The Transcription Factor GLI1 Modulates the Inflammatory Response during Pancreatic Tissue Remodeling*

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Background: Gli1 is induced during pancreatic injury; its function remains unknown.

Results: Loss of Gli1 in the stromal compartment impairs cytokine production, myeloid cell recruitment, and tissue repair.

Conclusion: Gli1 regulates remodeling during pancreatic tissue recovery.

Significance: This study defines a novel role for GLI1 in pancreas remodeling and expands the repertoire of target genes mediating GLI1 cellular functions.

Pancreatic cancer, one of the deadliest human malignancies, is almost uniformly associated with a mutant, constitutively active form of the oncogene Kras. Studies in genetically engineered mouse models have defined a requirement for oncogenic KRAS in both the formation of pancreatic intraepithelial neoplasias, the most common precursor lesions to pancreatic cancer, and in the maintenance and progression of these lesions. Previous work using an inducible model allowing tissue-specific and reversible expression of oncogenic Kras in the pancreas indicates that inactivation of this GTPase at the pancreatic intraepithelial neoplasia stage promotes pancreatic tissue repair. Here, we extend these findings to identify GLI1, a transcriptional effector of the Hedgehog pathway, as a central player in pancreatic tissue repair upon Kras inactivation. Deletion of a single allele of Gli1 results in improper stromal remodeling and perdurance of the inflammatory infiltrate characteristic of pancreatic tumorigenesis. Strikingly, this partial loss of Gli1 affects activated fibroblasts in the pancreas and the recruitment of immune cells that are vital for tissue recovery. Analysis of the mechanism using expression and chromatin immunoprecipitation assays identified a subset of cytokines, including IL-6, mIL-8, Mcp-1, and M-csf (Csf1), as direct GLI1 target genes potentially mediating this phenomenon. Finally, we demonstrate that canonical Hedgehog signaling, a known regulator of Gli1 activity, is required for pancreas recovery. Collectively, these data delineate a new pathway controlling tissue repair and highlight the importance of GLI1 in regulation of the pancreatic microenvironment during this cellular process.

Pancreatic cancer is among the deadliest of human malignancies. The median survival is less than 6 months, and this prognosis has not changed in almost 50 years (1). Pancreatic cancer is preceded by precursor lesions, the most frequent being pancreatic intraepithelial neoplasias (PanINs) (2, 3). Both PanINs and pancreatic cancer are almost uniformly associated with the presence of a mutant form of KRAS, most commonly KRASG12D (2–5). In mice, pancreatic cancer can be modeled by expressing oncogenic Kras in the pancreas epithelium (6, 7). The induction of pancreatitis, a known risk factor for the development of pancreatic cancer (8), in mice expressing oncogenic Kras leads to rapid PanIN formation (9–11). In wild type mice, the acute inflammatory response during pancreatitis is followed by rapid tissue repair over the course of several days (12). In contrast, mice bearing mutant Kras undergo rapid fibrosis and PanIN development upon induction of pancreatitis (11, 13). Although the biology of these phenomena is clearly established, the molecular mechanism modulating tissue repair remains elusive.

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The abbreviations used are: PanIN, pancreatic intraepithelial neoplasia; HH, Hedgehog; iKras*, inducible-KrasG12D (p48Cre,TetO-KrasG12D); Rosa26Gli1ΔTA-IREStEGF; KC, p48Cre,LSL-KrasG12D; Shh, sonic hedgehog; SMA, α-smooth muscle actin; DOX, doxycycline; qRT-PCR, real time quantitative PCR; iMC, immature myeloid cell; CCM, cell conditioned medium.
GLI1 in Pancreatic Remodeling

In this study, we identified a novel mechanism underlying pancreas repair after pancreatitis and Kras-driven, inflammation-induced pancreatic carcinogenesis. We demonstrate that the transcription factor GLI1 is required for pancreatic tissue remodeling after damage. Our studies revealed that a reduction in the dosage of Gli1, through genetic inactivation of one Gli1 allele, caused impaired tissue repair following induction of pancreatitis, altering the remodeling of the stroma. Furthermore, we identified a subset of known immune system regulators as GLI1 direct targets, including IL-6, mIL-8, Mcp-1, and M-csf (Csf1). Finally, we detected a reduction in the infiltration of macrophages, thus possibly explaining the impaired tissue remodeling. Our results define a novel mechanism controlling pancreatic tissue repair and identified a dosage-dependent role of the transcription factor GLI1 in the regulation of inflammatory responses in the pancreas.

EXPERIMENTAL PROCEDURES

Mice—Mice were housed in specific pathogen-free facilities at the University of Michigan Comprehensive Cancer Center. This study was approved by the University of Michigan University Committee on Use and Care of Animals (UCUCA) guidelines. p48Cre (Ptf1aCre) mice (14) were intercrossed with TetO-KrasG12D (15) and Rosa26ERTa/ERTa (16) to generate p48Cre;TetO-KrasG12D;Rosa26ERTa/ERTa (iKras*) triple mutants. Gli1lacZ/lacZ mice (17). Gli1lacZ/lacZ mice were bred with iKras* animals to generate iKras*Gli1lacZ/lacZ mice. LSL-KrasG12D mice were bred with p48Cre and Gli1lacZ/lacZ mice to create KC double transgenics (7) and KC;Gli1lacZ/+ triple transgenics. Doxycycline (DOX) (Sigma) was administered in the drinking water at a concentration of 0.2 g/liter in a solution of 5% sucrose and replaced every 3–4 days. Acute pancreatitis was induced by two 8-h series of intraperitoneal injections with caerulein (Sigma) at a concentration of 75 μg/kg over a 48-h period, as described previously (11).

