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Combined inhibition of Notch and JAK/STAT is superior to monotherapies and impairs pancreatic cancer progression

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with a high rate of metastasis. Recent studies have indicated that Notch and janus kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) signaling pathways are both important for the initiation and progression of PDAC. The purpose of this study was to determine the outcome of targeting these two tumor signaling pathways simultaneously both *in vitro* and *in vivo*. We assessed the combinational effects of the γ-secretase inhibitor IX (GSI IX) and JAK2 inhibitor (AG-490) on growth and epithelial plasticity of human pancreatic cancer cell lines, and in a genetically engineered mouse model (Pdx1-Cre, LSL-KrasG12D, p53$^{lox/+}$) of PDAC. Dual treatment with GSI IX and AG-490 significantly impaired cell proliferation, migration, invasion, soft agar growth and apoptosis when compared with monotherapies. Most importantly, combinational treatment significantly attenuates tumor progression *in vivo* and suppresses conversion from acinar-ductal-metaplasia to PDAC. Our results suggest that targeting Notch and JAK2/STAT3 signaling pathways simultaneously is superior to single inhibitions, supporting combined treatment by GSI IX and AG-490 as a potential therapeutic approach for PDAC. However, the study design limits the direct transfer into the clinic and the impact of dual treatment in patients with PDAC remains still to be determined.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with poor prognosis and a leading cause of mortality and morbidity with 5 year survival rate of 6% in Europe and the USA (1). Studies have described PDAC precursor lesions (pancreatic intraepithelial neoplasia, mucinous cystic and intraductal papillary mucinous neoplasms) and acinar-ductal-metaplasia (ADM) as components of neoplastic transformation (2). PDAC is characterized by early metastasis at the time of diagnosis, which makes it often unsuitable for curative surgery (3–5). In spite of large numbers of conventional and targeted therapies, current treatments have a limited impact on the course of the disease (6). The magnitude of this problem commands the need for new strategies to overcome this deadly disease.

Pancreatic cancer has been recognized as highly heterogeneous tumor with a large number of genetic alterations that affect a core set of 12 signaling pathways (7). Hence, it is essential to target more than one pathway to improve the therapeutic effects of cancer treatment. One of the known pathways to be deregulated in PDAC is the Notch signaling pathway. Notch is activated when the Notch ligands interact with their receptors and release the Notch intracellular domain, which then translocates to the nucleus and induces target genes like Hairy enhancer of split (Hes1) (8,9). It is well established that this pathway plays a crucial role during the embryonic development of the pancreas but remains only active in the centroacinar cells of adult tissues (10). On the contrary, the same pathway is known to be upregulated in PDAC and is involved in tumor progression (11,12). It has been shown that pharmacological and genetic inhibition of Notch signaling suppresses pancreatic intraepithelial neoplasia (PanIns) and PDAC development in genetically engineered mouse models (12–19). Combinational treatment with a γ-secretase inhibitor (GSI) and gemcitabine showed enhanced antitumor effects (19,20).

Similarly, aberrant expression of signal transducers and activators of transcription 3 (STAT3) is implicated in PDACs (21,22). STAT3 belongs to the family of latent cytosolic transcription factors. The activation of STAT3 critically depends on the phosphorylation of a conserved tyrosine residue by upstream kinases such as Janus kinase 2 (JAK2), which further triggers the dimerization and nuclear localization of STAT3 (23,24). It also has been reported that a wide range of human malignancies exhibit persistent activation of the JAK2/STAT3 signaling pathway (25,26). Some studies revealed that STAT3 proteins are involved in cell proliferation, metastasis, angiogenesis, host immune evasion and resistance to apoptosis in many tumor types as well as in the maintenance of the self-renewal capacity of embryonic stem cells (27–29). It was recently shown that STAT3 is critical for pancreatic carcinogenesis and is required for ADM development (30,31).

The prevalence of many activated signal transduction pathways that support the cancer phenotype is a major challenge for the treatment of PDAC. However, it is increasingly understood that combinational treatment of cancer might be crucial and superior to monotherapies in overcoming this challenge (32–34). Taking into account that both Notch and JAK2/STAT3 signaling pathways are playing important roles for the tumor initiation and progression of PDAC, we sought to analyze the effect of inhibiting these two pathways simultaneously in pancreatic cancer cell lines and in an established genetically engineered mouse model.

In this study, we targeted both Notch and JAK2/STAT3 signaling pathways by application of the GSI IX and the JAK2 inhibitor AG-490 to maximize the overall therapeutic effect against pancreatic tumors. Most importantly, we observed that *in vivo* combinational treatment significantly suppressed conversion from ADM to PDAC. Our finding highlights the potential of targeting these two tumor signaling pathways (Notch and JAK2/STAT3) simultaneously and constitutes a rationale for clinical translation.

Materials and methods

Cell culture

Human pancreatic cancer cell lines Panc1, Kp4, Kp1NL, Capan2, Hupt3, HPAC and BxPC3 were obtained from the laboratory of Nabeel Bardeesy, MGH Cancer Center, Boston. The cell lines were maintained at 37°C under a 5% CO_2 environment in RPMI 1640 + L-glutamine + 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Invitrogen, Karlsruhe, Germany) enriched with 10% fetal calf serum (Biochrom, Berlin, Germany) and antibiotics of penicillin/streptomycin (100 units/ml) (Invitrogen).

Drugs and treatment

For the *in vitro* experiments, GSI IX (Calbiochem, EMD Chemicals, Darmstadt, Germany) and AG-490 (Calbiochem) were prepared as a 10 mM stock in dimethyl sulfoxide (DMSO; AppliChem, Darmstadt, Germany). Cells were treated with DMSO or GSI IX (10 µM) or AG-490 (10, 30 and 60 µM) or in combination of GSI IX and AG-490 (10 + 10 µM) and were analyzed after 72 h. The concentrations of the drugs used for combinational treatment were optimized after a careful titration by cytotoxicity tests.

Abbreviations: ADM, acinar-ductal-metaplasia; DMSO, dimethyl sulfoxide; GSI IX, γ-secretase inhibitor IX; JAK2, Janus kinase 2; n.s., non-significant; PDAC, pancreatic ductal adenocarcinoma; pSTAT3, phospho-STAT3; siRNA, small interfering RNA; STAT3, signal transducers and activators of transcription 3.

