, which translocates to the nucleus and activates transcription. We investigated the role of this pathway in PDAC progression.

Methods—We tested the effects of a γ-secretase inhibitor (GSI) that blocks Notch signaling in PDAC cell lines and a genetically engineered mouse model of PDAC (Kras p53 L/+ mice).

Results—Notch signaling was activated in PDAC precursors and advanced tumors. The GSI inhibited the growth of premalignant pancreatic duct-derived cells in a Notchdependent manner. Additionally, in a panel of over 400 human solid tumor-derived cell lines, PDAC cells, as a group, were more sensitive to the GSI than any other tumor type. Finally, the GSI completely inhibited tumor development in the genetically engineered model of invasive PDAC ($p<0.005$ χ^2 test; compared with mice exposed to vehicle).

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Conclusions—These results suggest that Notch signaling is required for PDAC progression. Pharmacologic targeting of this pathway offers therapeutic potential in this treatment-refractory malignancy.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancerrelated mortality in the United States with over 34,000 deaths predicted in 2008.[1] It is characterized by its treatment-refractory behavior and dismal 5-year survival. The current standard of care for advanced disease, gemcitabine, results in modest clinical benefit, with a median increase in overall patient survival of 5 weeks.[2] To date, clinical trials using a range of targeted therapies have failed to appreciably extend survival.

The cell of origin and molecular pathogenesis of PDAC have yet to be fully defined. PDAC evolves from duct-like tubular complexes (metaplasias) and ductal precursor lesions known as Pancreatic Intraepithelial Neoplasias (PanINs) that occur in association with Kras mutations.[3] It is not clear in which cells the Kras mutations arise, leading to PDAC,[4–6] although recent evidence supports the notion that acinar cells can serve as a cell of origin.[7, 8] Furthermore, while a number of developmental signaling pathways have been proposed to contribute to PanIN initiation and progression, most have yet to be functionally tested. Defining the processes that are required for progression from PanIN precursor lesions to PDAC could lead to the design of improved treatment approaches to this disease. The validation of new targets is of particular significance in PDAC since progress in the therapeutic targeting of RAS has lagged compared to advances in targeting other commonly detected oncoproteins (e.g. EGFR, KIT, etc)[9].

The Notch signaling pathway has been implicated in the pathogenesis of a number of malignancies, including PDAC[10]. Notch signaling is activated by interaction of Notch ligands with their receptors, promoting a γ-secretase-dependent cleavage of the Notch receptor and release of the Notch intracellular domain (Notch-IC)[10, 11]. Notch-IC translocates to the nucleus where it serves to activate transcription by binding to the CSL transcription factor and Mastermind-like transcriptional co-activator MAML. This complex activates the transcription of a number of target genes, including members of the Hairy enhancer of split (Hes) family.

During pancreatic development, the Notch signaling pathway promotes expansion of pancreatic progenitors as reflected by gain- and loss-of-function studies[12–16]. In the adult pancreas the Notch target gene Hes1 is expressed in centroacinar cells and some ductal cells indicating likely Notch signaling activity in these cells[17]. The centroacinar localization is notable as these cells have been proposed to have progenitor-like properties and are candidates for the PDAC cell-of-origin[6]. In response to pancreatic damage or oncogene expression, Hes1 is upregulated in association with the development of duct-like tubular complexes[7, 17–21], and multiple components of the Notch signaling pathway are expressed or upregulated in advanced PDAC[7, 17, 22]. In addition, Notch signaling is required for normal pancreatic exocrine regeneration in response to pancreatic injury provoked by cholecystokinin analogue, cerulein[23], and may contribute to the proliferation of PDAC cell lines[24]. In sum, these data point to a potential contribution of Notch signaling in PDAC pathogenesis from its earliest to latest stages.

The proteolytic cleavage by γ-secretase required to activate Notch provides a therapeutically vulnerable point in the pathway[25], and a number of γ-secretase inhibitors (GSIs) have been developed that effectively block Notch activity and can inhibit growth of some tumor cell lines. In this study, we have directly monitored Notch activity in the normal

and neoplastic pancreas and studied the capacity of a GSI to block PDAC growth in a large panel of human cell lines and a genetically engineered mouse model (GEMM) that recapitulates the genetics and histopathogenesis of the human disease.

Methods

Cell culture

Human PDAC cell lines were obtained from ATTC, from Anirban Maitra, and from Christine Iacobuzio-Donahue. Derivation and cultivation of murine pancreatic duct cells and PanIN cells from the pancreata of wild type or Pdx1-Cre LSL-KRASG12D mice, respectively, were as described previously[26]. PDAC cell lines from Pdx-Cre p53 Kras mice were established and cultivated as described[27].

Animals

Therapeutic studies were performed with Pdx1-Cre; LSL-KrasG12D; p53$^{lox/+}$ mice[28]. To examine activation of Notch signaling *in vivo*, these mice were crossed to a transgenic GFP reporter strain[29]. qRT-PCR analysis of Notch pathway components was performed with Pdx1-Cre; LSL-KrasG12D; Ink4a/Arf$^{lox/+}$+ mice, which develop PanIN lesions and PDAC with similar kinetics[28].