Immunohistochemistry and Immunofluorescence—These assays were performed as described previously (17). Primary antibodies used were β-Gal/lacZ (1:200, Abcam), CD45 (1:200, Pharmingen), CK19 (1:100, Iowa Developmental Hybridoma Bank), F4/80 (1:100, BMA Biomedicals), phospho-ERK1/2 (1:100, Cell Signaling), Ki67 (1:100, Vector Laboratories), SMA (1:1000, Sigma), Vimentin (1:100, Cell Signaling). Images were taken with an Olympus BX-51 microscope, Olympus DP71 digital camera, and CellSens standard version 1.6 software. For immunofluorescence, Alexa Fluor (Invitrogen) secondary antibodies were used. Cell nuclei were counterstained with DAPI (Invitrogen). The images were acquired using an Olympus IX-71 confocal microscope and FluoView FV500/IX software or a Leica inverted SP5X confocal microscope with Leica Applications Suite Advanced Fluorescence (LAS AF) software.

Histopathology—Briefly, de-identified slides were examined by an expert pathologist (W. Y.). For each slide, five low magnification pictures were taken from the center, top right, top left, bottom right, and bottom left locations to cover most of the area of each section. A minimum of 50 total acinar or ductal clusters was counted from at least three independent animals for each group, as described previously (19). Each cluster counted was classified as acinar, acinar-ductal metaplasia, PanIN1A, -1B, -2, or -3 based on the classification consensus (20). The data were expressed as percentage of total counted clusters. Error bars represent S.E.

Quantification of Trichrome Staining—Images of iKras* and iKras*Gli1lacZ/+ mice. Gomori Trichrome-stained sections were taken with a Leica MZFIII dissection microscope and Olympus DP72 camera. Trichrome-positive areas were quantified with Image Pro Plus version 4 software (Media Cybernetics). The data were expressed as a percent of trichrome-positive area and averaged per time point. Error bars represent S.E.

Reverse Transcription Real Time-Quantitative PCR (qRT-PCR)—Tissue for RNA extraction was prepared through overnight incubation in RNAlater-ICE (Ambion) at –20 °C and then isolated using RNeasy Protect (Qiagen) according to the manufacturer’s instructions. Reverse transcription reactions were conducted using a High Capacity cDNA reverse transcription kit (Applied Biosystems). Samples for qRT-PCR were prepared with 1× SYBR Green PCR Master Mix (Applied Biosystems) and various primers as follows: TREKras, 5′-caagagcagattgccaggtacctgc-3′ (forward) and 5′-ggctgccagcgccttcgg3′ (reverse); Gli1, 5′-gagcagtaaatcagcttgctgc-3′ (forward) and 5′-agcagttcagagttcagctgg3′ (reverse); Ptch1, 5′-ttgagagccacccgtctcc-3′ (forward) and 5′-ggattagttaggctgctgc-3′ (reverse); Hhip1, 5′-gctctgtgttaaaggctgtacctg-3′ (forward) and 5′-cacaactggctgtctcaggg-3′ (reverse); Gli2, 5′-gtgcaacaggcccacacctctc-3′ (forward) and 5′-ggaatagttggaaggctg-3′ (reverse); Shh, 5′-caacaggctcatcatctctcgtg-3′ (forward) and 5′-ggaacagcggcgtatt-3′ (reverse); IL-6, 5′-ttcatctcctgtagttcctggg-3′ (forward) and 5′-ttcctatcctcctctctc-3′ (reverse); M-csf, 5′-gctctgtgttaaaggctgtacctg-3′ (forward) and 5′-ggaatagttggaaggctg-3′ (reverse); M-csf, 5′-gact- catcagagttgcctc-3′ (forward) and 5′-ggtgtcctgaggtc-3′ (reverse); Ccn11, 5′-tagagacagctcagttcctca-3′ (forward) and 5′-caacagctgaagttgctg-3′ (reverse). All primers were optimized for amplification under reaction conditions as follows: 95 °C 10 min, followed by 40 cycles of 95 °C 15 s and 60 °C 1 min. Melt curve analysis was performed for all samples after completion of the amplification protocol. CyclinH was used as the housekeeping gene expression control.

Cell Culture—Mouse-derived tumor cell lines were cultured for 3 days after which conditioned media were collected, centrifuged, and filtered. For cell stimulation, conditioned media from each group, as described previously (19). Each cluster counted was classified as acinar, acinar-ductal metaplasia, PanIN1A, -1B, -2, or -3 based on the classification consensus (20). The data were expressed as percentage of total counted clusters. Error bars represent S.E.

