Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and usually lethal disease of uncertain etiology. It has been proposed that IPF results from an aberrant reactivation of developmental pathways, notably the Hedgehog–Glioma-associated oncogene homolog (GLI) pathway. In this study, we determined whether the Hedgehog pathway was activated in bleomycin-induced lung injury in mice, and whether targeting the Hedgehog–Gli pathway could decrease bleomycin-induced lung fibrosis. After intratracheal injection of bleomycin on Day 0, C57Bl6 mice received GDC-0449 (an inhibitor of Smoothened, the transducer of the pathway), or 2,2'-[[Dihydro-2-(4-pyridinyl)-1,3(2H,4H)-pyrimidinediy]bis(methylene)]bis[N,N dimethylbenzenamine (GANT61; an inhibitor of GLI transcription factors in the nucleus), from Day 7 to Day 13. At Day 14, whole-lung homogenates were obtained for morphological analysis, assessment of cell apoptosis and proliferation, collagen quantification, and evaluation of profibrotic (transforming growth factor-β, connective tissue growth factor, plasminogen activator inhibitor 1, vascular endothelial growth factor-A) and proinflammatory mediators (IL-1β) expression. We showed that the Hedgehog pathway was activated in bleomycin-induced lung fibrosis on Day 14 after injury, with an increased lung expression of the ligand, Sonic Hedgehog, and with increased messenger RNA expression and nuclear localization of GLI1 and GLI2. Inhibition of Smoothened with GDC-0449 did not influence the development of bleomycin-induced lung fibrosis. By contrast, the inhibition of GLI activity with GANT61 decreased lung fibrosis and lung collagen accumulation, and promoted an antiﬁbrotic and anti-inﬂammatory environment. Our results identify the hedgehog-Gli pathway as a profibrotic pathway in experimental fibrosis. Inhibition of the Hedgehog–Gli pathway at the level of GLI transcriptional activity could be a therapeutic option in fibrotic lung diseases.

Keywords: lung fibrosis; Sonic Hedgehog pathway; bleomycin; mouse model

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and usually lethal disease of uncertain etiology. It has been proposed that IPF results from an aberrant activation of alveolar epithelial cells after injury that provokes the migration, proliferation, and activation of mesenchymal cells with the formation of myofibroblastic foci (1). The molecular mechanisms that determine the nature of IPF are still poorly known and there is as yet no efﬁcient therapeutic. IPF was associated with aberrant activation of developmental signaling pathways (2). The Hedgehog pathway is
Clinical Relevance

This study shows for the first time that pharmacological modulation of the interaction of Glioma-associated oncogene homolog (GLI) transcription factors, the final effectors of Hedgehog pathway, with DNA protects mice from bleomycin-induced lung fibrosis suggesting that the Hedgehog-Gli pathway is profibrotic in vivo and might be a target for lung fibrosis treatment.

required for lung development, as it controls epithelial–mesenchymal interactions during early lung development and proper lung branching morphogenesis (3, 4). In vertebrates, three secreted factors have been identified as three mammal orthologs of Drosophila Hedgehog proteins: Sonic Hedgehog (SHH), Desert Hedgehog (DHH), and Indian Hedgehog (IHH) (5). In fetal lung, SHH is secreted by epithelial cells, and is required for mesenchymal differentiation and proliferation (3).

At the cellular level in mammals, the canonical Hedgehog signaling pathway occurs in the primary cilium, which is a sensory, nonmotile organelle, and is described as a microtubule-based, antenna-like structure of most higher eukaryote cells (5). In the absence of the ligand SHH, the Hedgehog transmembrane domain receptor, Patched-1 (Ptc), is localized at the primary cilium, and prevents the translocation of the seven transmembrane protein, Smoothened (SMO), from cytoplasmic vesicles into the cilium (6). SMO, which is considered as an obligatory signal transducer, modulates the activities of members of the Glioma-associated oncogene homolog (GLI) transcription factor family (7, 8). In the absence of the ligand SHH, GLI1 is not expressed, whereas GLI2 and GLI3 are processed into, respectively, a weak and a strong transcriptional repressor (5). The binding of the ligand SHH to its receptor, PTC, will exclude PTC from the primary cilium. Therefore, SHH relieves the inhibition of PTC upon SMO that will translocate into the primary cilium. SMO then promotes the formation of GLI2 transcriptional activator. After translocation into the nucleus, GLI2 induces the expression of GLI downstream targets, such as the activator, Gli1, the inhibitory receptor, Ptc, and the decay receptor, Hedgehog interacting protein (Hhip) (5). Indeed, Hhip is a membrane receptor, which acts as an antagonist of SHH (9).