Compound

MRK-003 was synthesized according to standard medicinal chemistry procedures and was provided by Merck Research Laboratories, Boston, MA. For the *in vitro* experiments, stocks were prepared at 10mM in DMSO and dilutions were made directly before use. For the *in vivo* experiments, MRK-003 was dosed as a suspension in 0.5% methylcellulose (Sigma) made fresh daily. Animals were treated by gavage with 100mg/kg once daily, using a 3-days on and 4-days off intermittent dose schedule. Control animals were treated with vehicle alone using an equivalent dosing schedule.

Histology and immunohistochemistry

Tissues were processed as reported previously[28]. Antibodies and conditions for immunohistochemistry are presented in the Supplementary Methods. The histology was reviewed by a pancreatic pathologist. For the quantitation of the PanIN to total pancreas tissue ratio we counted the percentage of cells in 5–10 100× microsope fields / sample. In each field the different tissues (normal or diseased) were marked using Photoshop and finally compared with each other. One total microscope field was calculated as 100%.

Cellular, molecular, and statistical analyses

Detailed descriptions of procedures are provided in Supplementary Methods.

Results

Activation of the Notch pathway in Murine PDAC

Previous studies have suggested that Notch signaling is activated throughout the course of PDAC progression based on the expression of Notch pathway components[17]. To directly assess the activity of the pathway in PDAC pathogenesis, and to validate the use of Hes1 expression as a surrogate for Notch activity, we crossed a transgenic Notch-responsive GFP reporter strain[29] onto the Pdx1-Cre LSL-Kras^{G12D} p53$^{Lox/+}$ mouse model of PDAC (from hereon, designated Kras p53 L/+ mice)[28]. In the normal pancreas, strong Notch signaling, as visualized by immunohistochemical (IHC) staining for the GFP reporter, was most pronounced in cells occupying a centroacinar position (Fig. 1A), consistent with the reported

centroacinar staining pattern of Hes1[17, 20]. The majority of premalignant PanIN lesions, previously reported to exhibit strong Hes1 staining, also showed activation of the Notch reporter (Fig. 1B). Hence, as assessed by an independent measure of pathway function, Notch signaling is active in centroacinar cells, PanIN, and PDAC.

Next we wished to further define Notch pathway activity in the mouse model of advanced PDAC. By immunoblot analysis 8/9 primary PDACs were found to overexpress Hes1, and IHC confirmed that the tumor cells were the primary source of expression (Figure 1C and D). Notably, the only tumor lacking Hes1 expression (T4) displayed undifferentiated histopathology (data not shown). In accord with previous findings of Notch activation in early human pancreatic lesions and PDAC[17], expression profiling and quantitative reverse-transcriptase PCR (qRT-PCR) revealed that the Notch receptors, their ligands (Jagged and Delta families), and known target genes (Hes1, Hey1) were broadly upregulated in murine PanIN and PDAC relative to normal murine pancreas (Supplementary Figure 1).

To confirm that the Notch pathway was activated in the neoplastic epithelium, we performed immunoblot and qRT-PCR analysis of primary pancreatic ductal cells derived from Pdx1-Cre LSL-Kras[G12D] mice[27, 30] (PanIN cell lines) and PDAC cell lines from the Kras p53 L/+ model. All PanIN cell lines tested exhibited readily detectable Hes1 levels (Fig. 2A and data not shown), and 7/8 PDAC cell lines also overexpressed Hes1 (Fig. 1E). Moreover, induction of multiple Notch pathway components was observed in cultured PanIN and PDAC cells relative to cultured primary duct cells (Fig. 1F). Notch3 showed the most pronounced induction of all pathway components tested, consistent with prior observations from human pancreatic tumors[17]. The induction of several Notch ligands during PanIN and PDAC progression suggests that paracrine or autocrine signaling by tumor cells may be at least partially responsible for pathway activation *in vivo*.

Murine and human PDAC cell lines are sensitive to GSI

Based on the observed activation of the Notch pathway throughout the course of PDAC progression, we sought to assess the sensitivity to Notch inhibition of PanIN and PDAC cell lines by treatment with the cyclic sulfamide GSI, MRK-003[31]. MRK-003 is a potent (0.72 nM for NICD cleavage in vitro) and specific GSI that is orally bioavailable, with a plasma half-life of 12hr at 100mg/kg. MRK-003 treatment led to a rapid reduction in Hes1 expression in PanIN cell lines at concentrations as low as 0.75 μM, consistent with an inhibition of Notch activity (Fig. 2A left panel). Treatment of PanIN cells with 0.75–2.5 μM MRK-003 effectively blocked proliferation after 3–4 days exposure (Fig. 2A right panel). Next we selected a set of human PDAC-derived cell lines (N=3) and those derived from the Kras p53 mouse model (N=4) that showed robust Hes1 expression. These cells lines also demonstrated substantially reduced cell proliferation upon MRK-003 treatment, accompanied by loss of Hes1 expression (Fig. 2B, Supplementary Fig. 2, and data not shown). Furthermore, MRK-003 strongly inhibited the ability of murine and human PDAC cells to form clones in soft agar compared to vehicle treated cells, indicating that active Notch signaling is necessary for anchorage-independent clonogenic growth of these cells (Fig. 2C and data not shown).

