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Hedgehog Signaling Regulates Bladder Cancer Growth and Tumorigenicity

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Abstract

The role of Hedgehog (HH) signaling in bladder cancer remains controversial. The gene encoding the HH receptor and negative regulator *PATCHED1* (*PTCH1*) resides on a region of chromosome 9q, one copy of which is frequently lost in bladder cancer. Inconsistent with *PTCH1* functioning as a classic tumor suppressor gene, loss-of-function mutations in the remaining copy of *PTCH1* are not commonly found. Here, we provide direct evidence for a critical role of HH signaling in bladder carcinogenesis. We show that transformed human urothelial cells and many urothelial carcinoma cell lines exhibit constitutive HH signaling, which is required for their growth and tumorigenic properties. Surprisingly, rather than originating from loss of *PTCH1*, the constitutive HH activity observed in urothelial carcinoma cell lines was HH ligand dependent. Consistent with this finding, increased levels of HH and the HH target gene product GLI1 were found in resected human primary bladder tumors. Furthermore, on the basis of the difference in intrinsic HH dependence of urothelial carcinoma cell lines, a gene expression signature was identified that correlated with bladder cancer progression. Our findings therefore indicate that therapeutic targeting of the HH signaling pathway may be beneficial in the clinical management of bladder cancer. *Cancer Res; 72(17); 4449–58. ©2012 AACR.*

Introduction

Cancer of the urinary bladder is one of the most common malignancies worldwide, with a lifetime risk of 1 in 42 (1). Greater than 80% of bladder cancers originate from the urothelium of the bladder and are referred to as urothelial carcinoma (2). Urothelial carcinoma is classified into 2 subtypes based on whether or not the cancer cells infiltrate into the muscle layer of the bladder (2). Nonmuscle–invasive urothelial carcinoma (NMIUC) is a less aggressive type of cancer with good prognosis, but with a greater than 50% recurrence rate (3). Muscle-invasive urothelial carcinoma (MIUC) frequently metastasizes, resulting in a poor 5-year survival rate (4). The high recurrence rate and increased risk of metastasis of the 2 major urothelial carcinoma subtypes make the effective clinical management of urothelial carcinoma an important goal.

The deregulation of Hedgehog (HH) signaling has been linked to the etiology of many cancers, in which it is thought to play an initiating or maintenance role (5–7). Constitutive HH pathway activity is thought to result from mutations in key regulators of the pathway, overexpression of the HH ligands, or noncanonical activation of HH target genes (8, 9). The vertebrate HH ligands consist of Sonic Hedgehog (SHH), Indian HEDGEHOG (IHH), and Desert Hedgehog (DHH), which engage a signaling cascade by binding to their common cellular receptor PATCHED1 (PTCH1; ref. 10). Upon HH binding, PTCH1 releases its inhibitory effect on the transmembrane protein Smoothened (SMO), ultimately leading to activation of GLI family transcription factors (GLI1–3) (11). Among the GLIs, GLI1 is both a transcriptional activator and HH target gene (11–13). Furthermore, GLI1 is thought to be the most reliable biomarker of HH pathway activity (13, 14). The steady-state levels of GLIs are highly regulated via proteolysis, stabilization of which is thought important for cancer progression (15). Unlike GLI1, GLI2 and GLI3 are also regulated by

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proteolytic cleavage to convert them from transcriptional repressor forms to activator forms in response to HH. Ultimately, the overall activation status of GLIs determines the output of HH pathway activity (11, 16).

The role HH signaling plays in human bladder cancer has been quite controversial. Loss of certain regions of chromosome 9q is one of the most common and earliest genetic alterations in urothelial carcinoma (17–19). The *PTCH1* gene was reported to reside in a minimal deletion region of 9q by one group but not by others (20–22). Inconsistent with *PTCH1* functioning as a classic tumor suppressor gene for bladder cancer, mutations of *PTCH1* were rarely found in the remaining allele of urothelial carcinoma samples in which one copy of *PTCH1* was deleted, nor in other bladder cancers whose *PTCH1* LOH status were unclear (20–24). Furthermore, 2 groups reported on insensitivity of urothelial carcinoma cell lines to SMO antagonists, inconsistent with HH signaling playing a major role in urothelial carcinoma (25, 26). We recently showed that the bladder carcinogen arsenic activated HH signaling, and that GLI1 protein was highly expressed in the vast majority of human urothelial carcinoma specimens (27). Here, we provide direct experimental evidence, *in vitro* and *in vivo*, for a critical role of HH signaling in bladder carcinogenesis.

Materials and Methods

Cell lines, reagents, and assays

All cells were grown and maintained as described previously (25, 28). Stable cells were selected under G418 (Invitrogen) and were used as either polyclonal or monoclonal lines as indicated. Lentiviruses expressing various short hairpin RNAs (shRNA; Supplementary Table S3) were used to transduce target cells as previously described (29). Equal viral titers were determined as previously described (30). Urothelial carcinoma cells were seeded in 96-well plates, transduced with various shRNAs, and incubated for 3 to 4 days before determining cell proliferation and apoptosis using CellTiter-Glo and Caspase-3/7 Assays (Promega). The soft agar assay, GLI1 enrichment for immunoblotting, HH-reporter assay, and TaqMan-based quantitative reverse transcriptase PCR analysis were carried out as previously described (27, 28). Details for primary cilia staining, genome wide copy number, and expression profiling of urothelial carcinoma cells, gene signature generation, meta-analysis, and methylation analysis are provided in the Supplementary Materials and Methods.

Immunohistochemical staining

Resected human urinary bladder tissues were fixed in formalin, paraffin embedded and sectioned at 4 μm thickness under an approved Institutional Review Board protocol at the University of Miami, FL. Immunohistochemical staining for GLI1 and SHH was carried out by a DAKO autostainer and scored by a board certified pathologist (M.J.). The scoring criteria were based on an estimate of the intensity of tumor cells stained positive for GLI1 or SHH: − (negative); + (weakly positive); ++ (moderately positive); +++ (strongly positive). The primary antibodies for immunohistochemistry (IHC) were rabbit polyclonal GLI1 (27) and rabbit polyclonal SHH (Santa Cruz).