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Cell Culture—Mouse-derived tumor cell lines were cultured for 3 days after which conditioned media were collected, centrifuged, and filtered. For cell stimulation, conditioned media were mixed with low serum media (0.5%) at a 1:1 ratio. Mouse pancreatic fibroblast lines were derived from WT and Gli1lacZ/+ pancreata. Briefly, freshly isolated pancreata were minced with sterile scissors and then digested in 1 mg/ml collagenase. Digested samples were then filtered through a 100 μm strainer and cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). Cells were serum-starved (Iscove’s modified Dulbecco’s medium supplemented with 0.5% serum) for 36 h prior to addition of conditioned media, and samples were collected 0, 6, and 24 h following stimulation. Mouse NIH/3T3 cells (ATCC, Manassas, VA) were plated at 200,000 cells/well in 6-well plates in DMEM + 10% FBS + penicillin/streptomycin. After 24 h, cells were switched to low serum media (DMEM + 0.5% FBS) and treated with 100 μl of conditioned media from
control vector (pcDNA3) or Sonic Hedgehog N-terminal (NShh-pcDNA3) transfected COS7 cells. RNA was extracted after 24 h of treatment and subjected to qRT-PCR as described above. The mouse embryonic fibroblast line was a gift from Dr. Shilatifard (Stowers Institute for Medical Research, Kansas City, MO) (21), and it was cultured in DMEM (Invitrogen) supplemented with 10% FBS (SAFC BioScience).

Flow Cytometry—Single cell suspensions from the pancreas and spleen were prepared as follows: freshly isolated organs were minced with sterile scalpels prior to incubation in 1 mg/ml collagenase (Sigma) in Hanks’ buffered saline solution for 15 min at 37 °C. Cell suspensions were then passed through a 40 μm strainer. Single cell suspensions were prepared in Hanks’ buffered saline solution, 2% FBS. Antibodies used were as follows: CD45-Pacific Orange (1:50, Invitrogen); Gr1-FITC (1:50, Pharmingen); F4/80-PE-CY5 (1:50, eBioscience); CD11b-APC-CY7 (1:50, Pharmingen); CD3-PE (1:50, Pharmingen); CD8-APC-CY7 (1:50, Pharmingen); CD4-Pacific Blue (1:50, Pharmingen); CD25-APC (1:50, Pharmingen); Foxp3-FITC (1:50, eBioscience). Flow cytometry analysis was performed on a Cyan™ ADP analyzer (Beckman Coulter), and data were analyzed using Summit 4.3 Software.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP was conducted following the Magna ChIP kit protocol (Upstate). Briefly, 4 × 10⁶ cells, cultured in DMEM (Invitrogen) and supplemented with 10% FBS (SAFC BioScience), were cross-linked with 1% formaldehyde directly into the media for 10 min at room temperature. The cells were then washed and scraped with phosphate-buffered saline and collected by centrifugation at 800 × g for 5 min at 4 °C, resuspended in cell lysis buffer, and incubated on ice for 15 min. The pellet was then resuspended in nuclear lysis buffer and sheared to fragment DNA to ~700 bp. Samples were then immunoprecipitated using a GLI1 antibody (Novus Biologicals), normal rabbit IgG (Upstate) overnight at 4 °C on a rotating wheel. Following immunoprecipitation, samples were washed and eluted using the chromatin immunoprecipitation kit in accordance with the manufacturer’s instructions. Cross-links were removed at 62 °C for 2 h, followed by 10 min at 95 °C, and immunoprecipitated DNA was purified (Upstate) and subsequently amplified by real time PCR. PCR was performed using primer sets for areas containing potential Gli1-binding sites in the M-cp1, M-csf, IL-6, and IL-8 promoter sequence as follows: M-cp1, 5'-catactctggaagttatgccgag-3' (forward) and 5'-gccacgctactctgtgct-3' (reverse); M-csf, 5'-gccgatactctggtgctcag-3' (forward) and 5'-ccacattcgcctcctgtaag-3' (reverse); M-IL-6, 5'-gctctggagtggctagaa-3' (forward) and 5'-taggaatcctcgcctgtaag-3' (reverse); M-IL-8, 5'-gctttgagcattgatagttgctcag-3' (forward) and 5'-gctttgagcattgatagttgctcag-3' (reverse); and M-GLI1-negative region in chromosome 11 5'-ccacggaagttatgcagttc-3' (forward) and 5'-gctttgagcattgatagttgctcag-3' (reverse). Quantitative SYBR PCR was performed in triplicate for each sample or control using the C1000 Thermal Cycler (Bio-Rad).

Statistical Analysis—The data are expressed as the mean ± S.D. One-way analysis of variance with a Tukey post-test was used to compare data between groups. A p value < 0.05 was considered statistically significant.

RESULTS

GLI1 Is Required for Pancreas Recovery Following Pancreatitis or Inactivation of Oncogenic Kras—To investigate the role of GLI1 in tissue repair following acute pancreatitis as well as during early stages of pancreatic carcinogenesis, we used a mouse model where one GLI1 allele was replaced with a lacZ cassette, generating a Gli1 hemizygous animal (18). We initially induced pancreatitis in wild type (WT) and Gli1lacZ/+ mice with the cholecystokinin agonist caerulein (22). Both WT and Gli1lacZ/+ mice exhibited the characteristic acinar damage and influx of immune cells and activated fibroblasts 2 days after pancreatitis induction (Fig. 1, A and B). However, although normal tissue architecture and function was restored in WT pancreata after 1 week (Fig. 1A), 6 out of 10 Gli1lacZ/+ mice failed to resolve the fibroinflammatory response (Fig. 1B, black arrows). Interestingly, the severity of the phenotype did not increase in Gli1lacZ/lacZ mice (Fig. 1C). Taking advantage of the lacZ reporter, we evaluated β-galactosidase (β-Gal) activity to identify which cell population expressed Gli1 both in the normal pancreas and following the induction of pancreatitis. Gli1 expression was confined to a subset of fibroblasts, most prominently perivascular fibroblasts (bottom row in Fig. 1, B and C).