We next examined whether the effects of MRK-003 seen in these assays were specific for pancreatic cancer, or reflected a more general ability to inhibit cell growth. We employed a high-throughput platform[32] to assess the sensitivity of 434 human cell lines derived from a variety of solid tumor types to MRK-003. Notably, we found that, as a group, PDAC-derived cell lines exhibit the greatest sensitivity to Notch inhibition compared to the other tumor types, with half of the lines (13/26) showing at least a 25% decrease in cell number after 3 days treatment with 5 μM MRK-003 relative to vehicle (Fig. 2D). In contrast, cell lines from other cancer types, including those reported to be sensitive to GSI, showed

considerably lower response rates, including breast (28%), kidney (24%), and non-small cell lung cancer (33%)[10, 33, 34]. These data suggest that γ-secretase inhibition may be particularly effective in the context of PDAC.

MRK-003 effectively inhibits Notch signaling *in vivo*

Given the strong induction of Notch signaling in PanIN and the sensitivity of PanIN cell lines to MRK-003, we wished to test the possibility that GSI treatment could block the development of PanIN and/or PDAC *in vivo*. Previous work evaluating GSIs for the treatment of Alzheimer's disease and recent findings in renal cell carcinoma, demonstrated that rapid weight loss is dose-limiting, in part due to the role of Notch signaling in the control of intestinal progenitor cell fate[33, 35–37]. We developed an intermittent dosing regimen consisting of three daily oral doses of 100mg/kg followed by four days recovery. Intermittent treatment maintains target inhibition associated with *in vivo* efficacy while achieving tolerable intestinal side effects (manuscript in preparation). This dose and schedule effectively attenuated Notch signaling as indicated by a decrease in Hes1 expression in subcutaneously implanted PDACs (Fig. 3A and Supplementary Fig. 3A).

To further establish the *in vivo* efficacy of this dosing regimen, we assessed two biomarkers of γ-secretase activity: processing of the Amyloid Precursor Protein (APP) and intestinal goblet cell differentiation[38]. APP is processed by a two-step proteolytic pathway with γ-secretase serving as the last step that releases the Aβ40 peptide[39]. MRK-003-treated animals showed a significant reduction in the serum levels of Aβ40 compared to vehicle-treated controls, indicating effective inhibition of γ-secretase (Supplementary Fig. 3B). γ-secretase activity is also required for normal intestinal homeostasis, and inhibition of the Notch pathway within the intestinal crypt cells promotes goblet cell differentiation[35, 38]. Consistent with an inhibitory effect on Notch signaling, MRK-003 provoked goblet cell differentiation (Fig 3B). Nevertheless, the intestinal epithelium returned to normal after the four-day recovery period and both wildtype and Kras p53 mice treated with GSI showed normal weight gain and overall health through multiple cycles of this dosing regimen (data not shown). Furthermore, thymus and spleen were grossly normal with no detectable changes in organ size. Hence, MRK-003 can effectively block GSI activity and inhibit Notch signaling in vivo without overtly deleterious side effects.

γ-secretase activity is required for PDAC progression

To determine whether γ-secretase activity is required for the growth of premalignant and malignant cells *in vivo*, we used a highly faithful GEMM of PDAC: the Kras p53 L/+ model[28]. We initiated a treatment trial in which Kras p53 L/+ mice and control animals were treated with MRK-003 or vehicle starting at age 6 weeks. At this time point, Kras p53 L/+ mice exhibit isolated PanIN lesions but do not harbor established PDAC (unpublished data). In untreated mice, PanIN lesions progress to advanced PDAC requiring euthanasia with a mean latency of ~ 18 weeks. Necropsy at earlier ages reveals focal PDAC as early as ~ 11 weeks (mean ~ 15 weeks; unpublished data). Mice were maintained on the dosing regimen until they were euthanized either at 11 or 13 weeks, enabling analysis of the impact of MRK-003 on PanIN-to-PDAC progression.

Autopsy revealed that 12 of 34 of the vehicle-treated mice had gross pancreatic tumors whereas none of 25 MRK-003 treated animals exhibited pancreatic tumors (Fig. 3C and D; p<0.005). There was also a marked effect of MRK-003 treatment on PanIN formation. Most untreated mice (65%) lacked any detectable normal pancreatic tissue, and rather had a mixture of PanIN, ductal metaplasia and fibrosis. In GSI-treated animals, by contrast, diseased areas were focal in nearly all cases. Overall, the area occupied by normal pancreas comprised the majority of pancreatic tissue in only 20% of untreated mice while 75% of

treated mice had predominantly normal pancreas (Fig. 3E; p<0.005). GSI-treatment suppressed abundance of PanINs (p<0.005) and but not metaplastic ducts (Supplementary Fig. 3C). This increase in the regions of diseased pancreas in untreated mice was also reflected in an increase in the mean pancreatic-to-body mass ratio (Supplementary Fig. 3D; p<0.005, see Methods). GSI treatment of wild type control animals had no effect on pancreatic mass or histology (data not shown), indicating a specific effect of MRK-003 on Kras-driven tumorigenesis. These results suggest that sustained Notch pathway activity is required for the development of premalignant lesions and their progression to advanced PDAC in mice.