Xenograft tumors in nude mice

Six-week-old female athymic nude mice (Charles River) were inoculated with Vmcub1 or HT1376 cells which had been transduced with control shRNAs or *SMO*- and *GLI1*-specific shRNAs. A total of 1 to 5 million live cells were mixed with an equal volume of Matrigel (BD Biosciences) and injected subcutaneously in the flanks of nude mice. Tumor growth was monitored for up to 40 days, and tumor volume was measured by the formula: Volume $= (S \times S \times L)/2$, in which S and L are the short and long dimensions (29).

Statistics

All experiments were independently conducted at least 3 times unless otherwise stated. Statistical significance was determined by Student t test. P value 0.05 or less was considered statistically significant. Statistical analysis for representative experiments was not provided.

Results

We have suggested that elevated HH activity might account for the etiology of arsenic-induced bladder cancer (27). As chronic arsenic exposure transforms human urothelial cells *in vitro* (28), we reasoned that HH activity might be required for arsenic-mediated urothelial cell transformation. Therefore, we compared arsenic-transformed urothelial cells (URO-MSC52) with their passage-matched immortalized parental cells (UROtsa), initially noting that the expression level of the HH target gene *GLI1* was higher in the arsenic-transformed cells (Fig. 1A). We next knocked down the expression of *SMO* or *GLI1* in these cells, using 2 independently targeted shRNAs, to examine whether URO-MSC52 cells require HH signaling to maintain their transformed phenotype. When compared with cells transduced with a control scramble shRNA (scramble shRNA#1), URO-MSC52 cells exhibited a greater than 50% reduction in their ability to grow in an anchorage-independent manner upon reduction of *SMO* or *GLI1* (Fig. 1B). Moreover, although parental UROtsa cells grew poorly in soft agar, their ability to do so was dramatically improved after SHH transduction (Fig. 1C). Consistent with UROtsa cells being HH responsive, we observed an induction in HH target gene expression in response to a HH pathway agonist (Fig. 1D). This modest HH target gene activation is in contrast to the strong induction in anchorage-independent growth, which likely results from the different biologic properties measured in these mechanistically distinct assays. Collectively, these results suggested a critical role for HH signaling in urothelial cell transformation.

On the basis of our results with transformed urothelial cells, we hypothesized that increased HH pathway activity might be required for the growth of bladder cancer cells. To test this hypothesis, we measured the proliferation of a large panel of well-characterized human urothelial carcinoma cell lines and attenuated HH signaling in these cells using shRNA-based RNA interference to knockdown the expression of key HH pathway

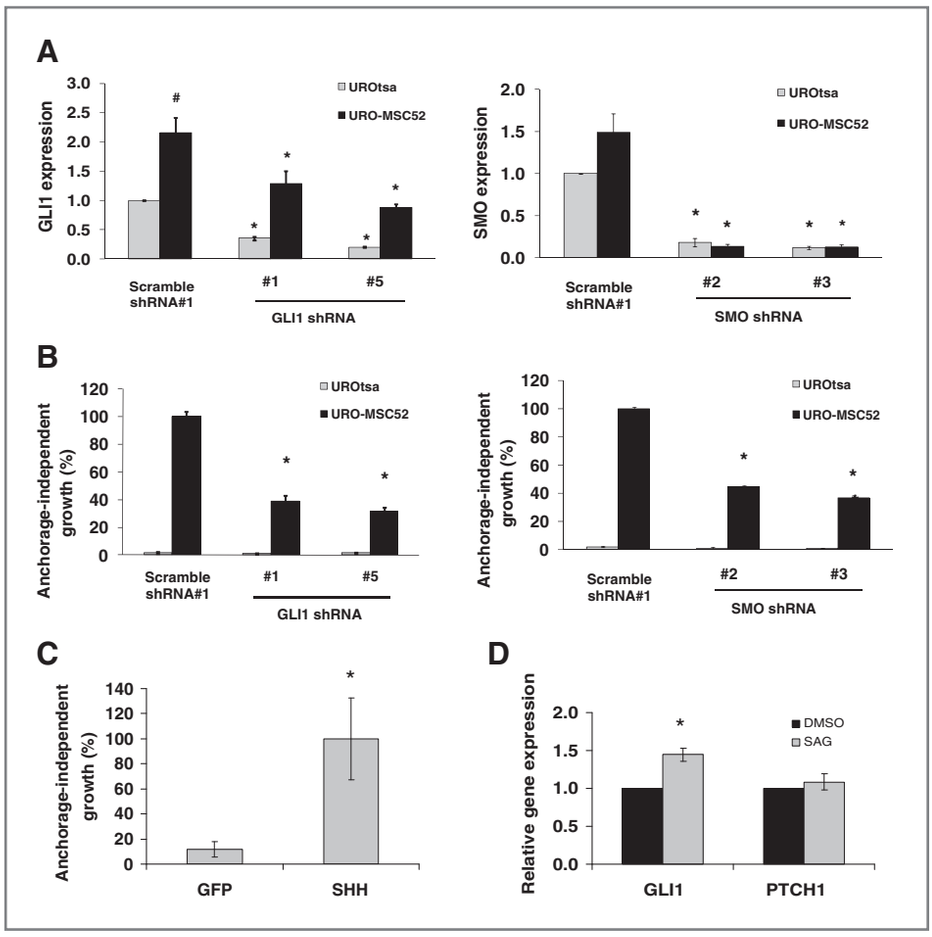

Figure 1. HH signaling is required for urothelial cell transformation. A, relative expression of *GLI1* (left) or *SMO* (right) was examined by qPCR in arsenic-transformed urothelial cells (URO-MSC52) and passage-matched control cells (UROtsa) after transduction of scramble shRNA#1 or shRNAs specific for *GLI1* or *SMO*. B, knockdown of *GLI1* (left) or *SMO* (right) attenuates the anchorage-independent growth of URO-MSC52 cells. C, UROtsa cells stably overexpressing SHH exhibit enhanced anchorage-independent growth. D, UROtsa cells were treated with SAG (200 nmol/L) or dimethyl sulfoxide for 48 hours before examining the expression of HH target genes by qPCR. Error bars, SEM. #, statistically significant changes when comparing UROtsa with URO-MSC52 cells; *, statistically significant changes when comparing within each cell line. DMSO, dimethyl sulfoxide.