Together, these results support a role for Gli1 expression in fibroblasts for proper tissue repair in the adult pancreas.

To determine whether Gli1 was similarly required to mediate pancreas repair following oncogenic KrasG12D inactivation, we crossed Gli1lacZ/lacZ mice with the iKras mouse model of pancreatic cancer, which allows for tissue-specific and reversible control of oncogenic KrasG12D, to generate iKrasGli1lacZ/+ animals. In iKras mice, activation and inactivation of the oncogene is obtained by the addition or removal of doxycycline (DOX) from the drinking water (Fig. 2A). The iKras mouse develops PanINs and a pronounced desmoplastic stroma upon oncogenic Kras activation followed by the induction of acute pancreatitis (17). Conversely, inactivation of oncogenic Kras expression in low grade PanINs leads to complete tissue recovery. We administered DOX to 4–6-week-old iKras and iKrasGli1lacZ/+ mice to express KrasG12D and then induced pancreatitis. We harvested pancreata 3 weeks later (n = 3–5) (Fig. 2B). Both iKras and iKrasGli1lacZ/+ mice exhibited a substantial acinar cell loss and PanIN formation with accumulation of desmoplastic stroma (Fig. 2, C and D). Thus, inactivation of one allele of Gli1 did not affect pancreatitis-driven PanIN formation in this model.

We then inactivated KrasG12D expression, by removing DOX from the water, and harvested tissue after 3 days and 2 and 5 weeks (n = 3–5). Notably, upon KrasG12D inactivation, recovery of the acinar cell compartment was apparent after only 3 days in iKras mice, whereas iKrasGli1lacZ/+ mice displayed delayed recovery of the acinar compartment (Fig. 2, C and D). Two weeks following KrasG12D inactivation, recovery of the acinar compartment as well as comprehensive remodeling of the stroma were complete in iKras mice. In contrast, iKrasGli1lacZ/+ mice retained low grade PanIN lesions and displayed a prominent amount of stroma even at the 5-week time point (Fig. 2C). Using β-Gal staining to follow Gli1 expression, we found that Gli1 levels increased throughout the stroma after 3
weeks of oncogenic Kras activation and persisted even after Kras inactivation (Fig. 2C, bottom row). These data suggest a critical role for Gli1 dosage during tissue recovery following both caerulein-induced pancreatitis and after inactivation of oncogenic Kras.

**FIGURE 1. Reduced expression of Gli1 delays tissue repair following pancreatitis.** H&E (scale bars, 50 μm) and β-Gal staining (scale bars, 20 μm) for WT (A), Gli1lacZ/+/H11001 (B), and Gli1lacZ/lacZ (C) mice either untreated or treated 2 days (2d) or 1 week (1w) following pancreatitis. Both Gli1lacZ/+/H11001 and Gli1lacZ/lacZ pancreata exhibit a delay in tissue recovery compared with WT, with some fibrotic areas remaining (black arrows) 1-week post-pancreatitis.

**Loss of Gli1 Impairs Stromal Remodeling during Tissue Repair—**To determine which cell types in the pancreas express Gli1, we performed co-immunofluorescence for β-Gal along with various epithelial, fibroblast, and immune cell markers in iKras*Gli1lacZ/+ mice. First, we observed a distinct segregation
FIGURE 2. Reduced expression of Gli1 impairs tissue recovery following oncogenic Kras inactivation. A, genetic make-up of iKras*Gli1lacZ/+ mice. B, experimental design. Kras* = KrasG12D. C, H&E (scale bars, 50 μm) and β-Gal staining (scale bars, 20 μm) for iKras* and iKras*Gli1lacZ/+ pancreata with Kras* active (Kras* ON) for 3 weeks (w) and following Kras* inactivation (Kras* OFF) 3 days (d) and 2 and 5 weeks. D, histopathological analysis of iKras* and iKras*Gli1lacZ/+ tissues at the indicated time points; data represent mean ± S.E.
of β-Gal+ cells and CK19, a marker for pancreatic ducts and PanINs, indicating that ducts and neoplastic epithelial cells did not express Gli1 (Fig. 3A). In contrast, we identified widespread co-expression of β-Gal with SMA, a marker of activated fibroblasts, as well as co-localization with Vimentin, another fibroblast marker (Fig. 3B, and data not shown). We additionally observed co-expression of Gli1 with a subset of the macrophages within the tissue, identified by the marker F4/80 (Fig. 3C). To determine whether the expression pattern of Gli1 was conserved in a different model of KrasG12D-driven pancreatic carcinogenesis, we crossed Gli1lacZ+/H11001 reported mice with the KC mouse (7). We collected pancreata from KC;Gli1lacZ+/H11001 mice 3 weeks following the induction of pancreatitis, when widespread PanINs are expected. Similar to our findings in the iKras*Gli1lacZ/+ mice, β-Gal expression in KC;Gli1lacZ/+ mice was detected in activated fibroblasts and in a subset of immune cells (expressing CD45) but not in epithelial cells (Fig. 4, A–D). Thus, stromal expression of Gli1 is common to caerulein-induced pancreatitis and two independent models of pancreatic cancer.

