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Priority Report

Tgfbr1 Haploinsufficiency Inhibits the Development of Murine Mutant *Kras*-Induced Pancreatic Precancer

Kevin Adrian,[1] Matthew J. Strouch,[1] Qinghua Zeng,[3] Morgan R. Barron,[1] Eric C. Cheon,[4] Akilesh Honasoge,[1] Yanfei Xu,[2] Sharbani Phukan,[2] Maureen Sadim,[2] David J. Bentrem,[1] Boris Pasche,[3] and Paul J. Grippo[1]

[1]Department of Surgery and [2]Cancer Genetics Program, Division of Hematology/Oncology, Department of Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois; [3]Division of Hematology/Oncology, Department of Medicine and Comprehensive Cancer Center, The University of Alabama at Birmingham, Birmingham, Alabama; and [4]Department of Surgery, Mount Sinai Hospital, University of Illinois at Chicago, Chicago, Illinois

Abstract

To dissect the role of constitutively altered *Tgfbr1* signaling in pancreatic cancer development, we crossed *Elastase-Kras*^{G12D} (*EL-Kras*) mice with *Tgfbr1* haploinsufficient mice to generate *EL-Kras/Tgfbr1*$^{+/-}$ mice. Mice were euthanized at 6 to 9 months to compare the incidence, frequency, and size of precancerous lesions in the pancreas. Only 50% of all *EL-Kras/Tgfbr1*$^{+/-}$ mice developed preinvasive lesions compared with 100% of *EL-Kras* (wild-type *Tgfbr1*) mice. The frequency of precancerous lesions was 4-fold lower in haploinsufficient than in control mice. Paradoxically, the precancerous lesions of *EL-Kras/Tgfbr1*$^{+/-}$ mice were considerably larger than those in *EL-Kras* mice. Yet, the mitotic index of precancerous cells and the observable levels of fibrosis, lipoatrophy, and lymphocytic infiltration were reduced in *EL-Kras/Tgfbr1*$^{+/-}$ mice. We conclude that *Tgfbr1* signaling promotes the development of precancerous lesions in mice. These findings suggest that individuals with constitutively decreased TGFBR1 expression may have a decreased risk of pancreatic cancer. [Cancer Res 2009;69(24):9169–74]

Introduction

Transforming growth factor-β (TGF-β) signaling plays dual roles in the etiology of pancreatic cancer (PC). Loss of TGF-β signals in both human (1–3) and mouse (4–6) pancreas has been associated with aggressive disease. Conversely, reduced TGFBR1 expression in human PC cells prevents TGF-β–mediated growth inhibition (7), and decreased TGFBR1 kinase activity in Panc1 cells attenuates growth, invasion, and metastasis *in vitro* and *in vivo* (8). The mechanism by which altered TGF-β signals can promote or inhibit PC development requires further investigation beginning at the receptor level.

In PC, the TGF-β pathway is altered at various levels including changes at the ligand, receptor, and intracellular messenger levels. Increased expression of all three TGF-β isoforms has been shown in PC, with the presence of TGFB2 being associated with advanced disease (9). In addition, decreased expression of TGF-β receptors has been shown in some human PC (7, 10). Most of these alterations are infrequent as shown by 1% and 4% mutations in *TGFBR1* and *TGFBR2*, respectively (11). The most common TGF-β–related mutation in PC (in excess of 50%) is loss of *SMAD4* (12). Furthermore, a global genomic analysis of human PC has recently shown that at least one member of the TGF-β signaling pathway (including TGFBR2, SMAD3, and SMAD4) is mutated in all PC (2). These findings highlight the central role played by alterations in the TGF-β pathway during PC development and progression, although the mechanisms involved are not fully understood.

Most mouse models of PC show that loss of TGF-β pathway genes in mutant *Kras*-induced precancer leads to the development of more aggressive disease. LSL-*Kras*^{G12D} mice with heterozygous or homozygous loss of either *Smad4* (4, 6) or *Tgfbr2* (5) alleles develop more aggressive PC at a greater incidence and a shorter latency. However, the effects of reduced *Tgfbr1* on mutant *Kras*-induced precancerous lesions have not been established *in vivo*. Given the central role played by *Tgfbr1*, which initiates the TGF-β signaling cascade, and the novel evidence that constitutively decreased *Tgfbr1* expression is a potent modifier of mouse (13) and human (14) colorectal cancer, and the recent findings that a *TGFBR1* haplotype is associated with non–small cell cancer risk (15), we undertook this study to characterize the role of decreased TGFBR1 signaling in PC.