Our group and others (10, 11) recently showed that the Hedgehog pathway was activated in the lung in patients with IPF, and that the Hedgehog–Gli pathway was crucial for fibroblast function and differentiation in vitro, particularly at the level of GLI transcription factors. Here, we hypothesized that targeting the Hedgehog–Gli pathway might be a clue to inhibiting fibrosis in vivo. Therefore, the aims of this study were: (1) to evaluate the activation of the Hedgehog–Gli pathway in bleomycin-induced lung fibrosis in mice; and (2) to demonstrate that inhibition of the Hedgehog–Gli pathway could inhibit the development of lung fibrosis in that experimental model.

Materials and Methods

Bleomycin-Induced Lung Fibrosis
All experiments were performed using male C57BL/6 mice and intratracheal bleomycin administration, as previously described (12).

To investigate the involvement of SHH pathway, the mice were treated with inhibitors of the SHH pathway. GDC-0449 (vismodegib) is a small-molecule inhibitor that binds to SMO. This agent was selected because of its human safety profile in phase 1 trials, as well as its effectiveness in solid organ tumors, like basal cell carcinoma and medulloblastoma (13, 14). The dose of GDC-0449 was adapted from Philips and colleagues (15). GDC-0449 (Selleck Chemicals, Houston, TX) was freshly reconstituted daily in DMSO. Mice were given intratracheal bleomycin on Day 1 and were assigned to treatment with either DMSO (control) or 40 mg/kg GDC-0449. All mice were given a subcutaneous injection every other day beginning on Day 7 after bleomycin injection.

A second group of mice was treated with 2,2’-[(Dihydro-2-(4-pyridinyl)-1,3 (2H,4H)-pyrimidinediy1]bis(methylene)]bis[N,N-dimethylbenzenamine (GLI antagonist [GANT] 61). GANT61 is known to inhibit the Hedgehog pathway by directly blocking the binding of GLI1 and GLI2 to their DNA targets (16). GANT61 was dissolved in DMSO. GANT61 (25 mg/kg) or vehicle was injected subcutaneously every other day from Day 7 to Day 13. This dose of GANT61 was adapted from the work of Lauth and colleagues (16). Mice were killed at Day 14, and lungs were removed for further analysis. In preliminary experiments, we verified that GDC-0449 and GANT61 did inhibit the expression of Gli1 mRNA, a major Hedgehog downstream target, in the lung when given to naïve mice every other day for a week (see the online supplement for further details).

Semiquantitative histological assessment of lung injury used the grading system described by Inoshima and colleagues (17). Hematoxylin and eosin staining and picrosirius staining were performed routinely to evaluate the morphology of the lung.

mRNA Analysis
Total mRNA was extracted from mouse lung homogenates, and the expression of the genes of interest was quantified by real-time PCR, as previously described (12) (see the online supplement for further details, including primer sequences in Table E1).

Western Blot Analysis
The ligand SHH, the receptor, PTC, and cleaved poly (ADP-ribose) polymerase (PARP) were quantified by Western blot in the left mouse lung by standard techniques (10). α-Actin and β-tubulin were used as loading controls (see the online supplement for further details).

Lung Collagen and Transforming Growth Factor–β1 Assay
Soluble lung collagen content was measured in the frozen, unlavaged right lung with the Sircol assay (Biocolor Ltd., Carrickfergus, UK). Results were expressed as micrograms per milligram of protein. Transforming growth factor (TGF)-β1 was measured in lung homogenates with a commercially available ELISA assay (QuantiKine R&D Systems, Abingdon, UK).

Immunohistochemistry
For immunohistochemistry, the paraffin-embedded sections were treated as described previously (12) (see the online supplement for further details and references for all antibodies used). For semiquantitative assessment of cleaved caspase-3 and anti–Ki-67 labeling, the positive cells and...
the total number of cells (nuclei) were enumerated in the alveolar regions of the lung, excluding the vessels and bronchi, in at least five representative fields per animal, and expressed as the ratio of positive cells: total number of cells. In every animal, 1,000–3,000 nuclei were routinely counted.

**Immunofluorescence**

To determine whether some myofibroblasts and some epithelial cells express GLI1, GLI2, and GLI3 in the nucleus, we performed double immunofluorescence experiments on samples from bleomycin-treated animals (Day 14). Alveolar epithelial cells were identified as ABCA3-positive cells, whereas myofibroblasts were identified as α-smooth muscle actin (α-SMA)–positive cells. The immunofluorescence protocol was adapted from Cigna and colleagues (10). See the online supplement for further details and references to all antibodies used.