Next, we wanted to assess the stroma that failed to remodel in iKras*Gli1lacZ/+ mice. Thus, we performed a Gomori Trichrome stain to visualize and quantify collagen deposition. Three weeks after KrasG12D activation, both iKras* and iKras*Gli1lacZ/+ mice pancreatea had an abundance of collagen deposition, which is a hallmark of the desmoplastic response in the neoplastic pancreas (23). The collagen deposition persisted at the 3-day time point but was fully resolved by 2 weeks following KrasG12D inactivation in iKras* mice. In contrast, in iKras*Gli1lacZ/+ mice, abundant collagen deposition was still observed throughout the stroma at 2 weeks, indicating a delay or impairment of the remodeling process (Fig. 5, A and B).

Both in iKras* and iKras*Gli1lacZ/+ pancreatic tissues, active proliferation (measured by Ki67 immunostaining) was observed both in the epithelial and stromal compartments 3 weeks following KrasG12D activation (Fig. 5C). Subsequent inactivation of oncogenic Kras shifted the majority of cell proliferation to the epithelial compartment in both genotypes (Fig. 5C). Additionally, in the presence of oncogenic Kras, phospho-ERK1/2 (pERK1/2), a downstream effector of Kras, was elevated in the epithelial compartment (Fig. 5D). Upon KrasG12D inactivation, pERK1/2 levels decreased in the epithelium, and there was a transient increase in the stroma of iKras* pancreata, as described previously (17). Interestingly, in iKras*Gli1lacZ/+ mice, elevated pERK1/2 levels in the stroma persisted even at 5 weeks following KrasG12D inactivation (Fig. 5D). Finally, we assessed SMA expression to mark the reactive stromal compartment. Three weeks following induction of KrasG12D expression, robust SMA expression was detected in the stroma of both sets of mice (Fig. 6A, black arrows). This expression rapidly decreased in iKras* animals upon KrasG12D inactivation. However, in iKras*Gli1lacZ/+ mice, SMA expression persisted longer than in iKras* mice. By 2 weeks, however, most of the fibroblasts persisting in the pancreas did not express SMA (Fig.
GLI1 in Pancreatic Remodeling

To investigate potential mechanisms by which GLI1 regulates pancreatic recovery, we assessed the expression of cytokines involved in tissue remodeling. First, we performed qRT-PCR analysis to assess differences in cytokine production between iKras* and iKras*GLI1lacZ/+ pancreatic tissue (scheme in Fig. 7A). We detected a significant decrease in IL-6 expression in the iKras*GLI1lacZ/+ pancreas (Fig. 7B). Likewise, we detected a significant reduction in both Mcp-1 and the murine homolog of IL8 (mIL-8) in iKras*GLI1lacZ/+ pancreata (Fig. 7, C and D). These cytokines all regulate leukocyte accumulation and function (25); however, only IL-6 was described previously as a GLI1 target (26). Additionally, transcription of M-csf, a critical factor for macrophage differentiation and function (27), was reduced in the iKras*GLI1lacZ/+ pancreas (Fig. 7E). These data suggest that GLI1 may regulate recovery of the pancreas through the expression of secreted factors involved in immune system function.

To test whether GLI1 loss affected cytokine production specifically in fibroblasts, we extracted pancreatic fibroblasts from untreated WT and Gli1lacZ/+ mice (scheme in Fig. 7F), followed by serum starvation and treatment with either cancer cell conditioned medium (CCM) from primary iKras* tumor cells (28) or control media for 0, 6, and 24 h; we then collected cells for RNA extraction (scheme in Fig. 7F). In parallel, fibroblasts were treated with conditioned medium from control or SHH-expressing COS7 cells. As expected, Gli1 and Ptch1 expression increased in WT fibroblasts treated with SHH-expressing COS7 cell conditioned medium, as measured by qRT-PCR. Of note, Gli1 had a higher basal expression level but reduced Shh-dependent activation in Gli1lacZ/+ fibroblasts. Moreover, the extent of Ptch1 activation was reduced in Gli1lacZ/+ fibroblasts (Fig. 7G). Over the course of experimental repeats, we found that Gli1 expression in WT fibroblasts was inconsistently increased after 6 h but always significantly increased by 24 h. Furthermore, transcription of IL-6 was significantly increased 6 h after exposure to CCM, in both WT and Gli1lacZ/+ cells. At 24 h however, WT fibroblasts sustained significantly greater levels of IL-6 transcription than Gli1lacZ/+ cells (Fig. 7H). Expression of both Mcp-1 and mIL-8 was also significantly reduced in Gli1lacZ/+ fibroblasts at both 6 and 24 h post-treatment (Fig. 7, I and J). Moreover, transcription of M-csf was also significantly reduced at 6 h in Gli1lacZ/+ fibroblasts compared with their WT counterparts; however, by 24 h the levels of M-csf transcription were the same (Fig. 7K). Of note, as both WT and Gli1lacZ/+ fibroblasts express a basal level of GLI proteins, activation of target genes could occur even before new Gli1 is transcribed. These results identify multiple potential GLI1-dependent cytokine targets in pancreatic fibroblasts.