We chose to evaluate *Tgfbr1* haploinsufficiency in a background of mutant *Kras*-induced pancreatic precancer. We hypothesized that partial loss of *Tgfbr1* would lead to a more severe phenotype including increased frequency of precancerous lesions, perhaps acting as a potent modifier of cancer development. However, our results show that *EL-Kras/Tgfbr1* heterozygous null mice have a significantly reduced propensity of developing carcinoma *in situ*, although lesions that do form are larger and more advanced. This paradoxical response may be explained by the fact that loss of certain TGFBR1 function can inhibit the aggressive nature of cultured PC cells while suppressing tumor growth *in vivo* (8). These findings highlight the central role of TGFBR1 and underscore the dichotomous nature of TGF-β signals with respect to pancreatic precancer development.

Materials and Methods

Mice. *EL-Kras* transgenic and *Tgfbr1*$^{+/-}$ mice were generated as previously described (13, 16). *EL-Kras* FVB male mice were bred to *Tgfbr1*$^{+/-}$ C57/BL6 females.

Histology and immunohistochemistry. Mouse pancreas was stained with H&E and scored for the presence of pancreatic precancer. Incidence

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

K. Adrian and M.J. Strouch contributed equally to this work.

Requests for reprints: Paul J. Grippo, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, 303 East Superior Street, Lurie 3-105, Chicago, IL 60611. Phone: 312-503-0732; Fax: 312-503-0386; E-mail: p-grippo@northwestern.edu or Boris Pasche, UAB Comprehensive Cancer Center, The University of Alabama at Birmingham, 1802 6th Avenue South, NP 2566, Birmingham, AL 35294-3300. E-mail: Boris.Pasche@ccc.uab.edu.

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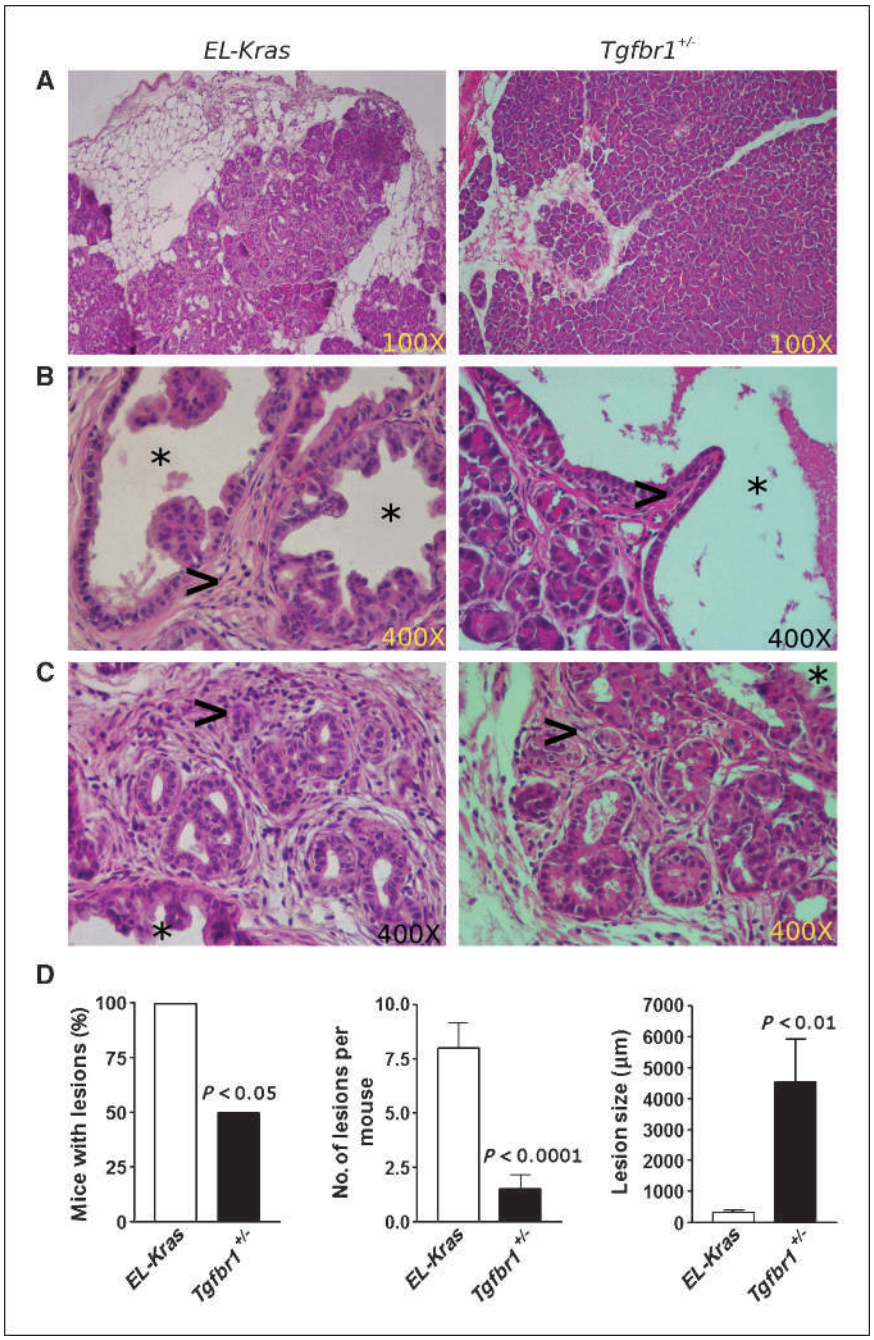

