GLI1 Inhibition Promotes Epithelial-to-Mesenchymal Transition in Pancreatic Cancer Cells

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Abstract

The Hedgehog (HH) pathway has been identified as an important deregulated signal transduction pathway in pancreatic ductal adenocarcinoma (PDAC), a cancer type characterized by a highly metastatic phenotype. In PDAC, the canonical HH pathway activity is restricted to the stromal compartment while HH signaling in the tumor cells is reduced as a consequence of constitutive KRAS activation. Here, we report that in the tumor compartment of PDAC the HH pathway effector transcription factor GLI1 regulates epithelial differentiation. RNAi-mediated knockdown of GLI1 abolished characteristics of epithelial differentiation, increased cell motility, and synergized with TGFβ to induce an epithelial-to-mesenchymal transition (EMT). Notably, EMT conversion in PDAC cells occurred in the absence of induction of SNAIL or SLUG, two canonical inducers of EMT in many other settings. Further mechanistic analysis revealed that GLI1 directly regulated the transcription of E-cadherin, a key determinant of epithelial tissue organization. Collectively, our findings identify GLI1 as an important positive regulator of epithelial differentiation, and they offer an explanation for how decreased levels of GLI1 are likely to contribute to the highly metastatic phenotype of PDAC.

Introduction

With a 5-year survival rate around 5% and about 37,000 deaths per year, pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancers and ranks 4th among cancer-related deaths in the United States (1, 2). The extremely poor clinical prognosis of PDAC is partially attributed to its high tendency to metastasize (3). However, the molecular mechanisms underlying invasion and metastasis in pancreatic cancer remain poorly understood.

Epithelial-to-mesenchymal transition (EMT) is a process in which cells lose their epithelial character and acquire a migratory mesenchymal phenotype (4). Although being crucial for normal metazoan development, EMT (and the reverse process mesenchymal-to-epithelial transition MET) is thought to be recapitulated in metastasizing cancer cells (5).

Loss of the homophilic cell adhesion molecule E-Cadherin, which is a main determinant of epithelial tissue organization and cell polarity, is considered a hallmark of EMT (6). A multitude of in vitro and in vivo models show enhanced invasiveness and metastasis upon E-Cadherin (CDH1) repression, whereas E-Cadherin overexpression conversely leads to a significant decrease in tumor malignancy (7–9). Repression of E-Cadherin occurs primarily on the transcriptional level and is in many instances mediated by direct binding of transcriptional repressors like SNAIL, SLUG, or TWIST to E-Box consensus sequences in the CDH1 promoter (10). Early invasion and metastasis as prevalent traits of pancreatic cancer suggest a prominent role for EMT and its upstream activators in the pathogenesis of PDAC (11–13).

Hedgehog (HH) signaling is one of 12 deregulated signal transduction pathways in pancreatic cancer (14, 15). Recent work shows that HH pathway activity in this cancer type is asymptomatically distributed. Although the epithelial tumor compartment constitutes the source of HH ligands, high HH pathway activity is predominantly associated with the stroma (16–19). The HH-activated stroma is in turn responsible for the production of tumor growth-promoting factors (17). HH pathway activity is not absent in the tumor cells, but is significantly lower compared with the stroma of human and mouse PDAC (16). Despite its low abundance in tumor cells, GLI1, a member and a transcriptional target of HH signaling, contributes to in vitro cell proliferation, anchorage-independent growth and cancer cell chemoresistance (18, 20, 21). The low HH/GLI activity in PDAC tumor cells are, at least to some extent, the result of mechanisms activated by mutant KRAS, which is a key driver of malignant development in the pancreas. First, KRAS leads to the abrogation of primary cilia on PDAC cells, an organelle crucial for the reception and transmission of signaling induced by HH ligands (22). Second, KRAS actively suppresses signaling events downstream of the primary cilium.
Here, we provide evidence that the low GLI1 level usually found in the epithelium of pancreatic carcinoma primes the cells toward an EMT. GLI1 is a transcriptional activator of E-Cadherin (CDH1), a main determinant of epithelial tissue organization and cell polarity (6). Lowering the endogenous GLI1 levels in PDAC cells experimentally results in loss of E-Cadherin expression, changes in cell morphology typically associated with a mesenchymal phenotype and an increase in cancer cell motility. We find that GLI1 levels significantly correlate with CDH1 expression in pancreatic cancer cells and in primary patient material. Interestingly, the effects of GLI1 on CDH1 expression do not require the upregulation of several well-established EMT inducers including SNAIL and SLUG and are instead mediated by the direct binding of GLI1 to the CDH1 promoter. Moreover, moderately decreased GLI1 expression significantly synergizes with stroma-derived EMT- and migration-inducing factors such as TGFβ and hepatocyte growth factor (HGF). These data ascribe a functional role for HH pathway suppression in cancer and propose that the reduced expression of an oncogene might be of functional relevance for certain aspects of tumor development.

