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# Targeting Notch signaling in pancreatic cancer patients – rationale for new therapy

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## ABSTRACT

Pancreatic cancer is one of the most aggressive and devastating human malignancies. Despite new knowledge in the molecular profile of pancreatic cancer and its precursor lesions, survival rates have changed very little over the last 40 years. Therefore, a better understanding of the detailed mechanisms underlying the pathogenesis of this disease is critical if we expect to develop new and effective strategies for prevention, early diagnosis and treatment of pancreatic cancer. The review herein focuses on a distinctive signaling pathway, the Notch pathway, which has recently been associated with carcinogenesis, including pancreatic cancer. It is aimed at summarizing key results which support a role for this pathway in the initiation, progression and maintenance of pancreatic cancer as a rationale for targeting and inhibiting this pathway in pancreatic cancer patients.

**Key words:** Notch, pancreatic cancer, pancreas development

## INTRODUCTION

With an overall 5-year survival rate under 5% and a median survival of 6 months, pancreatic cancer is one of the most devastating human malignancies. Moreover, the almost identical annual incidence and mortality rates make it the fourth leading cause of cancer-related death worldwide [1-4]. Despite enormous and important progress in medicine, the survival of patients with pancreatic cancer has not significantly improved over the last 40 years. The main reasons for the dismal prognosis of this type of cancer remains the late diagnosis of the disease, hence rendering surgical resection rates exceedingly low, in addition to yielding poor clinical response to gemcitabine (23.8%), a nucleoside analog used as a first-line palliative treatment [2,5,6]. This raises the urgent need for the identification of biomarkers to aid in the early detection of pancreatic cancer as well as in the development of new and effective treatment strategies. More importantly, a better understanding of the detailed mechanisms of disease pathogenesis is critical if we are to develop effective strategies for treatment, prevention and new detection methods for early diagnosis.

Recent findings suggest that developmental signaling pathways such as the Notch and Hedgehog pathways are involved in carcinogenesis including pancreatic cancer [7-24]. In fact, many recent studies have suggested that cancer is a disease triggered by the re-activation of developmental signaling pathways that are typically down-regulated following the completion of embryonic development [4,8,9]. Given that cancer progression and organ development are driven by similar mechanisms (i.e. both involve rapid bursts of proliferation, angiogenesis, tissue remodeling and cell migration), it is not surprising that both share the same signaling machinery. The following is aimed at outlining the various studies supporting a role for Notch signaling in pancreatic carcinogenesis as a rationale for targeting and inhibiting this pathway in pancreatic cancer patients.

## THE NOTCH SIGNALING PATHWAY

In vertebrates, there are four Notch genes (Notch 1-4), each of which encodes a single pass transmembrane receptor [25-27]. Binding of transmembrane ligands, namely Delta-

like-1,-3,-4, and Jagged 1, Jagged 2, renders membrane-bound Notch susceptible to a first cleavage by ADAM-family metalloproteases leading to a short-lived intermediate form that is rapidly cleaved within the transmembrane domain by γ-secretase. This second cleavage, blocked by γ-secretase inhibitors, releases the Notch intracellular domain (NIC) which then translocates to the nucleus where it associates with the DNA-binding protein CSL (also known as CBF1/RBPkJ) to induce expression of target genes [25-27]. Indeed, upon Notch activation, nuclear NIC binds to CSL which displaces co-repressors from CSL and converts the latter into a transcriptional activator [28]. The ensuing binding of Mastermind to NIC/CSL renders the ternary complex poised to activate transcription. This ternary complex also recruits general transcription factors to up-regulate transcription of Notch target genes [28]. To date, few transcription targets of Notch have been identified, although the hairy enhancer of split (Hes) as well as the Hes-related (Hey) family of genes are certainly the best characterized Notch targets [29-30]. These genes encode nuclear proteins that repress transcription. Finally, phosphorylation of NIC within its C-terminal PEST domain eventually leads to proteasome-dependent degradation of NIC which ensures the disassembly of the Notch enhancer complex [31,32].

## NOTCH SIGNALING DURING PANCREATIC DEVELOPMENT

In mice, pancreas specification begins at approximately embryonic day 8.5 (e8.5) with the expression of the IPF1/PDX1 transcription factor in distinct dorsal and ventral regions of the posterior foregut [33-35]. At e10, epithelial budding of this endodermal layer is clearly evident and pancreatic progenitor cells actively proliferate before reaching e13.5 where the majority of progenitor cells are now fully committed and undergo a differentiation program into one of the pancreatic cell types [36]. At maturity, the pancreas is composed of a branching network of acinar and duct cells (exocrine pancreas) which produce and deliver digestive enzymes into the gastrointestinal tract in response to cues from the stomach and duodenum [1-3]. There is also an endocrine component of the gland consisting in specialized endocrine cells (α,β,δ and PP) gathered together into clusters called islets of Langerhans. The endocrine function of the pancreas is to regulate metabolism and glucose homeostasis through secretion of hormones into the bloodstream [33].