Figure 1. Effect of *Tgfbr1* haploinsufficiency on pancreatic phenotype and precancer. H&E sections showing lipoatrophy (*A*), fibrosis (*B, arrowhead*), and lymphocytic infiltration (*C, arrowhead*) adjacent to a precancer lesion (*) in pancreata from *EL-Kras* (*left*) and *EL-Kras/Tgfbr1*$^{+/-}$ (*right*) mice. *D,* the pancreata of *EL-Kras* and *EL-Kras/Tgfbr1*$^{+/-}$ (*Tgfbr1*$^{+/-}$) mice were scored for incidence, frequency, and size of precancerous lesions.

(mice with lesions/all mice), frequency (lesions/random section), size (μm^2) of the lesions, and accompanying phenotypic features were assessed.

Antibodies for immunohistochemistry included pSMAD2 and pSMAD3 antibodies (Cell Signaling), Smad4, Tgfbr1, and Tgfbr2 (Santa Cruz Biotechnology), cleaved caspase-3 (Cell Signaling), and bromodeoxyuridine (BrdUrd) antibody (Chemicon/Millipore). TUNEL staining was performed using an ApopTag Peroxidase *In situ* Apoptosis Detection Kit (Millipore). pSmad2 and pSmad3 staining was graded on a 0 to 3+ scale in a blinded manner by two investigators (M.J. Strouch and P.J. Grippo). BrdUrd and TUNEL were calculated as percentages of positive nuclei/cells per total nucleated cells.

Western analysis. Protein lysates were loaded onto a gradient SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride Immobilon-P membrane (Millipore Corporation) which was blocked and incubated overnight with either Tgfbr1 or Tgfbr2 antibodies. The secondary antibodies used were either horseradish peroxidase–linked antirabbit IgG (Cell Signaling Technology) or horseradish peroxidase–linked antimouse IgG (Cell Signaling Technology). Blots were visualized by Supersignal West Femto Maximum Sensitivity Substrate (Pierce) and densitometric scanning.

Statistics. Data were expressed as mean ± SEM. Unpaired two-tailed *t* tests were used to analyze differences in mouse lesion incidence, frequency, size, and BrdUrd and TUNEL counts. Analysis of pSMAD2 and pSMAD3 staining was performed with a Pearson χ^2 analysis.