Materials and Methods

Cell lines and regents

PDAC cell lines were obtained from American Type Culture Collection and were cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (high Glucose) plus 10% heat-inactivated FBS plus 1 mM/L Na-Pyruvate and Penicillin/Streptomycin. Cell lines were not passed longer than 6 months. Recombinant TGF-β1 and recombinant HGF (both R&D Systems) were used at final concentrations of 5 ng/mL and 10 ng/mL, respectively. SB-431542 (Sigma) was employed at a final concentration of 10 μmol/L; corresponding amounts of dimethyl sulfoxide were added to the untreated samples. Cells were usually treated 24 hours after siRNA transfection and treatment was maintained for 48 hours. The E-Cadherin luciferase reporter construct was a kind gift of Lluis Lajas (INSERM).

Transfection with siRNA

Cells were seeded at 50% to 70% confluency at transfected with siRNA using Dharmafect1 according to the instructions of the manufacturer. Fresh medium was added 24 hours after the beginning of the transfection. If not stated otherwise, cells were harvested 72 hours after transfection.

Immunoblotting

Cultured cells were lysed in SDS-Buffer, proteins were separated on SDS gels and transferred onto polyvinylidene difluoride membranes. Detection of blotted proteins was by incubation of the membranes using the following antibodies: anti-β-Actin (Sigma, A5441); anti-HA Tag (Cell Signaling, #2367); anti-E-Cadherin (BD Biosciences, #610181); anti-β-Catenin (Cell Signaling, #9587), anti-α-E-Catenin (Cell Signaling, #3236); anti-Twist1/2 (Santa Cruz, sc-15393); anti-Zeb1 (Cell Signaling, #3396); anti-Zeb2 (Sip1; Santa Cruz, sc-271984); anti-Snail (Cell Signaling, #3879); anti-Slug (Cell Signaling, #9585).

Immunofluorescence

Cells were seeded on cover slips or chamber slides (Nunc) and were fixed for 10 minutes in 4% formaldehyde/PBS at room temperature. Staining of permeabilized cells was carried out using the following antibodies: α-E-Cadherin (Cell Signaling, #4065); α-β-Catenin (Cell Signaling, #9587); anti-α-E-Catenin (Cell Signaling, #3236); α-Cytokeratin (CAM5.2; BD Biosciences, #347653); α-Keratin 14 (Covance, #PRB 155-P); Texas Red Phalloidin (Invitrogen). Subsequently, cells were mounted in Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Labs). Microscopy was carried out on a Leica differentially methylated region fluorescence microscope, pictures were taken with a QuantiFire XI camera (Intas) and processed with Photoshop (Adobe).

