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Hedgehog Signaling in Normal Urothelial Cells and in Urothelial Carcinoma Cell Lines

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Constitutive activation of hedgehog signaling, often caused by *PTCH1* inactivation and leading to inappropriate activation of GLI target genes, is crucial for the development of several human tumors including basal cell carcinoma of the skin and medulloblastoma. The *PTCH1* gene at 9q22 is also considered as a candidate tumor suppressor in transitional cell carcinoma (TCC), of which >50% show LOH in this region. However, only rare mutations have been found in *PTCH1*. We have therefore investigated GLI-dependent promoter activity and expression of hedgehog pathway components in TCC cell lines and proliferating normal urothelial cells. Normal urothelial cells cultured in serum-free medium, but not TCC lines exhibited low, but significant promoter activity under standard growth conditions. Accordingly, GLI1-3 and *PTCH1* mRNAs were expressed at moderate levels, and sonic hedgehog (SHH) mRNA expression was low to undetectable. In co-transfection experiments GLI1 increased promoter activity significantly in one TCC line and further in normal urothelial cells, but less strongly in other TCC lines. Expression patterns of GLI factor mRNAs did not correlate with inducibility. No significant effects of SHH or cyclopamine on proliferation were observed, ruling out autocrine effects. However, SHH induced GLI-dependent promoter activity in normal urothelial cells. Taken together, our data suggest that the hedgehog pathway is weakly active in normal adult urothelial cells and of limited importance in TCC.
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Inappropriate activation of intracellular signal transduction pathways is a central event in the development of many human cancers. Activation of the hedgehog pathway is crucially involved in specific human cancers, such as basal cell carcinoma of the skin and medulloblastoma (Toftgard, 2000; Ruiz i Altaba et al., 2002). Normally, this pathway regulates cell growth and differentiation during embryonic tissue patterning as well as adult tissue homeostasis and may, in particular, be important for the maintenance of stem cell compartments in certain epithelia (Taipale and Beachy, 2001; Zhang and Kalderon, 2001). Hedgehog signaling results in enhanced activity of GLI transcription factors through a signaling cascade that is only partly elucidated. Hedgehog growth factors like sonic hedgehog (SHH) relieve the Smoothened (SMO) protein from inhibition by the tumor suppressor Patched (PTCH). Activated SMO releases GLI transcriptional activators from a microtubule associated multiprotein complex containing the kinase Fused, Suppressor of Fused (SUFU), and the kinesin-like protein Costal2 (Taipale and Beachy, 2001). It is not completely understood whether SMO acts via G-proteins or direct interaction between SMO and the Costal2/Fused complex (DeCamp et al., 2000; Norris et al., 2000; Jia et al., 2003). GLI proteins then enter the nucleus and activate specific target genes. Three GLI factors are known in humans and expressed in a cell-type specific manner. In many cells, GLI1 and GLI2 are activators, whereas GLI3 rather acts as a repressor (Sasaki et al., 1999; Shin et al., 1999; Aza-Blanc et al., 2000; Persson et al., 2002; Buttitta et al., 2003). *GLI1*, *GLI2*, and *PTCH1* are themselves target genes of the pathway (Regl et al., 2002).

Constitutive activation of the hedgehog pathway in basal cell carcinoma and medulloblastoma is caused by different genetic alterations including inactivation of *PTCH1* and *SUFU* genes as well as point mutations activating *SMO* (Wolter et al., 1997; Reifenberger et al., 1998; Xie et al., 1998; Taylor et al., 2002). Since the pathway is not completely known, however, alterations in further components cannot be excluded. Recently, a second mechanism leading to hedgehog pathway activation has been observed in small cell lung cancer (Watkins et al., 2003) and in some gastrointestinal cancer cell lines (Berman et al., 2003; Thayer et al., 2003). In these cancers, an autocrine loop may be initiated by SHH overexpression.