**Statistical Analysis**

Data were expressed as medians (25th–75th percentile; minimum and maximum values). Statistical analysis was performed with GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Experimental groups were compared first with a nonparametric ANOVA equivalent (the Kruskall-Wallis test) and, if significant, pairwise comparisons were performed with the Mann-Whitney U test. Comparison of histological scores on Day 14 was performed with Fisher’s exact test after grouping the scores 0 and 1 and the scores 2 and 3. *P* values below 0.05 were considered significant.

**Results**

**Expression of the Hedgehog Pathway in Bleomycin-Induced Lung Fibrosis**

To determine whether and how the Hedgehog pathway was modulated during bleomycin-induced lung fibrosis, we evaluated the expression of the different components of the pathway in the lung after intratracheal instillation of bleomycin. The levels of *Shh*, *Ihh*, *Dhh*, Gli1, Gli2, Gli3, Ptc, Smo, and Hhip mRNA were measured by

![Figure 1. Expression of the Hedgehog ligands in bleomycin-induced lung injury in mice. (A) Sonic Hedgehog (Shh), (B) Indian Hedgehog (Ihh), (C) and Desert Hedgehog (Dhh) messenger RNA (mRNA) expression was examined by quantitative PCR in control lung and in bleomycin-induced lung injury in mice, on Days 3, 7, and 14 after bleomycin. Data are medians (25th–75th percentile; minimum and maximum) from five to seven mice per condition. *P* < 0.05; **P** < 0.01. Sonic Hedgehog and Indian Hedgehog mRNA increased at Day 14 during the fibrotic phase of bleomycin-induced lung injury. (D) Immunolabeling of Sonic Hedgehog (SHH) in control lung (naive untreated mice) and on Day 14 after bleomycin injury (scale bar, 100 μm). Note that SHH protein increased strongly on Day 14.**
In naive mice, all the components of the pathway were detected in the lung (Figures 1–3). After bleomycin treatment, Shh mRNA expression decreased by 40% on Day 3, and was induced by 60% on Day 14 (Figure 1A). Ihh mRNA expression was induced threefold on Day 14 (Figure 1B). Dhh mRNA decreased by 50% on Day 3 and remained at a low level until Day 14 (Figure 1C). Immunohistochemistry showed that bronchial and alveolar epithelial cells and alveolar macrophages were the main sources of SHH in the control lung (Figure 1D). After bleomycin, inflammatory cells also expressed SHH. In summary, although there was a reduced expression of the ligands of the pathway during the inflammatory phase of bleomycin-induced lung injury, there was an induction of the expression of SHH and Ihh in the fibrotic phase.

The mRNA expression of the receptor, Ptc, was decreased by 60% early after bleomycin injection (Day 3) and its low expression was maintained until Day 14 (Figure 2A). Immunohistochemistry localized PTC at the level of bronchial and alveolar epithelial cells in the control lung (Figure 2B). Western blot analysis showed that the lung content of the protein PTC was profoundly decreased on Days 7 and 14 after bleomycin (Figure 2C). Decreased PTC was confirmed by immunohistochemistry performed on fibrotic lung samples obtained at Day 14 after bleomycin (Figure 2B). In parallel, the lung content of Hhip mRNA, the SHH decoy receptor, was similarly decreased (Figure 2D). SMO is the obligate transducer of the Hedgehog–Gli pathway. There was a 50% inhibition of Smo mRNA expression, which began on Day 3 and was maintained until Day 14 (Figure 2E). Decreased expression of the protein SMO was also detected by immunohistochemistry (Figure 2F). In summary, these data show...
that the expression of the receptor, PTC, and the transducer, SMO, is decreased during the inflammatory and fibrotic phases of bleomycin-induced lung injury. We then analyzed the expression of GLI transcription factors, as these are both the main effectors and the targets of the Hedgehog pathway. Lung Gli1 mRNA content was transiently decreased on Day 7, and increased to basal level on Day 14 (Figure 3A). In parallel, Gli2 and Gli3 mRNA levels were stable on Days 3 and 7, but increased fourfold on Day 14 during the fibrotic phase. Immunohistochemistry allowed us to identify the GLI-positive cells and the subcellular localization of the Gli factors (Figure 3B). In naive mice, Gli1 and Gli2 were detected in bronchial and alveolar epithelial cells and in some macrophages, most often in the cytoplasm. The increased expression of both factors was evident on Days 7 and 14 after bleomycin, with a preferential nuclear localization. By contrast, Gli3, the inhibitory Gli, was localized in the nucleus in naive mice, whereas it was mostly cytoplasmic in the bleomycin-treated mice. These data indicate an activation of the Gli pathway in bleomycin-induced lung injury with nuclear translocation of Gli1 and Gli2.