Scratch assay

In vitro scratch assay was carried out according to Liang and colleagues (23). Pancreatic cancer cell lines suitable for this assay were chosen according to growth characteristics and their ability to form monolayers in the applied time frame. Cells seeded in 24-well cell culture plates were transfected at 60% to 70% confluence in triplicates. Scratching was carried out 48 hours posttransfection. Growth medium was removed and straight incisions were made with a P200 pipette tip at a 90° angle. Cells were washed several times with PBS to remove detached cells and supplied with new growth medium. Pictures of the scratches were taken at 0 and 24 hours using an Axiovert 35 inverted microscope (Zeiss) equipped with a Scion digital camera. To ensure the recording of the same scratch area, reference points were made on the culture plate with a marker pen prior to photography. Migration was measured as closure of the scratched area as suggested in ref. (23). Images were processed using TScratch (CSelab, ETH Zurich; http://www. cse-lab.ethz.ch/; ref. 24). Open scratch area was determined automatically according to developer’s suggestions and—to acquire higher accuracy—adjusted manually. Migration was subsequently defined as ratio of open scratch area after 24 hours and initial scratch area. At least 3 independent experiments were carried out in triplicates.

Results

GLI transcription factors are regulators of CDH1 expression in pancreatic cancer cells

On the basis of the high propensity of pancreatic cancer cells for early metastasis it is generally assumed that these cells harbor intrinsic alterations which prime them toward an EMT, an important promigratory and prometastatic driving force in tumors (6). Because HH/GLI activity has been linked to the induction of an EMT in other settings (25–27), we were interested to elucidate the role of GLI transcription factors in pancreatic cancer. We transiently transfected PDAC cell lines

(19). It is currently unclear if the asymmetrical distribution of HH/GLI activity in pancreatic cancer is of pathophysiologic significance or if it represents a mere byproduct of additional cancerous alterations.
with siRNA targeting either of the 2 activating GLI isoforms, GLI1 or GLI2 (Fig. 1A). Knocking down endogenous GLI1 or GLI2 in Panc1, Su86.86, AsPC1, or HS766T cells induced a striking reduction of CDH1 expression. As a positive control, we applied recombinant TGFβ, a potent EMT inducer in PDAC cells (11, 13). Interestingly, CDH1 reduction was more pronounced in siGLI1-transfected cells than in TGFβ-treated samples, suggesting a powerful contribution of GLI factors to the regulation of CDH1 expression. Given that GLI2 and GLI1 can induce each other’s expression (28, 29), it was possible that the effects of siGLI2 were, at least in part, mediated by a reduction in GLI1 expression. However, this GLI1/GLI2 regulatory circuit was not fully functional in AsPC1 and HS766T cells, allowing for the selective knockdown of GLI1 without changes in GLI2 expression (Fig. 1A). These experiments showed that knocking down GLI1 alone was sufficient for the repressive effects on CDH1 expression.

The significant reduction of CDH1 expression upon loss of the GLI transcription factors could also be verified on the protein level using immunoblotting (Fig. 1B) and immunofluorescent staining experiments (Fig. 1C). Supporting these findings and excluding off-target effects, similar results were obtained using additional siRNA constructs to knock down GLI1 (Supplementary Fig. S1). Conversely, overexpression of GLI1 strongly induced the transcription of CDH1 compared with mock-transfected cells. The increased CDH1 expression in these cells could not be documented on the protein level, suggesting that MiaPaCa2 cells harbor additional posttranscriptional mechanisms to decrease CDH1 protein amounts. Similar findings were recently reported by Song and colleagues (30). Interestingly, we were unable to detect a further CDH1 induction upon transfection of GLI1 in Panc1 and Su86.86 cells (Supplementary Fig. S2), suggesting that the GLI1-CDH1 axis is saturated in these cells. Hypothesizing that limiting amounts of transcriptional GLI-cofactors could be the reason for the lack of CDH1 induction after GLI1 transfection, we assumed that titrating out these cofactors should interfere with the capability of endogenous GLI1 to induce E-Cadherin expression. To address this question we cloned a GLI1 construct lacking the 5 zinc-finger domains which are required for DNA binding (GLI1AZF, Supplementary Fig. S3A). As expected and in contrast to full-length GLI1, this construct was unable to activate a luciferase reporter containing GLI-binding sites (Supplementary Fig. 3B). Despite the inability to bind to DNA however, Panc1 cells stably expressing GLI1AZF showed a prominent scattering and mesenchymal phenotype (Supplementary Fig. S3C). In line with the morphologic appearance, GLI1AZF-expressing cells had significantly reduced levels of E-Cadherin mRNA and protein (Supplementary Fig. S3D and S3E). Taken together, these results strongly suggest that transcriptional GLI cofactors are limiting and set the upper limit for GLI-induced CDH1 induction.