In transitional cell carcinoma (TCC), the major histological subtype of bladder cancer, the role of hedgehog signaling is debated. The *PTCH1* gene at 9q22 is a tumor suppressor candidate, since 9q LOH occurs in >50% of TCC (Linnenbach et al., 1993; Spruck et al., 1994; Habuchi et al., 1995). However, only rare mutations could be detected in the retained *PTCH1* allele (McGarvey et al., 1998; LaRue et al., 2003). These findings do not rule out an involvement of the hedgehog pathway by other mechanisms such as haploinsufficiency of *PTCH1*, mutations in other known or unknown genes regulating hedgehog signaling, or an autocrine

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loop mechanism, the more so as hedgehog signaling is involved in urothelial development (Barnett et al., 2002). However, its function in the adult tissue and its activity in normal urothelial cells has not been studied yet.

Constitutive activation of the hedgehog pathway ought to be most apparent in established TCC cell lines. These harbor genetic changes and gene expression patterns typical of advanced TCC and provide an established, well-characterized experimental system to study properties of this tumor (Rieger et al., 1995; Williams et al., 2002). Specifically, they maintain proliferation in low serum (Swiatkowski et al., 2003) indicating that they have become largely independent of external signals for proliferation, likely by activation of cell-autonomous mechanisms of growth regulation. However, neither the canonical MAPK pathway nor WNT/β-catenin signaling are constitutively activated (Swiatkowski et al., 2003; Thievessen et al., 2003). We have therefore studied hedgehog pathway activity in TCC cell lines as well as in cultured normal urothelial cells. These proliferate spontaneously in primary cultures or more strongly upon stimulation by growth factors such as epidermal growth factor (EGF) or bovine pituitary extract (BPE). Together, our data support the assumption that activation of the hedgehog pathway is infrequently involved in TCC.

MATERIALS AND METHODS

Tissues

For the analysis of SHH expression in TCC tissues, RNA samples from a previous study (Kimura et al., 2003) were used. Use of human tissues was permitted by the ethics committee of the HHU medical faculty.

Cell lines and culture

The bladder carcinoma cell lines VMCub1, VMCub2, SW1710, SD, HT1376, 5637, and BFTC905, obtained from the DSMZ, Braunschweig, Germany, were cultured in Dulbecco's Minimal Essential Medium (Gibco Life Technologies, Karlsruhe, Germany), supplemented with 15% fetal calf serum and 100 μg/ml penicillin/streptomycin as described (Grimm et al., 1995). Some relevant properties of the TCC lines used in this study are compiled in Table 1. The hepatoma cell line HepG2 was cultured as described (Schulz et al., 1988). Normal uroepithelial cells (NUEC) were prepared using ureters from nephrectomy patients by a standard method (Southgate et al., 1994) with minor modifications (Swiatkowski et al., 2003). They were routinely maintained in keratinocyte serum-free medium (KSFM, Gibco Life Technologies) supplemented with 50 μg/ml BPE, 5 ng/ml EGF and 30 ng/ml cholera toxin. After the first passage, they were used for experiments as described (Swiatkowski et al., 2003; Thievessen et al., 2003).

DNA synthesis rates were determined 24 and 48 h after application of SHH (25–100 nM) or cyclopamine (300 nM) using a commercially available BrdU incorporation assay (Roche, Basel, Switzerland).

Plasmids

The reporter plasmids p106A and p106B, containing wild-type or mutant GLI binding sites, respectively, in front of the chicken crystallin minimal promoter driving the *Photinus pyralis* luciferase gene, were kindly provided by Dr. Rüther, Düsseldorf, Germany. The *Renilla reniformis* luciferase reporter plasmid, pTKRL, used as internal control for transfection efficiency, and the negative control vector pGL3 were purchased from Promega, Mannheim, Germany. pLINE-luc, constructed by insertion of bps −193 to +661 from the active LINE-1 element L1.2B into pGL3 (Steinhoff et al., 2002) was used as positive control. The expression plasmids p167 and p196, kindly donated by Dr. Rüther, contain GLI1 and GLI3 cDNAs, respectively, under control of the CMV promoter.