MRK-003 blocks epithelial proliferation

Next, we assessed whether MRK-003 affected cell proliferation or apoptosis in evolving PanINs in specimens analyzed after 4 weeks of treatment. In vehicle-treated mice, Ki-67 staining was abundant in regions of ductal metaplasia and PanIN, relative to normal pancreatic ducts (N=6 mice in each group, data not shown). MRK-003-treatment resulted in a significant decrease in Ki-67 staining in these lesions compared to the vehicle-treated animals (Fig. 3F; p<0.05). By contrast, comparable proportions of apoptotic nuclei were noted by TUNEL staining in PanIN lesions and tubular complexes from MRK-003- and vehicle-treated animals (Supplementary Fig. 4A). These results suggest that γ–secretase inhibition reduces the proliferative rate of premalignant cells but does not strongly influence apoptosis during PDAC progression *in vivo*.

MRK-003 exerts its growth effects through Notch signaling

Although these results indicate that MRK-003 effectively inhibits the Notch pathway, it is possible that its effects on cell proliferation are independent of this activity. To determine whether inhibition of Notch signaling mediates the growth inhibitory activity of the compound, we used MigR1 retroviral infection[40] to reactivate Notch signaling in PDAC cells exposed to MRK-003. Infectivity was monitored with an IRES-GFP contained within the viral vector, and infection rates reproducibly achieved 40–50%. Viral infection with the parental vector had no effect on the dose-responsiveness of NB507 PDAC cells to MRK-003 (Fig. 4A), and constitutively active Notch1 (ICN1) or Notch3 (ICN3) did not augment cell growth on their own (Fig. 4B). By contrast, infected cells were rescued from the growth-inhibitory effects of MRK-003 upon expression of ICN1 and ICN3 (Fig. 4C). These results suggest that MRK-003 inhibits PDAC cell growth through its effects on Notch signaling. Notably, we found that all five GSI-sensitive PDAC cell lines tested had robust expression of Hes1, while Hes1 was reduced or absent in a set of five GSI-resistant lines (Supplementary Fig. 5). Hence, relative Hes1 expression appears to be associated with GSI-sensitivity in PDAC cell lines. Infection of PDAC cells with retroviruses expressing the Notch target genes, c-Myc- or Hes1, did not prevent GSI-induced growth inhibition (Supp. Fig. 6), indicating that these genes are not sufficient to mediate the rescue effects of ICN.

Discussion

In this study, we used Kras p53 L/+ mice – which recapitulate most features of human pancreatic cancer, including progression from PanIN to PDAC, distant metastases, and reproducible genomic changes – to determine whether Notch signaling is required for the development of advanced lesions. We have confirmed that the Notch pathway is activated from the earliest stages of murine PDAC and that human and murine premalignant and malignant cells depend upon Notch signaling for growth. Indeed, human PDAC-derived cell lines – which commonly exhibit resistance to chemotherapeutic agents – demonstrate increased sensitivity to blockade of the pathway in comparison with other solid tumors. Most significantly, treatment of Kras p53 L/+ mice with the GSI, MRK-003, attenuates the

progression of PanIN to PDAC. Therefore, the Notch signaling pathway appears to be required for initiation of murine PDAC and may be a viable therapeutic target in this treatment-refractory malignancy in humans.

Since we treated mice early in the PanIN-to-PDAC sequence, it is possible that Notch signaling is required at multiple stages of disease progression. For example, MRK-003 might block the initial development of PanIN lesions and/or tubular complexes. Previous work has implicated Notch signaling in PanIN formation and ductal metaplasia, a process by which tubular complexes arise from either acinar or Hes1-expressing centroacinar cells[17, 20, 41]. Alternatively, enhanced Notch signaling may allow established tubular complexes or PanIN lesions to proliferate and acquire more aggressive characteristics. Consistent with this notion, Ki67 staining of similarly-graded PanIN lesions from GSI- and vehicle-treated pancreata revealed that MRK-003 constrains the proliferation of these cells. Future studies will be needed to define the relative importance of effects on the initiation of tubular complexes and PanIN versus the progression of PanIN to PDAC.

Although the observed activity of MRK-003 in PDAC-derived cell lines is consistent with a cell autonomous role for Notch signaling, it is possible that GSI blocks tumor progression through non cell-autonomous effects. Recent studies have shown that Notch signaling can contribute to tumorigenesis by restraining non-productive angiogenesis[42, 43]. Correspondingly, inactivation of Notch signaling can promote the formation of non-functional vessels resulting in poor tumor perfusion and decreased tumorigenesis. Similarly, immune surveillance is thought to play an important role in tumor progression. GSI could conceivably mediate an anti-tumor effect by changing the balance of growth-promoting tumor associated macrophages or immunosuppressive regulatory T-cells[44, 45]. In limited studies of tumor vasculature and lymphocyte infiltration, we did not observe an effect of GSI treatment on the tumor vasculature or stroma (Supplementary Fig. 4B and data not shown). Finally, we cannot rule out the possibility that inhibition of a γ-secretase sensitive pathway other than Notch is responsible for blocking tumor progression.

Molecular "crosstalk" between Ras and Notch signaling is well known to occur during development[46]. The two pathways also exhibit cooperativity in tumor cells, where Notch appears to be a common and critical downstream effector of oncogenic Ras signaling[47]. Consistent with the notion that Notch signaling plays an important role in the progression of murine PDAC, recent work has shown that ectopic activation of Notch signaling promotes PanIN initiation and progression[8]. The mechanism of Notch activation in evolving PDAC is not yet defined. Oncogenic Kras could contribute to Notch induction in since, as we have shown, Kras[G12D] expressing PanIN-derived cells exhibit increased expression of Notch signaling components relative to wildtype duct cells. Alternatively, previous studies have shown that EGFR signaling can activate Notch in cultured acinar cells[17]. EGFR is markedly upregulated in metaplastic regions and in PanINs from Pdx1-Cre Kras[G12D] mice and is a potential inducer of Notch in this setting.

