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Sonic hedgehog signaling promotes motility and invasiveness of gastric cancer cells through TGF-β-mediated activation of the ALK5–Smad 3 pathway

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It is known that the activation of hedgehog (Hh) signaling is involved in the progression and invasion of various tumors, including gastric carcinoma. In this study, we investigated the impact of transforming growth factor (TGF)-β signaling on the sonic hedgehog (Shh)-mediated invasion of gastric cancer cells. We found that higher concentrations of N-Shh enhanced cell motility and invasiveness in gastric cancer cells, whereas no increase was observed in cells that were treated with KAAD-cyclopamine (a Shh signaling inhibitor) or anti-Shh blocking antibodies. In addition, the N-Shh-induced migration and invasiveness of gastric cancer cells were reduced by treatment with anti-TGF-β blocking antibody or TGF-β1 small interfering RNA (siRNA) in presence of N-Shh when compared with control groups. Furthermore, TGF-β1 secretion, TGF-β-mediated transcriptional response, expression of activin receptor-like kinase (ALK) 5 protein and phosphorylation of Smad 3 were also enhanced by treatment with N-Shh, but not KAAD-cyclopamine, anti-Shh or TGF-β1 blocking antibodies. Blockade of the ALK5 kinase in the presence of N-Shh significantly inhibited phosphorylation of Smad 3, activity of matrix metalloproteinases and Shh-induced cell motility/invasiveness. Importantly, transient expression of ALK5 siRNA or Smad 3 siRNA reduced the ability of N-Shh to stimulate migration and invasion of those cells compared with the cells treated with nonspecific control siRNA. In summary, these results indicate that Shh promotes motility and invasiveness of gastric cancer cells through TGF-β-mediated activation of the ALK5–Smad 3 pathway. Additionally, our findings are the first to suggest a role and mechanism for Shh signaling as it relates to the metastatic potential of gastric cancer, thereby indicating potential therapeutic molecular targets to decrease metastasis.

Introduction

The hedgehog (Hh) signaling pathway plays a critical role in organized cell growth and differentiation during embryonic tissue patterning; however, it may also be involved in the maintenance of stem cell compartments in adults (1–4). The mammalian Hh ligand, sonic hedgehog (Shh), activates Hh signaling by binding to their receptor, Patched (Ptch) 1 and 2, to relieve Ptch repression of Smoothened (Smo), which is a membrane protein related to G protein-coupled receptors. Upon activation, Smo promotes nuclear translocation of a family of transcription factors, Ci in *Drosophila* and Glis (Gli1, Gli2 and Gli3) in vertebrates, to active Hh target genes (3,5). A variety of human cancers are caused by mutations that lead to inappropriate Hh pathway activation. For example, excessive activation mutations in the *Smo* gene and loss-of-function mutations in the *ptch* gene cause the majority of human and murine cancers (6,7), and inhibition of the pathway with a ligand-blocking antibody or Smo inhibitor, KAAD-cyclopamine, has been shown to retard the growth of tumors and to decrease tumor size (8,9).

It was recently reported that the Hh signaling pathway was involved in not only *de novo* vascularization of certain embryonic tissues but also in induction of angiogenesis in an adult mammalian system (10). In addition, observations by other groups have shown that Hh signaling plays a role in the control of the motility and migration of multiple cell types (11–13). Furthermore, studies conducted by Hochman *et al.* (14) have suggested that the components of the Shh signaling pathway directly participate in cell migration and angiogenesis processes, whereas inhibition of this pathway blocks Shh-induced cell migration and angiogenesis. Taken together, these observations imply that the Hh activity is correlated with the severity of the associated tumor and that this activity is both necessary and sufficient to maintain metastatic behavior.

Transforming growth factor (TGF)-β is a pleiotropic cytokine that plays a critical role in the modulation of cell growth, differentiation, plasticity and migration. TGF-β primarily signals by binding to distinct receptors with intrinsic serine/threonine kinase activity, TGF-β type I receptor and TGF-β type II receptor (TβRII). The binding of TGF-β to TβRII leads to recruitment and transphosphorylation of TGF-β type I receptor I [also termed activin receptor-like kinase (ALK)] and to activation of receptor-activated Smads (15–17). TGF-β signaling is mediated by either ALK1 or ALK5 (18), which occurs through the phosphorylation of Smad 1, 5 and 8 or Smad 2 and 3, respectively. TGF-β receptors, which function as tumor suppressors in normal and preneoplastic tissues, acquire oncogenic functions during tumor progression. In addition, TGF-β receptors are mutated or expressed at substantially attenuated levels in a variety of human cancers and are correlated with the acquisition of resistance to growth suppression by TGF-β (19–21). Additionally, it has been reported that the expression of constitutively active ALK5 mutants enhances tumor invasion and angiogenesis in some cells by regulating the expression of matrix metalloproteinases (MMPs) (22,23). In addition, activation of mitogen-activated protein kinases and PI3K/Akt signaling cascades by TGF-β receptors can also potentially contribute to cell migration and invasion (24–26).

Despite the critical role that Hh signaling plays in the promotion of tumorigenesis, the molecular and cellular mechanisms behind Hh regulation in tumor metastatic behavior are unknown. Here, we report that the Shh signaling pathway, acting through the TGF-β–ALK5 pathway, may selectively contribute to tumor cell motility and invasion in gastric cancer. In addition, we show that disruption of TGF-β–ALK5 signaling by anti-TGF-β neutralizing antibody or ALK5 kinase inhibitor results in suppression of Shh-mediated cell migration and invasion. Based on these findings, we suggest that the Shh signaling pathway induces metastatic functions of gastric cancer through the recruitment of TGF-β–ALK5–Smad 3 signaling.

Abbreviations: ALK, activin receptor-like kinase; Hh, hedgehog; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; siRNA, small interfering RNA; smo, smoothened; Shh, sonic hedgehog; TGF, transforming growth factor; TβRII, TGF-β type II receptor.

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Materials and methods

Cell and tissues

The human gastric carcinoma cell lines, AGS and MKN-28, were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to the American Type Culture Collection's instructions. The human gastric cancer tissue obtained from the tissue bank of Korea University Guro Hospital. Forty-one patients with gastric cancer were selected to this study. Thirty-two lymph node metastasis specimens and nine lymph node-negative gastric cancer specimens were included in this study. This protocol was reviewed and permitted by Institutional Review Board of Guro Hospital.

Reagents and antibodies

Anti-Shh N-terminal peptide antibody, recombinant human N-Shh, recombinant human TGF-β1 and monoclonal anti-TGF-β antibody, which completely neutralizes the activities of the isomeric forms TGF-β1, TGF-β2 and TGF-β3, were purchased from R&D Systems (Minneapolis, MN). KAAD-cyclopamine was purchased from Calbiochem (San Diego, CA) and diluted in dimethyl sulfoxide (control vehicle). SB 431542 was purchased from TOCRIS Bioscience (Ellisville, MO). Rabbit anti-ALK5, rabbit anti-phospho-Smad 3 (Ser433/435) and rabbit anti-Smad 3 were obtained from Cell Signaling Technology (Beverly, MA). Rabbit anti-ALK1 and mouse β-actin antibody were obtained from Sigma. Rabbit anti-TGF-β receptor II was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell proliferation assay

Cells were seeded at a concentration of 4×10^3 cells per 100 μl of culture medium per well in 96-well plates. N-Shh was added to some cells, and after 24 or 48 h, the number of viable cells was determined in triplicate wells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche) according to the manufacturer's instructions.

Wound healing assay

AGS and MKN-28 cells were seeded at 1×10^5 cells/well in 12-well plates and then preincubated for 24 h in serum-free RPMI (Invitrogen) before wounding across the cell monolayer with a plastic tip. Cells were then grown in culture medium that was untreated or treated with various drugs in the presence or absence of N-Shh. Cell migration into the wound surface was then monitored by microscopy after 24 h and reported as the estimated ratio of the remaining wounded area relative to the initial wound area. Quantitation of the closure of the monolayer was performed using the NIH Image program and results are expressed as the percentage of wound closure. This assay was repeated three times independently.