Transfection experiments and reporter gene assays

Transient transfections were carried out with cells grown in 6-well plates to 30% confluence using FuGene (Roche, Mannheim, Germany). Per well, 1 μg of reporter plasmid, 0.5 μg of expression plasmid and 0.15 μg pTKRL were transfected. At 80% confluence cells were lysed and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Heidelberg, Germany). Plasmids without a promoter (pGL3) or with a cell-type independent retrotransposon promoter (pLINEluc) were included in each experiment as quality controls. Each experiment was repeated with at least five independent passages or cultures.

RNA isolation and RT-PCR

Total RNA was isolated from cultures grown to 80% confluence, using the RNeasy® Midi Kit (Qiagen, Hilden, Germany). After quantification, mRNA was transcribed into first strand cDNA using AMV-RT (Promega, Mannheim, Germany).

Conventional PCR reactions were carried out in a total 20 μl volume containing 1× PCR-buffer (Biometra, Göttingen, Germany), 150 μM of each nucleotide, 10 pmoles of each SHH (Barnett et al., 2002) or β-actin primer, and 1 U of Taq polymerase (Biometra). Each PCR cycle consisted of 30 sec denaturation at 95°C, 30 sec at 56°C, and 1 min at 72°C. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

The expression levels of *PTCH1*, *GLI1*, *GLI2*, and *GLI3* were determined by real-time RT-PCR analysis using the ABI PRISM 5700 (Applied Biosystems, Weiterstadt, Germany) sequence detection system. Continuous quantitative measurement of the PCR product was achieved by incorporation of SYBR Green fluorescent dye (Invitrogen, Karlsruhe, Germany) into the double-stranded DNA. The transcript level of each gene was normalized to the transcript level of *ARF1* (ADP-ribosylation factor 1). The following primer sequences were employed: *PTCH1*; sense 5′-gccagcggctacttactcatg, and anti-sense 5′-gccactgacagtgcaaccag (GenBank accession no. NM_000264; product 119 bp); *GLI1*, sense 5′-gccacacaagtgcacgtttg, and anti-sense 5′-ggtgcgtcttcaggttttcg (NM_005269; 106 bp); *GLI2*, sense 5′-acgttcgagggctgctcg, and anti-sense 5′-gtccgaggcgttggagaag (NM_030379; 132 bp); *GLI3*, sense 5′-gtggctggactgctcaagag, and anti-sense 5′-ctagtctcgagtaggccttgtg (NM_000168; 150 bp); *ARF1*, sense 5′-gaccacgatcctctacaagc, and anti-sense 5′-tcccacacagtgaagctgatg. All measurements were performed in triplicate. As controls for

TABLE 1. Properties of TCC lines

TCC line	TP53 mutation in	RB1 status Western blot	CDKN2A mutation in	WNT basal	WNT inducible	Hedgehog basal	Hedgehog inducible
VmCub1	Mutant exon 5	+	Mutant exon 2	–	–	–	–
VmCub2	Mutant exon 5	+	Deleted	–	(+)	–	–
SW1710	Mutant exon 8	+	Deleted	–	+	–	+
SD	Mutant exon 4	+	Deleted	–	–	–	–
HT1376	Mutant exon 7	–	+	–	–	–	–
5637	Mutant exon 8	–	+	–	+	–	–
BFTC905	wild-type (MDM2↑↑)	+	Deleted	–	–	–	(+)

For each cell line, *TP53*, RB, and *CDKN2A* status are given as previously described (Steinhoff et al., 2002; Florl and Schulz, 2003; Swiatkowski et al., 2003). +, present; −, not detectable; (+), weakly detectable.

the cell lines two independent cultures of normal urothelial cells were used, and as reference tissues for the medulloblastoma samples, non-neoplastic cerebellar tissue samples from two different patients.

RESULTS

Reporter gene analysis of endogenous and inducible hedgehog signaling activity

First, endogenous activity of the hedgehog pathway was determined by measuring the activity of a GLI-dependent promoter in proliferating normal urothelial cells and seven TCC lines under standard growth conditions. These were transfected with either p106A or p106B plasmids, which are identical but for mutations in the GLI binding sites next to a basal promoter driving luciferase expression. The ratio of luciferase expression of p106A to p106B is therefore an indicator of hedgehog pathway activity. Differences in transfection efficiencies were corrected by co-transfection of a *Renilla* luciferase plasmid.