GLI1 modulates CDH1 expression in PDAC cells by regulating its promoter

Given the fact that a knockdown of GLI1 was sufficient to regulate CDH1 expression without alterations in GLI2 levels, we focused on GLI1 in our further studies. To expand our findings on the GLI1-CDH1 interplay we expanded our analysis and measured GLI1 and CDH1 expression in 15 PDAC cell lines. Figure 2A (and Supplementary Fig. S4) shows a statistically significant positive correlation between GLI1 and CDH1 expression in this panel. Interestingly, cell lines which harbor wild-type KRAS did not fully fit into the correlation (Fig. 2A). However, the correlation data suggested a more direct interaction between GLI1 and CDH1. Bioinformatics studies identified 13 candidate binding sites for GLI transcription factors located in 5 different clusters within 3,500 bp upstream of the transcriptional start site in the CDH1 gene (Fig. 2B). In agreement with our previous results, overexpression of GLI1 in Panc1 cells showed that this transcription factor is able to increase the activity of a luciferase construct driven by a 3 kb fragment of the CDH1 promoter (Fig. 2C). Finally, chromatin immunoprecipitation experiments showed binding of endogenous GLI1 to the CDH1 promoter (cluster #4) in Panc1 cells (Fig. 2D). To investigate if this GLI1-CDH1 relationship can also be found in vivo, we analyzed 144 primary tumor samples from pancreatic cancer patients and found a highly significant positive correlation between the GLI1 and CDH1 expression levels (Fig. 2E).

Taken together, these results establish CDH1 as a novel transcriptional target of GLI1 in PDAC cells.

Repression of CDH1 after GLI1 silencing does not depend on canonical EMT inducers