Matrigel invasion assay

In all, 1×10^5 cells/well were seeded in the upper chamber, which was coated with Matrigel (Calbiochem), and serum-free medium containing different concentrations of N-Shh was added to the lower chamber. After 24 h of incubation, non-migrating cells were removed from the upper chamber with a cotton swab and cells present on the lower surface of the insert were stained with Diff-Quik Stain (Biochemical Sciences, Swedesboro, NJ). The invading cells were then counted by microscopy. All experiments were repeated three times.

Small interfering RNA transfection

Small interfering RNA (siRNA) duplex specific to TGF-β1, ALK5 or Smad 3 were synthesized at Invitrogen. The siRNA sequences used were as follows: TGF-β1 siRNA, 5′-GGG CUA CCA UGC CAA CUU CTT-3′ and 5 ′-GAA GUU GGC AUG GUA GCC CTT-3′, ALK5 siRNA, 5′-CAU AUU GCU GCA ACC AGG ATT-3′ and 5′-UCC UGG UUG CAG CAA UAU GTT-3′ and Smad 3 siRNA, 5′-GAU CUU CAA CAA CCA GGA GTT-3′ and 5′-CUC CUG GUU GUU GAA GAU GAU CTT-3′. As a non-specific control siRNA, scrambled siRNA duplex was used. Transfection was done using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instruction.

TGF-β1 quantitation

Cells were plated at a density of 5×10^4 cells in six-well plates, cultured under serum-free conditions and then was untreated or treated with various drugs in the presence or absence of N-Shh for 24 h. Serum-free conditions were used to prepare conditioned medium to avoid carryover of latent TGF-β1 in serum. Following exposure to various drugs with or without N-Shh, amounts of TGF-β1 released into cell culture supernatants were measured using Quantikine human TGF-β1 Immunoassay kits (R&D Systems, Milwaukee, WI). Briefly, acid-activated samples and standards in triplicate were added to the precoated microplates and incubated at room temperature for 2 h. A second antibody, anti-TGF-β1 polyclonal antibody, was then added to complete the sandwich followed by TGF-β1–horseradish peroxidase conjugate. TGF-β1 was detected by adding the chromogenic substrate and this was followed by stop solution. Absorbance was determined at optical density 450 nm.

Luciferase assay

Cells were plated at a density of 3×10^4 cells/well in six-well plates and then transfected with 3TP-Lux (1 μg) or CMV–renilla (1 μg) using Lipofectamine 2000. Six hours after transfection, the media was replaced with new media that had been treated with either N-Shh or inhibitors, as indicated in the figure legends, for 24 h before harvesting. The luciferase activity was then measured using luciferase assay kits (Promega) and normalized to *Renilla* luciferase activity.

Western blotting

Whole-cell extraction was conducted using RIPA buffer [50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate and 1% Na deoxycholate (pH 7.4)] supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml peptasin A, 10 μg/ml aprotinin and 5 μg/ml leupeptin). Protein concentrations were then measured using Bio-Rad protein assay kits (Bio-Rad, Hercules, CA). Next, the protein lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, then transferred onto nitrocellulose membranes (Hybond^{TM}-P; Amersham Biosciences, Piscataway, NJ), blocked with phosphate-buffered saline (PBS) containing 0.2% Tween 20 and 5% non-fat dry milk, incubated with primary antibody and then with horseradish peroxidase-labeled secondary antibody. The signals were then detected using X-ray film.

Gelatin zymography

The activities of MMP-2 and -9 in the conditioned media were determined by gelatin zymography. Briefly, the conditioned media were collected and centrifuged. Samples were then loaded onto sodium dodecyl sulfate–polyacrylamide gels that were polymerized with gelatin at a final concentration of 1 mg/ml. After electrophoresis, the gels were renaturated in 2.5% Triton X-100 and then incubated in developing buffer [50 mM Tris–HCl buffer (pH 7.4) in the presence of 5 mM $CaCl_2$ overnight at 37°C. The gels were then stained with 0.5% Coomassie Blue R-250 and destained in 10% methanol and 5% acetic acid in water. The gelatinase activity was then visualized as transparent bands on a blue background, and the molecular sizes were determined based on mobility using gelatin zymography standards (Chemicon). Three individual experiments were conducted using independently obtained protein samples.

Immunohistochemistry

Sections (5 m thick) of formalin-fixed, paraffin-embedded tumor specimens were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed with microwave oven irradiation for 20 min in the jar containing 0.01 M citrate buffer (pH 6.0) and placed into 0.025% trypsin and 50 mM Tris buffer for 5 min. Endogenous peroxidase was blocked using 3% hydrogen peroxide in PBS for 12 min. The specimens were incubated with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal donkey serum for 30 min at room temperature and then incubated at 4°C overnight in a 1:50 dilution of a rabbit polyclonal antibody against human Gli (Cell Signaling Technology, Danvers, MA). In case of ALK5 stain, the slide incubated with 1:100 dilution of a rabbit polyclonal antibody against human ALK5 (Santa Cruz Biotechnology). The samples were then rinsed and incubated with peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Next, the slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics, Huntsville, AL). The sections were washed three times with distilled water, counterstained with Mayer's hematoxylin (Biogenex Laboratories, San Ramon, CA) and washed once each with distilled water and PBS. Afterward, the slides were mounted using a Universal Mount (Research Genetics) and examined using a bright-field microscope. Depending on the percentage of positive cells and staining intensity, Gli and ALK5 staining was classified into three groups: negative, weak and strong expression. Specifically, the percentage of positive cells was divided into five grades (percentage scores): 0 ($<$10%), 1 (10–25%), 2 (26–50%), 3 (51–75%) and 4 ($>$75%). The staining intensity was divided into four grades (intensity scores): 0 (no staining), 1 (light brown), 2 (brown) and 3 (dark brown). Positive staining was determined using the following formula: overall score = percentage score × intensity score. An overall score of $\leq$3, $>$3 to $\leq$6, $>$6 to $\leq$9 and $>$9 was defined as negative, weak, moderate and strong expression, respectively.

Statistical analysis

The two-tailed χ^2 test was performed to determine the significance of the difference between the covariates. P values less than 0.05 were defined as statistically significant. The SPSS software program (version 13.0, SPSS, Chicago, IL) was used for the analyses.

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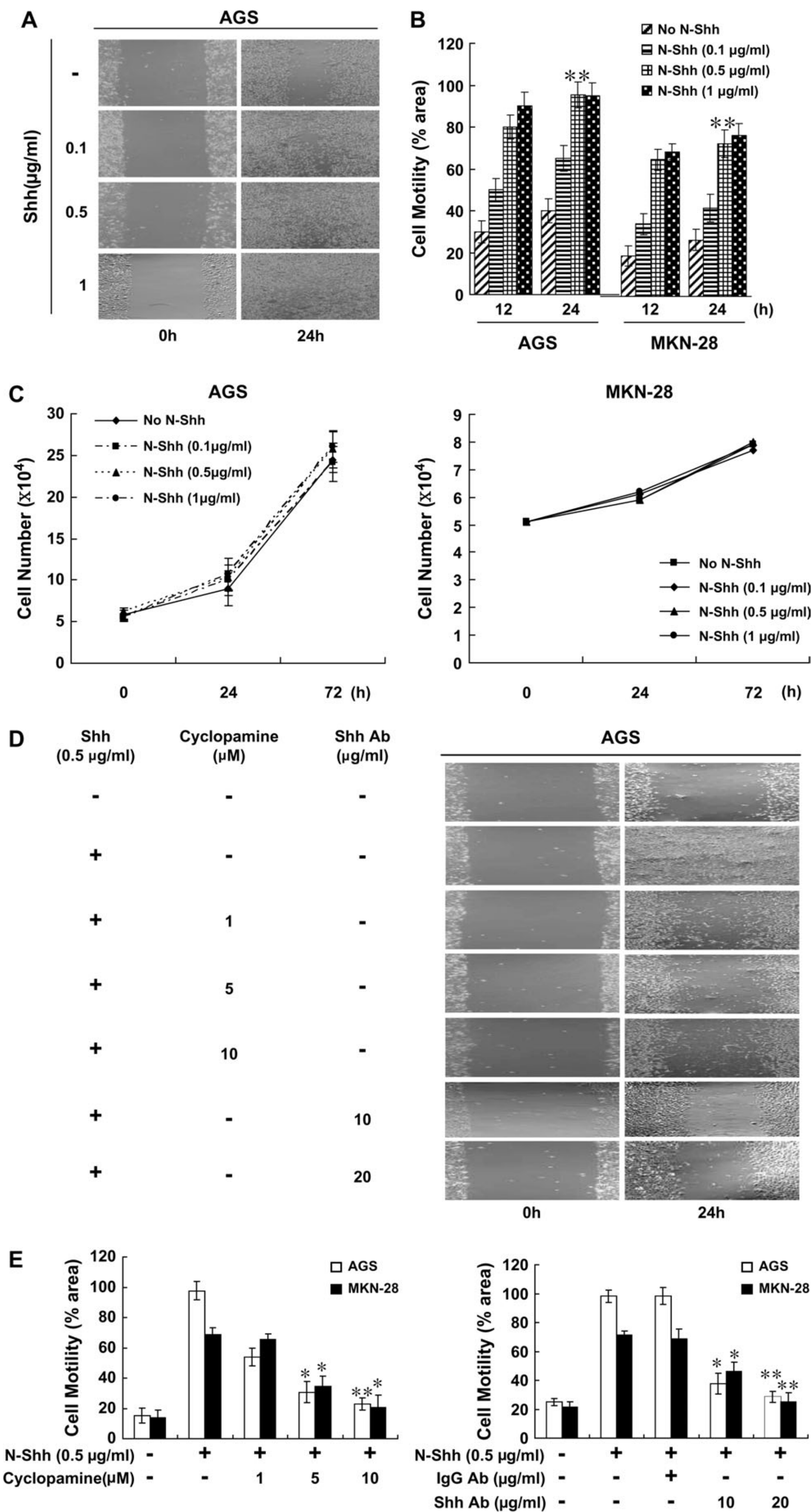