Figure 1A shows the p106A to p106B activity ratio in seven TCC lines and in normal cells. Significant activity was found in normal urothelial cells (3.0 ± 0.07, $P < 0.05$). Furthermore, slightly increased, but more variable activity (2.6 ± 1.5) was seen in the HT1376 cell line, which was not statistically significant. In contrast, ratios of 1 and lower found in the other TCC lines (range: 0.42 ± 0.22–1.16 ± 0.08) indicate a lack of pathway activity. In additional experiments, the source of the hedgehog activity in normal cells was investigated. Omission of various components from the growth medium which might affect activity of the pathway, such as BPE or cholera toxin, did not cause significant decreases in GLI-dependent promoter activity (data not shown) suggesting an endogenous origin.

Next, we tested whether co-transfection of GLI1 could induce hedgehog-specific promoter activity in TCC lines and NUEC in co-transfection experiments. Figure 1B displays ratios of p106A activity with versus without the GLI1 expression construct p167 in cell lines and NUEC. A statistically significant ($P < 0.01$) 40-fold increase took place in SW1710 cells. The 10-fold increase in BFTC905, a cell line derived from a high-grade papillary tumor, bordered on significance ($0.05 < P < 0.1$), as did the further increase over the higher basal level in normal urothelial cells. In the other TCC cell lines, GLI1 elicited only slight increases over the basal levels. No significant increases were observed upon co-transfection of GLI1 with the control plasmid p106B.

To test whether the different inducibilities in the TCC lines might depend on differences in GLI3 levels, GLI1 and GLI3 were co-transfected into the SW1710 or VMCub1 lines, which were inducible and non-inducible, respectively, by GLI1 alone (Fig. 1C). Co-transfection of both factors did not induce promoter activity in VMCub1 cells, which were non-inducible by GLI1 on its own.