Because the repression of CDH1 expression during an EMT is normally executed by transcriptional repressors such as SNAIL, which directly bind to the CDH1 promoter, we wondered if the well-established EMT repressors are induced in pancreatic cancer cells after GLI1 knockdown. As can be seen in Fig. 3A, neither SNAIL (SNAI1) nor Slug (SNAI2) are induced by siGLI1. A lack of SNAIL induction was also verified on the protein level using siGLI1-transfected Panc1 cells (Fig. 3B). In contrast, TGFβ was capable of significantly inducing SNAIL expression on the mRNA and protein level in these cells (Fig. 3A and B). In line with these results, other established CDH1 repressors (TWIST1, TWIST2, ZEB1, ZEB2, TCF3, TCF4, GSC) were not induced after siGLI1 transfection, strongly suggesting a mechanism which does not rely on the induction of these CDH1 repressors (Supplementary Fig. SSA). Because TGFβ is a very potent EMT inducer and many PDAC cells express endogenous TGFβ (31), we wanted to investigate if a reduction in GLI1 levels would sensitize the cells to TGFβ. However, using a selective inhibitor of TGFβ signaling (SB4–31542) we found no effect on the ability of siGLI1 to repress CDH1 expression regardless if only endogenous or additional recombinant TGFβ was present (Fig. 3C). The functionality of the TGFβ antagonist was verified by measuring the expression of the TGFβ target gene PAI1 (Fig. 3C). Taken together, the effects of siGLI1 on CDH1 do not require the transcriptional induction of classical EMT inducers. Given that some EMT molecules are regulated on the protein level, we further analyzed the protein levels of SLUG, TWIST1/2, ZEB1, and ZEB2 in Panc1 cells transfected with siGLI1 (Supplementary Fig. S5B). In particular, ZEB1 was stabilized in cells with GLI1 knockdown. However, these alterations were also observable in cells with CDH1 knockdown (Supplementary Fig. S5B), arguing that the changes in ZEB1...
GLI1 regulates CDH1 expression. A, QPCR analysis of Panc1, Su86.86, AsPC1, and Hs766T cells transiently transfected with control siRNA (siLuc, targeting firefly luciferase) or siRNA targeting GLI1 (siGLI1) or GLI2 (siGLI2). The EMT inducer TGFβ (5 ng/mL for 48 hours) was applied as a positive control. B, immunoblot detecting of E-Cadherin expression in siGLI-transfected PDAc cell lines. Shown is a representative blot of at least 3 independent experiments. C, immunofluorescence staining of E-Cadherin (white) expression in siRNA-transfected Su86.86 cells. The nuclei are stained white. The inset shows a magnification. Shown is a representative picture of at least 3 independent experiments. D, MiaPaCa2 cells were stably transfected with EGFP (mock) or a GLI1 expression plasmid. QPCR analysis reveals the induction of CDH1 by exogenous GLI1.
Figure 2. CDH1 is a GLI1 target gene in PDAC cells. A, heatmap of GLI1 and CDH1 expression in 15 PDAC cell lines as measured by qPCR of triplicate samples. The positive correlation is statistically significant. B, schematic drawing depicting the predicted GLI-binding sites (G) in the human CDH1 promoter. The transcription start site is at +1. C, transient transfection of GLI1 into Panc1 cells results in a significant induction of a luciferase reporter construct containing the human CDH1 promoter. D, chromatin immunoprecipitation (CHIP) using an antibody against endogenous GLI1 in Panc1 cells. E, correlation of CDH1 and GLI1 mRNA copy number in 144 human primary pancreatic cancer samples.
protein amount are most likely associated with the induction of an EMT phenotype per se and are not GLI mediated.

Decreased GLI1 expression results in widespread loss of epithelial markers and the induction of a mesenchymal-like cellular morphology

The cell–cell adhesion molecule E-Cadherin is a major determinant of the organization of epithelial tissues and its loss is a hallmark of an EMT. E-Cadherin loss is associated with characteristic changes in cellular morphology resulting from reduced cell–cell contact and the gain of mesenchymal traits (7, 8). As shown in Fig. 4A, similar to TGFβ, the GLI1 knockdown led to specific alterations in PDAC cell morphology. These cells adopted a more mesenchymal, spindle-like shape, and left the epithelial cell clusters which are normally found in control samples. Staining the actin cytoskeleton with phalloidin furthermore revealed a rearrangement of F-actin filaments from the cortical periphery into internal stress fibers, similar to what can usually be found in mesenchymal cells. The changes in morphology could also be observed by transient cotransfection of cells with a short hairpin RNA (shRNA) targeting GLI1 (shGLI1; Fig. 4B).

Next, we analyzed changes in epithelial marker expression upon knockdown of GLI1. In line with the loss of E-Cadherin in siGLI1-transfected cells, additional important epithelial marker genes were also significantly decreased in numerous PDAC cell lines, such as Keratin 19 (KRT19) or the adherens junctions components EVAI1 and PTPRM (Fig. 4C; ref. 32). In contrast, Integrin β1 (ITGB1), which mediates the interaction with the extracellular matrix (ECM) and which has previously been implicated in cancer cell metastasis (33), was clearly upregulated (Fig. 4C). Moreover, global analysis of gene expression by means of cDNA microarray revealed a widespread reduction of cytokeratins in Panc1 cells transfected with GLI1 siRNA compared with cells transfected with control siRNA (Fig. 4D). Reduction of cytokeratin expression was also verified by immunostaining in Su86.86 cells (Fig. 4E). Thus, together these findings define GLI1 as a gatekeeper of the epithelial phenotype in PDAC cells.