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Results

Shh signaling stimulates gastric cancer cell migration and invasion

To investigate the role of Shh signaling as it relates to invasive phenotypes and the motility of gastric cancer cells, we tested the effects of Shh signaling on the motility of AGS and MKN-28 cells in a wound closure assay. Prior to evaluating the motility, we ensured that the Hh target genes, *Shh*, *PTCH*, *Smo* and *Gli*, were expressed in the AGS and MKN-28 cells in accordance to those reported in previous studies (data not shown) (27). Those cells then were treated with increasing concentrations of N-Shh, and their ability to close the wound over time was assessed. A significant increase in the percentage of total area that was covered by cells that were subjected to N-Shh treatment was apparent 12 h after the wound was produced, and the average increase in motility of the N-Shh-treated cells (0.5 μg/ml) was 2.5-fold greater ($P < 0.001$) than that of the untreated control cells after 24 h. In addition, our results also showed that N-Shh caused a dose-dependent regulation on migration. Cells treated with N-Shh at doses of 0.1–0.5 μg/ml for 24 h a significant increase in cell motility, whether the response to higher doses of N-Shh (0.5–1 μg/ml) was insignificant. Taken together, these results indicate that the maximum effect of N-Shh on migration occurred in cells that were treated with a concentration of 0.5 μg/ml for 24 h (Figure 1A and B). The overall cell growth was not affected by treatment with N-Shh when cells were treated with the same concentration and for the same length of time, which indicates that the difference in motility was not due to an effect on proliferation (Figure 1C). To confirm the contribution of Shh signaling to the motility of gastric cancer cells, the cells were treated with selective inhibitors of the Shh signaling pathway. The additional incubation of N-Shh-treated cells with control vehicle or KAAD-cyclopamine, a specific inhibitor of Smo, reversed the stimulatory effect of N-Shh on cell migration by 71% and 63.1% inhibition, respectively, in AGS and MKN-28, versus cells treated with N-Shh plus control vehicle (Figure 1D and E). Similar results were obtained when cells were treated with anti-Shh blocking antibody, with by 70% and 68.7% inhibition, respectively, being observed in AGS and MKN-28, versus cells treated with N-Shh plus IgG antibody (Figure 1D and E). Taken together, these results demonstrate that Shh signaling has an essential role in the motility of gastric cancer cells.

Next, the effect of Shh signaling on the invasion of gastric cancer cells was measured using a Matrigel invasion assay. The ability of gastric cancer cells to invade Matrigel was markedly enhanced by treatment with increasing concentrations of N-Shh, as shown in the wound closure assay (Figure 2A). Conversely, the N-Shh-induced invasiveness of AGS and MKN-28 cells was reduced by nearly 97% and 91%, respectively, in cells that were treated with KAAD-cyclopamine, and the invasiveness was reduced by 87% and 81%, respectively, in cells that were treated with anti-Shh blocking antibody when compared with cells that were treated with N-Shh plus control vehicle and IgG antibody, respectively (Figure 2B and C), demonstrating that Shh signaling plays an important role in the invasiveness of gastric cancer cells.

TGF-β signaling mediates Shh-induced migration and invasion of gastric cancer cells

Numerous studies have shown that more aggressive forms of tumors are growth-stimulated by TGF-β (28,29). Wang *et al.* (30) reported that TGF-β might modulate the metastatic potential of gastric cancer cells by breaking down basement membrane barriers and promoting their motility. Our above results also demonstrate that the Shh signaling pathway plays a critical role in the motility and invasiveness of gastric cancer cells, which indicates that activation of the TGF-β signaling pathway may affect Shh-mediated cellular motility and invasion (Figures 1 and 2). To examine this possibility, we next tested the role of TGF-β signaling in Shh-mediated migration properties. AGS and MKN-28 cells were treated with increasing concentrations of N-Shh with or without TGF-β1 (5 ng/ml), and their motility and invasive capacities were then assessed. Our results showed that TGF-β1, in the absence of Shh, slightly stimulated the migration and invasiveness of those cells, whereas a combination of increasing concentrations of N-Shh and TGF-β1 (5 ng/ml) significantly stimulated the migration and invasiveness of those cells (Figure 3A and D). To determine if abrogation of TGF-β signaling could block the Shh-mediated cellular motility and invasion, we blocked TGF-β signaling using anti-TGF-β blocking antibody or TGF-β1 siRNA. As shown in Figure 3, the stimulatory effects of N-Shh on cell migration and invasion were completely abolished by anti-TGF-β blocking antibody (Figures 3B and E) and by TGF-β1 siRNA (Figure 3C and F) treatment, but not by the control IgG antibody or non-specific siRNA. Taken together, these findings show that Shh signaling modulates the migration and invasion of gastric cancer cells through activation of the TGF-β signals.

The Shh signaling pathway induces TGF-β secretion and activates the TGF-β transcriptional response in gastric cancer cells

Next, to determine if N-Shh-stimulated cell migration and invasion resulted from elevated levels of TGF-β protein, we examined the secretion of TGF-β1 protein using specific enzyme-linked immunosorbent assays to quantitate the amount of TGF-β1 in medium in which AGS and MKN-28 cells were cultured. TGF-β1 secretion was measured 0.1, 0.5, 1, 6, 12 and 24 h after both cell types were treated with or without increasing concentrations of N-Shh and we found to be elevated in a dose-dependent manner as early as 0.5 h after N-Shh stimulation (Figure 4A). In response to the N-Shh addition, maximal increases in TGF-β1 secretion were observed at 12 and 24 h after N-Shh stimulation, with levels 13-fold greater than untreated cells being observed after 24 h. Conversely, TGF-β1 secretion was markedly reduced in the presence of either KAAD-cyclopamine or anti-Shh blocking antibody (Figure 4B). However, control experiments in which control vehicle or IgG antibody was used showed negligible effects.