To test whether these putative GLI1 targets were common to other fibroblast populations, we treated serum-starved NIH/3T3 cells with control-conditioned medium (COS7-CM) or NSHH-conditioned medium (COS7-SHH) for 24 h followed by assessment of cytokine expression by qRT-PCR (scheme in Fig. 8A). We also included untreated cells to exclude any effects of the control-conditioned media. Gli1 and Ptch1 were up-regulated in response to NSHH treatment, confirming increased expression (Fig. 6B), consistent with a role for this gene in pancreatic recovery and remodeling.
HH pathway activity in these cells (Fig. 8B). Although we did not observe significant changes in the expression of Mcp-1 or M-csf, both IL-6 and mIL-8 were up-regulated in response to NSHH treatment (Fig. 8B). Therefore, IL-6 and mIL-8 may be general HH targets in fibroblasts, whereas Mcp-1 and M-csf might be pancreas-specific.

Finally, we determined whether these cytokines were direct GLI1 target genes. Bioinformatic analysis identified multiple putative GLI1-binding sites in the promoters of these cytokines (Fig. 8C). To assess GLI1 binding to these sites, we performed ChIP assays in mouse embryonic fibroblasts. Endogenous GLI1 bound to the promoter of IL-6, mIL-8, Mcp-1, and M-csf (Fig. 8, D–G). We also observed GLI1 binding on the Cend1 promoter (Fig. 8H), used as a positive control (29, 30). To control for the specificity of GLI1 binding, we performed PCR in a region lacking canonical GLI1-binding sites. Importantly, GLI1 binding was not detected in this region (Fig. 8I). Taken together, these data indicate that several cytokines important in immune cell recruitment and function are direct GLI1 transcriptional targets whose expression is reduced upon loss of a single Gli1 allele.

Immune Cell Infiltration Is Regulated in a Gli1-dependent Manner—To define whether the altered cytokine expression profiles detected in Gli1lacZ/+ animals affected immune cell recruitment in vivo, we performed flow cytometry analysis on iKras+ and iKras+Gli1lacZ/+ mice 3 weeks after pancreatitis. We quantified both the myeloid and T lymphocyte subsets recruited to the neoplastic pancreas. Because B lymphocytes

FIGURE 5. Gli1 is required for stroma remodeling. A, Gomori Trichrome staining of iKras+ and iKras+Gli1lacZ/+ pancreata. Scale bar, 50 μm. B, quantification of Trichrome-positive area (expressed as percentage of total area). Data represent mean ± S.E. Immunohistochemistry for Ki67 (C) and pERK1/2 in iKras+ and iKras+Gli1lacZ/+ (D) pancreata. Scale bars, 20 μm. d, day; w, week.
FIGURE 6. Characterization of iKras* and iKras*Gli1lacZ/+ tissues upon Kras* inactivation. A, immunohistochemistry for SMA in iKras* and iKras*Gli1lacZ/+ pancreata. Black arrows indicate fibroblasts positive for SMA expression, and white arrows indicate fibroblasts negative for SMA expression. Scale bars, 20 μm. qRT-PCR for HH signaling pathway components are shown for Gli1 (B), Ptc1 (C), Hhip1 (D), Gli2 (E), and Shh (F). d, day; w, week.
are not reported to function in the wound-healing process, we did not quantify their numbers in this analysis (31). We detected no difference in the overall population of CD45+/H11001 hematopoietic cells (Fig. 9A). Previous work characterizing the immune infiltrate of pancreatic cancer revealed a prominent leukocyte population, predominantly F4/80+/H11001/CD11b+/H11001 macrophages, that developed during PanIN formation and persisted through tumor development (32). Comparison of
iKras* and iKras*Gli1lacZ/+ pancreata 3 weeks after KrasG12D activation revealed lower F4/80 staining in the stroma of mice with reduced Gli1 dosage (Fig. 9B). Flow cytometry analysis corroborated the decrease in F4/80+ macrophages (Fig. 9C). Additional characterization of the myeloid populations revealed that immature myeloid cells (iMCs), a heterogeneous population of immature monocytes and granulocytes, were decreased in iKras*Gli1lacZ/+ samples compared with iKras* tissues (Fig. 9D). Both polymorphonucleus-derived iMCs and mononucleus-derived iMCs were reduced in iKras*Gli1lacZ/+ pancreata (Fig. 9, E and F).

Analysis of T cell subsets revealed significantly elevated CD3+ lymphocyte populations in the iKras*Gli1lacZ/+ pancreas (Fig. 9G). More specifically, both CD3+/CD4+ Helper T cells (Fig. 9H) and CD3+/CD8+ cytotoxic T cells (Fig. 9I) were significantly increased in the iKras*Gli1lacZ/+ pancreas. However, levels of CD3+/CD4+/CD25+/FoxP3+ regulatory T cells were equivalent between iKras* and iKras*Gli1lacZ/+ pancreas (Fig. 9J). Thus, the changes in the myeloid cell subset correlated with the changes observed in the T cell populations. Moreover, upon matching qRT-PCR Gli1 expression with flow-analyzed immature myeloid cell populations for individual iKras*Gli1lacZ/+ mice, we found a positive correlation between Gli1 levels and myeloid cell infiltration (Fig. 9K). Taken together, these data indicate a novel role for Gli1 in regulating the immune cell response in the neoplastic pancreas.