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Ethics statement

The mice used in this study were maintained in the animal care of Medizinische Universitätsklinik Klinik Tuebingen (UKT), Germany. All experimental protocols were reviewed and approved by institutional guidelines for animal care of UKT and Baden Württemberg (protocol number: M16/11), and all studies were performed according to the methods approved in the protocol.

Animals and treatment

Therapeutic studies were performed with Pdx1-Cre, LSL-KrasG12D, $p53^{lox/+}$ mice. The transgenic Pdx1-Cre mice and $p53^{lox/+}$ mice were obtained from Aram Hezel (University of Rochester Medical Center). Kras mice were kindly provided by Lars Zender (UKT, Germany). The mice were crossed and genotyped to obtain the mice with background Pdx1-Cre, LSL-KrasG12D, $p53^{lox/+}$. We initiated an animal trial with 5 mice per every investigated group. Mice were treated intraperitoneally with GSI (10 mg/kg/body wt), AG-490 (10 mg/kg/body wt), vehicle or a combination of GSI + AG-490 starting at an age of 14 weeks. Treatment regime was performed for 6 weeks, using a 3 days on and 4 days off intermittent dose schedule as described previously (12). Health status and treatment side effects were inspected every second day. After 6 weeks of treatment, the mice were killed; sizes of pancreas and of visible tumor were measured by caliper and organ tissues were harvested. Tumor volume was calculated using the following formula: Tumor volume $V = [(\eth\backslash 6) \times (\text{Length}) \times (\text{Width}^2)]$. The tumor tissues were snap frozen for protein analysis and fixed in 4% formalin for histology. The other organs like liver, lung and spleen tissues were collected for further histological analysis. All animals were analyzed for metastases and ascites. Animal data are summarized in Table I.

Cell proliferation assay, apoptosis assay, cell cycle, invasion assay, migration assay and anchorage-independent growth assay

The protocols used in this study are described in Supplementary Materials and Methods, available at *Carcinogenesis* Online.

Construction of tissue microarrays, histology, immunoblotting, small interfering RNA transfection and enzyme-linked immunosorbent assay

For detailed information of antibodies, primers and protocols for histology can be found in the Supplementary Materials and Methods, available at *Carcinogenesis* Online.

Statistical analysis

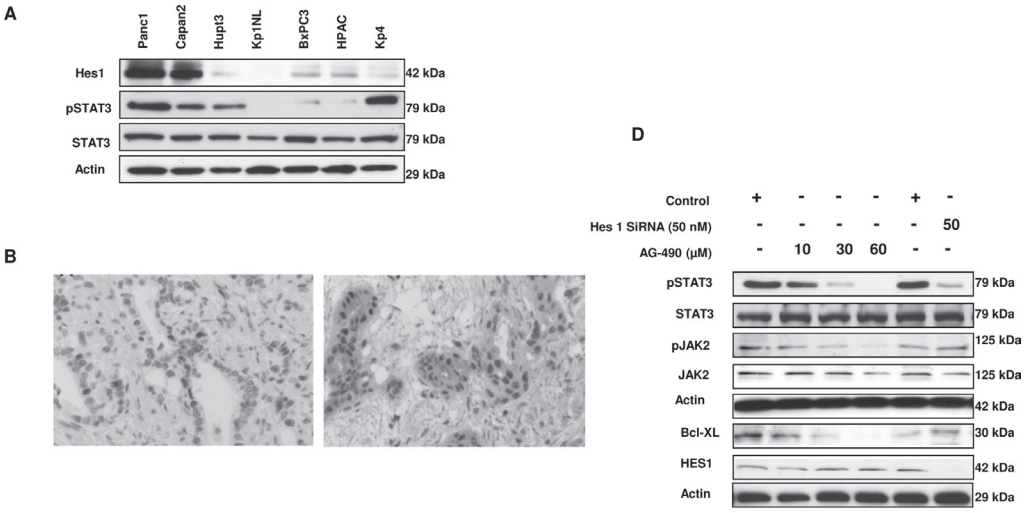
All the experiments were repeated 2–3 times. The results were analyzed using software GraphPad prism version 5.0 (GraphPad Software, San Diego, CA) and SPSS version 11.0 (SPSS, Chicago, IL). The tests include one-way analysis of variance and Student's *t*-test along with Bonferroni post-test and paired and unpaired *t*-tests. Differences were considered as statistically significant when the *P*-value was <0.05 (*), or as non-significant (n.s.).

Results

Inhibition of Hes1 downregulates the phosphorylation of STAT3

Notch and STAT3 are deregulated in PDAC (12–16,24–26). In order to confirm the activation of these pathways, we screened a panel of seven human PDAC cell lines to analyze the levels of phospho-STAT3 (pSTAT3) and the Notch target gene Hes1 (Figure 1A). The tested cell lines have a variable genetic background and are derived from different tumor stages. The results from immunoblot analysis indicated that two cell lines (Panc1 and Capan2) had high levels of both pSTAT3 and Hes1. In contrast, Kp4 cells showed a strong pSTAT3 and only a weak Hes1 expression. Kp1NL was the only PDAC cell line with negligible activity of both pathways. To determine whether Notch and JAK/STAT signaling is also present in human PDAC tissues, we have analyzed sections from 65 patients (Figure 1B and C). We found a co-expression (Hes1/pSTAT3) in 50% of the analyzed tissue microarrays. Hes1 had a stronger expression in tissues taken from patients with G_1 differentiation compared with G_2 and G_3 tumors. In contrast pSTAT3 expression was highest in G_2 differentiated PDACs.

Several studies have established an association between Notch signaling and a diversity of other pathways known to have a role in pancreatic cancer. Previous publications have shown a link between Notch and STAT pathways in mouse neuroepithelial tissues and a monkey kidney cell line (35). It has also been reported that small interfering RNA (siRNA) inhibition of Hes1 resulted in reduced pSTAT3 levels, suggesting that its presence is necessary for STAT3



C

PDAC	Hes1	pStat3	Co-expression Hes1/pStat3
G1	19/21	11/19 *	9/19
G2	15/22	14/20 *	12/20
G3	17/22	8/18 *	8/18

Fig. 1. Target genes of Notch and JAK/STAT signaling are expressed in human PDAC and are modulated by Hes1 siRNA. (**A**) Western blot analysis of Hes1, pSTAT3 and total STAT3 in a panel of seven PDAC cell lines. β-actin was used as a loading control. (**B**) Expression of pSTAT3 and Hes1 in the same representative human PDAC tissues from a tissue microarray detected by immunohistochemistry (×400 magnification). (**C**) Table showing the data of 65 analyzed human PDAC tissues, including single and co-expression of Hes1 and pSTAT3. * missing cases were not evaluated because of unstained internal positive control. (**D**) Panc1 cells were treated with DMSO (control), AG-490 (10, 30 and 60 μM), control siRNA, Hes1 siRNA (50 nM) for 72 h. The expression of pSTAT3, STAT3, pJAK2, JAK2, Bcl-XL and Hes1 was analyzed by western blot. β-actin was used as a loading control.