To determine if N-Shh causes transcriptional activity at the TGF-β regulated promoter in gastric cancer cells, we assayed the TGF-β luciferase activity using a plasminogen activator inhibitor-I promoter report construct, 3TP-Lux. AGS cells that were transfected with 3TP-Lux were incubated in medium supplemented with increasing concentrations of N-Shh for 24 h. As seen in Figure 4C, treatment with N-Shh led to a dose-dependent increase in the TGF-β-induced 3TP-Lux activity; however, stimulation of the cells with greater than 0.5 μg/ml N-Shh did not result in an additional increase in luciferase activity. Exogenous TGF-β1 pretreatment also increased the 3TP-Lux activity in AGS cells when compared with the untreated control (Figure 4D). It is important to note that AGS cells cotreated with 5 ng/ml TGF-β1 and increasing concentrations of N-Shh showed higher 3TP-Lux activation than AGS cells treated with TGF-β1 alone. Therefore, to further test the effect of Shh signaling on TGF-β transcriptional

Fig. 1. Shh signaling promotes gastric cancer cell migration. (**A**) Confluent AGS and MKN-28 cell monolayers were wounded with a pipette tip and then treated with or without increasing concentrations of N-Shh. Cell migration to the wound area was then monitored by microscopy for 24 h post-wound. (**B**) The percentage of total area covered by cells was assessed using the NIH Image program. Bars represent the standard deviation of three independent experiments conducted in triplicate. $^{**}P < 0.001$, compared with untreated control groups. (**C**) The proliferation of AGS and MKN-28 cells treated with indicated concentrations of N-Shh was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assays. Bars represent the standard deviation of three independent experiments conducted in triplicate. (**D**) Confluent AGS and MKN-28 monolayers were wounded with a pipette tip and then treated with N-Shh (0.5 μg/ml) alone or together with an increasing concentration of KAAD-cyclopamine (cyclopamine), control vehicle (dimethyl sulfoxide), anti-Shh blocking antibody or control IgG antibody in serum-free medium. Cell migration to the wound area was monitored by microscopy for 24 h post-wound. (**E**) The percentage of total area covered by cells was then assessed using the NIH Image program. Bars represent the standard deviation of three independent experiments conducted in triplicate. $^{*}P < 0.01$ and $^{**}P < 0.001$, compared with cells that were treated with N-Shh plus control vehicle and IgG antibody, respectively.

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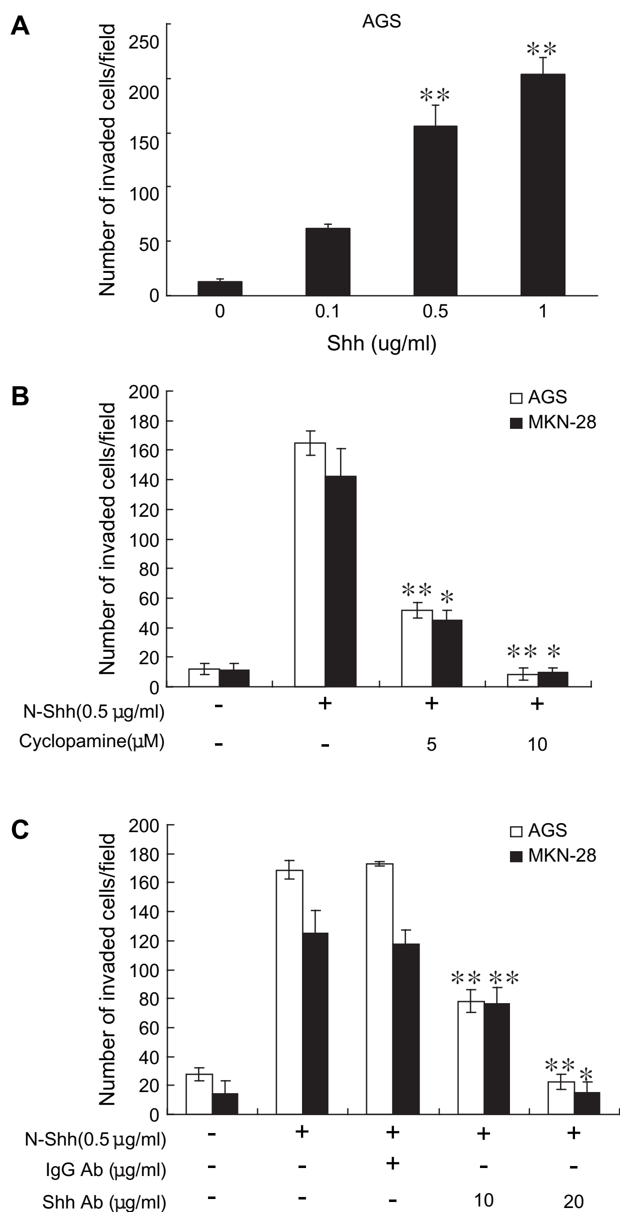

Fig. 2. Shh signaling promotes gastric cancer cell invasion. (**A**) AGS cells (1×10^5 cells/well) were seeded in the upper chamber, which was coated with Matrigel, and serum-free medium containing different concentrations of N-Shh was added to the lower chamber. After 24 h of incubation, the cells that invaded the lower surface of the insert were stained with Diff-Quik Stain and counted by microscopy. The data are expressed as the means of three independent experiments ± standard deviation. $^{**}P < 0.001$, compared with untreated control groups. Growth medium treated with N-Shh (0.5 µg/ml) alone or together with increasing concentrations of KAAD-cyclopamine, control vehicle (dimethyl sulfoxide) (**B**), anti-Shh blocking or control IgG antibody (**C**) was added to the lower chamber, and AGS and MKN-28 cells were seeded into the upper chamber. After 24 h of incubation, cells that invaded to the lower surface of the insert were stained with Diff-Quik Stain and counted by microscopy. Five different areas of invaded cells were counted for each data point ($n = 5$). The data are expressed as the means of three independent experiments ± standard deviation. $^{*}P < 0.01$ and $^{**}P < 0.001$, compared with cells that were treated with N-Shh plus control vehicle and IgG antibody, respectively.

activity, AGS cells transfected with 3TP-Lux reporter plasmid were stimulated for 24 h either with anti-Shh blocking antibody or control IgG antibody in the presence of N-Shh. As shown in Figure 4E, N-Shh-stimulated 3TP-Lux reporter activity was blocked in the presence of anti-Shh blocking antibody, but not in the presence of control IgG antibody. Consistent with reduced 3TP-Lux reporter activity that was observed in the presence of anti-Shh blocking antibody, KAAD-cyclopamine completely reversed the enhancement of the TGF-β-induced 3TP-Lux reporter activity that was induced by N-Shh stimulation. Similar results were obtained when MKN-28 cells were treated with anti-Shh blocking antibody or KAAD-cyclopamine (Figure 4F), and these results matched well with the results of the cellular migration and invasion experiments (Figure 3), suggesting that Shh-mediated induction of the TGF-β signaling may play a role in Shh-enhanced cell migration and invasion in gastric cancer cells.

Shh promotes motility and invasiveness of gastric cancer cells through TGF-β-mediated activation of the ALK5–Smad 3 pathway

Next, to elucidate the molecular mechanisms underlying the Shh-stimulated TGF-β response in cellular motility and invasion of gastric cancer cells, we evaluated TβRII, ALK5, ALK1 and Smad protein expression and activation by TGF-β to determine if it they were affected by N-Shh. The expressions of the above listed proteins were tested 0, 0.5, 1, 6, 12 and 24 h prior to stimulation with N-Shh by western blot. As shown in Figure 5A, the expression of ALK5 protein was elevated as early as 0.5 h after treatment and this elevation was sustained for 24 h following N-Shh stimulation. However, there was no change in the expression of ALK1 or TβRII protein, irrespective of the concentration of N-Shh. Similar to the observed increases in the expression of ALK5, phosphorylation of Smad 3 was found to be significantly enhanced as a result of N-Shh stimulation. In addition, stimulation of AGS cells with increasing concentrations of N-Shh revealed that the expressions of ALK5 and phosphorylation of Smad 3 increased in a dose-dependent fashion (Figure 5C). Furthermore, reprobing with an antibody directed against total Smad 3 did not reveal any significant change in protein levels, which indicates that the observed increases in expression did not occur as a result of an increase in the amount of total Smad 3 protein. However, there was no change observed in the expression of Smad 2, 4, 6 or 7 irrespective of the presence of different concentrations of N-Shh (data not shown). In order to determine whether treatment with N-Shh enhanced the expression of ALK5 and phosphorylation of Smad 3 by TGF-β, AGS cells were treated with either TGF-β1 alone or with increasing amounts of N-Shh. As expected, the expression levels of ALK5 and phosphorylation of Smad 3 in cells that were cotreated with both TGF-β1 and N-Shh were higher than those of cells treated with TGF-β alone (Figure 5B). To further confirm the effect of Shh signaling on the expression or activation of the ALK5–Smad 3 protein, we inhibited Shh signaling by treating cells with anti-Shh blocking antibody or KAAD-cyclopamine and then performed western blot analysis. Both the expression of ALK5 and phosphorylation of Smad 3 were markedly reduced in cells that were cotreated with KAAD-cyclopamine when compared with cells treated with N-Shh plus control vehicle (Figure 5C). Similarly, when the Shh signaling pathway was blocked using anti-Shh blocking antibody, a reduced expression of these proteins was observed (Figure 5D), suggesting that Shh signaling plays a critical role in the induction of ALK5 and phosphorylation of Smad 3.