components (*SMO*, *GLI1*, *GLI2*, and *GLI3*). All the shRNA constructs used here were validated at the mRNA and protein levels (Supplementary Fig. S1). To further control the stringency of this shRNA-mediated approach, we used 2 independent shRNAs to target each HH component and 4 sequence-distinct control shRNAs. In general, urothelial carcinoma cell lines showed a wide range of sensitivity to knockdown of HH signaling components (Fig. 2A and Supplementary Fig. S2A). For example, *GLI1* knockdown decreased the proliferation of T24 and Vmcub1 cells by 60% but had little effect on the proliferation of HT1376 and J82 cells, although the knockdown efficiency of the targeted genes was similar among the cell lines tested (Supplementary Fig. S1B). Interestingly, *GLI3* attenuation showed similar inhibitory effects on cell proliferation as other positive HH regulators did, indicating that the GLI3 activator form likely predominated in the sensitive cells. Knocking down the expression of *SMO*, *GLI1*, *GLI2*, or *GLI3* showed similar inhibitory effects on proliferation in any given urothelial carcinoma cell line, implying that the HH signaling rather than any individual pathway component might be important for urothelial carcinoma cell proliferation. Overexpression of *GLI1* was able to rescue the proliferation defects rendered by *SMO* shRNA in T24 cells, suggesting the shRNA-mediated *SMO* knockdown was specific (Fig. 2B). The incomplete rescue observed might also implicate GLI1-independent, SMO-dependent signaling mechanisms (such as those through GLI2 or GLI3) or an HH-dependent GLI1 modification may also be important for cell proliferation (31). Similar attenuation of proliferation was also observed in a subset of urothelial carcinoma cells treated with the SMO antagonist GDC-0449 (Supplementary Fig. S2B). Overall, these results suggested that HH signaling is required for cell proliferation in at least a subset of urothelial carcinoma cell lines.

We further characterized the role of HH signaling in urothelial carcinoma cells focusing on 4 cell lines (HT1376, J82, Vmcub1, and T24 cells), as they represent cells in which the proliferation was least or most affected when HH signaling was inhibited. We first confirmed their relative sensitivity to HH

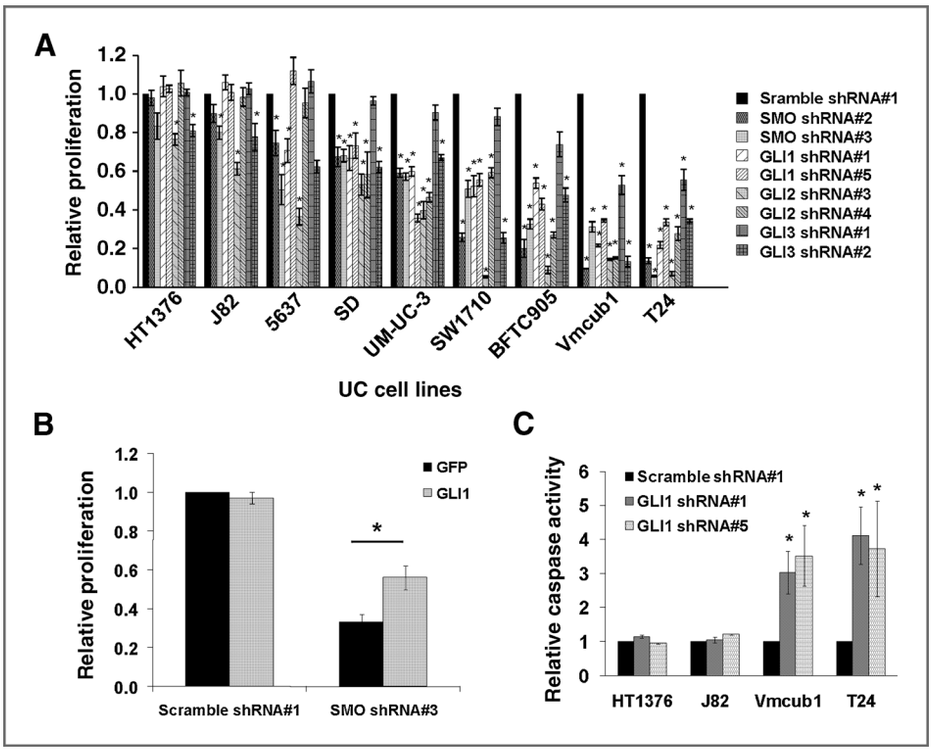

Figure 2. HH signaling mediates the proliferation and survival of human urothelial carcinoma cell lines. A, urothelial carcinoma cell lines were transduced with indicated shRNAs. Cell proliferation was determined 4 days later and normalized to cells receiving scramble shRNA#1. B, T24 cells overexpressing GFP or GLI1 were transduced with scramble shRNA#1 or *SMO* shRNA#3. Cell proliferation was determined 3 days after shRNA transduction. C, urothelial carcinoma cell lines were transduced with indicated shRNAs and caspase-3/7 activity was measured 4 days later. Error bars, SEM. *, statistically significant changes compared with control cells.

inhibition using a second distinct proliferation assay (Supplementary Fig. S2C). We next examined whether HH signaling could act as a survival factor for urothelial carcinoma cells, which had been suggested for other HH-dependent cancer cell lines (32–35). We therefore attenuated the expression of *GLI1* or *SMO* and then measured cell apoptosis. Caspase-3/7 activity was induced when *GLI1* expression was attenuated in the 2 cell lines whose proliferation was most dependent on HH signaling (Vmcub1 and T24 cells), but not in cells that showed the least dependence on HH signaling for proliferation (HT1376 and J82 cells; Fig. 2C). Similar results were obtained when *SMO* levels were reduced, using the cleavage of PARP as an indicator of apoptosis (Supplementary Fig. S3). PARP cleavage was evident when HT1376 and J82 cells were exposed to puromycin, suggesting that these cells have a functional apoptotic pathway (Supplementary Fig. S3 and data not shown). These results indicated that HH signaling is required to maintain the viability of a subset of urothelial carcinoma cell lines.