*Notch1* expression is detected in scattered cells of the e9.5 pancreatic epithelium [37]. Its expression becomes more uniform across the pancreatic epithelium by e10.5 up until e14.5 where *Notch1* expression begins to decline [37]. In the e11.5 embryo, *Notch2* expression overlaps with *Notch1* expression; however, by day e15.5, *Notch2* expression becomes restricted to ductal cells [37]. As for *Notch3* and *Notch4*, their expression is excluded from the epithelium but is found in endothelial cells of the e15.5 pancreas [37]. The Notch ligand Delta-like1 is also detected in scattered cells of the e15.5 duct epithelium [37]. As for the well-known Notch target gene *Hes1*, it is expressed in a limited number of cells of the e10.5-e12 pancreatic epithelium [37-38] suggesting that only a subset of *Notch1/2* positive cells display activation of Notch signaling. This pattern of expression of Notch signaling molecules coincides with the massive proliferation of pancreatic progenitors during embryonic development.

Genetic studies have shown that mice with reduced Notch signaling in the developing pancreas i.e. mice with a generalized knock-out of Delta-like1 [39], Hes1 [46], CSL [39] or specific pancreatic CSL knock-out [40,41], exhibit premature endocrine differentiation [38-41] and pancreatic hypoplasia [38,40,41]. Conversely, ectopic expression of activated Notch1 (NIC) in the developing pancreas inhibits both exocrine and endocrine differentiation, with the majority of pancreatic epithelial cells remaining IPF1/PDX1-positive in newborns [42-43], at a time when IPF1/PDX1 expression is normally restricted to differentiated β-cells [33]. Collectively, these results suggest that Notch signaling plays an important role early in development by maintaining pancreatic epithelial cells in a progenitor state and thereby delaying their differentiation until timely appropriate. One proposed mechanism is that the Notch target Hes1 prevents cell cycle exit [44], by suppressing the expression of the cell cycle inhibitor *p57*, whilst simultaneously avoiding premature endocrine differentiation of pancreatic progenitors [45], by repressing the expression of the differentiation factor *Ngn3*.

## NOTCH SIGNALING IN THE ADULT PANCREAS

In the adult mouse pancreas, little-to-no expression of *Notch1 and Notch2* has been detected in exocrine pancreatic cells [46-47]. As for Hes1, it is expressed in the adult mouse pancreas but only in centroacinar cells and a few duct cells [10-12, 48]. Of note, increased expression levels of *Notch1*, *Notch2*, *Jagged2* and *Hes1* have been detected in the mouse exocrine tissue of caerulein-induced regenerating pancreas [46] and caerulein-induced acute pancreatitis [49], comparatively to the pancreas of control mice. Nevertheless, mRNA expression levels of these Notch components returned to control levels by the end of the recovery process [46, 49]. In addition, pancreatic-specific *Notch1* conditional knockout mice exhibit impaired regeneration after caerulein-induced pancreatitis [50], suggesting that Notch1 downstream signaling actively participates in the regeneration process following pancreas injury. In essence, the Notch signaling pathway, under normal conditions, is relatively inactive in the adult pancreas. However, under particular situations associated with acinar dedifferentiation and/or epithelial cell proliferation, transient re-activation of the Notch signaling pathway can be observed.

## NOTCH SIGNALING IN PANCREATIC CARCINOGENESIS

On the other hand, cumulative observations now suggest that sustained re-activation of the Notch signaling pathway may contribute to initiation, progression and maintenance of pancreatic ductal adenocarcinoma (PDA). It is generally accepted that pancreatic intraepithelial neoplasms (PanIN) represent a preinvasive form of PDA [1-4]. Moreover, genetic alterations have now been associated with histological progression from low-grade PanIN (called PanIN-1) to intermediate grade PanIN (PanIN-2), to high-grade PanIN (PanIN-3) and, finally, to invasive PDA. Interestingly, compared to little-or-no expression in normal pancreatic tissue, moderate-to-high level expression of Notch receptors (Notch1 and Notch2), Notch ligands (Jagged-1 and Jagged-2) and Notch target genes (Hes1, Hes4, Hey1, HeyL) have been observed in metaplastic ductal epithelium, PanIN-2 as well as PDA tissues in humans [10,19]. Moreover, several genetically engineered mutant mouse models of exocrine pancreatic cancer display aberrant Notch1 and/or Hes1 expression in metaplastic lesions, PanIN-1 to PanIN-3 and PDA [10-18], thus supporting a role for sustained Notch signaling activity in pancreatic cancer initiation and progression in both humans and mice.