To further elucidate the role of ALK5–Smad 3 signaling in Shh-mediated migration and invasion, we blocked TGF-β signaling using the ALK5 kinase inhibitor, SB 431542. As shown in Figure 5E, in spite of the presence of added N-Shh, treatment of the AGS cells with SB 431542 significantly decreased phosphorylation of Smad 3. In addition, wound closure and invasive activity in response to SB 431542 by AGS and MKN-28 cells treated with N-Shh was blocked (Figure 5F). We also knocked down ALK5 or Smad 3 in AGS and MKN-28 cells using ALK5- or Smad 3-specific siRNA and analyzed for cellular motility and invasion. The effectiveness of these siRNA to delete ALK5 or Smad 3 expression was confirmed by western blot (Figure 5G). Importantly, transient expression of ALK5 siRNA or Smad 3 siRNA reduced the ability of N-Shh to stimulate migration and invasion of those cells compared with the cells treated with

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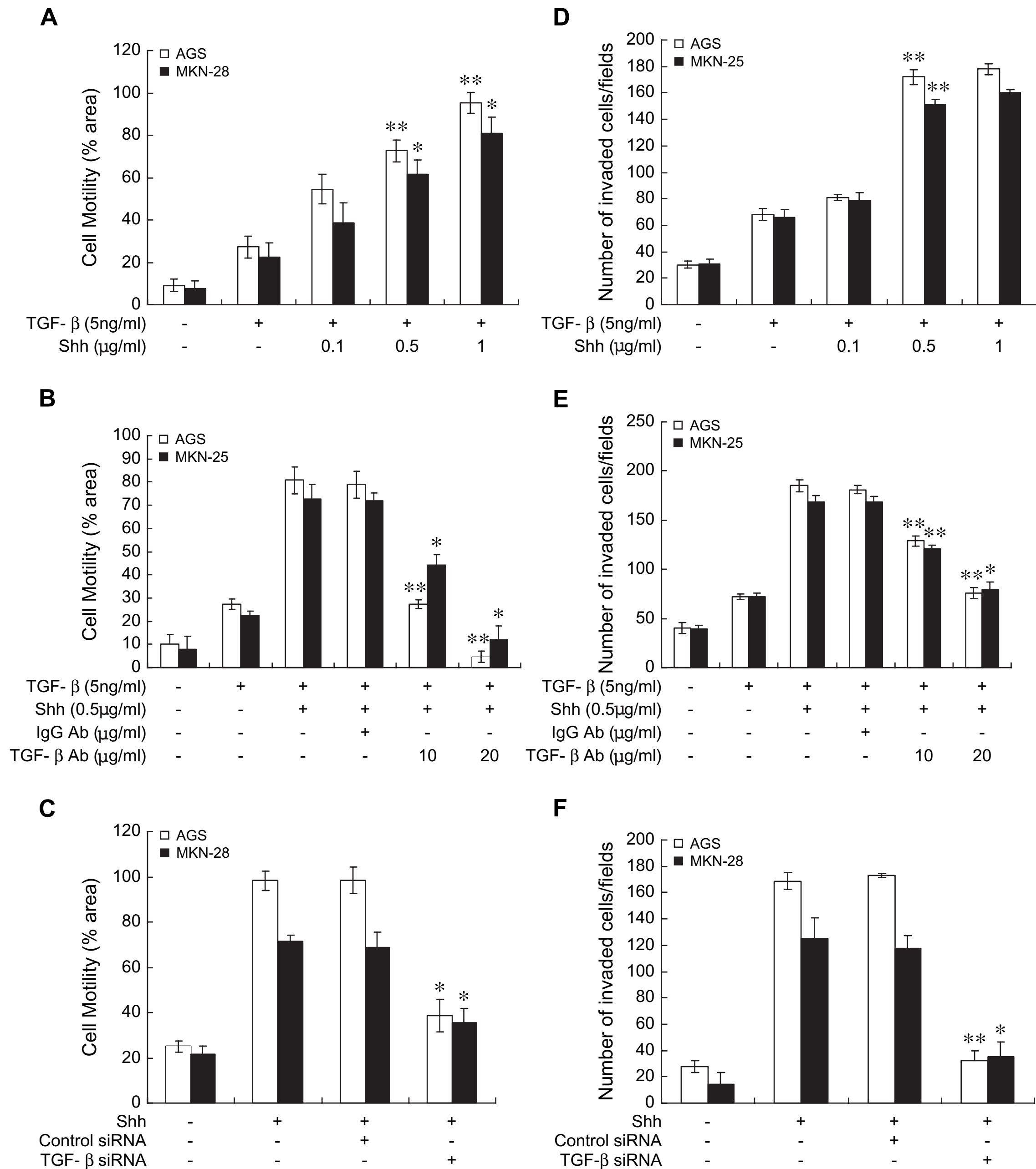

Fig. 3. TGF-β signaling mediates Shh-induced cellular migration and invasion. (**A**) Confluent AGS and MKN-28 cell monolayers were wounded with a pipette tip and then treated with increasing concentrations of N-Shh with or without TGF-β (5 ng/ml) and cell migration to the wound area was then monitored for 24 h post-wound. The percentage of total area covered by cells was then assessed using the NIH Image program. Bars represent the standard deviation of three independent experiments conducted in triplicate. $^{*}P < 0.01$, $^{**}P < 0.001$, compared with 5 ng/ml TGF-β groups. Confluent AGS and MKN-28 cells were wounded with a pipette tip and then treated with N-Shh (0.5 μg/ml) alone or together with an increasing anti-TGF-β blocking antibody, control IgG antibody (**B**), TGF-β siRNA (100 nM) or non-specific siRNA (**C**) in serum-free medium. Cell migration to the wound area was then monitored for 24 h post-wound and the percentage of total area covered by cells was then assessed using the NIH Image program. Bars represent the standard deviation of three independent experiments conducted in triplicate. $^{*}P < 0.01$ and $^{**}P < 0.001$, compared with control groups (IgG antibody for anti-TGF-β blocking antibody and non-specific siRNA for TGF-β siRNA, respectively). (**D**) Growth medium treated with increasing concentrations of N-Shh in the presence or absence of TGF-β (5 ng/ml) was then added into the lower chamber, and AGS and MKN-28 cells were seeded into the upper chamber. After 24 h of incubation, cells that migrated to the lower surface of the insert were stained with Diff-Quik Stain and counted by microscopy. Five different areas of invaded cells were counted for each data point ($n = 5$). The data are expressed as the means of three independent experiments ± standard deviation. $^{**}P < 0.001$, compared with 5 ng/ml TGF-β groups. AGS and MKN-28 cells treated with N-Shh (0.5 μg/ml) alone or together with an increasing anti-TGF-β blocking antibody, control IgG antibody (**E**), TGF-β siRNA (100 nM) or non-specific siRNA (**F**) were then evaluated in a performance invasion assay. The data are expressed as the means of three independent experiments ± standard deviation. $^{*}P < 0.01$ and $^{**}P < 0.001$, compared with control groups (IgG antibody for anti-TGF-β blocking antibody and non-specific siRNA for TGF-β siRNA, respectively).

non-specific control siRNA (Figure 5H). Taken together, these findings show that Shh signaling modulates the migration and invasion of gastric cancer cells through TGF-β signals, especially the activation of ALK5–Smad 3.