We further characterized the localization of Gli1, Gli2, and Gli3 in alveolar epithelial cells and in myofibroblasts in the fibrotic lung using a double immunofluorescence method. Alveolar epithelial cells were identified as ABCA3 positive, and myofibroblasts as α-SMA-positive cells. Features of Hedgehog activation, such as nuclear localization of all Gli1 and Gli2 transcription factors, could be shown in alveolar epithelial cells and myofibroblasts (Figure 4).

Inhibition of SMO Activity with GDC-0449 Did Not Inhibit Bleomycin-Induced Fibrosis

To evaluate the impact of the SHH pathway in bleomycin-induced fibrosis, we treated mice with GDC-0449, an inhibitor of SMO, from Day 7 to Day 13, during the fibrotic phase of the model. In preliminary experiments, we observed that GDC-0449 significantly decreased Gli1 mRNA content in whole-lung homogenates in naive mice treated every other day for 1 week (see Figure E1 in the online supplement for further details). As mentioned previously here, the Hedgehog pathway was reactivated in the lung from Day 7, during the fibrotic phase after bleomycin instillation. Hence, we decided to treat the animals with GDC-0449 from Day 7 to Day 13 after bleomycin instillation.

As compared with vehicle, GDC-0449 did not influence the lung injury score.
(Figures 5A and 5B), and did not inhibit the development of lung fibrosis. Indeed, the lung soluble collagen content was similar in control and treated animals (Figure 5C). Similarly, picrosirius red staining for collagen, and collagen-1 and collagen-3 immunohistochemistry, gave similar results in control and treated animals (Figure 5D). Nevertheless, Col1a1 and Col3a1 mRNA, as well as Acta2 mRNA content, was decreased in GDC-0449-treated mice compared with vehicle (Figure 5E). With respect to lung fibrosis, these results suggest that the bleomycin-treated mice partially responded to SMO inhibition, but GDC-0449 failed to fully counteract lung fibrogenesis in this model.

GDC-0449 reduced Gli1 mRNA, but did not significantly modify Gli2 mRNA content in treated animals (Figure 6A). Immunohistochemistry showed that most of the cells were GLI1 positive in fibrotic areas of the lung, with a prominent nuclear localization of GLI1. The percentage of GLI1-positive cells was similar in control and treated animals (Figure 6B). The ligand SHH content was increased in lung homogenates from treated animals as compared with controls (Figure 6C), whereas PTC protein level was still very low on Day 14, and similar in control and treated animals (Figure 6D).

To better understand the effect of GDC-0449, we evaluated the expression of some key profibrotic mediators in GDC-0449- and vehicle-treated mice. As shown in Figure 7, inhibition of SMO did not influence the expression of those profibrotic mediators on Day 14 after bleomycin instillation. Interestingly, the concentration of TGF-β1 in lung homogenates was increased in GDC-0449-treated animals. As the balance of proliferation and apoptosis is central to the pathophysiology of fibrotic lung diseases, we first evaluated the number of cells expressing Ki-67 as a marker of proliferation. GDC-0449 decreased the number of GLI1-positive cells in the lung (Figure 7C). In addition, we investigated cleaved caspase-3 as a marker of cell apoptosis (Figures 7D and 7E). As compared with vehicle-treated animals, immunolabeling showed that GDC-0449 decreased the number of cleaved caspase-3. However, we also used cleaved PARP as readout for caspase-3 activity to confirm these findings. PARP is one of the main cleavage targets of caspase-3 and caspase-7 downstream of both intrinsic and extrinsic apoptotic pathways (18). Overall, we showed that GDC-0449 did not influence apoptosis, as the amounts of cleaved PARP were similar in the lung of vehicle- and GDC-0449-treated mice assayed by Western blot at Day 14 after bleomycin instillation.

Altogether, these results demonstrate that the inhibition of SMO does not inhibit the development of bleomycin-induced lung fibrosis in mice.

GANT61 Reduced Bleomycin-Induced Pulmonary Fibrosis

The GLI transcription factors are the final effectors of the Hedgehog pathway. We asked whether the inhibition of the GLI transcription factors could modulate the fibrotic process. GANT61 is known to inhibit the Hedgehog pathway by directly blocking the binding of GLI1 and GLI2 on their DNA targets (16). In preliminary experiments, we observed that GANT61 significantly decreased the Gli1 and Gli2 mRNA content in whole-lung homogenates in naïve mice treated with GANT61 every other day for 1 week (see Figure E1 and data in the online supplement for further details). As we observed that Gli1 and Gli2 mRNA content was induced in the lung after Day 7, during the fibrotic phase after bleomycin instillation, we decided to treat the animals with GANT61 from Day 7 to Day 13 after bleomycin instillation.