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activation (35). Doucas *et al.* (36) also reported a significant correlation between Notch3 and both level of STAT3 and pSTAT3 in pancreatic tumors. Based on these reports, we attempted to determine the role of Hes1 in phosphorylation of STAT3 in PDAC cell lines. Therefore, we both transfected Panc1 cells with Hes1 siRNA to inhibit its expression or control siRNA and treated with different dosages of AG-490 (10, 30 and 60 µM) or DMSO (control). Notably, depletion of Hes1 resulted in the downregulation of pSTAT3 on the protein level (Figure 1D). The decrease of pSTAT3 was highest under monotherapy with 60 µM AG-490 and was associated with a downregulation of the antiapoptotic protein Bcl-XL, an established STAT3 target (Figure 1D) (37). However, no change was observed in phosphorylated JAK2 under knock down of Hes1 by siRNA, but under AG-490 treatment, a decrease was observed (Figure 1D). Hes1 expression remained unchanged under AG-490 monotherapy, but a significant decrease was observed in the presence of Hes1 siRNA (Figure 1D). STAT3 expression remained unchanged in all treatment regimes. To exclude off target effects, the experiment was repeated with a second siRNA (Supplementary Figure 1A, available at *Carcinogenesis* Online). Furthermore, we quantified the expression of interleukin-6 (IL-6) under Notch inhibition to determine if Notch could influence IL-6 expression, which leads to an activation of JAK/STAT pathway. We observed a downregulation of IL-6 expression after treatment with GSI at 72 h as determined by enzyme-linked immunosorbent assay (Supplementary Figure 1B, available at *Carcinogenesis* Online). Simultaneous targeting of Notch and STAT3 by different class of inhibitors might potentially decrease PDAC carcinogenesis by inhibiting the cross talks between both oncogenic pathways.

The GSI IX and the JAK2 inhibitor AG-490 cooperatively attenuates cell proliferation and induces apoptosis

It is evident from our first *in vitro* experiment that Hes1 depletion can downregulate phosphorylation of STAT3. We hypothesized that targeting both Notch and JAK2/STAT3 signaling pathway together would be more effective than single treatment alone. Accordingly, we selected three human PDAC cell lines for our further experiments: (i) Panc1 that exhibited elevated pSTAT3 and Hes1; (ii) Kp4 with high pSTAT3 expression; (iii) Kp1NL that has negligible expression for both signaling proteins. All cell lines are treated with DMSO (control), 10 µM GSI alone, 10 µM AG-490 alone or a combination of 10 µM GSI + 10 µM AG-490 in order to determine cell proliferation (Figure 2A). Testing these possibilities, we found a correlation between high levels of pSTAT3, Hes1 and sensitivity to GSI and AG-490. We observed that in Panc1 cells, proliferation was inhibited to a greater degree than in Kp4 and Kp1NL cells especially under combinational treatment (Figure 2A). In addition, we performed annexin V assay to check whether the inhibition of the tumor signaling pathways leads to apoptosis. As shown in Figure 2B, for Panc1