Despite the γ-secretase-sensitive role that Notch signaling plays in controlling cell differentiation in multiple adult tissues, including the intestine[35, 48, 49], mice treated with a 3-day on 4-day off dosing regimen for over 5 months showed no overt ill effects. Hence, intermittent treatment provides a potent anti-tumor effect with minimal toxicity. Future studies with MRK-003, used as either a single agent or in combination with other drugs, will further delineate the mechanism and efficacy of this and similar compounds. Faithful GEMMs that recapitulate the molecular pathogenesis of human cancer provide a stringent platform for testing the efficacy of candidate therapeutic agents. Such models have the potential to play an especially crucial role in the preclinical evaluation of therapeutics for cancers that have no effective treatment. While the effects we observed on tumor

progression were dramatic, additional investigation of potential efficacy in the setting of more advanced pancreatic malignancy is clearly warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

GSI	γ-secretase inhibitor
PDAC	pancreatic ductal adenocarcinoma
PanIN	Pancreatic Intraepithelial Neoplasia

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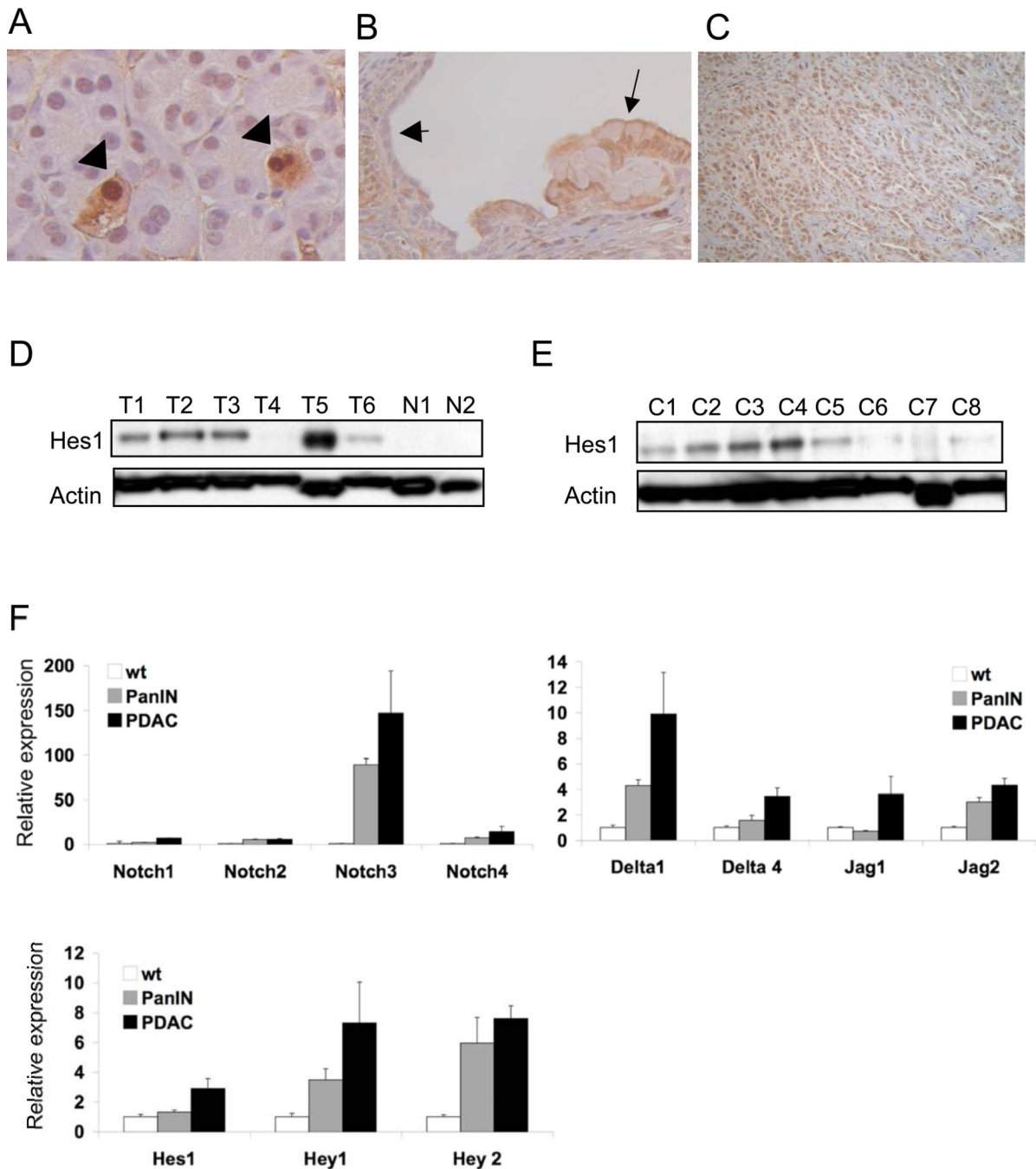
A