As compared with vehicle, GANT61 strongly reduced the extent of lung lesions on Day 14 (Figures 8A and 8B), decreased lung collagen content as assessed with the
Figure 5. GDC-0449 (vismodegib) did not prevent lung fibrosis in bleomycin-induced lung injury. Morphological assessment of lung injury on Day 14 after bleomycin showed that GDC-0449 did not influence lung injury as compared with vehicle-treated mice (A) hematoxylin and eosin staining; scale bar, 100 μm). (B) The pathological grade of inflammation and fibrosis was evaluated under ×40 magnification and determined according to the following criteria: 0, no lung abnormality; 1, presence of inflammation and fibrosis involving less than 25% of the lung parenchyma; 2, lesions involving 25–50% of the lung; and 3, lesions involving over 50% of the lung (according to Inoshima and colleagues [17]). (C) Soluble collagen content was measured with the Sircol assay in lung homogenates, and reached similar levels in GDC-0449– and vehicle-treated mice (n = 5 mice per group); evaluation of collagen accumulation with picrosirius staining, and collagen 1 and collagen 3 immunolabeling, gave similar results in both groups (D) (scale bar, 100 μm). Col1a1, Col3a1, and Acta2 mRNA content was decreased in GDC-0449–treated mice as compared with vehicle-treated mice. (E) Quantitative PCR analysis; data are medians (25th–75 percentile; minimum and maximum) from five to seven mice per condition. *P < 0.05.
Figure 6. GDC-0449 modulates the Hedgehog pathway. Components of the Hedgehog pathway were analyzed on Day 14 after bleomycin, in control and GDC-0449–treated mice. (A) Gli1 mRNA content was decreased in the GDC-0449–treated group, whereas Gli2 mRNA content was similar to the control group. (B) GLI1 immunolabeling showed that the percentages of GLI1-positive cells and nuclear-positive cells were similar in GDC-0449– and vehicle-treated mice (scale bar, 100 μm). (C) Western blot quantification of SHH in lung homogenates. SHH protein levels were increased in lung homogenates in GDC-treated mice. (D) Western blot quantification of Patched-1 (PTC) in lung homogenates. PTC was similar in control and GDC-0449–treated mice. Data are from five to seven mice per condition. * P < 0.05; ** P < 0.01.
Sircol assay (Figure 8C), with picrosirius staining, and by immunohistochemistry (Figure 8D), and decreased Col1a1, Col3a1, and Acta2 mRNA content by 50% (Figure 8E). GANT61 decreased Gli1 and Gli2 mRNA content (Figure 9A) and the percentage of GLI1-positive cells (Figure 9B), whereas the SHH ligand and its receptor, PTC, were unchanged, as assessed by Western blot (Figures 9C and 9D). GANT61 induced an antifibrotic and anti-inflammatory environment in the lung with a decreased expression of Ctgf, Vegfa, Pai1, and Il1b mRNA (Figure 10A). TGF-β1 mRNA and protein levels were similar in control and treated animals (Figure 10B). GANT61 inhibited the proliferation of alveolar cells, as assessed by the number of Ki-67–positive cells in the lung on Day 14 after bleomycin (Figure 10C). In addition, immunolabeling showed that GANT61 profoundly decreased the number of cleaved caspase-3–positive cells (a marker of apoptosis), although cleaved PARP protein level was unchanged (Figures 10D and 10E).

Altogether, these results demonstrate that inhibition of GLI transcription activity with GANT61 stimulates an antifibrotic environment and protects from bleomycin-induced lung fibrosis.