Knockdown of GLI1 leads to the disassembly of adherens junctions

In epithelial cells, E-Cadherin is physically linked to catenins, which mediate the connection to the actin cytoskeleton. EMT induction in pancreatic cancer cells by exposure to TGFβ results in the disassembly of these adherens junctions concomitant with a destabilization of α- and β-Catenin (34). In line with these findings, α- and β-Catenin were destabilized in siGLI1-transfected PDAC cells and were grossly reduced in immunofluorescence and Western blotting experiments (Fig. 5A and B). Importantly, the remaining β-Catenin in siGLI1-transfected cells was mainly localized to the cytoplasm and was absent from the nucleus (Fig. 5A). In line with these observations, knockdown of GLI1 did not result in an induction of WNT signaling as measured by means of WNT luciferase reporter assays (Fig. 5C). In support of these findings, the WNT target gene AXIN2 was not induced upon transfection of cells with siGLI1 (data not shown). Similar results were recently
Figure 4. Knockdown of GLI1 expression leads to loss of epithelial markers. A, morphologic changes of Panc1 (left) and Su86.86 (right) cells after transfection with siGLI1 or exposure to TGFβ. The upper panel shows bright field images whereas the lower panel depicts fluorescence images after staining the f-actin network with Phalloidin-Texas Red. Nuclei appear in blue. B, Panc1 cells were transiently transfected with control (scramble) shRNA plasmid (shSCR) or GLI1-specific shRNA (shGLI1). For verification of shRNA efficacy see Nolan-Stevaux and colleagues (18). Cells were cotransfected with GFP-F to label membranes and to better visualize cell morphology. TGFβ was applied as a positive control. C, QPCR data showing the decrease in epithelial gene (KRT19, EVA1, PTPRM) expression upon knockdown of GLI1 in several cell lines. In contrast, Integrin β1 (ITGB1) is upregulated. D, microarray data showing a widespread reduction in Keratin gene expression in siGLI1 (Pool of 4 different siRNAs)-transfected compared to control (siLuc)-transfected Panc1 cells. Two independent experiments are shown. E, immunostaining of siRNA-transfected Su86.86 cells for Keratin 14 (KRT14) and Keratin 7/8 (KRT7/8).
described by Herzig and colleagues in a model of β-cell carcinogenesis showing lack of WNT pathway activation after loss of E-Cadherin (35). These data support a function for GLI1 in the maintenance of adherens junctions and in WNT pathway responsiveness, thus, further emphasizing its role as an epithelial differentiation factor.

**Decreased GLI1 levels promote pancreatic cancer cell migration**

The loss of CDH1 has been shown to promote cancer cell motility (7, 8). To elucidate if low GLI1 levels enhance cellular migration we carried out in vitro scratch assays with confluent PDAC cells which had been transfected with control siRNA, siCDH1 (as a positive control), or siGLI1. In agreement with our previous finding that knockdown of GLI1 results in decreased CDH1 expression, PDAC cells transfected with siGLI1 closed the scratch area much more efficiently than control cells (Fig. 6A and B). With respect to their migratory potential, the siGLI1-transfected cancer cells were equipotent to the positive control, siCDH1-transfected cells (Fig. 6A and B). Similar results were obtained using an independent set of GLI1-specific siRNA (Supplementary Fig. S6). Importantly, the increased closure of the scratch was not due to changes in cell number, as shown in Fig. 6C.