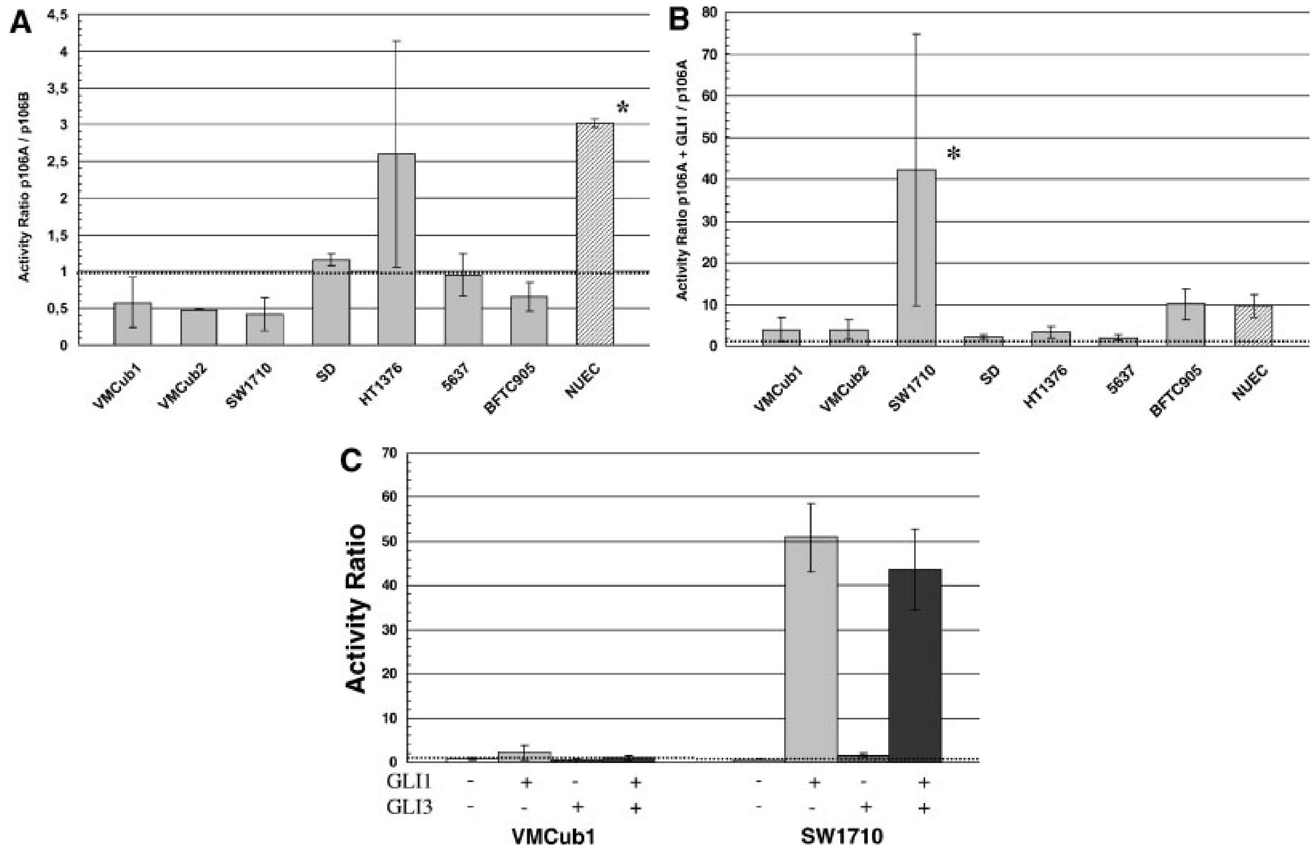

Fig. 1. Basal and inducible activities of a GLI-dependent promoter in TCC lines and normal uroepithelial cells (NUEC). The indicated TCC lines or NUEC were transfected with reporter plasmids and luciferase activity was measured 2-days later. All data are derived from at least five independent triplicate experiments. **A**: Basal activity of a GLI-dependent promoter (contained in p106A) in TCC lines. Mean ± SD of the p106A/p106B activity ratio are shown. The dotted line indicates a ratio of 1 corresponding to lack of activity. The ratio is significantly different from 1 in normal urothelial cells (*t-test; $P < 0.05$). **B**: Induction of p106A reporter activity by GLI1 contained in the expression plasmid p167. Data are mean ± SD of the ratio p106A + p167/p106. The dotted line indicates a ratio of 1 indicating lack of inducibility. Statistically significant inducibility (*t-test; $P < 0.05$) was observed in SW1710 cells. **C**: Effect of GLI1 and GLI3 cotransfection (indicated by + or −) on GLI1-dependent promoter activity in the TCC lines VMCub1 and SW1710. Data are mean ± SD SD of the p106A:p106B ratio from four independent experiments, each in triplicate.

Instead, it tended to slightly diminish the strong activation by GLI1 in SW1710 cells, but this effect was not statistically significant, in spite of multiple repeats. In neither cell line did GLI3 alone induce luciferase expression from p106A.

Expression of GLI factors and PTCH1 mRNAs

Since in the transfection experiments GLI1, but not GLI3, behaved as an activator, in line with expectations (Sasaki et al., 1999; Shin et al., 1999; Aza-Blanc et al., 2000; Persson et al., 2002; Buttitta et al., 2003), we investigated whether the observed different inducibilities between the TCC lines might be caused by different patterns in GLI factor expression. Since commercially available antibodies against GLI proteins did not yield specific Western blots in our hands, quantitative real-time RT-PCR assays were performed to determine mRNA levels. In addition, expression of PTCH1 mRNA was determined, as constitutive activation of hedgehog signaling results in increased expression of PTCH1 as well as GLI1 mRNAs. As controls and for comparison, in these experiments, two medulloblastoma specimens with PTCH1 mutations and normal cerebellum were included. In addition, the hepatoma cell line HepG2 was investigated which shows constitutive activation of WNT/β-catenin signaling (Thievessen et al., 2003), but not of the hedgehog pathway.

The results are presented in Figure 2. Normal cerebellum and normal urothelial cells were each set as 1 and the tissue samples and cell lines related to these, respectively. As expected (Reifenberger et al., 1998), the medulloblastoma specimens expressed increased levels of mRNAs of all four components of hedgehog signaling, in particular of GLI2 mRNA and also of PTCH1 mRNA.