As the 4 urothelial carcinoma cell lines showed distinct dependence on HH signaling for proliferation, we reasoned that the HH signaling pathway might be differentially regulated in these cells. By comparing the expression of several HH pathway components, we observed that the HH-dependent cells (Vmcub1 and T24) expressed higher levels of *SMO*, but not other genes examined (Fig. 3A and data not shown). We also compared the protein level of GLI1 in these 4 cell lines as a second determinant of HH activity, as stabilization of GLI1 protein is a key event for HH signaling in cancer (15). GLI1 was immunoblotted from lysates of these 4 urothelial carcinoma cell lines after GLI enrichment, using Sepharose beads conjugated to a defined GLI-binding DNA oligonucleotide. High levels of GLI1 were only detected in HH-dependent cells (Fig. 3B). Notably, J82 cells expressed comparable high levels of *GLI1* mRNA, but unlike the HH-dependent cells, failed to accumulate significant amount of GLI1 protein (compare Fig. 3B with Fig. 3A). The accumulation of *SMO* and GLI1 indicated that Vmcub1 and T24 cells harbor a higher level of HH pathway activity, correlating well with their dependence on HH signaling for proliferation.

We further compared the genome-wide expression profiles of these 2 groups of cell lines to understand the biologic significance of HH dependence. We searched for genes that were commonly expressed in HH-dependent cells or in HH-independent cells, but were differentially expressed between these 2 groups of cells (Supplementary Fig. S4A and Table S1). Such analysis identified 2,507 genes and this gene signature was able to predict the HH dependence in urothelial carcinoma cell lines by carrying out a meta-analysis. When searching for correlations between our gene signature and the published gene expression profiles of 5 urothelial carcinoma cell lines in a public dataset (36), we were able to identify 2 HH-independent cell lines used to generate this signature, HT1376 and J82, and 2 HH-dependent cell lines that were not used to generate this signature, UM-UC-3 and BFTC905 cells (Supplementary Fig. S4B and compare with Fig. 2A). We then used a similar approach to correlate our gene signature to publicly available gene signatures in 5 bladder cancer studies. This analysis identified significant positive associations with published gene signatures obtained by comparing urothelial carcinoma to normal urothelium and by comparing metastatic urothelial

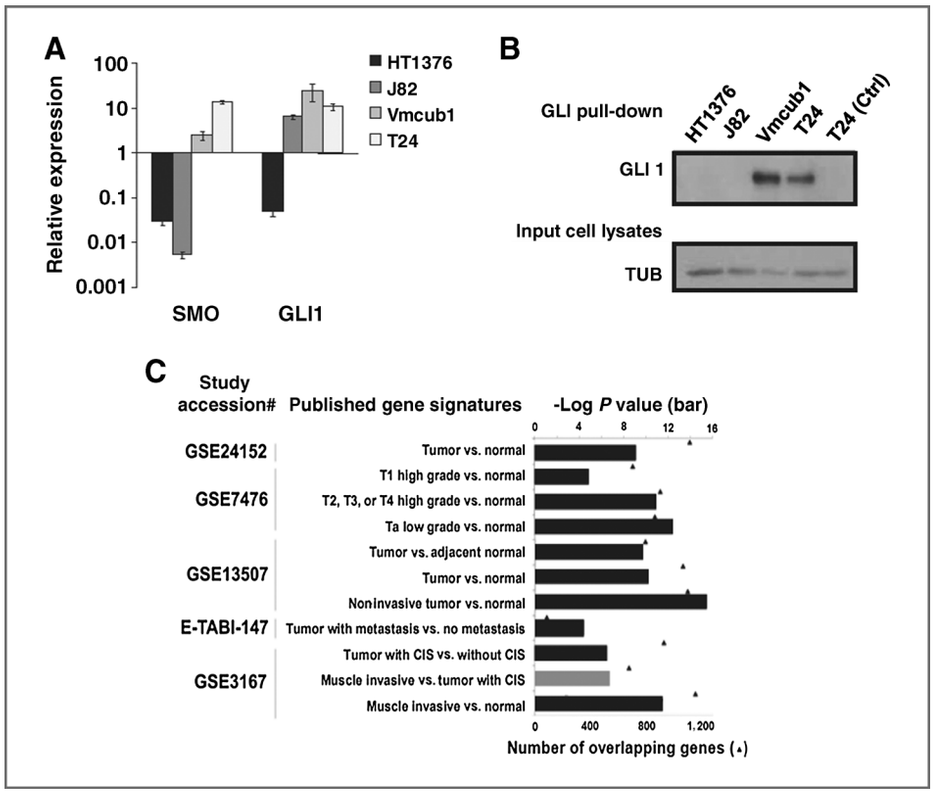

Figure 3. Levels of HH pathway activity correlate with urothelial carcinoma progression. A, the expression of *SMO* and *GLI1* was examined by qPCR in HH-independent cell lines (HT1376 and J82) and HH-dependent cell lines (Vmcub1 and T24), normalizing to the expression values of UROtsa cells (set to 1). Error bars, SEM. B, an immunoblot for GLI1 from the indicated cell lines was conducted after enriching for GLI proteins using the GLI-binding oligonucleotide beads (first 4 lanes) or the nonspecific oligonucleotide (Ctrl, last lane). Equal loading was verified by immunoblotting for α-tubulin (TUB) in the original cell lysates. C, a gene signature, consisting of 2,507 genes differentially expressed between HH-dependent and HH-independent urothelial carcinoma cell lines, correlated with urothelial carcinoma progression. A meta-analysis was carried out to compare this gene signature with gene signatures from available bladder cancer studies. Black bar, positive correlation; gray bar, negative correlation; triangle, number of overlapping genes; CIS, carcinoma *in situ*.

carcinoma to nonmetastatic urothelial carcinoma (Fig. 3C). Such associations were lost when a scrambled gene signature, consisting of 2,601 differentially expressed genes from a random grouping (T24 and HT1376 versus Vmcub1 and J82), were used to carry out the meta-analysis (data not shown). These results suggested that the HH-dependent cell lines represent a more tumorigenic cell population. Moreover, this 2,507-gene signature seems able to predict urothelial carcinoma progression and might prove of prognostic value.