**Figure 7.** Effect of GDC-0449 on the profibrotic environment in the lung. (A) GDC-0449 had no effect on connective tissue growth factor (Ctgf), vascular endothelial growth factor (Vegfa), plasminogen activator inhibitor-1 (Pai-1), or IL-1β (Il1b) mRNA content, as assessed by quantitative PCR and compared with vehicle-treated animals at Day 14. Transforming growth factor (TGF) -β1 (tgfb1) mRNA was slightly decreased. Data are medians (25th–75th percentile; minimum and maximum) from five to seven mice per condition; *P < 0.05. (B) TGF-β1 concentration in lung homogenates assayed by ELISA was similar in GDC-0449– and vehicle-treated mice. (C) Immunolabeling of Ki-67 (a marker of proliferation) in cyclopamine-treated mice and in mice treated with the vehicle. The percentage of Ki-67–positive cells was similar in the two groups of animals. (D) Immunolabeling of cleaved caspase-3 (a marker of apoptosis). The lungs were harvested on Day 14 after bleomycin injury. GDC-0449 decreased caspase-3 activation (data are means ± SD from five fields per slide from at least four different mice per condition). Scale bar, 100 μm. (E) Western Blot analysis for cleaved poly (ADP-ribose) polymerase (PARP) in lung homogenates from control and treated mice. Cleaved PARP protein level was at similar levels in both groups.
Figure 8. 2,2’-[Dihydro-2-(4-pyridinyl)-1,3(2H,4H)-pyrimidinediyl]bis(methylene)]bis[N,N dimethylbenzamine (GLI antagonist [GANT] 61) decreased bleomycin-induced lung injury in mice. Morphological assessment of lung injury on Day 14 after bleomycin showed that GANT61 decreased lung injury in mice as compared with vehicle-treated mice. (A) Hematoxylin eosin staining. (B) The pathological grade of inflammation and fibrosis was evaluated under x40 magnification and determined according to the following criteria: 0, no lung abnormality; 1, presence of inflammation and fibrosis involving less than 25% of the lung parenchyma; 2, lesions involving 25–50% of the lung; and 3, lesions involving over 50% of the lung, according to Inoshima and colleagues (17); scale bar, 100 μm. (C) Soluble collagen content, assessed by the Sircol assay in lung homogenates, was decreased in GANT61 as compared with vehicle-treated mice (n = 5 mice per group); picrosirius staining, and collagen 1 and collagen 3 immunolabeling, confirmed the decreased collagen content (D); scale bar, 100 μm. Lung Col1a1, Col3a1, and Acta2 mRNA content decreased with GANT61 treatment, as assessed by quantitative PCR, and was decreased in GANT61-treated mice (E). Data are medians (25th–75th percentile; minimum and maximum) from five to seven mice per condition. *P < 0.05.
induced fibrosis when given 7 days after bleomycin.

**Discussion**

This is the first study to evaluate the effect of pharmacologic inhibition of the Hedgehog-Gli pathway in lung fibrosis. Our results demonstrate that (1) the Hedgehog-Gli pathway is activated in bleomycin-induced lung fibrosis in mice and (2) inhibition of SMO with GDC-0449 does not modulate lung fibrosis, whereas (3) inhibition of GLI with GANT61 inhibits lung fibrosis development and promotes an antifibrotic environment. Our results identify the GLI transcription factors as central to the pathophysiology of lung fibrosis in vivo in mice, and, together with our previous in vitro results in humans, suggest that the interaction of GLI

Figure 9. GANT61 modulates the Hedgehog pathway. Components of the Hedgehog pathway were analyzed on Day 14 after bleomycin, in control and GANT61-treated mice. (A) Gli1 mRNA content was decreased in the GANT61-treated group, whereas Gli2 mRNA was similar to the control group. (B) The percentage of GLI1-positive cells was decreased in GANT61-treated mice, whereas the percentage of nuclear-positive cells was similar to vehicle-treated mice; scale bar, 100 μm. (C) Western blot quantification of SHH in lung homogenates. SHH levels were similar in GANT61- and vehicle-treated mice. (D) Western blot quantification of PTC in lung homogenates. PTC levels were similar in GANT61- and vehicle-treated mice. Data are from five to seven mice per condition; **P < 0.01.
transcription factors with DNA might be a new therapeutic target in fibrotic lung disorders in humans.

**Reactivation of the Hedgehog Pathway during Pulmonary Fibrosis**

Regulated function of the Hedgehog signaling pathway is critical during embryonic development, whereas deregulated Hedgehog pathway is documented in a variety of human diseases, including lung fibrosis (10, 19). In the current study, we demonstrate an activation of the Hedgehog–Gli pathway in the lung after bleomycin injury. Indeed, we observed that the expression of SHH, the main ligand of the pathway, was increased, whereas the expression of the receptor, PTC, was profoundly decreased, at the mRNA and protein levels. As PTC controls the activation of SMO, any decrease of PTC is supposed to increase the global activity of SMO, the transducer of the pathway, thus contributing to the activation of the pathway. This has been clearly demonstrated in PTC-heterozygous (Ptc+/−) mice, which demonstrate an overactivation of the pathway (20). The best evidence for activation of the pathway comes from the demonstration of the