Shh-TGF-β signaling is required for enhanced MMP-2 and -9 activity

We next evaluated Shh-mediated cell migration and invasion to determine if it resulted from elevated levels of MMPs, which are well-documented extracellular matrix-degrading enzymes whose activity is

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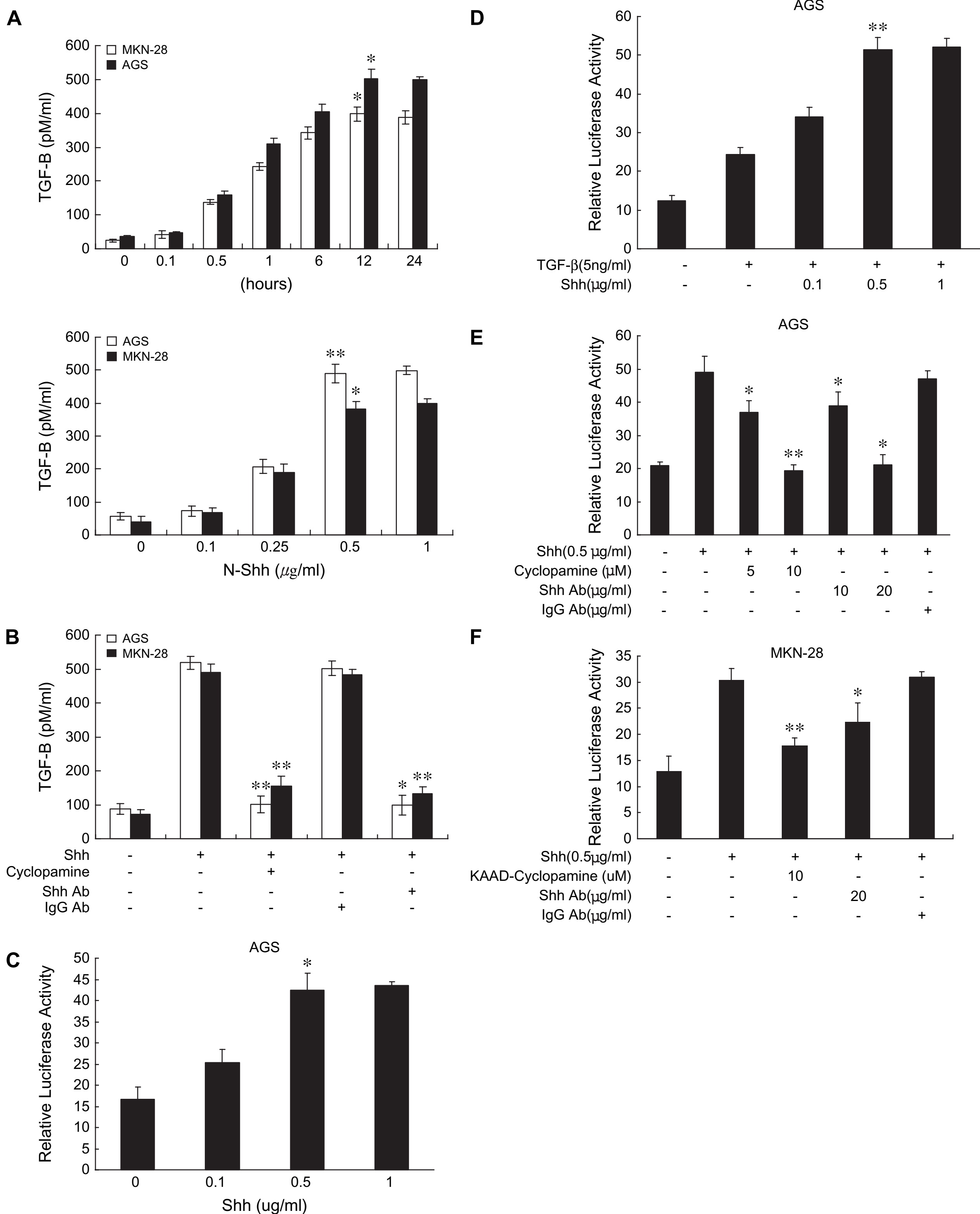

Fig. 4. The Shh signaling pathway induces TGF-β secretion and activates a TGF-β-induced transcriptional response in gastric cancer cells. (**A**) AGS and MKN-28 cells were plated in six-well plates, cultured under serum-free conditions and then treated with increasing concentrations of N-Shh for the times indicated. Following exposure to N-Shh, the samples assayed were acid activated, and the levels of secreted TGF-β were then measured as described in the Materials and methods section. Bars represent standard deviation of three independent experiments conducted in triplicate. The *P* values were calculated by comparing treatment cells to untreated control cells; $^{*}P < 0.01$, $^{**}P < 0.001$. (**B**) Using growth medium treated with N-Shh (0.5 μg/ml) alone or together with increasing concentrations of KAAD-cyclopamine or anti-Shh blocking or control IgG antibody, we measured the levels of secreted TGF-β. Bars represent the standard deviation of three independent experiments conducted in triplicate. $^{*}P < 0.01$ and $^{**}P < 0.001$, compared with cells that were treated with N-Shh plus control vehicle and IgG antibody, respectively. (**C**) AGS cells were transiently transfected with 3TP-Lux (1 μg) or CMV–renilla (1 μg), followed by treatment with an increasing concentrations of N-Shh. After 24 h, 3TP-Lux reporter activities were measured by a luminometer and normalized to *Renilla* luciferase activity.

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associated with tumor invasiveness. To measure the effect of Shh signaling on MMPs, we performed gelatin zymography using conditioned media obtained from AGS cells. We found that the supernatants of AGS cells treated with N-Shh contained higher levels of the active form of MMP-2 (66 kDa) and MMP-9 (86 kDa) (Figure 6A). To further demonstrate that Shh signaling plays a critical role in the activity of MMP-2 and -9, AGS cells were treated with either anti-Shh blocking antibody or KAAD-cyclopamine and then tested for activity of these proteinases. A clear decrease in MMP-2 and -9 activities occurred after abrogating Shh signaling (Figure 6C), suggesting that Shh-mediated induction of MMP-2 and -9 may play a role in Shh-enhanced cell migration and invasion.

The above results (Figure 5) indicate that Shh signaling modulates the migration and invasion of gastric cancer cells through TGF-β–ALK5–Smad 3 signaling. To determine whether the effect of Shh signaling on MMP-2 and -9 activities was the result of increased TGF-β signaling, AGS cells were treated with either TGF-β1 alone or with increasing amounts of N-Shh. Gelatin zymography showed elevated levels of the active form of MMP-2 and -9 in response to cotreatment with increasing amounts of N-Shh when compared with TGF-β1 alone (Figure 6B). Moreover, MMP-2 and -9 activity was completely abolished by exposure of the cells to anti-TGF-β blocking antibody or SB 431542, even in the presence of N-Shh (Figure 6C). Taken together, the results of these experiments revealed that TGF-β–ALK5–Smad 3 signaling is required for enhanced MMP-2 and -9 activity by Shh signaling.

Gli and ALK5 expression in human tumor tissue according to lymph node metastasis

To investigate the role of Shh-TGF-β signaling in the metastasis of human gastric cancers, we performed Gli or ALK5 immunohistochemistry on tumor biopsies obtained from a total of 41 gastric cancer patients, including 32 lymph node metastasis specimens and 9 no lymph node-negative gastric cancer specimens. ALK5 expression was classified as strong, moderate, weak and negative in 1 (2%), 9 (21%), 22 (53%) and 9 (21%) cases. Gli expression was classified as strong, moderate, weak and negative in 2 (4.8%), 16 (39%), 20 (48%) and 3 (7.3%) cases, respectively. The expression pattern of Gli was similar to ALK5 expression in human gastric tumor tissue (Figure 7). Expression pattern between the Gli and ALK5 was strongly correlated ($r = 0.439$, $P = 0.004$). Despite of non-significant expression pattern of ALK5 expression in human gastric cancer tissue according to lymph node metastasis ($P = 0.752$), Gli demonstrated the trend of high expression in human gastric tissues that had lymph node metastasis compared with no lymph node metastasis ($P = 0.094$).