We next determined the ability of these 2 groups of urothelial carcinoma cell lines to grow in an anchorage-independent manner. When these cells were transduced with shRNAs targeting *SMO* or *GLI1* and then embedded in soft agar, the HH-dependent T24 cells and Vmcub1 cells showed a dramatic reduction in their ability to form soft agar colonies (Supplementary Fig. S5A). Surprisingly, the HH-independent HT1376 and J82 cells also showed significant reduction in colony formation. This reduction in soft-agar growth was specific to *SMO* and *GLI1* shRNAs, as 4 control shRNAs failed to elicit the same inhibitory effect (Supplementary Fig. S5B). This unexpected result indicated that the anchorage-independent growth of these latter cells require HH signaling. Indeed, HT1376 and J82 cells exhibited 50- to 100-fold increase in *GLI1* expression when they were grown in soft agar relative to when these cells were grown in monolayer culture, which might explain this switch in HH dependence (Supplementary Fig. S5C).

We further evaluated the dependence of urothelial carcinoma cells on HH signaling during tumor development in a xenograft tumor model. Vmcub1 and HT1376 were chosen because they grew well *in vivo* (data not shown). These cells were similarly transduced with 2 control shRNAs or 2 shRNAs independently targeting either *SMO* or *GLI1* and were injected subcutaneously into athymic nude mice. Tumor volume was then monitored for up to 40 days. Although palpable tumors were observed in control shRNA–treated cells, approximately 2 weeks after implantation, *SMO* or *GLI1* shRNAs greatly repressed the tumor growth originating from Vmcub1 cells, but had no effect on the growth of HT1376 tumors (Fig. 4). These results are consistent with Vmcub1 cells being more sensitive than HT1376 cells to HH pathway inhibition for proliferation. Overall, our *in vitro* and *in vivo* results highlight the importance of HH signaling in mediating the tumorigenic properties of a subset of urothelial carcinoma cell lines.

Because HH signaling is required for various aspects of tumorigenicity in certain urothelial carcinoma cells, we sought

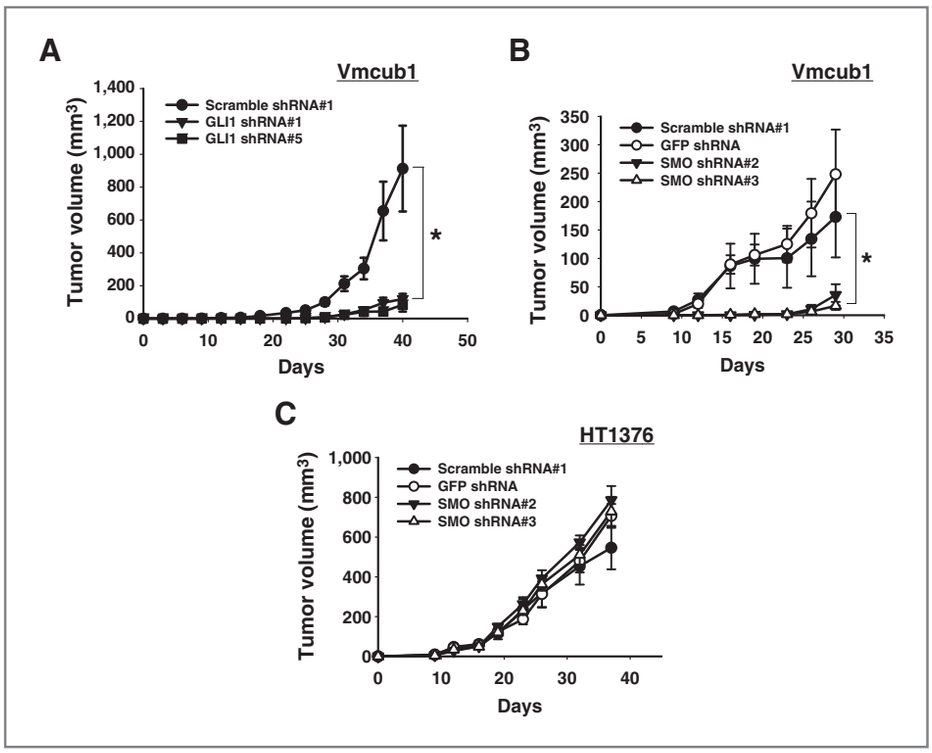

Figure 4. HH signaling is required for urothelial carcinoma tumor growth *in vivo*. Vmcub1 (A and B) and HT1376 (C) cells were transduced with control shRNAs or 2 shRNAs targeting either *GLI1* (A) or *SMO* (B and C). Equal amount of viable cells were then injected subcutaneously in the flanks of athymic nude mice ($N = 7$–10 mice per group). Tumor volumes were measured for a total of 40 days. Error bars, SEM. *, statistically significant changes comparing with control shRNA–transduced cells.

to explore the mechanism that drives this constitutive HH signaling. Deletion of the entire *PTCH1* locus and *PTCH1* mutations were reported in some primary human bladder cancers (20, 23). As *PTCH1* loss-of-function would result in increased HH pathway activity, we reasoned this could account for the constitutive HH signaling in urothelial carcinoma cell lines. Therefore, we examined the chromosomal integrity along 9q in the 4 urothelial carcinoma cell lines using a high-density single-nucleotide polymorphism (SNP) array. Unexpectedly, the *PTCH1* locus is intact in all 4 urothelial carcinoma cell lines (Fig. 5A). Moreover, *PTCH1* does not seem to be epigenetically silenced or mutated (Fig. 5B and data not shown). Collectively, these results argued against the contribution of *PTCH1* alterations to constitutive HH signaling in any of the urothelial carcinoma cell lines tested. We further examined the copy number changes of all known HH pathway components, including loss of the negative regulator *SUFU* and amplification of *GLI1*, but failed to find obvious genetic changes that might account for the HH pathway activity in these cells (data not shown).