Results

***Tgfbr1* haploinsufficiency and pancreatic precancer.** Upon histologic examination, we noted a general decrease in lipoatrophy (Fig. 1*A*), focal fibrosis (Fig. 1*B*), and lymphocytic infiltration (Fig. 1*C*) in the haploinsufficient group. All 6-month-old *EL-Kras*

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mice have pancreatic precancer (unpublished findings from 75 mice). In this study, six out of six *EL-Kras* mice and three out of six *EL-Kras/Tgfbr1*$^{+/-}$ mice had precancerous lesions ($P < 0.05$; Fig. 1*D*). There was also a significantly higher frequency of precancerous lesions found in *EL-Kras* compared with *EL-Kras/Tgfbr1*$^{+/-}$ mice (8.00 ± 1.18 versus 1.50 ± 0.67, respectively; $P < 0.0001$; Fig. 1*D*). However, when *EL-Kras/Tgfbr1*$^{+/-}$ mice developed lesions, they were significantly larger than those seen in *EL-Kras* mice (4,522 ± 1,417 versus 334 ± 56 μm^2, respectively; $P < 0.01$; Fig. 1*D*).

Effect of *Tgfbr1* haploinsufficiency on precancerous cellular proliferation and apoptosis. We next sought to determine if the decrease in frequency and increase in size of precancerous lesions was the result of altered mitotic and/or apoptotic indices between *Tgfbr1* haploinsufficient and control mice. The rate of BrdUrd incorporation (cell mitosis) was assessed in cells within precancerous lesions from *EL-Kras* and *EL-Kras/Tgfbr1*$^{+/-}$ mice. Immunohistochemistry for BrdUrd and TUNEL was scored as a percentage of positive nuclei/cells over total cells with nuclei per lesion per mouse (representative staining: BrdUrd in Fig. 2*A* and TUNEL in Fig. 2*C*). There was a trend towards reduced proliferation in *EL-Kras/Tgfbr1*$^{+/-}$ mice compared with *EL-Kras* mice, which did not reach significance (7.65 ± 1.10 versus 4.90 ± 0.20, respectively; $P = 0.067$; Fig. 2*B*). The apoptotic rate of *EL-Kras* mice was significantly higher than that observed in *EL-Kras/Tgfbr1*$^{+/-}$ mice (8.04 ± 0.56 versus 2.37 ± 0.51; $P < 0.001$; Fig. 2*D*), representing a nearly 3.5-fold difference. Samples were also stained with cleaved caspase-3 (data not shown) to verify TUNEL staining.

Analysis of Tgfbr1/Tgfbr2 ratio in whole mouse pancreas from *EL-Kras/Tgfbr1*$^{+/-}$ mice. Western analysis was used to determine the relative levels of Tgfbr1 (Fig. 3*A*) compared with