In contrast, none of the TCC lines showed as pronounced and consistent increases in the levels of the four mRNAs compared to normal urothelial cells. Levels of GLI1 mRNA or GLI2 mRNA were clearly increased in some lines (e.g., BFTC905, 5637, or VMCub2), whereas PTCH1 mRNA was generally only slightly elevated, up to 2.5-fold. Importantly, none of the differences between the cell lines, singly or combined appeared related to the differences in GLI1-dependent promoter activity and inducibility between normal urothelial cells and the TCC lines SW170 and BFTC905 on one hand and the other TCC lines on the other hand.

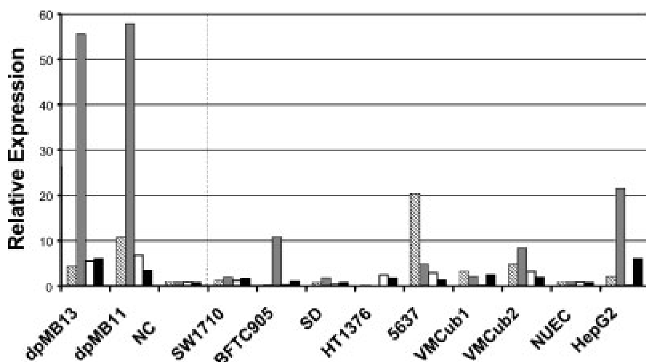

Fig. 2. Expression of hedgehog pathway component mRNAs. Expression of mRNAs for GLI1 (hatched bars), GLI2 (grey bars), GLI3 (white bars), and PTCH1 (black bars) was measured by quantitative real-time RT-PCR in TCC lines, HepG2 cells and NUEC as indicated. NUEC values were set as 1. For comparison, the same parameters were determined in two medulloblastoma tissues with hedgehog pathway activation (dpMB13 and dpMB11) compared to normal cerebellum tissues (NC). For these samples, NC values were set as 1.

Expression of SHH mRNA in TCC and effect of SHH and cyclopamine on TCC lines and normal urothelial cells

To investigate the possibility of hedgehog signaling activation by an autocrine mechanism, expression of SHH mRNA was investigated. The mRNA was detected at very low levels in the TCC lines and in normal urothelial cells (Fig. 3). Additionally, we analyzed RNA from 17 TCC tissues of various stages by the same method. In 12/17 specimens, SHH mRNA was undetectable, and it was as weakly expressed in the five further specimens as in the TCC lines (data not shown).

Furthermore, we tested whether SHH was capable of inducing GLI1-dependent reporter gene activity in normal urothelial cells, in the GLI1 responsive TCC line SW1710, and in the VMCub1 line non-reactive to GLI1 (cf. Fig. 1B). A SHH concentration of 25 nM was used, which is active with gastrointestinal cell lines (Berman et al., 2003). In normal urothelial cells, SHH induced p106A GLI-reporter activity 2.14 ± 0.13-fold, compared to a 1.87 ± 0.09-fold induction by GLI1 cotransfection in the same series of experiments, over the already enhanced activity of the GLI-reporter construct (cf. Fig. 1). In VMCub1, p106A activity increased 1.37 ± 0.07-fold upon cotransfection of GLI1 and decreased to 0.93 ± 0.08-fold upon SHH treatment. While these results were as expected, SHH was non-efficacious in SW1710 cells, which is surprising, since these cells respond to transfected GLI1. In this particular set of experiments, GLI1 induced a 9.2 ± 0.23-fold increase in p106A activity, whereas a decrease to 0.56 ± 0.10-fold was seen with SHH.

Likewise, neither SHH nor cyclopamine significantly affected BrdU-incorporation in the TCC lines VMCub1 and SW1710 or in normal urothelial cells. In several independent experiments, SHH at concentrations up to 100 nM and cyclopamine at 0.3 μM, a concentration shown to be active in gastrointestinal cell lines (Berman et al., 2003), were applied for 1 to 3 days, each with and without serum and growth factor supplements. In none of these experiments was a significant effect on BrdU-incorporation observed (data not shown).