Besides loss of *PTCH1* or *SUFU*, constitutive HH signaling in cancer cells commonly results from expression of the HH ligands (35, 37, 38). Indeed, overexpression of *SHH* has been observed in many urothelial carcinoma cell lines, including the majority used in this study (25). We confirmed the expression of all 3 HH ligands in the urothelial carcinoma cell lines (Supplementary Fig. S6A). To determine the biological significance of HH ligand production, we attenuated the expression of each of the HH ligands using shRNAs and then examined the subsequent changes in the proliferation of urothelial carcinoma cells. Consistent with the proliferation of Vmcub1 and T24 cells being most dependent on levels of HH pathway activity, these 2 cell lines were similarly sensitive to knockdown of HH ligands (Fig. 5C). These results suggested that production of HH ligands could account for the constitutive HH signaling that is required for the tumorigenic properties of urothelial carcinoma cells.

We next used several loss-of-function and gain-of-function approaches to confirm the causative effect of HH ligand expression on the constitutive HH signaling we observed in urothelial carcinoma cells. We first knocked down the highest expressed HH ligand in each of the 4 urothelial carcinoma cell lines and then monitored the expression of the HH target genes *GLI1* and *PTCH1* as indicators of HH activity. HH attenuation reduced the expression of *GLI1*, and in one case also *PTCH1,* in T24 and Vmcub1 cells (Fig. 6A and B). Similar results were obtained using GLI1 protein as a readout of HH activity. HH inhibition also decreased the expression of *GLI1* and *PTCH1* in HT1376 and J82 cells, though to a lesser extent (Supplementary Fig. S6B and C). Next, we examined whether the intrinsic HH pathway activity of the urothelial carcinoma cells would be enhanced by exogenous HH stimuli. Indeed, a HH-driven luciferase reporter gene was readily activated when T24 and Vmcub1 cells were engineered to overexpress *SHH* or exposed to a SMO agonist (Fig. 6C and data not shown). Furthermore, this increased HH activation correlated with a dramatic increase in colony size when such cells were grown in an anchorage-independent manner (Fig. 6D). These results suggested that HH pathway activity is maintained in urothelial carcinoma cell lines via the production of HH ligands.

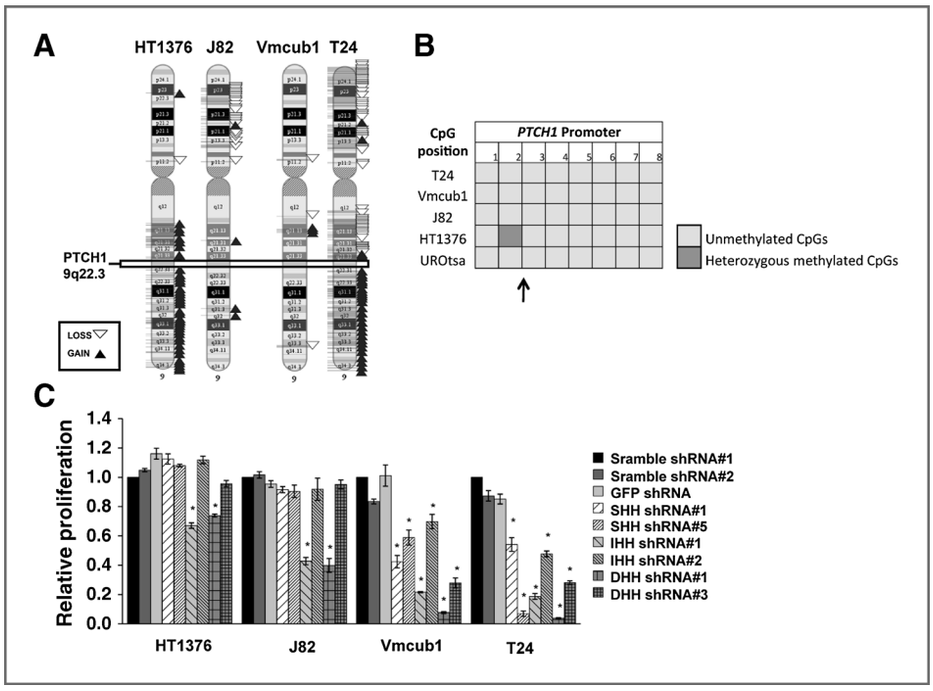

Figure 5. HH ligand production is required for urothelial carcinoma cell proliferation. A, the *PTCH1* locus (highlighted in the box) is retained in urothelial carcinoma cell lines. Virtual karyograms of chromosome 9 were generated based on a high-density SNP array analysis. Triangles denote loss or gain of chromosomal regions respectively. B, methylation analysis for a region of the *PTCH1* promoter. Numbers denote CpG islands within this promoter region. An arrow denotes the position of the GLI binding sites. C, the indicated urothelial carcinoma cell lines were transduced with either control shRNAs or shRNAs targeting *SHH*, *IHH*, or *DHH*. Cell proliferation was determined 4 days after shRNA transduction and normalized to the cells receiving scramble shRNA#1. Error bars, SEM. *, statistically significant changes comparing with control shRNA–transduced cells.