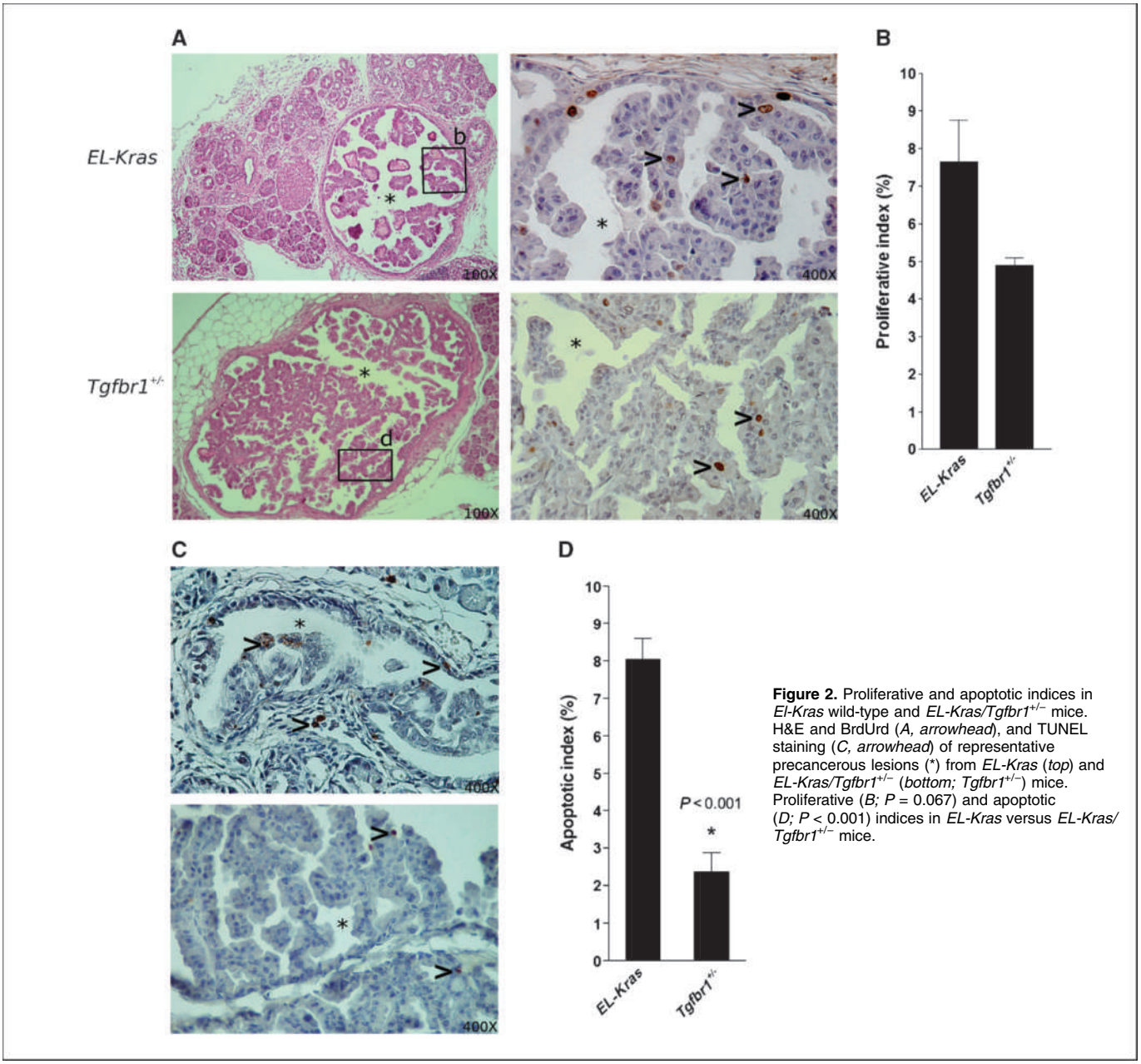

Figure 2. Proliferative and apoptotic indices in *El-Kras* wild-type and *EL-Kras/Tgfbr1*$^{+/-}$ mice. H&E and BrdUrd (*A, arrowhead*), and TUNEL staining (*C, arrowhead*) of representative precancerous lesions (*) from *EL-Kras* (*top*) and *EL-Kras/Tgfbr1*$^{+/-}$ (*bottom; Tgfbr1*$^{+/-}$) mice. Proliferative (*B; P* = 0.067) and apoptotic (*D; P* < 0.001) indices in *EL-Kras* versus *EL-Kras/Tgfbr1*$^{+/-}$ mice.

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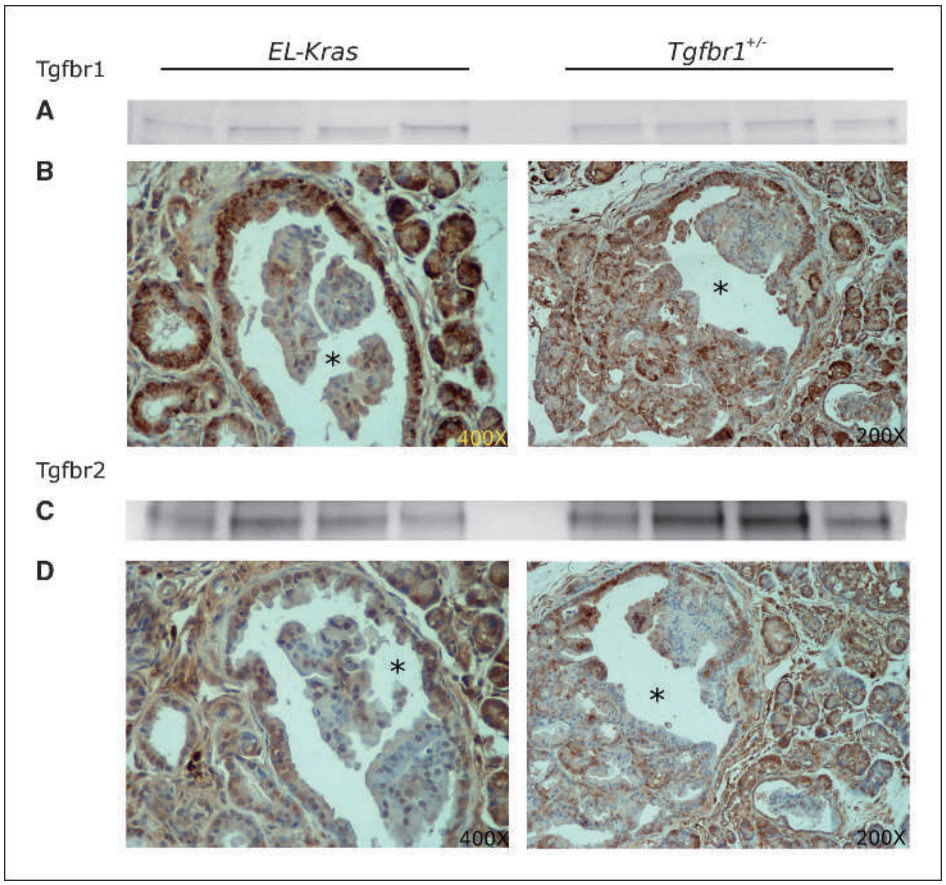