Although it is not clear from which epithelial cell population PanIN arises, some studies support a model whereby acinar cells are progressively replaced by duct-like epithelia (acinar-to-ductal metaplasia) which can ultimately progress into PanIN and PDA [1-4,13,15-16,51]. In support of this hypothesis, adenovirus-mediated overexpression of NIC in primary acinar cell cultures has been shown to be sufficient to induce acinar-to-ductal metaplasia [10,14]. In addition, treatment of primary acinar cell cultures with TGFα causes acinar cell transdifferentiation [10,14], a conversion associated with Notch1 cleavage [14] (activation) as well as Hes1 and Hey1 expression [10,14]. Prevention of Notch activation by γ-secretase inhibitors in TGFα-treated cells prevents acinar-to-ductal metaplasia [10,14]. Moreover, transgenic overexpression of TGFα driven by an acinar promoter has been shown to result in Notch pathway activation in premalignant pancreatic epithelium and malignant pancreatic cancer [10]. With increased EGF/TGFα signaling being proposed as an initiating event [2,3,52], these results suggest that sustained re-activation of the Notch pathway within the pancreas may represent an early event leading to PDA. However, it will nonetheless be crucial to define the level of Notch activation needed as well as the Notch targets required to promote pre- and malignant pancreatic lesions. Indeed, adenovirus-mediated overexpression of some Notch target genes in primary acinar cell cultures, namely Hes1, Hey1 and Hey2, have failed to recreate the effects of NIC on acinar-to-ductal metaplasia [10] suggesting that Hes1 expression (or Hey1, Hey2) alone is insufficient to induce metaplasia. Combined with the observations that Hes1 expression can be observed without Notch receptor cleavage [53-56] (activation), this suggests that solely investigating the expression of Hes1 in pancreatic cells is both inadequate and insufficient to conclusively confirm Notch re-activation and predict progression of an abnormal pancreatic epithelium into PDA. A better understanding of the molecular and cellular events downstream of Notch activation will likely help in identifying combinations of target genes and/or additional downstream effectors needed for progression of pancreatic metaplastic lesions into PanIN and PDA.

Despite the fact that overexpression of NIC has been shown to be sufficient to promote acinar-to-ductal metaplasia in primary acinar cell cultures [10,14], a recent report has revealed that expression of NIC, either driven by IPF1/PDX1 (pancreatic progenitors) or elastase (acinar) promoters, while inducing *Hes1* and *HeyL* expression, was nevertheless insufficient to initiate pancreatic metaplastic lesions or PanIN after 4 months [16]. A possible explanation for this failure to induce metaplasia may reside in the fact that adenoviral vectors used to express NIC [10,14] in isolated acinar cells could have resulted in higher NIC expression levels than the knock-in strategy [16]. Moreover, careful examination of pancreatic tissues from older NIC-expressing mice would be mandatory to completely exclude NIC *per se* as a potential pancreatic metaplastic inducer since longer latency times may be needed. Another possibility would be that placing acinar cells in culture render them more susceptible to NIC-induced transformation i.e. acinar cells extracted from their normal environment may have dysregulated signaling pathways compared to the tightly-controlled pathways present in normal pancreatic tissue. This would suggest that NIC cooperates with other signaling pathway(s) to initiate pancreatic carcinogenesis. Of note, co-expression of NIC with an oncogenic KRas, either in pancreatic progenitors or mature acini, was recently shown to result in the formation of metaplastic and PanIN lesions at a time point where no such lesions were observed in either NIC or oncogenic KRas expressing mice [16]. These results are in accordance with a model of pancreatic carcinogenesis in which both activated KRas signaling and aberrant Notch activation act cooperatively to initiate pancreatic carcinogenesis. In keeping with such a model and through the use of transgenic mice, it would be interesting to induce NIC expression only after oncogenic KRas has provoked metaplastic or PanIN lesions to verify whether NIC also participates in PanIN-to-PDA progression. Interestingly, a recent article elegantly provided evidences that γ-secretase activity, probably by inducing Notch signaling, was required for the progression of pre-malignant to malignant pancreatic cells *in vivo*. To test this hypothesis, the authors used a highly reliable genetically-engineered mouse model of PDA and began treating the mice with γ-secretase inhibitor at a time frame when the animals exhibited isolated PanIN lesions without detectable PDA. After 11-13 weeks of treatment, autopsies revealed that 12 out of the 34 vehicle-treated mice developed PDA while none of the 25 γ-secretase inhibitor-treated animals exhibited PDA [20]. Such data,

together with the observation that Notch receptors, ligands and target genes are expressed in metaplastic lesions, PanIN and PDA [10,20], suggest that Notch signaling could play an active role in pancreatic cancer progression as well.