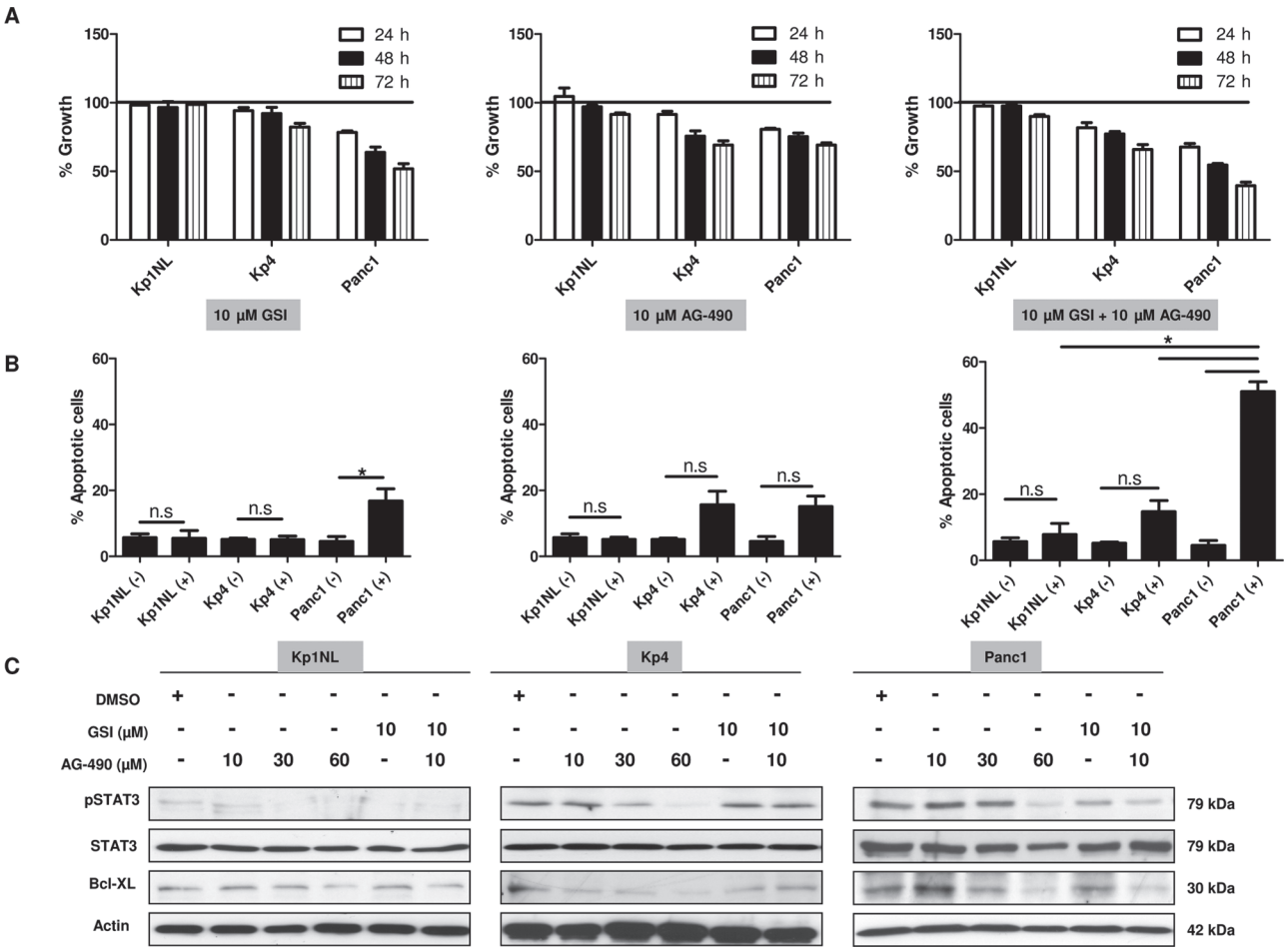


Fig. 2. Combinational treatment inhibits cell proliferation, pSTAT3 expression and induces apoptosis. (**A**) Panc1, Kp4 and Kp1NL cells were treated in triplicates with DMSO, GSI (10 µM), AG-490 (10 µM) and in combination of 10 µM GSI + 10 µM AG-490 for 72 h. Cell viability was measured by WST (water soluble tetrazolium) assay and average is shown relative to untreated controls of each cell line. (**B**) Panc1, Kp4 and Kp1NL cells were treated as indicated previously for 72 h and apoptosis was quantified by staining with annexin V and propidium iodide using flow cytometry. Differences were considered as statistically significant when the *P*-value was <0.05 and n.s. when *P*-value was >0.05. The error bar represents standard deviation. (**C**) Panc1 cells were treated with AG-490 (10, 30 and 60 µM), GSI (10 µM) and also in combination of 10 µM GSI + 10 µM AG-490 for 72 h. The expression of pSTAT3, STAT3, Bcl-XL and Hes1 was analyzed by western blot. **P* < 0.05. β-actin was used as a loading control.

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cells, the percentage of cells undergoing apoptosis were 12–18% under 10 µM GSI, 15–20% in AG-490 and 50–55% in combinational treatment. Furthermore, this induction correlated with downregulation of pSTAT3 and Bcl-XL (Figure 2C). In contrary, Kp4 cells that express high pSTAT3 levels did not undergo apoptosis when treated with 10 µM GSI but, 10–15% apoptotic cell were observed at 10 µM AG-490 and in combinational treatment (Figure 2B), which was associated with no changes in pSTAT3 and Bcl-XL at 10 µM AG-490 (Figure 2C). Whereas in Kp1NL cells, no apoptosis was observed nor had changes in pSTAT3 or Bcl-XL expression (Figure 2B and C).

We also performed cell viability assays, apoptotic assays and cell cycle analysis for Panc1 cells with DMSO, different concentrations of AG-490 (10, 30 and 60 µM) and in combinational treatment with AG-490 and GSI (10 + 10 µM; Supplementary Figure 2A–C, available at *Carcinogenesis* Online). The inhibition was confirmed by phase contrast images (Supplementary Figures 2A–C and 3C, available at *Carcinogenesis* Online). Subsequently, the effect of drugs on the cell cycle distribution was examined. Consistent with apoptotic profiles, a substantial increase in the sub-G_1 population was observed in AG-490 (60 µM) and in combinational treatment with AG-490 and GSI (10 + 10 µM) (Supplementary Figure 3E, available at *Carcinogenesis* Online). Next, we analyzed the phosphorylation of JAK2 and Hes1 expression. Notably, for these experiments, we observed significant differences in phosphorylated JAK2 under AG-490 monotherapy in a dose-dependent manner, but not under GSI treatment. Hes1 downregulation was observed in presence of GSI as well as in combinational treatment (Supplementary Figure 3D, available at *Carcinogenesis* Online).

Similarly, cell viability and apoptosis assays were also done for Kp4 and Kp1NL cells with different drug concentrations. The treatment of Kp4 cells with AG-490 showed a gradual decrease in cell viability and induced apoptosis (Supplementary Figures 2A, B, D and 3B, available at *Carcinogenesis* Online). The decrease was confirmed by phase contrast images of Kp4 cells (Supplementary Figure 2D, available at *Carcinogenesis* Online). As expected, Kp1NL cell line did not have any affect on cell proliferation (Supplementary Figure 2A, B and E, available at *Carcinogenesis* Online) nor on apoptosis (Supplementary Figure 3A, available at *Carcinogenesis* Online). Overall, our results suggest that there is a correlation between high levels of pSTAT3, Hes1 and sensitivity to GSI and AG-490. Importantly, we observed that combinational treatment decreases the growth and provokes cell death through apoptosis at doses that had negligible effects when used as single agents.

Combinational treatment inhibits the ability of Panc1 cells to grow in soft agar and affects the cell migration and invasion

We next examined the effect of DMSO, AG-490 (10, 30 and 60 µM) and the combination of AG-490 and GSI (10 + 10 µM) treatment on anchorage-independent growth by assaying colony formation on soft agar by using Panc1 cells. Results indicated that under all treatments, cells were inhibited from forming clones in soft agar compared with the vehicle in a dose-dependent manner (Supplementary Figure 4A and B, available at *Carcinogenesis* Online). However, combinational treatment by AG-490 and GSI showed the strongest ($P < 0.05$) inhibition of colony formation. Furthermore, we have studied cell motility