Ligand-dependent HH signaling is thought to require primary cilium (39). We examined and identified the presence of obvious primary cilia structures in at least one urothelial carcinoma cell line, T24, and in the immortalized UROtsa cells. We next engineered 2 independent clonal T24 cell lines stably expressing a SMO-GFP fusion protein and asked whether SMO accumulated in primary cilia and whether this localization could be regulated by modulators of HH pathway activity. In such T24-derived cell lines, SMO-GFP enriched in primary cilia in about 50% of the cells, whereas GFP alone failed to accumulate at the primary cilia (Supplementary Fig. S7A). The cilium-localized SMO was also activated, as it could be detected by an antiserum specific for activated phospho-SMO (Supplementary Fig. S7B). These results were in accordance with the constitutive active HH signaling observed in T24 cells. We further measured SMO-cilia translocation in response to small molecule modulators of SMO. Consistent with what had been previously reported (40, 41), the SMO antagonist SANT1 decreased the accumulation of SMO in primary cilia whereas the SMO agonist SAG increased this localization (Fig. 6E). These results supported the model of ligand-dependent HH pathway activation in urothelial carcinoma cells. Similar results were observed in UROtsa cells in which SMO localized to primary cilia in response to SAG treatment (Supplementary Fig. S7C and D), consistent with them also being HH responsive (See Fig. 1C and D).

We extended our findings to examine the level of GLI1 and SHH in human urinary bladders and urothelial carcinomas by IHC and *in situ* hybridization (Fig. 7, Supplementary Fig. S8 and Table S2). In general, GLI1 and SHH were either negative or weakly positive in normal bladder urothelium, although strong positivity was sometimes observed in individuals with cystitis cystica syndromes (Supplementary Fig. S9). However, enhanced GLI1 and SHH levels were observed in the majority of the urothelial carcinoma samples examined, and they tended to enrich in similar areas of the tumors. Particularly, in the cases in which adjacent normal urothelium was available, GLI1 and SHH staining were either absent or confined to the basal layer of normal urothelium, but more enriched in the tumor cells. These results suggested that ligand-dependent HH pathway activation likely occurs in primary human urothelial carcinoma samples.

Discussion

We show here that HH signaling plays an important role in mediating the tumorigenic properties of urothelial carcinoma cells both *in vitro* and *in vivo*. These urothelial carcinoma cell lines seemed to maintain their intrinsic HH activity through the expression of HH ligands in an autocrine-like fashion. Our results from the urothelial carcinoma cell lines were further validated in primary human bladder cancer samples, in which SHH and GLI1 levels were frequently found elevated in tumor cells but not in normal urothelial cells, consistent with the model of ligand-dependent HH pathway activation in primary human urothelial carcinoma.

Our model of autocrine-like signaling in urothelial carcinoma is inconsistent with the conclusions from a recent publication in which HH signaling was proposed to regulate the regenerative proliferation of murine urothelial cells through an indirect paracrine-like mechanism (42). In this later model, SHH production from the urothelial compartment acts on stromal cells to provide a feedback mechanism that regulates the proliferation of urothelial cells. They further suggested that

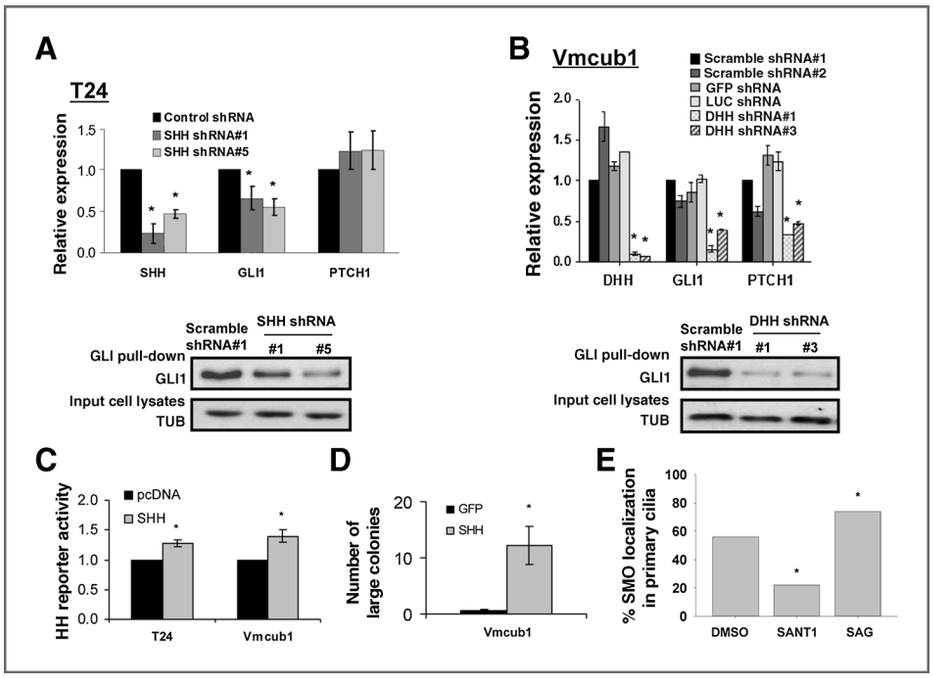

Figure 6. The constitutive HH signaling in urothelial carcinoma cell lines is ligand dependent. *SHH* or *DHH* levels were knocked down in T24 (A) or Vmcub1 (B) cells, respectively. Changes in the expression of the HH target genes, as well as the shRNA-targeted transcripts, were measured by qPCR (top). The immunoblots for GLI1 were conducted after GLI enrichment (bottom). Equal loading was verified by immunoblotting for α-tubulin (TUB) in the original cell lysates. C, a GLI-driven luciferase plasmid and a constitutive *Renilla* plasmid were cotransfected with or without *SHH* overexpression in T24 or Vmcub1 cells. Luciferase activity was measured 48 hours after transfection and normalized to *Renilla* activity. D, Vmcub1 cells stably overexpressing either GFP or SHH were grown in soft agar. Shown is the quantification for the number of colonies larger than 500 μm in diameter (per 35-mm dish). E, quantification of SMO localization to primary cilia in a T24 clone isolate stably expressing SMO-GFP. Cells were treated with dimethyl sulfoxide, SANT1 (100 nmol/L), or SAG (100 nmol/L) before carrying out the immunostaining and quantification. At least 150 ciliated cells were counted in each treatment group. Error bars, SEM. *, statistically significant changes comparing with the control cells. DMSO, dimethyl sulfoxide.

this epithelial-to-mesenchymal HH signaling is how the pathway functions in mediating human bladder cancer. We show here that primary human urothelial carcinoma cells express both SHH and GLI1, consistent with an epithelial-to-epithelial HH signaling mechanism. This result is reached by us and by another group (43) using both IHC and *in situ* hybridization as detection methods. Moreover, unlike mouse urothelial stem cells that lack *GLI1* expression, *GLI1* is the most commonly expressed biomarker in human bladder cancer–initiating cells (44). This difference in HH signaling between human and mouse is not restricted to that found in the urinary bladder, as similar observations have been made in other tissues such as prostate and colon (35, 45). However, our results cannot rule out the contribution epithelial-to-mesenchymal HH signaling might play in human urothelial carcinoma.