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**Figure 10.** GANT61 promoted an antifibrotic environment in the lung. (A) Tgfb1, Vegfa, plasminogen activator inhibitor-1 (Pai-1), and Il1b mRNA content, assessed by quantitative PCR, was decreased in GANT61-treated mice as compared with vehicle-treated animals, whereas Ctgf mRNA was unchanged at Day 14. Data are medians (25th–75th percentile; minimum and maximum) from five to seven mice per condition. *P < 0.05; **P < 0.01; ***P < 0.001. (B) TGF-β1 content in lung homogenates assayed by ELISA was similar in the treated and control groups. (C) Immunolabeling of Ki-67 (a marker of proliferation) in GANT61-treated mice and in mice treated with the vehicle (DMSO) from Day 7 to Day 13. GANT61 decreased cell proliferation at Day 14 (scale bar, 100 μm). (D) Immunolabeling of cleaved caspase-3 (a marker of apoptosis), GANT61 profoundly decreased caspase-3 activation. The percent of positive cells for each condition is presented in the graph (data are means ± SD from five fields per slide from four different mice per condition; scale bar, 100 μm). (E) Western blot analysis for cleaved PARP in lung homogenates from control and GANT61-treated mice. Cleaved PARP was at similar level in both groups.
selective nuclear localization of GLI1 and GLI2, the GLI activators, which was observed in alveolar and bronchial epithelial cells and in fibroblasts in the fibrotic lung on Day 14 after bleomycin administration. At the same time, GLI3, the GLI inhibitor, was preferentially localized to the cytoplasm in the same cells. Our results are supported by those of Liu and colleagues (21), who observed that there are abundant GLI1-positive mesenchymal cells in fibrotic lesions and increased numbers of GLI1-positive cells in preserved alveolar septa in bleomycin-induced lung injury in mice. Activation of the Hedgehog pathway has also been demonstrated in FITC-induced lung fibrosis (22) and in hyperoxia-induced lung injury (23). Most importantly, activation of the Hedgehog pathway observed in experimental lung fibrosis in mice fits very well with the observations made in the lungs of patients with IPF. Indeed, we previously described in lung samples from patients with IPF a decreased expression of PTC and Hhip mRNA, as well as the nuclear localization of GLI1 and GLI2, particularly in hyperplastic alveolar epithelial cells and within the fibroblastic foci (10).

Apoptosis of epithelial cells is central to the pathophysiology of fibrotic lung diseases. Immunolabeling showed that GDC-0449 and GANT61 decreased the number of cleaved caspase-3–positive cells on Day 14, but neither GDC-0449 nor GANT61 modulated cleaved PARP accumulation. By contrast, GANT61 had an inhibitory effect on the proliferation of alveolar cells, as assessed by the number of Ki-67–positive cells in the lung, whereas GDC-0449 did not. SHH pathway is well known to control cell cycle progression in proliferative cells (5). The Hedgehog pathway has been shown previously to modulate the differentiation of epithelial cells and, more specifically, to contribute to the epithelial–mesenchymal transition of epithelial cells in the kidney (24) and the liver, where this phenomenon is thought to contribute to the development of hepatic and biliary fibrosis (20, 25). At present, there is no evidence that activation of the Hedgehog pathway contributes to epithelial–mesenchymal transition in alveolar epithelial cells, although it is conceivable.

Activation of the Hedgehog pathway in fibroblasts might also be important for pathogenesis. Indeed, Hedgehog signaling potently stimulates the release of collagen and myofibroblast differentiation in vitro by skin fibroblasts (26). We have shown that the Hedgehog–GLI pathway was required for basal proliferation, extracellular matrix synthesis, and α-SMA expression, and for TGF-β–stimulated myofibroblast differentiation of human lung fibroblasts (10). Similarly, Bolaños and colleagues (11) observed that SHH increased the proliferation, migration, extracellular matrix production, and survival of fibroblasts in vitro.

There is emerging evidence that Hedgehog signaling in vivo promotes fibrosis under some circumstances and in different organs. For example, paracrine Hedgehog signaling causes fibrotic reactions in zebrafish pancreas and in pancreatic carcinoma (27, 28). Hedgehog signaling also promotes kidney fibrosis (24, 29), liver fibrosis (20), and skin fibrosis in scleroderma (26, 30). Adenoviral overexpression of SHH in the skin of mice was sufficient to induce skin fibrosis (26). Recently, adenoviral overexpression of SHH in the lung has been shown to promote local deposition of collagen (31). Furthermore, the Ptc–/– mice, which display excessive activation of the Hedgehog pathway, are more susceptible to experimental induced dermal fibrosis (26).

**Figure 11.** Simplified overview of the Hedgehog pathway. The binding of SHH to its receptor, PTC relieves (SMO) inhibition by PTC and activates the pathway. The GLI transcription factors are then processed into transcriptional activators. The GLI transcription factors can also be induced in an SMO-independent manner by other signaling pathways (e.g., epidermal growth factor [EGF], fibroblast growth factor [FGF], TGF-β). Cyclopamine (CYC) and GDC-0449 (vismodegib) inhibit the Hedgehog pathway at the level of SMO. Meanwhile, the small molecule, GANT61, inhibits the pathway at the level of GLI transcription factors in the nucleus, downstream of both SMO and the other GLI-inducing signaling pathways. GLI-act, activator GLI.