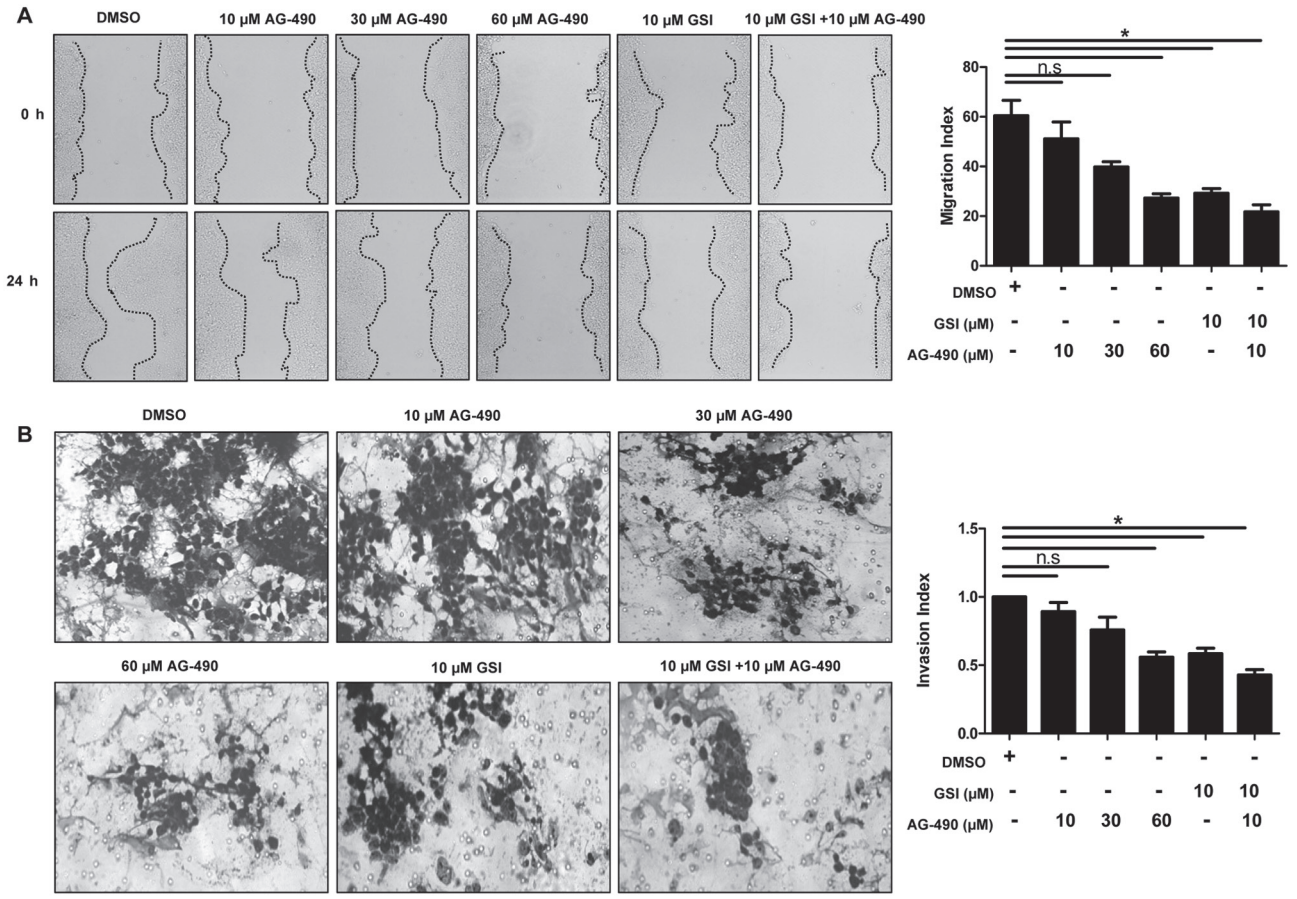


Fig. 3. Pharmacological inhibition of Notch and JAK2 downregulates migration and invasion. (**A**) Wound healing experiments were done for Panc1 cells cultured with DMSO, AG-490 (10, 30 and 60 µM), GSI (10 µM) or in combination of 10 µM GSI + 10 µM AG-490. The dotted lines are representing the edges of the wound. Photographs were taken at 0 and 24 h under light microscope (×10 magnification). The migration index was calculated as described in Materials and methods and plotted in bar graphs. (**B**) Panc1 cells were treated as indicated previously for 48 h to investigate the effect on invasiveness. The number of cells that invaded through the membrane was determined by light microscope (×20 magnification) and invasion index was calculated as described in Materials and methods and plotted in bar graphs. Differences were considered as statistically significant when the *P*-value was <0.05 and n.s. when *P*-value was >0.05. The error bar represents standard deviation. *$P < 0.05$.

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by employing *in vitro* wound healing assays. Cells were treated with similar concentrations and combinations of drugs as mentioned previously. Significant ($P < 0.05$) inhibition of wound healing was observed with 60 µM AG-490, 10 µM GSI and in combination of GSI and AG-490 (10 + 10 µM; Figure 3A). In contrast, 60–70% wound healing was seen after 24 h in untreated cells. Furthermore, we performed cell invasion using Matrigel-coated transwell chambers under DMSO, single and double GSI and AG-490 treatments. As shown in Figure 3B, the invasion of Panc1 cells was significantly reduced ($P < 0.05$) with 60 µM AG-490, 10 µM GSI and in combinational treatment (10 µM AG-490 + 10 µM GSI). Thus, single and combinational treatment can inhibit colony formation, migration and has an anti-invasive effect on human Panc1 cells.

Dual inhibition of Notch and JAK2/STAT3 signaling effectively impairs pancreatic tumor progression in vivo

The role of STAT3 signaling in both tumor initiation and persistent propagation of advanced PDAC has been reported in other publications. Based on these results, it is known that activation of STAT3 occurs at the earliest stages of pancreatic tumorigenesis and is maintained in invasive cancers. Recent work demonstrated that in genetically engineered mice, pSTAT3 expression was present at all stages of PDAC progression, in both ADM and PanIns as well as in invasive PDAC (26,27). The Notch pathway, which is deactivated in adult pancreas, is often activated in PDAC and it is also reported that in Pdx1-Cre, LSL-KrasG12D, $p53^{lox/+}$ mice, sustained Notch pathway activity is required for the development of premalignant lesions and their progression to advanced PDAC (12–16). We hypothesized that combinational inhibition of Notch and JAK2/STAT3 signaling would improve the treatment efficacy and attenuate pancreatic tumorigenesis. Thus, to further investigate the potential efficacy of combinational treatment, we treated genetically engineered mice (Pdx1-Cre, LSL-KrasG12D, $p53^{lox/+}$) with vehicle (control), GSI, AG-490 and in combination. Treatment was started at an age (14 weeks) where animals had already proven precursor lesions (PanIns and ADMs) and PDAC (Figure 4A). Five mice were analyzed at 14 weeks to study the onset tumor load. Despite the fact that mice analyzed showed a slight variations in the tumor size, however, the difference was not significant (data not shown). Consequently, we have used 5 mice per group in all drug- or vehicle-treated animals. After 6 weeks of treatment, all control mice exhibited severe tumor burden with macroscopic detectable huge solid tumors along with ascites (Figures 4B, 5A and B, Table I). Out of five control mice, one animal showed liver and lung metastases (Supplementary Figure 5B and C, available at *Carcinogenesis* Online, Table I). In the group treated with GSI only, three mice out of five had visible, but small solid tumors and two exhibited microscopically tumor formation (Figures 4B, 5A and B, Table I). Similarly, in the group treated with AG-490 only, three mice had visible, but small solid tumors and two exhibited microscopical tumor formation (Figures 4B, 5A and B, Table I). In contrast, mice treated with a combination of GSI and AG-490 did not reveal any macroscopic tumors and the pancreas volume was comparable with wild-type pancreas (Figures 4B, 5A and B, Table I). However, histological analysis showed that two mice exhibited two small areas with PDAC. Autopsy revealed that all