**Low GLI1 levels synergize with TGFβ or HGF to promote PDAC EMT and cell migration**

Pancreatic tumor and stroma cells express a variety of EMT-inducing factors, such as TGFβ and HGF (12). To investigate the functional interplay of lowered GLI1 levels with these factors, we used siRNA concentrations which minimally affect GLI1 expression. In addition, we applied recombinant TGFβ in a concentration range between 0.1 and 1 ng/mL, which had no discernable effect on CDH1 or GLI1 expression when applied alone (Fig. 7A). However, the combination of low siGLI1 and low TGFβ concentration had a synergistic effect on both, CDH1 expression (Fig. 7A) and cellular motility (Fig. 7B and C). A similar synergistic effect on E-Cadherin was also obtained using Su86.86 cells (not shown). To expand our analysis on synergism, we tested HGF as another motility-promoting factor present in the PDAC microenvironment. As can be seen in Fig. 7B and C, 2.5 ng/mL HGF (which on its own had no effect on cell migration) potently synergized with low siGLI1 in the in vitro scratch assay. Taken together, we could observe a synergistic behavior of only slightly reduced GLI1 levels with TGFβ or HGF on pancreatic cancer cell motility.

**Discussion**

The strong propensity of pancreatic cancer cells for invasion and metastasis implies that these cells are primed to undergo an EMT due to intrinsic alterations and/or extrinsic signals provided by the abundant microenvironment called desmoplasia. The developmental HH signaling pathway, which is normally inactive in the adult pancreas, has been shown to be reactivated in PDAC where it promotes stromal hyperplasia, myofibroblast differentiation, and production of ECM (36, 37). Contrasting an earlier perception, canonical HH signaling in tumor cells is dispensable for cancer development as was recently shown by the epithelium-specific deletion of Smo, a critical bottleneck of HH signaling, in a PDAC mouse model (18). Interestingly, mice with a homozygous Smo deletion in the pancreatic epithelium had a significantly reduced survival compared with heterozygotes (18), arguing that a reduction in basal HH signaling caused a higher mortality in these mice. In this respect, pancreatic cancer contrasts other malignancies such as basal cell carcinoma or medulloblastoma, in which high GLI1 levels in the tumor cells are necessary and sufficient for disease progression (38, 39). In PDAC, stromal GLI1 levels are up to 120- to 150-fold higher than in the neighboring
epithelium (16), possibly being the result of mutant KRAS acting on primary cilia and on downstream HH pathway components (19, 22). We could show for the first time that the low GLI1 levels associated with the epithelial compartment prime tumor cells toward the induction of an EMT. In PDAC cells, GLI1 is a positive regulator of E-Cadherin, one of the major epithelial determinants. Reduced expression of GLI1 results in a widespread loss of epithelial markers and leads to the acquisition of a mesenchymal morphology. Similar effects might be expected from a manipulation of endogenous GLI2, of which the full-length activator form could be detected in PDAC cells (Supplementary Fig. S7). In contrast, transfected GLI3 behaves as a transcriptional repressor on a HH/GLI reporter and an E-Cadherin reporter construct (Supplementary Fig. S8). Thus, the possibility exists that, following GLI1 removal, endogenous GLI3 occupies the empty GLI binding sites in the CDH1 promoter contributing to E-Cadherin repression. Alternatively, GLI3 might repress E-Cadherin via lowering of GLI1 expression.

Surprisingly, we could not detect an upregulation of mesenchymal marker genes such as Vimentin, Fibronectin, or N-Cadherin in siGLI1-transfected cells. To rule out the possibility that the time frame of our transient siRNA transfection experiments precluded the detection of secondary alterations in mesenchymal gene expression, we carried out 2 successive rounds of siRNA transfection and thus expanded our analysis to 6 days posttransfection. However, no upregulation of mesenchymal genes was observed (not shown), arguing for a selective control of epithelial marker genes by GLI1. However, despite the lack of altered mesenchymal gene expression, significantly increased cell motility was observed in cells with low GLI1.