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Figure 1. Notch pathway activation accompanies PDAC progression
The Notch reporter strain [24] confirms pathway activation in normal pancreatic
centroacinar cells A, arrowheads) and PanIN lesions of Pdx1-Cre LSL-KrasG12D p53 L/+
mice (B). Note staining in PanIN epithelium (arrows) but not in adjacent normal ductal
epithelium arrowhead). (C) Hes1 is widely expressed in the tumor cells of advanced mouse
PDAC. D) Western blot analysis demonstrates a significant increase in Hes1 expression in
primary mouse PDAC tumors (T1–T6) and (E) PDAC cell lines (C1–C8) compared to
normal pancreatic tissue (N1, N2). (F) Quantitative reverse transcription-PCR (qRTPCR)
analysis of Notch pathway components in cultured wildtype duct cells, duct cells derived

from PanIN-bearing pancreata, and PDAC cells (mean +/− SEM of 3 independent lines for each group). A,B and C, original magnification 400×.

A

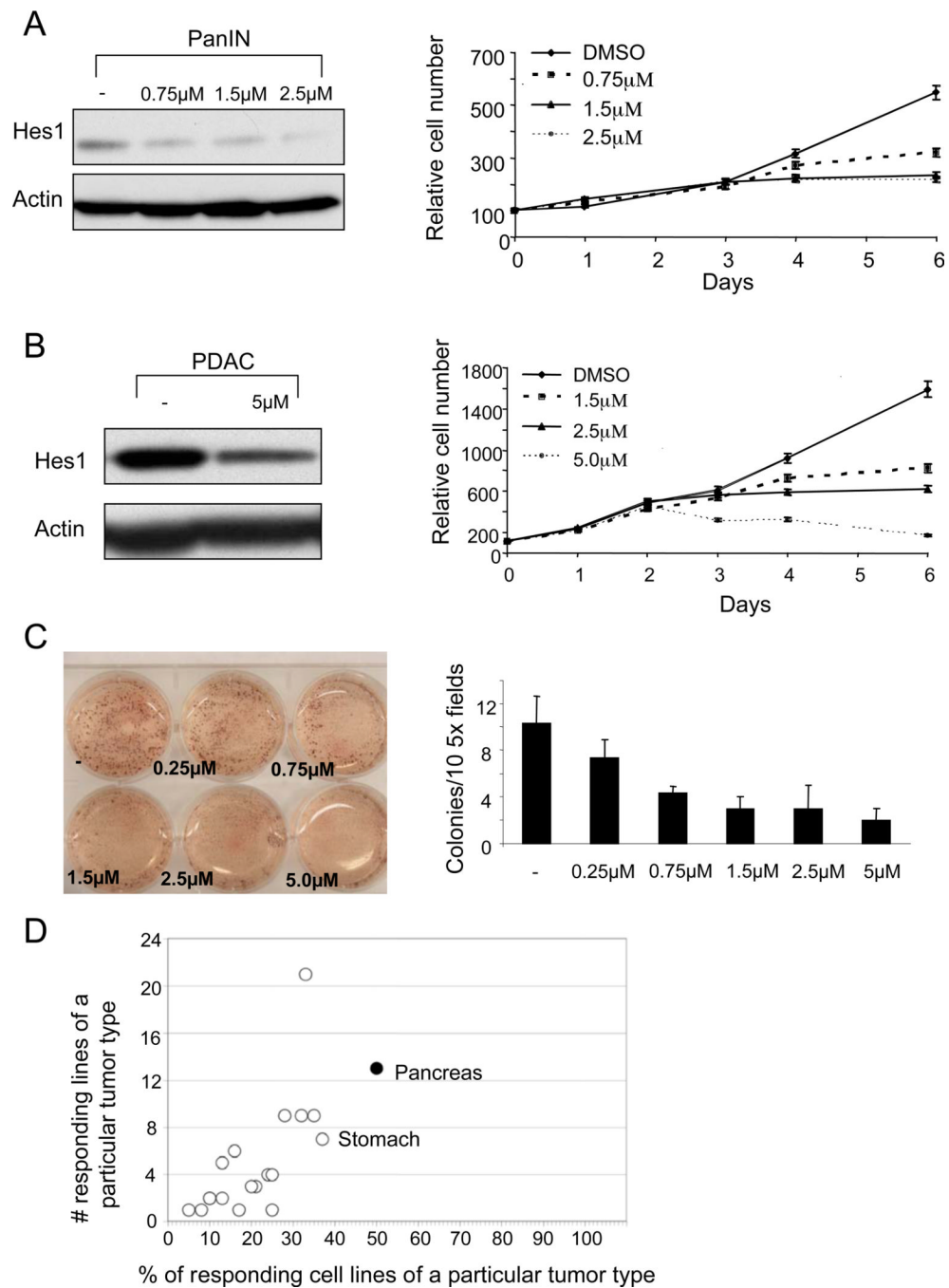PanIN

B

PDAC

C

D

Figure 2. PanIN and PDAC cell lines are sensitive to GSI treatment

(A) PanIN cells treated with the indicated concentrations of the GSI exhibit decreased levels of Hes1 protein by Western blot analysis (left) and a dose-dependent shift in the growth curve right). (B) Treatment of the mouse PDAC cell line (N490) with the GSI results in a similar decrease in Hes1 protein levels (left) and growth inhibition (right). Note that most treatment conditions led to a plateau in the growth curves, suggesting that the compound exerts static rather than toxic effects. (C) Soft agar assay of a GSI-treated human PDAC cell line (Panc1), with quantitation on right. Note significant difference between colony numbers in cells treated with 1.5μM GSI compared to vehicle-treated cells (p<0.005; Student's T test). (D) A set of 434 human cancer-derived cell lines comprising 23 different solid tumor