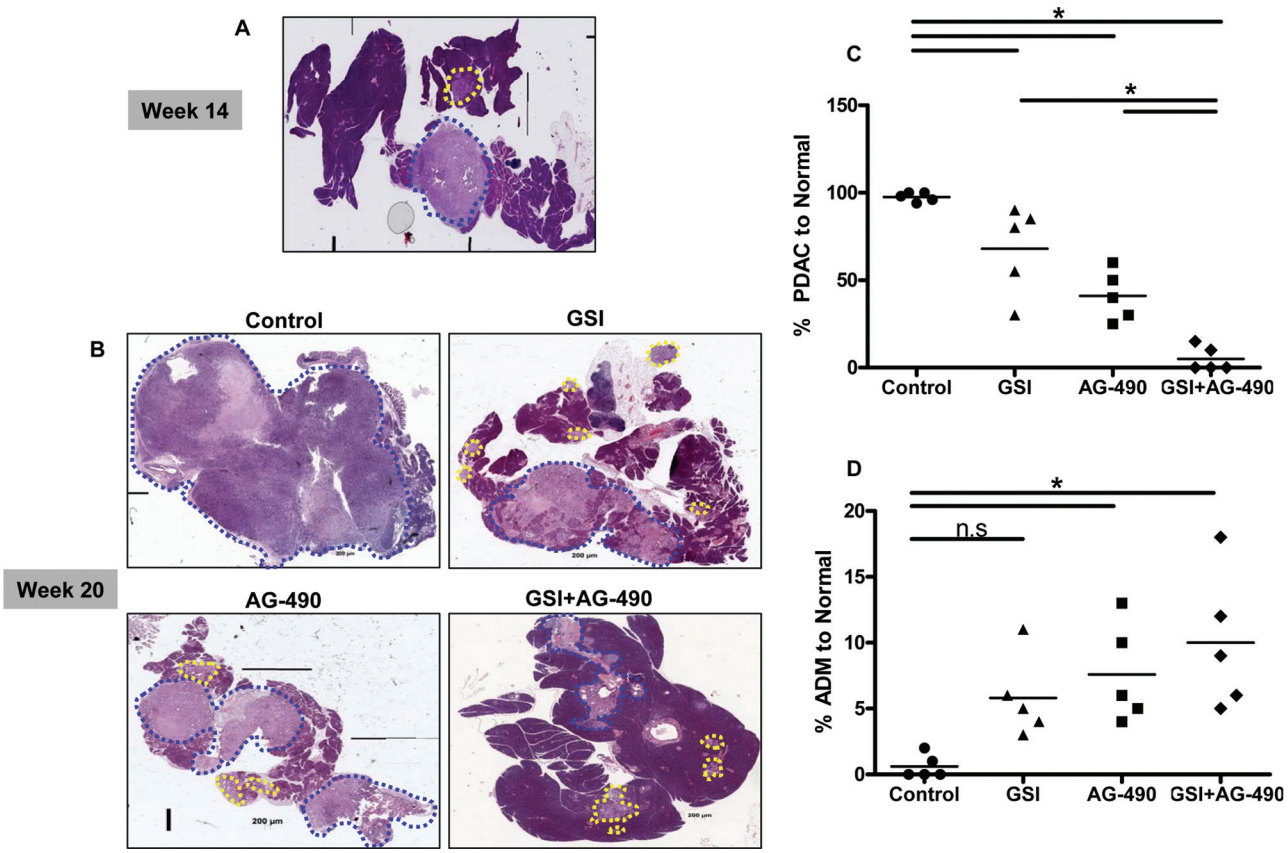


Fig. 4. Combinational therapy impairs pancreatic cancer progression in transgenic mice. (**A**) Representative image of full pancreas from a mouse before the start of the treatment (14 weeks). Representative images of pancreatic tumor tissues after 6 weeks of either treatment with (**B**) control, GSI, AG-490 or GSI + AG-490. Dashed blue lines sketch out the tumor tissues and yellow lines outlines the ADM areas. Areas without lining represent normal pancreatic tissues. (**C**) The graph illustrates the percentage of each pancreas occupied by the tumor in individual mice. Each symbol represents a mice and the horizontal line represents percentage of each group ($n = 5$). (**D**) The graph demonstrates the percentage of each pancreas occupied by the ADMs in individual mice. Each symbol represents a mice and the horizontal line represents percentage of each group ($n = 5$). Differences were considered as statistically significant when the P value was <0.05 and n.s. when P-value was >0.05. The error bar represents standard deviation. *$P < 0.05$.