Importantly, our data do not dispute a functional role for epithelial GLI1 in tumor growth and in fact, in vitro investigations document the importance for GLI1 in cell proliferation, anchorage-independent growth, and chemoresistance (18, 20, 21). Regarding the latter, we could find that Gemcitabine exposure to PDAC cells resulted in upregulation of both, GLI1 and CDH1. More work will have to unravel the exact interplay between GLI1, EMT and chemoresistance in pancreatic cancer. However, given the finding that even very modest reductions in GLI1 mRNA strongly synergized with TGFβ or HGF suggests that the cellular GLI1 expression level acts as a potent sensitizer regulating the epithelial
phenotype. These data imply that by adjusting the GLI1 expression levels, a cell could switch between a proproliferative (higher GLI1) and a promigratory (lower GLI1) state. In fact, it is known that cells undergoing an EMT proliferate less than control cells (40, 41). Therefore, it will be interesting to learn if GLI1 expression levels are undulating in vivo in phases of primary tumor growth, invasive growth, and metastasis. In this respect, it is interesting to see that MET (mesenchymal-to-epithelial transition), the reversal of EMT, is crucial for successful metastasis (42). In line with this concept and our findings, HH/GLI2 activation has been shown to induce CDH1 expression, maintain an epithelial phenotype in non-PDAC cell types and block keratinocyte invasiveness in 3D culture (43–45).

However, our findings in pancreatic cancer cells are to some degree in contrast to certain earlier reports in nontransformed kidney cells, prostate carcinoma, and nonalcoholic fatty liver disease (25–27). One potential reason being that the alimentary tract including the pancreas and the stomach are endodermally derived organs, suggesting that certain lineage- or tissue-specific effects might be of importance for the GLI1-CDH1 regulation. In fact, we could find a CDH1 decrease upon siGLI1 transfection also in carcinoma cells of the lung, another endodermally derived organ (not shown).

One recent report in PDAC cells claims a GLI1/MUC5AC-dependent E-Cadherin destruction process (46). However, this study describes a posttranslational mechanism which could only be detected under certain culture conditions and which utilized nonphysiologic GLI1 levels (46), raising doubts about the generalization of these events. Because our data show that overexpression of a mutant GLI1 lacking the zinc-finger domains is capable of inducing an EMT, similar indirect effects might be obtained by strong overexpression of full-length GLI1, giving a potential explanation for some of the differences also to another study in which ectopic GLI1 expression in normal pancreatic duct cells reduced CDH1 expression (47). In some instances, the in vitro use of pharmacologic inhibitors such as Cyclopamine might have been misleading given their...
HH-unspecific nature (17). In addition to potential off-target effects in the tumor cells, the possible influence of Smo inhibitors on the Gli-positive stroma in vivo might complicate the interpretation of the results (47). In an effort to address the reason for some of the discrepancies, we determined if the disease stage of the tumor cell (primary tumor versus metastasis) might play a role in the GLI1-responsiveness but could not find an altered CDH1 regulation in a primary tumor/metastasis cell line pair (Supplementary Fig. 59).

PDAC is characterized by the vast presence of stromal cells and the high content of extracellular matrix (ECM). In this context, it is very interesting that PDAC cells express Integrin β1 upon a reduction of GLI1. Integrins are key molecules implicated in the binding to and the communication with the ECM and the subsequent signaling by focal adhesion kinase (FAK; ref. 48). As a result, the cellular GLI1 levels might not only determine the cell-specific epithelial phenotype but also define its interactive potential with the environment. In fact, Integrin β1-FAK signaling has been shown to be essential for the proliferation of mammary carcinoma cells at metastatic niches (33).

Interestingly, TGFβ has recently been shown to act as an inducer of GLI2 and, as a result, also GLI1 (18, 49, 50). This raises the question of whether the EMT-promoting capability of TGFβ is simply stronger than the concomitant (MET-promoting) GLI induction or if the induction of GLI factors constitutes part of a negative feedback mechanism.

In summary, we identified GLI1 as a potent positive regulator of E-Cadherin (CDH1), the major gatekeeper of the epithelial phenotype in pancreatic cancer cells. Because this regulation involves direct GLI1 binding to the CDH1 promoter and does not depend on the upregulation of well-established CDH1 repressors like SNAIL, SLUG, or TWIST, it adds a novel mechanism to the regulatory circuits defining the differentiation state of tumor cells. Our findings place GLI1 in a central position within the tumor signaling network and associate key cellular decisions with the HH pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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