types were assessed for growth inhibition following 3 days of exposure to 5 μM MRK-003. The proportion of cell lines from each tumor type that showed >25% decreased growth is plotted on the X-axis (% responders) and the total number of responders is plotted on the y-axis. In addition to pancreatic cancer-derived cell lines (50%; 13/26), only stomach cancer-derived cell lines (37%; 7/19) also showed a response rate of >35%.
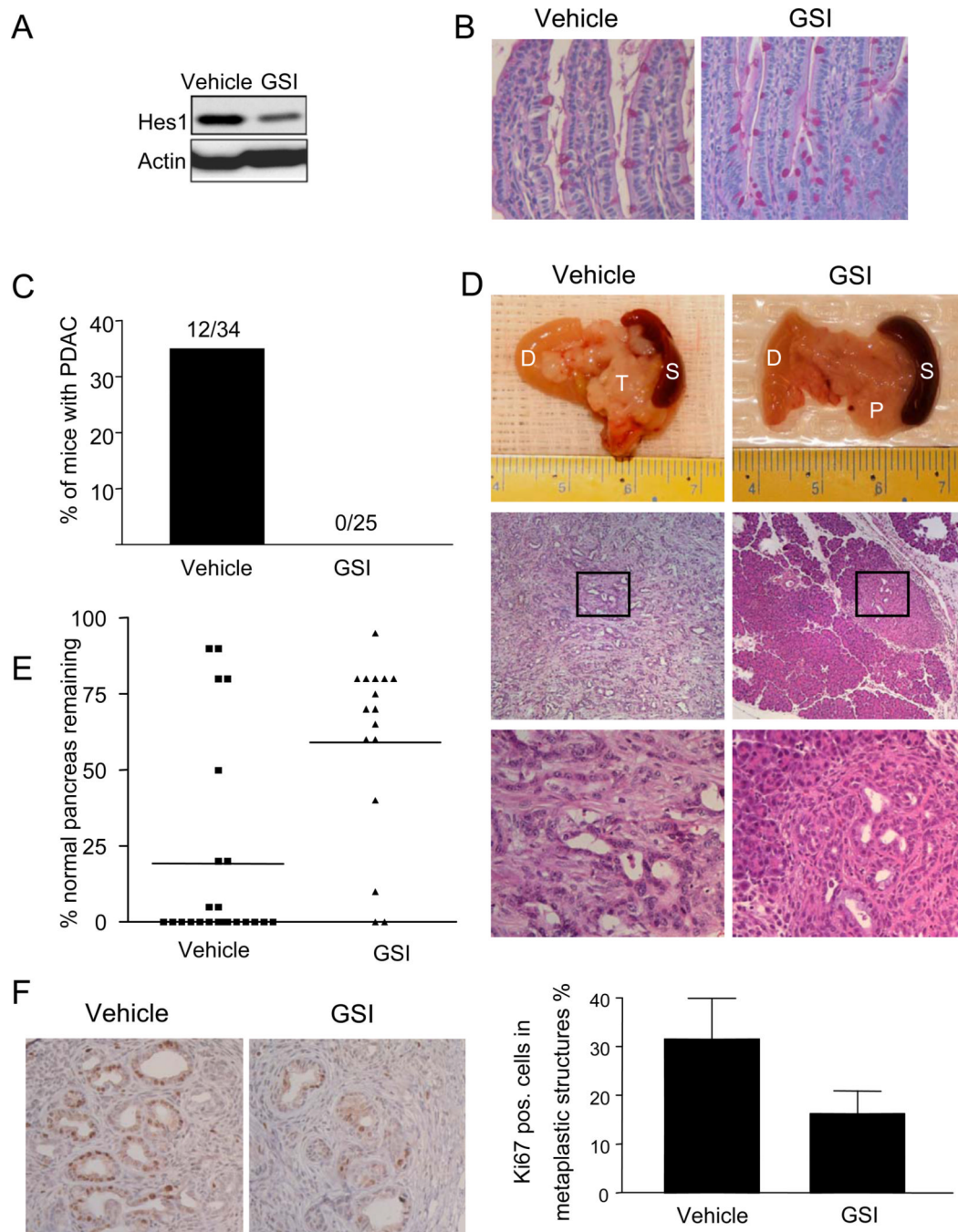
Figure 3. GSI attenuates PDAC development in Kras p53 mice