**Pharmacological Modulation of the Hedgehog Pathway Influences Lung Injury and Repair**

We observed that inhibition of SMO with GDC-0449 did not counteract the development of lung fibrosis when given during the fibrotic phase of bleomycin-induced lung injury, although treatment with GDC-0449 did decrease the content of Col1a1, Col3a1, and Acta2 mRNA in the lung as compared with vehicle. The absence of an antifibrotic effect of GDC-0449 in vivo might be due to pharmacological reasons, as we gave the drug subcutaneously every other day. Longer treatment duration, or higher doses of GDC-0449, could have had a greater antifibrotic effect. We rather believe that the nonprotective effect of GDC-0449 indicates that targeting SMO is probably not a valid option to treat fibrotic lung diseases, for two reasons. First, we showed that this treatment protocol inhibited the Hedgehog pathway in naive mice and bleomycin-treated mice at Day 14 compared with vehicle, as evidenced by decreased Gli1 lung mRNA content. Second, in preliminary experiments, we observed that cyclopamine, a classical inhibitor of SMO, did not prevent the development of bleomycin-induced lung fibrosis in mice when given subcutaneously for 13 days with an osmotic pump (data not shown).
shown), although cyclopamine was very active in vitro in inhibiting some of the effects of TGF-β on human control lung fibroblasts (10).

These results obtained either with GDC-0449 or cyclopamine were unexpected, as most of the literature suggests that inhibition of SMO has antifibrotic properties. For example, GDC-0449 (40 mg/kg/d) was shown to inhibit the development of liver fibrosis when given intraperitoneally for 9 days in mice (15). Other inhibitors of SMO have been studied in different models of skin fibrosis in mice. Inhibition of SMO, either by LDE223 or by small interfering RNA, prevented dermal thickening, myofibroblast differentiation, and accumulation of collagen in the skin upon challenge with bleomycin in mice (30). Similarly, inhibition of SMO with LDE223 limited the development of skin fibrosis in a murine model of chronic graft-versus-host disease (30). Targeting SMO also exerted potent antifibrotic effects in tight-skin-1 mice, prevented progression of fibrosis and induced regression of pre-established fibrosis (30). However, it is worth noting that the effect of SMO inhibition in renal fibrosis is controversial, as the reported effects of Hedgehog signaling inhibition are mixed. For instance, Ding and colleagues (24) showed that cyclopamine, a classical SMO inhibitor, reduced matrix expression and mitigated fibrotic lesions in ureteral obstruction.

Conversely, Fabian and colleagues (29) observed that the Hedgehog antagonist, IPI-926, abolished Gli1 induction in vivo, but did not decrease kidney fibrosis in vivo in a model of unilateral ureteral obstruction. Most importantly, however, Ochoa and colleagues (32) observed that SMO inhibition with cyclopamine was associated with a reduced survival after partial hepatectomy in mice, suggesting that the Hedgehog pathway was critical for repair. Similarly, endogenous Hedgehog signaling plays a critical role in the response to cardiac ischemia, both preserving cardiac function and minimizing infarct area (33).

Conversely, we observed that delayed treatment with GANT61 (from Day 7 after bleomycin administration), an inhibitor of the interaction of GLI with DNA, inhibited fibrosis and promoted an antifibrotic environment in the lung. Interestingly, we have previously shown that lung fibroblasts from patients with IPF were partially resistant to the effect of cyclopamine, whereas those cells responded to GANT61 (10). This suggests that inhibition of GLI1 in the nucleus might be more relevant than inhibition of SMO in terms of protection against fibrosis. Hence, we believe that inhibiting the interaction of GLI factors with DNA, as does GANT61, is an interesting approach to developing innovative antifibrotic therapies. Indeed, a growing body of evidence indicates that, in some conditions, Gli activation occurs through Hedgehog-independent pathways (34), as illustrated in Figure 11. For instance, GLI2 was identified as an early gene target of the TGF-β/SMAD cascade, independent of Hedgehog signaling (35). A reciprocal cross-talk between the Hedgehog and TGF-β pathways also occurs during embryonic lung development (36). Other signaling pathways capable of inducing the activation of GLI proteins are the fibroblast growth factor, epidermal growth factor, and mitogen-activated protein kinase pathways (34). Therefore, the GLI are at the crossroads of many different profibrotic pathways, and inhibiting the GLI will inhibit more than the Hedgehog pathway.

In summary, we demonstrate for the first time that inhibition of Hedgehog signaling by targeting the GLI transcription factors is highly effective at preventing lung fibrosis in a mouse model. Our results strongly suggest that inhibition of GLI activity could be a target for IPF treatment. The effectiveness of the late administration is particularly interesting if we consider treating human disease with this class of molecules in the future.

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**References**