Reduced Gli1 Expression Decreases M2 Macrophage Recruitment to the Pancreas—During tissue recovery, macrophages play an important role in multiple aspects of the wound healing process (33). In particular, alternatively activated macrophages (M2) can digest cell debris and extracellular matrix components to promote tissue remodeling (34, 35). Given the importance of M2 macrophages in tissue recovery, we tested whether a reduction in Gli1 affected their recruitment. We performed flow cytometry on the pancreas after 3 weeks of KrasG12D activation, as well as 3 days following subsequent KrasG12D inacti-

![Figure 8](image-url)
FIGURE 9. Tissues with reduced Gli1 expression have altered immune infiltration. A, flow cytometry analysis for CD45+ cells. B, immunohistochemistry for F4/80 macrophages in iKras* and iKras*Gli1lacZ+ pancreata. Flow cytometry analysis is shown for macrophages (C), immature myeloid cells (D), polymorphonucleus-derived immature myeloid cells (E), mononucleus-derived immature myeloid cells (F), CD3+ T cells (G), Helper T cells (H), cytotoxic T cells (I), and regulatory T cells (J). K, correlation between Gli1 expression and immature myeloid cell infiltration in iKras*Gli1lacZ+ pancreata. Flow cytometry analysis is shown for M2 macrophages (L) and M1 macrophages (M).
FIGURE 10. Canonical Hh signaling is essential for pancreatic tissue repair following injury. A, H&E for p48Cre;Shh/+/pancreata either untreated or 2 days (d) and 1 week (w) following pancreatitis. Fibrotic areas are highlighted with black arrows. B, experimental design. C, quantitation of tissue repair. D, H&E (scale bars, 50 μm), Gomori Trichrome (scale bars, 50 μm), SMA immunostaining (scale bars, 50 μm), and pERK1/2 immunostaining (scale bars, 50 μm) for iKras* pancreata with Kras* active (Kras* ON) for 3 weeks and subsequently inactivated (Kras* OFF) 2 weeks. Mice were treated with vehicle or Smo antagonist LDE-225 during the 2 weeks following Kras* inactivation. E, experimental design. F, qRT-PCR analysis for cytokines IL6, Mcp-1, mIL-8, and M-csf in WT pancreatic fibroblasts treated with CCM with and without concomitant HH inhibition.
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FIGURE 11. Working model of GLI1 regulation of pancreas remodeling following injury. Following injury, pancreatic fibroblasts up-regulate the expression of cytokines regulating myeloid cell recruitment and function. GLI1 dosage is critical for this response. Reduced GLI1 dosage leads to reduced cytokine expression and impaired recruitment of myeloid cells. Specifically, the recruitment of M2 macrophages (the myeloid population critical for injury repair and tissue remodeling) is reduced. Ultimately, reduction in GLI1 dosage results in improper pancreatic recovery.

viation to detect numbers of M2 (CD11b⁻;CD64⁺;F4/80⁻; CD11c⁻;CD206⁺) and M1 (CD11b⁺;CD64⁺;F4/80⁺;CD206⁻; CD11c⁺) macrophages. In both iKras⁺ and iKras⁺GlilacZ/⁺ mice, M2 macrophage numbers were comparably low at 3 weeks of KrasG12D activation. Interestingly, M2 macrophage numbers increased significantly upon KrasG12D inactivation in iKras⁺ mice. However, we detected a significant decrease in M2 macrophages in iKras⁺GlilacZ/⁺ mice at this time point (Fig. 9L). This population reduction was specific to M2 macrophages, as M1 macrophage populations were comparable between iKras⁺ and iKras⁺GlilacZ/⁺ mice at both time points tested (Fig. 9M). Thus, the specific macrophage population that is critical for tissue repair is decreased in iKras⁺GlilacZ/⁺ mice.

Canonical Hedgehog Signaling Is Required for Pancreas Recovery—To determine whether the loss of GLI1 affected pancreas tissue repair through the HH pathway (36), we used two complementary in vivo approaches. First, we investigated the ability of mice lacking expression of SHH ligand in the pancreatic epithelium (p48Cre;Shhf/−) to recover from pancreatitis. Similar to GlilacZ/⁺ mice, p48Cre;Shhf/− mice displayed defects in stromal remodeling 1 week after pancreatitis (Fig. 10A). Second, we used a pharmacological method to inhibit canonical HH signaling. PanIN-bearing iKras⁺ mice were treated with either the Smo antagonist LDE225 or with vehicle by oral gavage every 24 h for 2 weeks following KrasG12D inactivation (Fig. 10B). As expected, vehicle-treated iKras⁺ mice showed complete recovery of the epithelium and remodeling of the stroma. In contrast, the vast majority of LDE225-treated tissues did not recover (Fig. 10, C and D). Similar to iKras⁺GlilacZ/⁺ mice, LDE225-treated iKras⁺ pancreata showed improper stromal remodeling, with abundant residual collagen deposition (Fig. 10D, bottom row). LDE225-treated mice also displayed SMA and pERK1/2 positivity throughout the stroma, indicating a reactive stroma that failed to resolve (Fig. 10D). Thus, canonical HH signaling is necessary for PanIN regression and stromal remodeling in the pancreas.