We previously showed that the bladder carcinogen arsenic activates HH signaling (27). This finding was substantiated using a cohort of bladder cancer patient samples, correlating increased arsenic exposure with higher HH activity in primary bladder cancer samples. Here, we further ascertained the functional significance of increased HH activity by arsenic in urothelial cell transformation. HH induction was both sufficient and necessary to allow urothelial cells to grow in an anchorage-independent manner. Notably, *SMO* depletion could partially reverse the growth of these arsenic-transformed cells, which was contradictory to our previous conclusion that arsenic activated HH signaling downstream of *SMO* (27). We speculate that a second HH-activating event, such as HH ligand expression, might have occurred to sustain a high level of HH activity during UROtsa transformation by arsenic. Alternatively, it is possible that HH activation might arise from mechanistically distinct ways in response to the different arsenic species used in these studies (27, 28).

Here, we showed that a subset of urothelial carcinoma cell lines harbor an active HH signaling pathway driven by ligand production. The cell lines used to obtain these findings, where known, were derived from MIUC (46, 47). However, we had previously shown a highly significant association between arsenic exposure in bladder cancer patients and increased HH activity in NMIUC, which we proposed was through loss of GLI3 repressor (27). Consistent with a role of GLI3 in NMIUC, certain SNPs of GLI3 could serve as prognostic markers for poor survival of NMIUC patients (48). Moreover, loss of the *PTCH1* locus was also found primarily in NMIUC (20). Therefore, although HH pathway activity may be commonly required in human bladder cancer, such activation might result from multiple routes in different urothelial carcinoma subtypes: (i) NMIUC acquires HH

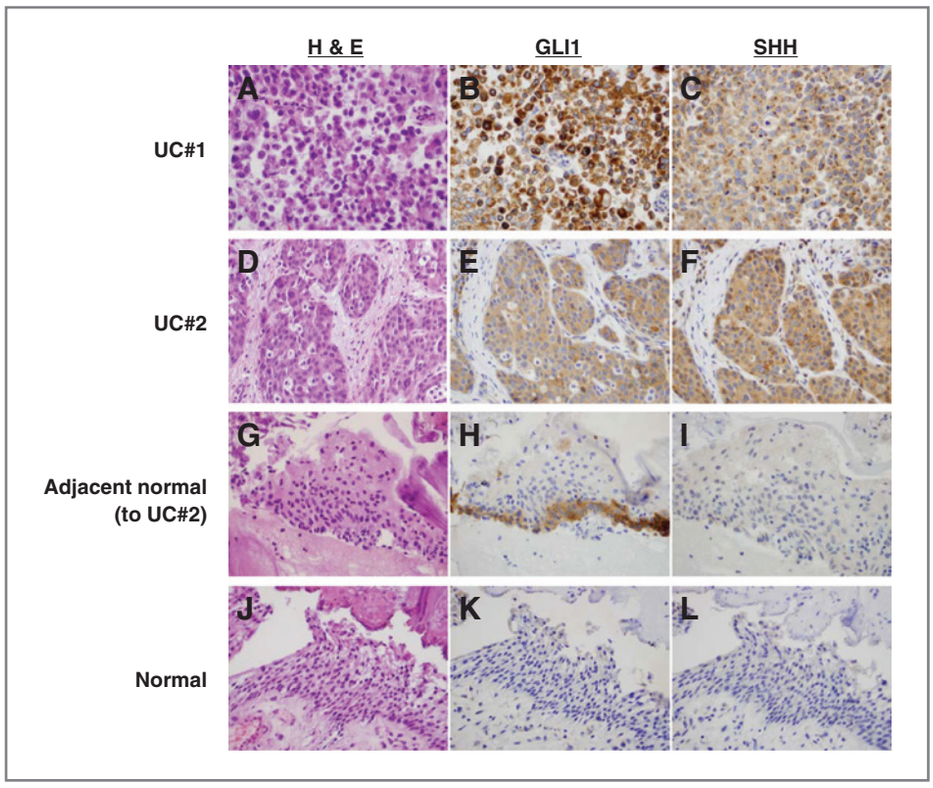

Figure 7. GLI1 and SHH are enriched in primary human urothelial carcinoma samples. Hematoxylin and eosin staining (H&E) or immunohistochemical staining for GLI1 and SHH was carried out in malignant and normal human bladder samples. Shown are representative cases of a high-grade T1 tumor (UC#1, A–C), a high-grade T2 tumor (UC#2, D–F), the matched adjacent normal urothelium to UC#2 (G–I), as well as one case of normal bladder (J–L). H&E (A, D, G, J); GLI1 staining (B, E, H, K); SHH staining (C, F, I, L). The staining results are summarized in Supplementary Table S2. UC, urothelial carcinoma.

signaling activity primarily by genetic alterations of HH pathway components; (ii) MIUC often elaborates HH signaling activity by HH ligand production. In this manner, HH signaling might play an initiating role in NMIUC but a maintenance role in MIUC, consistent with a suggestion that these 2 subtypes of UC might have distinct etiologies (49, 50). Regardless of the mechanism of activation, our results suggest that the therapeutic targeting of the HH signaling pathway will be beneficial for bladder cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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