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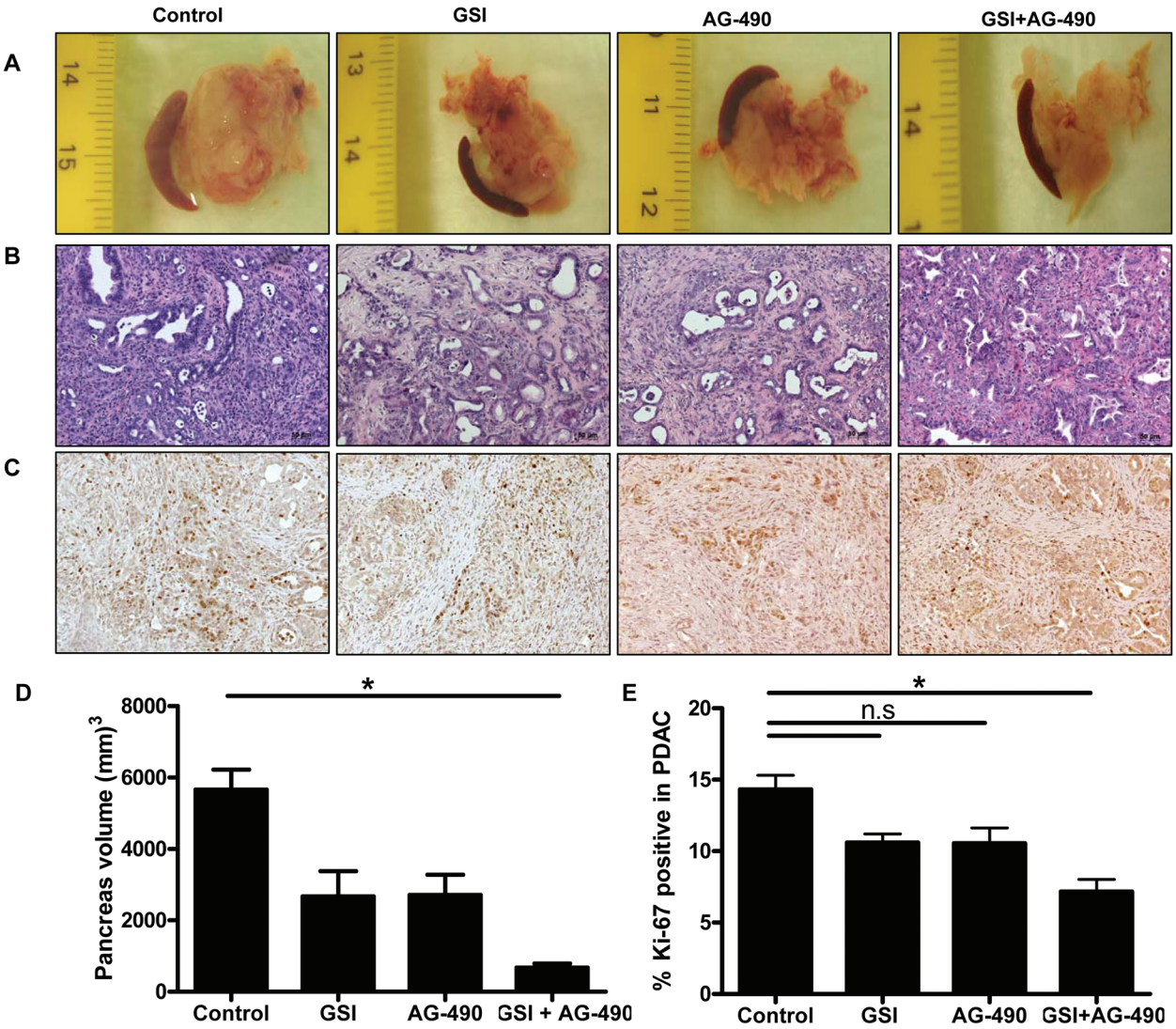


Fig. 5. Treatment with GSI and AG-490 influences pancreas volume and cell proliferation. Panel (**A**) represents macroscopic pictures of pancreas and spleen from control, GSI, AG-490 and combinational treated mice. Tissues were harvested after 6 weeks of each treatment. (**B**) Hematoxylin and eosin staining of representative tumors from each treatment group (×20 magnification). (**C**) Ki-67 staining of pancreatic tumors from each treatment group ($n = 5$; ×20 magnification). (**D**) Quantification of the pancreas volume ($n = 5$). (**E**) Quantification of Ki-67 staining in the tumor tissues ($n = 5$), showing reduction in Ki-67 positive cells following GSI + AG-490 treatment. Differences were considered as statistically significant when the *P*-value was <0.05 and n.s. when *P*-value was >0.05. The error bar represents standard deviation. *$P < 0.05$.

drug-treated animals showed no metastases and no ascites. To further evaluate the drug influence on precursor lesions and pancreatic progression, we calculated the areas of PDAC to normal pancreatic tissue and estimated ADM areas (Figure 4D). In line with our other data, we also found that control mice lacked ($P < 0.05$) any detectable normal pancreatic tissue and rather had big solid PDAC tissues (Figure 4C). Mice treated with GSI or AG-490 alone showed 30–75% normal pancreatic tissue (Figure 4C). In contrast, dual-treated mice had 90% ($P < 0.05$) normal pancreatic tissue (Figure 4C). Importantly, ADM formation was significantly ($P < 0.05$) higher in mice treated with GSI and AG-490 (Figure 4D). Control and single-treated animals showed less areas with ADMs, indicating that dual treatment suppresses conversion from ADM to PDAC (Figure 4D). Significant decrease was also observed in pancreas volume under all treatment regimes when compared with control (Figure 5D). To analyze the drug treatments effects on cell proliferation, we performed Ki-67 staining. Only combinational treatment resulted in a ($P < 0.05$) decrease in Ki-67 staining (Figure 5C). Single GSI- and AG-490-treated mice showed a decrease in Ki-67 staining but were not significant compared with control mice (Figure 5E). In general, drug applications were safe and showed no side effects. Mice weights were not significantly different between the analyzed groups (Supplementary Figure 5A, available at *Carcinogenesis* Online).

Finally, we determined whether combinational *in vivo* treatment impairs Notch and JAK2/STAT3 signaling. Analysis by immunoblot showed that single treatment with GSI or AG-490 as individual agents or in double showed a tendency to decrease in pSTAT3 compared with control mice (Supplementary Figure 6A, available at *Carcinogenesis* Online). In addition, this decrease correlated with the downregulation in phospho-JAK2, Bcl-XL and Hes1 (Supplementary Figure 6A, available at *Carcinogenesis* Online). In line with our *in vitro* data, no changes were observed in the full form of STAT3 and JAK2. Additionally, we checked the expression of Hes1 and pSTAT3 by immunohistochemistry (Supplementary Figure 6B, available at *Carcinogenesis* Online). However, co-expression was detected, but there was no significant difference between the treatment groups. Our data validate that simultaneous inhibition of Notch and JAK2/STAT3 signaling *in vivo* is more effective than single treatment and blocks conversion from ADM to PDAC.