Figure 3. Establishment of a Tgfbr1/Tgfbr2 ratio. Western blot analyses (*A* and *C*) of two sets of four protein lysates from *EL-Kras* (*left*) and *EL-Kras/Tgfbr1*$^{+/-}$ (*right; Tgfbr1*$^{+/-}$) mice with subsequent (*B* and *D*) immunohistochemistry of representative tissues probed for Tgfbr1 (*A* and *B*) and Tgfbr2 (*C* and *D*) expression.

Tgfbr2 (Fig. 3*C*). Immunohistochemical staining of precancerous lesions from *EL-Kras* and *EL-Kras/Tgfbr1*$^{+/-}$ (Fig. 3*B*) mice displays a modest reduction in Tgfbr1 staining in precancerous lesions, although the change is quite subtle. Overall Tgfbr1 immunostaining of normal parenchyma was similar between the groups. Immunostaining for Tgfbr2 was modestly increased throughout the pancreas and focally increased in regions of precancerous lesions when comparing *EL-Kras* to *EL-Kras/Tgfbr1*$^{+/-}$ (Fig. 3*D*) mice.

To establish a Tgfbr1/Tgfbr2 ratio, relative levels of Tgfbr1 (53 kDa) and Tgfbr2 (75 kDa) were determined in the same lane of total protein loaded (Fig. 3). The average of each group (four mice) was compared with each other to show that the Tgfbr1/Tgfbr2 ratio for *EL-Kras* and *EL-Kras/Tgfbr1*$^{+/-}$ were 1:2 and 1:3, respectively (Supplemental Fig. S1). Interestingly, this reduction was not due to reduced Tgfbr1 but to increased Tgfbr2 in *EL-Kras/Tgfbr1*$^{+/-}$ mouse pancreas.

Downstream effects of *Tgfbr1* haploinsufficiency. Next, we sought to determine whether *Tgfbr1* haploinsufficient mice had concomitant decreased levels of pSmad2 and pSmad3 in pancreatic parenchyma and precancerous lesions. Using immunohistochemistry, we observed decreased staining in both the pancreatic parenchyma and precancerous lesions of *EL-Kras/Tgfbr1*$^{+/-}$ mice compared with *EL-Kras* mice (Fig. 4*A* and *B*). χ^2 analysis of staining intensity for both pSmads showed a significantly stronger parenchymal staining in *EL-Kras* mice compared with *EL-Kras/Tgfbr1*$^{+/-}$ mice ($P < 0.01$ and $P < 0.05$, respectively; Supplemental Fig. S2). We observed Smad4 staining in pancreatic islets although with no detectable staining in exocrine tissue. The only difference was the presence of infrequent nuclear staining of islet cells in *EL-Kras* mice not observed in *EL-Kras/Tgfbr1*$^{+/-}$ mice (Fig. 4*C*).

Discussion

In PC, the role that TGF-β signaling plays, particularly regarding TGFBR1 and TGFBR2, is poorly understood. More information is available regarding the role of SMAD4, in which its loss occurs in about half of all PC (11) with reduced survival following surgery (3) and, in combination with mutant *Kras* expression, promotes PC in mice (4, 6). This indicates that abrogation of TGF-β signaling downstream of SMAD4 enhances disease aggressiveness. The role of decreased TGFBR1 expression during the early stages of PC development is essentially unknown.