Some reports also suggest that Notch signaling contributes to the maintenance of the transformed phenotype of pancreatic cancer cells. Indeed, down-regulation of Notch1 receptors using specific siRNA [21-24] or treatment with γ-secretase inhibitor [20] is correlated with reduced proliferative rates [20,23-24], increased apoptosis [23,24], decreased anchorage-independent growth [20,21] and decreased invasion properties [22] of pancreatic cancer cells. Although a considerable number of mechanistic issues remain to be determined, accumulated evidences clearly support a substantial role for Notch signaling in pancreatic cancer initiation, progression and maintenance. Inhibiting this signaling in pancreatic cancer patients could potentially represent an attractive new therapeutic strategy to improve their survival.

## TARGETING NOTCH SIGNALING AS A NEW THERAPEUTIC STRATEGY FOR PANCREATIC CANCER PATIENTS

Early clinical studies in healthy volunteers [57] and patients with Alzheimer's disease [58] have shown that oral administration of γ-secretase inhibitor for 6 weeks was generally well-tolerated, with diarrhea as the most common side effect. In agreement with preclinical studies, diarrhea was due to improper intestinal homeostasis since inhibition of Notch signaling favors goblet cell differentiation [59]. Nevertheless, this effect was reversible, probably because of the rapid and continuous regeneration of the intestinal epithelium, thus indicating that intermittent oral administration of the γ-secretase inhibitor greatly improves intestinal toxicity [60]. Furthermore, a recent phase 2 safety clinical trial demonstrated that a 14-week treatment with γ-secretase can be safely tolerated [61]. In light of this observation, it appears that the prolonged use of γ-secretase inhibitor in patients is potentially conceivable. However, it remains to be determined whether this inhibition of Notch signaling by a γ-secretase inhibitor would significantly benefit pancreatic cancer patients.

Certainly, inhibition of Notch signaling in the setting of pancreatic cancer treatment is particularly attractive because this pathway does not appear to play a major role in the normal adult pancreas and thus only cancerous cells should be affected. As stated earlier, little-to-no expression of Notch signaling components are present in the adult normal pancreas [38]. Moreover, intraperitoneal administration of the γ-secretase inhibitor dipenzazepine for 5 consecutive days in normal mice resulted in only moderate histological alterations in the exocrine pancreas *in vivo* [50] while β cell-specific CSL knock-out mice had normal β cell number and function [40]. These results suggest that inhibition of Notch signaling in the adult would not have deleterious effects on normal pancreatic exocrine and endocrine functions, hence substantiating an earlier study whereby mice treated on a "3-day on, 4-day off" regimen of the oral γ-secretase inhibitor MRK-003 for over 5 months showed no over-ill effects [20].

Finally, in a recent study, a high-throughput platform was used to assess the sensitivity of 434 human cell lines derived from a variety of solid tumor types to MRK-003 [20]. Of noted interest, it was found that PDA-derived cell lines exhibited the greatest sensitivity to Notch inhibition when compared to other tumor types including breast, kidney and non-small cell lung cancer [20], suggesting that γ-secretase inhibition could be particularly effective in the context of PDA. Moreover, inhibition of Notch signaling by γ-secretase treatment also suppressed the abundance of PanIN and blocked the progression of PanIN to PDA in a mouse model of PDA. Lastly, reduced Ki67 staining in comparable lesions from γ-secretase-treated mice versus vehicle-treated mice were observed with no differences in TUNEL staining, suggesting that inhibition of γ-secretase activity reduces the proliferative rate of pre-malignant cells but does not strongly influence apoptosis during PDA progression *in vivo* [20].

## CONCLUSIONS

Experimental studies to date have demonstrated that Notch activity is important in early pancreatic development but rather dispensable in normal adult pancreas homeostasis. However, descriptive studies have indicated that Notch signaling is aberrantly re-activated in pre- and malignant pancreatic lesions as well as in PDA [10-24]. Pancreatic overexpression of an activated Notch (NIC) accelerates the formation of oncogenic KRas-induced PanIN lesions [16]. Furthermore, oral administration of γ-secretase inhibitor in mice blocks the progression of PanIN to PDA [20]. Altogether, these increasing observations support a potentially significant role for activated Notch signaling in the initiation, progression and maintenance of pancreatic cancer. Recent data also suggest that blocking Notch activity could prevent PanIN-to PDA progression [20], thus yielding hope for pancreatic cancer patients. However, additional investigations are clearly warranted as many unanswered questions remain to be addressed. The molecular and cellular mechanisms underlying the contribution of Notch signaling in pancreatic carcinogenesis are still unclear. Moreover, further studies will be needed to evaluate the effectiveness of inhibiting Notch signaling for prevention of PanIN-to-PDA progression as well as in the setting of more advanced pancreatic malignancy. Finally, studies will be needed to test whether utilization of γ-secretase inhibitors truly represents the best approach to achieve inhibition of Notch signaling.

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