(A) Tumor cells were transplanted as xenografts into SCID mice, and the mice were treated systemically with GSI or vehicle, resulting in a decrease in Hes1 protein levels. (B) PAS staining of small intestine from control and GSI-treated mice, showing an increase in goblet cells in the GSI-treated animal. (C) PDAC incidence in GSI-treated and control mice at ages 11–13 weeks. (D) Macroscopic and histological images of representative pancreas (P), tumor (T), and surrounded duodenum (D) and spleen (S) from vehicle-treated and GSI-treated mice. The boxed region in the middle panels is shown at higher magnification in the lower panels. The vehicle-treated mouse has a PDAC, while the GSI-treated mouse has focal PanIN lesions. (E) The proportion of pancreatic tissue occupied by PanIN lesions was

quantified in vehicle- (N=23) and GSI-treated (N=16) pancreata. GSI treatment resulted in a significant (p<0.005; chi squared test) reduction in the abundance of PanINs. (F) Left panel. Ki67 staining of metaplastic ducts. Right panel. Quantitation of Ki67 staining in the neoplastic epithelium, showing significant (p<0.005; Student's T test) reduction in Ki67^{+} cells following GSI treatment. C, magnification 100× and 400×, E, magnification 400×.
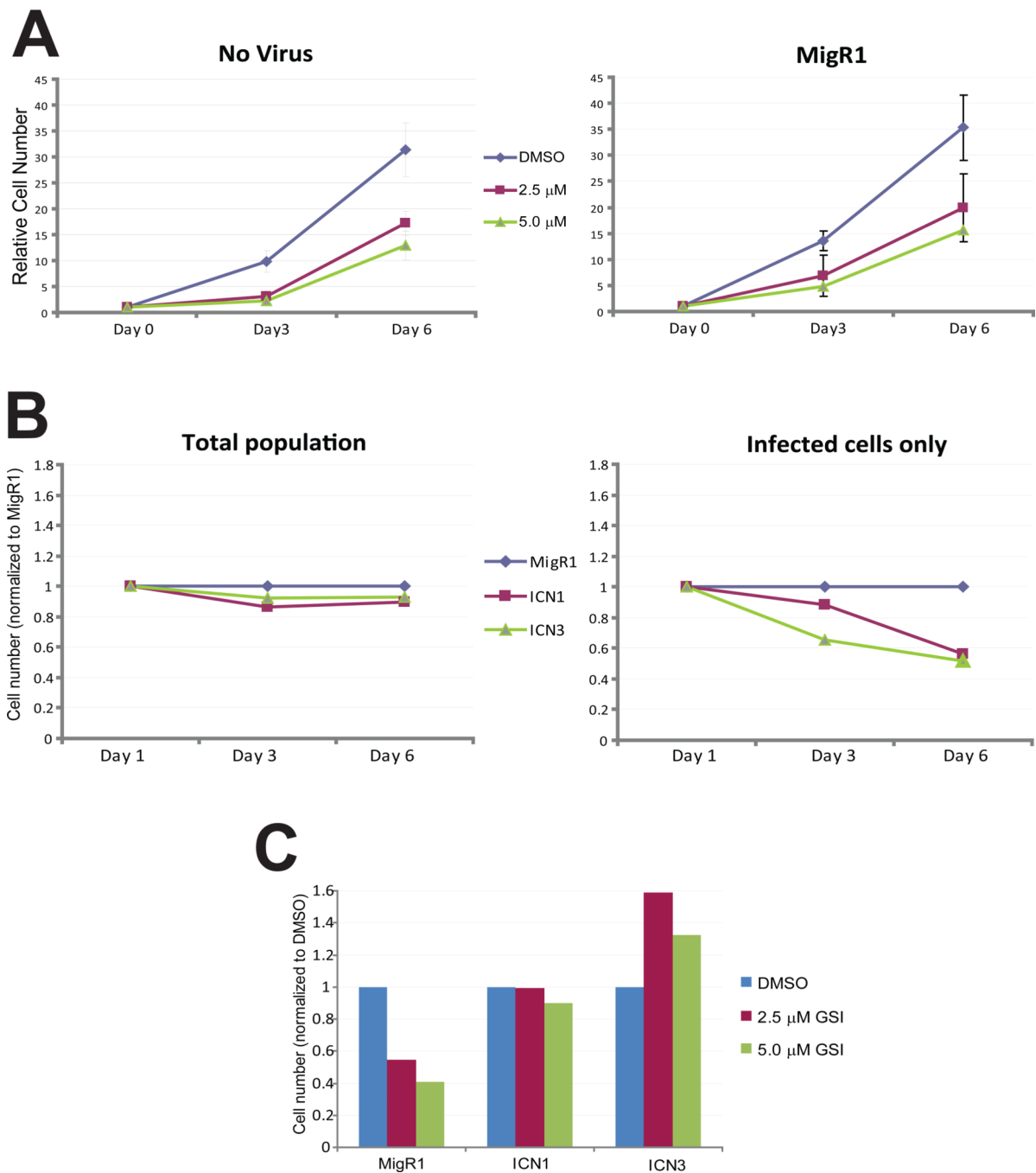
Figure 4. Notch signaling rescues cells from the growth inhibitory effects of GSI
(A) Retroviral infection has no effect on growth inhibition of NB507 PDAC cells by
MRK-003. Cell number was determined 3 days or 6 days following treatment with the GSI
(left panel); infection with MigR1 retrovirus did not alter the dose-response curve. (B) Cell
number was determined following infection with control (empty) MigR1 virus, or virus
encoding a constitutively active form of Notch1 (ICN1) or Notch3 (ICN3). ICN1 and ICN3
expression had minimal effects on NB507 cell number compared to MigR1 when the total
population was examined (left panel); when only infected cells were measured, ICN1and
ICN3 had a mild inhibitory effect on growth (right panel). (C) ICN1 and ICN3 prevented

GSI-induced growth inhibition after 6 days of treatment (only infected cells are shown). These results are representative of two independent experiments.