In this *in vivo* study, we examined the role of constitutively decreased Tgfbr1 signaling on mutant *Kras*-induced precancer. We observed a significantly decreased incidence and frequency of precancerous lesions, along with decreased fibrosis and inflammation, in *EL-Kras/Tgfbr1*$^{+/-}$ mice compared with *EL-Kras* mice. *Tgfbr1* haploinsufficiency recapitulates the human condition because reduced protein level is observed in cancerized ducts of the pancreas compared with robust levels in neighboring parenchyma (7). Two recent reports suggest that constitutively decreased TGFBR1 expression is a potent modifier of colon cancer risk in mice (13) and humans (14). These findings were the impetus behind our initial hypothesis. We have previously shown that *Tgfbr1* can promote mutant *Kras*-induced precancer yet suppress tumor formation while enhancing metastasis (8). The ability of TGF-β signals to both

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restrain and enhance cancer progression is a well-documented phenomenon (17, 18). In fact, there is evidence of a paradoxical nature within this *in vivo* investigation: reduced incidence and frequency but increased size of precancerous lesions in *EL-Kras/Tgfbr1*$^{+/-}$ mice. The presence of larger lesions implies an earlier time of onset and/or reduced apoptosis, which was evident in this study with a nearly 3.5-fold decrease in the apoptotic rate. Possible explanations for the phenotypic differences between *EL-Kras/Tgfbr1*$^{+/-}$ and *EL-Kras* mice include: cell types targeted with these acquired mutations, stage of cellular transformation, potential independent signaling of each receptor, additional genetic mutations, the *Tgfbr1/Tgfbr2* protein ratio, the interaction between mutant *Kras* and reduced *Tgfbr1* expression, or a combination of these mechanisms, which are currently being assessed in our laboratories.

In this study, *Tgfbr1* haploinsufficiency also resulted in decreased Smad signaling in pancreatic parenchyma and precancer. Findings in rat gastric epithelial cells indicate that the phosphorylation status of SMAD2 and SMAD3 can have a profound effect on cell phenotype. Activated ras leads to sustained c-*Jun*-NH_2-kinase activation, leading to reduced phosphorylation of Smad2 and Smad3 and subsequent enhanced invasive potential (17). In addition, *Tgfbr1*-dependent fibrogenesis is mediated through Smad1 (not Smad2/3) and requires Erk1/2 activation, which is a downstream ras event (19). Crosstalk between activated ras and altered *Tgfbr1*-mediated TGF-β signaling is likely responsible for the effects observed in preinvasive lesions in mouse pancreas and this interaction needs to be considered in future studies.

The *Tgfbr1/Tgfbr2* ratio may also play a key role in the effects observed in *EL-Kras/Tgfbr1*$^{+/-}$ mouse pancreas, as changes in this ratio may invoke cellular and tissue modifications (19), which were initially reported in skin epithelium (20). As for mutant *Kras*-induced pancreatic preinvasive lesions, reduced levels of *Tgfbr2* lead to aggressive cancer (5), and in our model system, increased frequency and severity of precancerous lesions (21). Hence, even a modest increase in the *Tgfbr1/Tgfbr2* ratio has a dramatic biological effect as early stage lesions advance or give rise to cancer. In this study, a decrease in this ratio seems to reverse the phenotype, although with some caveats (larger lesions). Findings in human PC are less clear, as reports vary regarding the expression of TGFBR1 and TGFBR2 in cancer cells. In 12 PC cell lines, an increase in TGFBR1 expression has been observed (22). In human cancer samples, initial reports indicated high levels of TGFBR1 in normal duct cells with low or nondetectable levels in cancer cells; the converse was true for TGFBR2 (7). Another report shows that high