Discussion

The Shh signaling pathway, although initially recognized for its role in regulating cell proliferation, tissue generation and cell differentiation during embryogenesis (1–4), has more recently been shown to play a role in the proliferation of a variety of human cancer cells, including BCCs, medulloblastomas, small cell lung cancer, pancreatic cancer, prostate cancer and gastric cancers. Previous studies have reported that aberrant activation of Shh signaling through Ptch 1 is frequently detected in cases of advanced gastric adenocarcinoma and that this signaling activation was associated with poorly differentiated gastric tumors. Experiments conducted on gastric cancer cells have also shown that cyclopamine, which inhibits cellular responses to Shh signaling by specifically binding to Smo, can inhibit the malignant growth of gastric cancer cells *in vitro* and *in vivo*. Taken together, these findings indicate that specific inhibitors of the Shh signaling pathway may be effective in the future treatment of gastric cancers (6,7). Despite the central role that Shh signaling plays in the development of gastric cancer, little is known about the putative function that Shh regulation plays on cell migration and invasion. Here, we provided the first evidence supporting a novel function of the Shh signaling pathway as a stimulating factor in the regulation of tumor metastasis.

Recently, several studies have suggested that Shh signaling plays a role in the control of the motility of multiple cell types. For example, Shh signaling has been shown to enhance endothelial progenitor cell-mediated microvascular remodeling during wound healing (11) and is essential for yolk sac and embryonic vasculogenesis (12,13). In addition, disruption of Shh signaling by cyclopamine has been shown to inhibit EMT and reduce metastasis in pancreatic cancer cells (31). Gli overexpression induced lymphatic metastasis, whereas the inhibition of Shh signaling reduced cell growth and motility (32). In addition, the results of this study also indicate that the activation of Shh signaling is associated with the migration and invasion of gastric cancer cells. Furthermore, we found that the migration and invasion of gastric cancer cells were induced by N-Shh stimulation and blocked by abrogating Shh signaling with KAAD-cyclopamine or anti-Shh blocking antibody (Figures 1 and 2). Taken together, these data suggest that abnormal activation of this signaling may contribute to metastasis in a variety of cancers, including gastric cancer, and that inhibition of the Shh signaling pathway may be useful for preventing metastasis. However, the mechanisms by which high expression of Shh signaling enhances cellular motility and invasion should be further investigated.

To become a more invasive phenotype, it is necessary for epithelial cancer cells to acquire migratory and invasive capabilities, which then leads to the capacities of the migration and invasion through degradation of the basement membranes and the extracellular matrix at the primary tumor site and establishment of metastatic colonies at distant organs (33,34). Generally, migration and invasive capacities are positively correlated with metastasis potential. At the molecular level, there is altered expression of epithelial markers and upregulated expression of the proteins involved in cell migration and invasion (35,36). A recent study demonstrated that Hh signaling is correlated with the severity of the associated tumor and that this activity is sufficient to maintain metastatic behavior (10–14). Here, we report that Shh signaling pathway may induce metastatic functions of gastric cancer through the mechanism of tumor cell motility and invasion. However, the metastatic effects of Shh signaling should be further investigated.

The processes of cell migration and invasion, as well as expression of their related proteins were also found to be regulated by several signaling pathways, including TGF-β (37,38). Although TGF-β elicits most of its tumor suppressor activity by potently inhibiting the proliferation of many cancer cells, several studies have previously implicated TGF-β in the progression of cancer based on its contribution to tumor invasion, its suppression of the immune response and its alteration of the tumor microenvironment (22,26,28). Wang *et al.* (30) reported that TGF-β might modulate the metastatic potential of gastric cancer cells by promoting their motile and invasive activities. In this study, we showed that Shh signaling plays an essential role in the motility and invasiveness of gastric cancer cells and that this occurs through the TGF-β signaling pathway. Based on these findings, we evaluated the intracellular cross talk between TGF-β and the Shh

Bars represented standard deviation from three independent experiments. $^{*}P < 0.01$, compared with untreated control cells. (**D**) AGS cells transfected with 3TP-Lux (1 μg) or CMV–renilla (1 μg) were treated with the indicated concentration of N-Shh in the presence of TGF-β (5 ng/ml). The cells were then harvested 24 h later and the levels of luciferase were measured. 3TP-Lux reporter activities were then measured and reported as described in (C). $^{**}P < 0.001$, compared with 5 ng/ml TGF-β groups. AGS (**E**) and MKN-28 (**F**) cells transfected with 3TP-Lux (1 μg) or CMV–renilla (1 μg) were treated with N-Shh (0.5 μg/ml) or together with increasing concentrations of KAAD-cyclopamine, control vehicle (dimethyl sulfoxide), anti-Shh, TGF-β blocking antibody or control IgG antibody and after 24 h, 3TP-Lux reporter activities were measured and reported as described in (C). $^{*}P < 0.01$ and $^{**}P < 0.001$, compared with cells that were treated with N-Shh plus control vehicle and IgG antibody, respectively.

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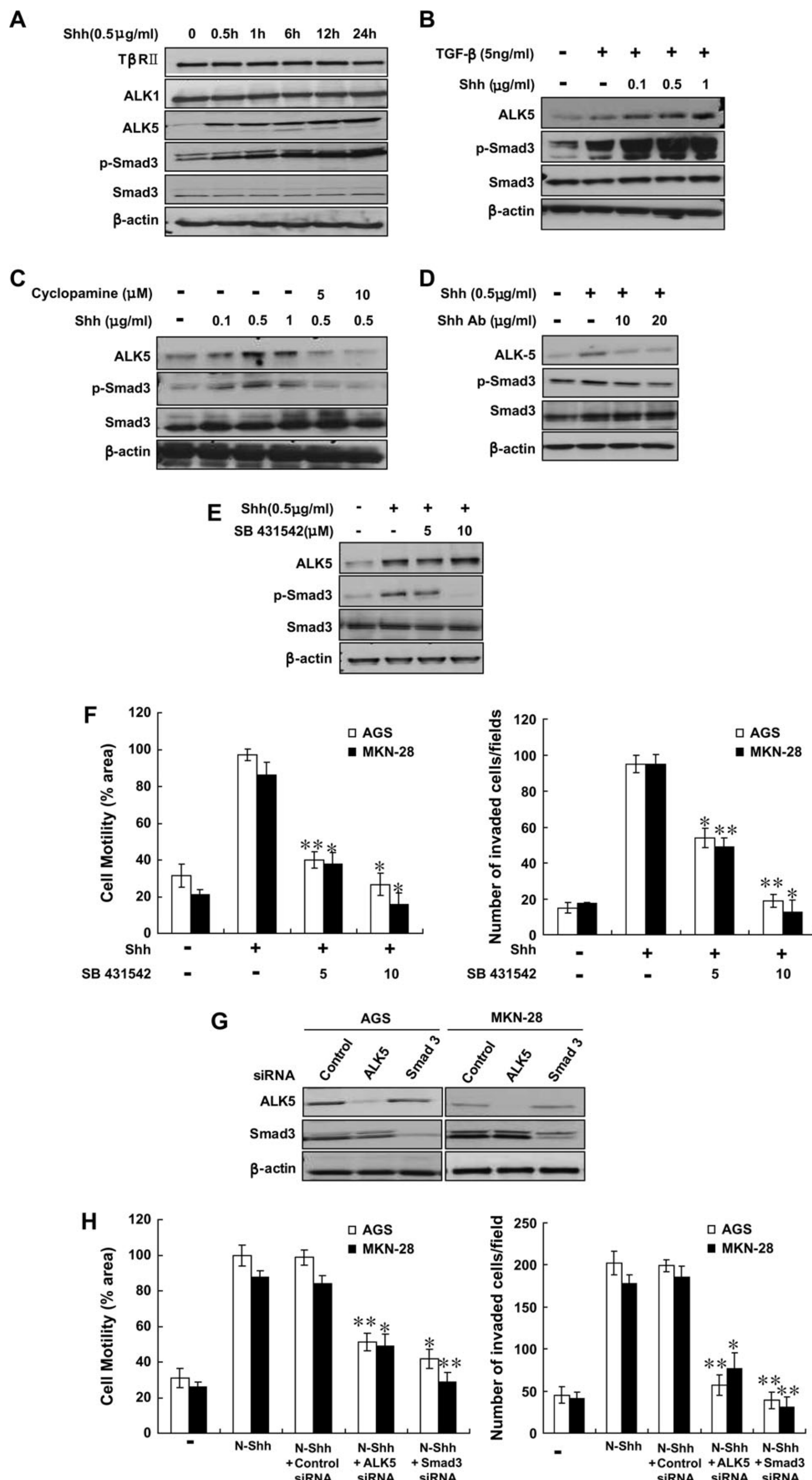