To determine whether the cytokines we identified as GLI1 targets were regulated by canonical HH signaling, we treated WT pancreatic fibroblasts with CCM alone or with CCM and the selective Smoothened inhibitors SANT-1 or SANT-2 (scheme in Fig. 10E) (37). After 6 h of treatment, cells were collected for qRT-PCR analysis. CCM treatment induced expression of IL-6, Mcp-1, mIl-8, and M-CSF, as seen previously. However, concomitant HH inhibition significantly reduced the level of induction, indicating that ligand-dependent HH signaling mediates activation of these targets (Fig. 10F). Collectively, our in vivo and in vitro data reveal a requirement for canonical HH signaling for cytokine expression in fibroblasts and for pancreas tissue repair.

DISCUSSION

The HH signaling pathway is essential for proper embryonic development and adult tissue homeostasis (38). HH signaling culminates in the modulation of the GLI1 family of transcription factors, which in turn regulate the gene expression mediating HH-dependent cellular responses (39). Mammals possess three GLI transcription factors, GLI1, GLI2, and GLI3. Although both Glil and Glil mutant mice die embryonically or perinatally due to a range of HH-dependent developmental defects, Glil mutant mice are viable and fertile, and reach adulthood with no apparent impairments (40). However, recent studies indicate that these mice have defects in regulating inflammation and tissue repair following injury (41). Specifically, mice hemizygous for Glil are more susceptible to intestinal inflammation and chemical-induced injury (42). Glil mice are also less susceptible to inflammation-induced metaplasia upon Helicobacter pylori infection (43). In humans, a variant of Glil with reduced transcriptional function confers higher susceptibility to irritable bowel disease mediated by an altered myeloid response (42). Thus, both in previous studies and in our work, Glil dosage appears to mediate critical aspects of myeloid cell function in the gastrointestinal system. These findings could be explained with a threshold model, wherein the overall ratio of GLI1 activator and repressor is crucial for transcriptional output. Future studies will be required to examine the requirement for other GLI factors in pancreatic cancer.
We have previously shown that GLI activity in pancreatic fibroblasts leads to expression of IL-6, an inflammatory cytokine that activates Stat3 in the pancreatic cancer cells (26). However, the regulation of Gli1 expression and its role in pancreatitis and tissue repair and during the onset of pancreatic cancer have not been comprehensively addressed.

Here, we investigated the role of Gli1 during tissue repair in the pancreas. Unlike the stomach and intestine, which have a basal level of active HH signaling, components of the HH pathway are undetectable in the normal pancreas both during development and in the adult tissue (24, 44). However, HH signaling is activated during pancreatitis (45), as well as in pancreatic cancer (46, 47). The specific role of Gli1 during the processes, however, has not been explored. We investigated the effect of reducing Gli1 dosage in two pathologic contexts as follows: induction of acute pancreatitis and pancreatitis-induced pancreatic carcinogenesis. Inactivation of even a single copy of Gli1 disrupted tissue repair following both pancreatitis and oncogenic Kras inactivation. We determined that Gli1 was primarily expressed in pancreatic fibroblasts. The primary function of fibroblasts is to secrete extracellular matrix proteins and thus provide structural integrity to connective tissues (48). However, these cells not only contribute to tissue homeostasis but also respond to epithelial damage. In addition to promoting epithelial recovery, fibroblasts secrete a range of immunomodulatory proteins, such as IL-6, MCP-1, and IL-8, which regulate immune cell function during both tissue damage and recovery (49, 50). This function is critical, as improper immune cell recruitment has adverse effects on various aspects of tissue healing. Although fibroblast-mediated regulation of immune response is important for wound recovery, few studies have focused on the ability of these stromal cells to coordinate immune cell recruitment and function during neoplasia (51). In this study, we found that Gli1lacZ/+ fibroblasts had a significantly altered transcriptional profile compared with their WT counterparts in response to cancer cell conditioned media. Notably, loss of Gli1 resulted in lower transcriptional levels of multiple potent factors that regulate immune cell migration and function, such as M-csf, Mcp-1, and mll-8. Consequently, we found that in vivo, the profile of immune cells in the PanIN-bearing pancreas is significantly altered; loss of Gli1 results in fewer myeloid cells and greater numbers of T cells.

The precise regulation of the immune response is critical for tissue recovery. In particular, the monocyte-macrophage population plays a vital role; these cells participate in wound debridement, the removal of extracellular matrix, and dead or damaged cells (31). In the liver, macrophage depletion during recovery from inflammatory injury resulted in impaired stromal remodeling. However, depletion of macrophages during injury induction led to an overall reduction in scar matrix (52). Likewise, our data suggest that the improper recruitment of myeloid cells, particularly M2 macrophages, leads to improper pancreatic repair in iKras+Gli1lacZ/+ mice.

In summary, our studies show that Gli1 is a key modulator of pancreatic inflammation, through transcriptional regulation of cytokine production in pancreatic fibroblasts. A working model summarizing our findings is provided in Fig. 11.

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