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Table I. Summary of observation in Pdx1-Cre, LSL-KrasG12D, $p53^{lox/+}$ mice under different treatment groups

ID	Treatment	Tumor		Ascites	Other
		Macroscopic	Microscopic		
75D	Control	Yes	Yes	+	
24E	Control	Yes	Yes	+	
24D	Control	Yes	Yes	+	
50E	Control	Yes	Yes	+	
59D	Control	Yes	Yes	+	Liver and lung metastasis
25D	GSI	Yes	Yes	–	
56D	GSI	Yes	Yes	–	Ear tumor
39E	GSI	Yes	Yes	+	
19E	GSI	No	Yes	–	
68D	GSI	No	Yes	–	
33D	AG-490	Yes	Yes	–	
42D	AG-490	Yes	Yes	–	
42E	AG-490	No	Yes	–	
63D	AG-490	Yes	Yes	–	
50D	AG-490	No	Yes	–	
43D	GSI + AG-490	No	Yes	–	
23D	GSI + AG-490	No	Yes	–	
64D	GSI + AG-490	No tumor		–	
79D	GSI + AG-490	No tumor		–	Mouth cyst
85D	GSI + AG-490	No tumor		–	

Macroscopic implies visible tumor and microscopic means no visible tumor by eyes, '+' indicates positive ascites and '–' indicates negative for ascites.

Discussion

Worldwide PDAC is one of the most aggressive human cancer diseases with early metastasis and high resistance to conventional chemotherapy (1,3–6). The complexity of pancreatic tumor-, vascular- and stroma cells and the known genetic alterations clearly suggest that targeting a single tumor pathway will not yield outstanding inhibition of tumor growth (2). Furthermore, targeting multiple signaling pathways with overlapping roles has the potential to overcome treatment resistances and increase the likelihood of treatment response by different mechanisms of action (32–34).

Several studies have highlighted the role of Notch and JAK2/STAT3 signaling in pancreatic cancer progression and in precursor lesions (12–18,20–22,30). Although single pharmacological inhibition of these tumor signaling pathways has been performed and has produced some *in vitro* and *in vivo* benefits (12–15,20–23), combinational treatment has not been tested yet. However, correlation between Notch3 and levels of both STAT3 and pSTAT3 in human PDAC tissues has been demonstrated (36). Thus, we sought to analyze the therapeutic efficiency for PDAC by simultaneous inhibition of Notch and JAK/STAT pathways both *in vitro* and *in vivo*.

We hypothesized that cultivated *in vitro* human pancreatic cancer cells might be more effectively treated under combination of GSI and AG-490. Here, we have confirmed that PDAC cell lines have activated Notch and JAK2/STAT3 signaling, but the expression was diverse. We showed that Hes1 and pSTAT3 are co-expressed in 50% of the analyzed human PDAC tissues. We found that inhibition of Hes1 by siRNA downregulates the phosphorylation of STAT3 and might reflect an overlap of Notch and JAK/STAT pathways. Furthermore, the combinational treatment (GSI + AG-490) leads to a significant decrease in cell proliferation with an increase in apoptotic cell death and colony formation ability. This correlated with downregulation of pSTAT3 and its target gene Bcl-XL. Our *in vitro* results are consistent with earlier findings, which have used single pharmacological inhibition (12,15,21,22). However, the new aspect of this study is combinational treatment, and we have shown that it has significant effects at doses that had negligible effects when used as single agents. In this study, we also demonstrated that the sensitivity of PDAC cells to JAK2 or GSI inhibition correlated with activation of pSTAT3 and Hes1. Additionally, we have shown that treatment by GSI and AG-490 significantly inhibits migration and invasion. We assume that there might be cross talk between Notch and JAK/STAT pathways in PDAC, which could be responsible for the significant effect of dual inhibition. Our hypothesis can be supported by the recently published data on breast carcinoma where the authors postulated that non-canonical Notch can upregulate IL-6, which in turn induces the activation of pSTAT3 (38). It is also evident from the work by Corcoran *et al.* (23) that IL-6 cytokine family ligands likely contribute to STAT3 activation in human PDACs (23). Indeed, we also found that single treatment with GSI resulted in downregulation of IL-6 and support the cross talk between Notch and JAK/STAT signaling.

For this *in vivo* study, we used a well-established mouse model (Pdx1-Cre, LSL-KrasG12D, $p53^{lox/+}$ mice), which recapitulate human pancreatic cancer progression to determine whether combinational inhibition of Notch and JAK2/STAT3 signaling is superior to monotherapies. In contrast with other published data, animals used in this study were treated at starting age of 14 weeks, a timepoint when mice have precursor lesions and already show PDAC formation. Most significantly, double treatment of mice drastically inhibited pancreatic tumorigenesis. Specifically, we analyzed for the first time that only double-treated mice developed small PDACs (2/5), but therefore had a high number of ADMs. ADM is the earliest change observed in models of KRAS-induced pancreatic tumorigenesis and is a potential precursor to PanIns (30,31). Based on the other publications, the severe inhibition of the tumor progression can be explained by the fact that STAT3 and Notch are required for the development of precursor lesions and the progression of PDAC in mouse models (12,13,16,18,26,30,31). Obviously, monotherapies are not having the same effects on pancreatic tumorigenesis as combinational treatments, and our data suggest that the antitumor effect of AG-490 is enhanced by simultaneous inhibition of Notch signaling. This study also clearly demonstrated that combinational treatment resulted in a macroscopically normal looking pancreas and reduction of cell proliferation. Although monotherapies have antitumor benefits, the advantage of the combinational therapeutic effect of GSI and AG-490 is promising. Single- and double-treated animals showed no organ metastases, and the treatment regime was well tolerated in all experimental groups. Nevertheless, we have to note that dual treatment was not able to completely eliminate the tumor burden. Limitations of our *in vivo* study included the small number of animals in each group and the absence of survival data, as well as the lack of imaging during treatment. However, 60% of our double-treated mice showed no tumor development and had distinct changes of pancreatic histology.

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As it is known that specimens of human PDAC show an expression of pSTAT3 from 30–100% and that Notch receptors and ligands are often upregulated, the combinational treatment seems even more attractive (11,30). The heterogeneity of genetic alterations associated with PDAC makes it unlikely that a single component approach will be more effective than multiple target therapies (7). However, it will be necessary to identify additional biological markers that can predict treatment response, and further investigations will be required to define the roles of Notch and JAK2/STAT3 signaling in this context. Nevertheless, it already looks quite promising to combine target genes of these two pathways for further therapeutic approaches and perhaps combine them with classical chemotherapeutic drugs. In conclusion, we provide first evidence that combinational treatment with GSI IX and AG-490 is superior to monotherapies and has a high potential to diminish tumor growth of PDAC. Overall, our findings provide evidence that targeting Notch and JAK2/STAT3 signaling have a significant combinatorial efficacy in a PDAC mouse model and strengthen the rationale for a dual therapeutic approach.

Supplementary material

Supplementary Figures 1–6 and Materials and Methods can be found at http://carcin.oxfordjournals.org/

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