Fig. 5. Loss of ALK5–Smad 3 signaling is correlated with Shh-induced cellular migration and invasion. (**A**) AGS cells were incubated in the presence or absence of 0.5 μg/ml N-Shh for the times indicated, and the total protein was harvested for western blot analysis using specific antibodies against TβRII, ALK1, ALK5, p-Smad 3, Smad 3 and β-actin. (**B**) Protein extracts of AGS cells treated with the indicated concentration of N-Shh in the presence of TGF-β (5 ng/ml) were prepared for western blot as in (A). AGS cells were treated with N-Shh (0.5 μg/ml) or together with increasing concentrations of KAAD-cyclopamine, control

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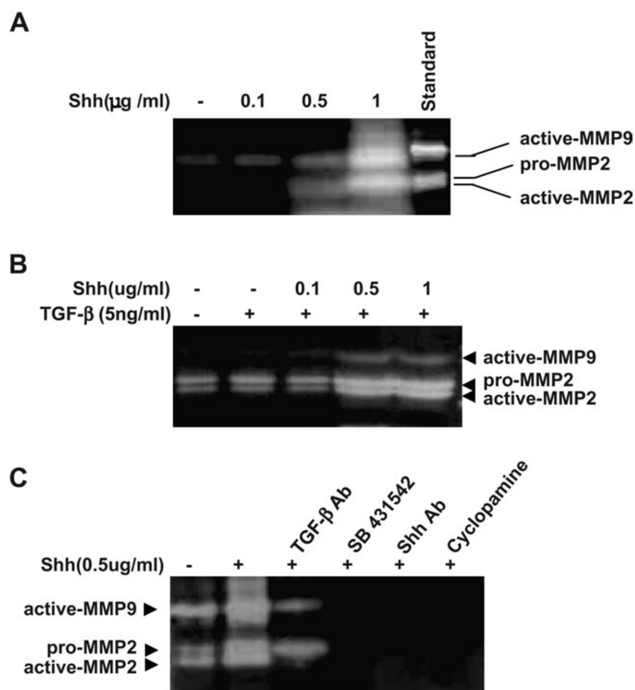

Fig. 6. Shh-TGF signaling upregulates MMP-2 and -9 activity. (**A**) Conditioned media from AGS cells treated with increasing concentrations of N-Shh were collected and analyzed for MMP activities by gelatin zymography. A human MMP-2 and -9 standard was used as a positive control. (**B**) AGS cell were treated with increasing concentrations of N-Shh with or without TGF-β (5 ng/ml) for 24 h and the conditioned media were then subjected to gelatin zymography. (**C**) AGS cells were treated with TGF-β blocking antibody (10 μg/ml), SB431542 (10 μM), Shh blocking antibody (20 μg/ml) or KAAD-cyclopamine (10 μM) in the presence of N-Shh (0.5 μg/ml) and the conditioned media were subjected to gelatin zymography.

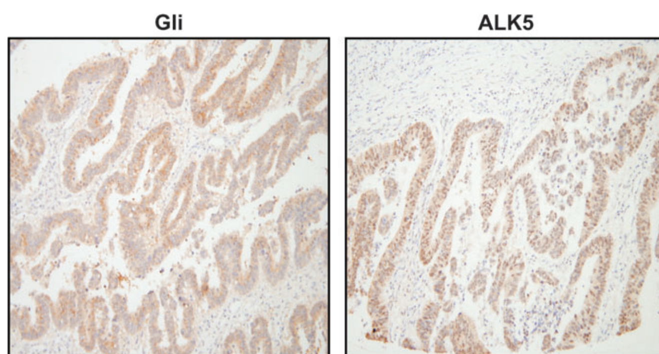

Fig. 7. Gli and TGF-R showed similar pattern of expression in gastric cancer tissue. (**A**) Gli stain, ×200 and (**B**) ALK5 stain showed high expression in the same area of gastric cancer, ×200.

signaling pathway to determine if it plays an important role in the metastatic potential of gastric cancer and found that a very close positive correlation between the TGF-β and Shh signaling pathways existed. Interestingly, we found that activation of the Shh signaling pathway in gastric cancer cells induced TGF-β1 secretion, TGF-β regulated transcriptional activity, TGF-β-mediated migration and TGF-β-mediated invasion following treatment with N-Shh (Figure 3). Furthermore, we found that blockage of TGF-β signaling by anti-TGF-β blocking antibody or TGF-β1 siRNA renders gastric cancer cells unresponsive to Shh-mediated migration and invasion, indicating that the TGF-β signaling pathway modulates Shh signaling in gastric cancer migration and invasion.

To better understand the mechanism by which TGF-β induced Shh-mediated migration and invasion, we evaluated these effects to determine if they were mediated through different TGF-β type I receptors. We found that N-Shh stimulation of gastric cancer cells induced expression of ALK5, but not TβRII and ALK1, whereas treatment with KAAD-cyclopamine or anti-Shh blocking antibody resulted in a significant decrease in ALK5 expression. ALK1 signals through Smad 1/5, whereas ALK5 signals through Smad 2/3. In particular, gastric cancer cells treated with N-Shh were found to display a significant increase in the phosphorylation of Smad 3 (Figure 5), but not in blocking the phosphorylation of Smad 2 or Smad 1/5 (data not shown). Additional treatment with SB 431542, ALK5 siRNA or Smad 3 siRNA, however, resulted in a significant decrease in the amount of phosphorylated Smad 3 and completely reversed the motile and invasive effects of Shh signaling on these cells. Taken together, these results strongly suggest that the motile and invasive activities of Shh on gastric cancer cells can be attributed to ALK5/Smad 3. However, the precise mechanism by which the Shh signaling pathway controls TGF-β–ALK5 function to regulate Shh-mediated metastatic potential needs to be further investigated.

Members of the Gli family that are transcriptional mediators of Shh signaling mediate the response of Shh. When Shh binds to the Ptch receptor, inactivated Ptch enables signaling via Smo. This causes Gli, which is released from a large protein complex by Smo, to translocate to the nucleus to activate the Shh target genes (3,5). Recently, Gli was shown to affect the gene expression of BMP-2, which is structurally related to the TGF-β superfamily in osteoblasts (39). It is interesting to note that we found that Shh signaling resulted in no changes in the gene expression of either the TGF-β receptor II or ALK1, whereas enhancement of ALK5 gene expression was induced by N-Shh stimulation (data not shown). Therefore, Shh signaling needs to be further evaluated to determine if it affects the activity of the ALK5 promoter through a physical interaction between the Gli protein and a specific region of the ALK5 promoter.

In summary, our findings emphasize the potential role of TGF-β–ALK5 signaling in Shh-induced cellular migration and invasion. These observations improve our understanding of the mechanism by which Shh signaling activation occurs as it relates to the metastatic behavior of gastric cancer cells, which may prove useful in identifying therapeutic molecular targets to inhibit Shh-dependent migration and invasion.

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vehicle (dimethyl sulfoxide) (**C**), anti-Shh blocking antibody or control IgG antibody (**D**) and western blots were then performed for the indicated antibodies. (**E**) Protein extracts of AGS cells treated with the indicated concentration of SB 431542, a selective ALK kinase inhibitor, in the presence of N-Shh (0.5 μg/ml), were prepared for western blot analysis using specific antibodies against ALK5, p-Smad 3, Smad 3 and β-actin. AGS and MKN-28 cells treated with increasing concentrations of SB 431542 (**F**), ALK5 siRNA, Smad 3 siRNA or non-specific siRNA (**H**) in the presence of N-Shh (0.5 μg/ml) were then evaluated in a performance migration and invasion assay. The data are expressed as the means of three independent experiments ± standard deviation. $^{*}P < 0.01$ and $^{**}P < 0.001$, compared with cells that were treated with N-Shh plus control vehicle and non-specific control siRNA, respectively. (**G**) Expression of these siRNA was confirmed by western blotting for ALK5 and Smad 3, respectively.

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