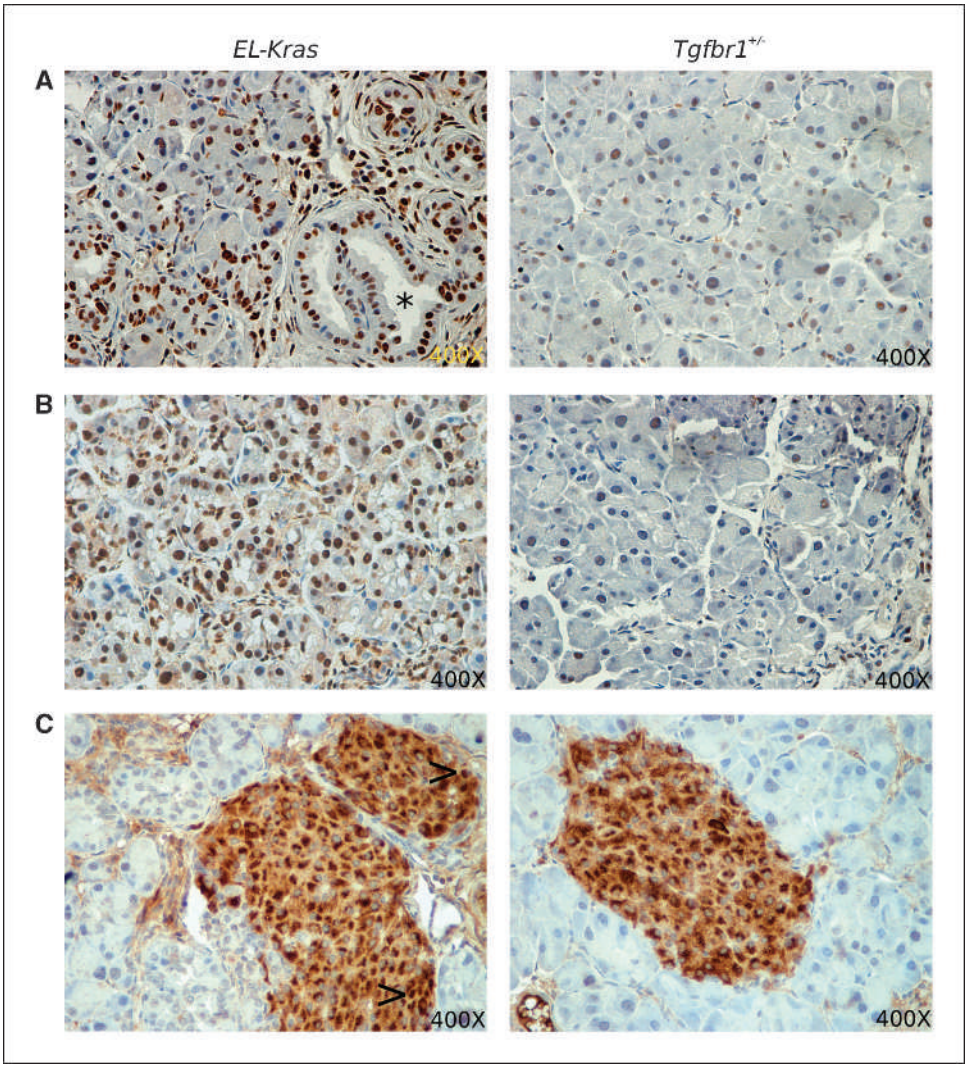

Figure 4. Effect of *Tgfbr1* haploinsufficiency on downstream TGF-β signaling. Representative pancreas staining of pSmad2 (*A*), pSmad3 (*B*), and Smad4 (*C*) in *EL-Kras* (*left*) and *EL-Kras/Tgfbr1*$^{+/-}$ (*right; Tgfbr1*$^{+/-}$) mice (*arrowhead,* infrequent nuclear staining).

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expression of both TGFBR1 and TGFBR2 in human PC samples correlates with advanced disease (23). Exactly how all this effects the *Tgfbr1/Tgfbr2* ratio has not been evaluated until this study, in which the loss of one allele only modestly affects the levels of Tgfbr1 but has a more profound effect in increasing the levels of Tgfbr2. In *EL-Kras* mice, decreased *Tgfbr1/Tgfbr 2* ratio may inhibit early precancer development but promote cell survival once lesions do arise.

The observation that *Tgfbr1* haploinsufficiency leads to a reduction of mutant *Kras*-derived preinvasive lesions of the pancreas supports the novel concept that a delicate balance in TGF-β signaling between its cancer-suppressing and cancer-promoting attributes plays a central role in the early stages of precancer development. These findings also suggest that individuals with constitutively decreased TGFBR1 expression may have a lower risk for developing PC.

Disclosure of Potential Conflicts of Interest

B. Pasche has filed a patent related to TGFBR1 signaling and colorectal cancer risk. The other authors disclosed no potential conflicts of interest.

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