DISCUSSION

Constitutive activation of the hedgehog pathway is a key event in the development of specific human cancers, specifically of basal cell carcinoma of the skin and medulloblastoma (Taipale and Beachy, 2001; Ruiz i Altaba et al., 2002). Activation of the pathway is most often caused by loss of PTCH function, less often by mutations activating SMO, and in rare cases by mutations inactivating SUFU or PTCH2 (Cohen, 2003). Conceivably, mutations in further components of the pathway could provoke its overactivity, for example, in cases of basal cell carcinoma or medulloblastoma, where none of the above alterations is detectable. Amplification

Fig. 3. Expression of SHH mRNA in TCC lines and NUEC. Expression of SHH mRNA was determined in the indicated TCC lines, NUEC and HepG2 hepatoma cells by RT-PCR using β-actin for comparison. Control refers to PCR without cDNA.

of *GLI1* is one of several possibilities under study. A further possibility is that loss of one *PTCH1* allele may be sufficient to promote tumor development by haploinsufficiency in certain tissues. This has been postulated for urothelial cancers, where loss of one *PTCH1* allele is frequent, but mutations of the remaining allele are extremely rare (McGarvey et al., 1998; LaRue et al., 2003). No mutations in other pathway components have been reported in urothelial cancers, but they are obviously difficult to exclude. More recently, the hedgehog pathway has been reported to become activated in small cell lung cancer, but not in other histological types of lung cancer, and in various gastrointestinal tract cancers by an autocrine mechanism (Berman et al., 2003; Thayer et al., 2003; Watkins et al., 2003).

Independent of the mode of activation, hedgehog pathway activation has been shown to be detectable by an increased expression of its target genes and enhanced activity of GLI-dependent promoters in several cancer types (Unden et al., 1997; Bonifas et al., 2001; Regl et al., 2002). According to such promoter assays, the hedgehog pathway is not activated in a broad range of TCC lines and in these appears to be less active than in normal urothelial cells. Moreover, co-transfection of GLI1 induced activity of a GLI-dependent promoter in some, but not all TCC lines, suggesting that the pathway is repressed by lack of a co-activating factor or by the presence of a repressor. Theoretically, these differences might be due to different expression patterns of GLI factors, as GLI3 in general tends to act as a repressor of hedgehog target genes, while GLI1 and GLI2 function as activators. This general rule may apply to cells of urothelial origin. In our experiments, GLI1 acted as an activator, and GLI3 had little effect, but, if anything, inhibited rather than promoted induction by GLI1 (Fig. 1C). However, the differences in GLI factor expression do not seem to account for the differences in response to GLI1, as no distinct differences between normal urothelial cells, the inducible cell lines SW1710 and BFTC905, and non-inducible lines were apparent (cf. Fig. 2). Since no specific antibodies were available to GLI proteins, the theoretical possibility remains that the differences in inducibility are due to different patterns of GLI factor expression at the protein level.

A second indicator of hedgehog pathway activity is the expression of specific target genes. In this regard, *GLI1* and *GLI2* as well as *PTCH1* have been described as targets (Regl et al., 2002). Accordingly, tumors with constitutive activation of the pathway often display overexpression of *GLI1* and *PTCH1* mRNAs (Reifenberger et al., 1998). Indeed, two medulloblastoma samples used as controls in the present study considerably overexpressed the mRNAs of all three GLI factors and of PTCH1 compared to normal brain tissue (Fig. 2). In cell lines from gastrointestinal tumors, expression of PTCH1 mRNA correlated with promoter activity in reporter assays (Berman et al., 2003). This was clearly not so in the urothelial system. Rather, our data indicate that normal urothelial cells express components of the pathway which are maintained in TCC cells at similar levels. In support of our data, expression of PTCH has been reported in normal bladder tissue, with an accordingly diminished level in tumors with half the gene dosage due to LOH at 9p22 (Aboulkassim et al., 2003).

A third indicator of pathway activation could be increased expression of SHH, leading to an autocrine loop, as in small-cell lung cancer and some gastrointestinal cancers (Berman et al., 2003; Thayer et al., 2003; Watkins et al., 2003). However, expression levels of SHH at the mRNA level were low in all TCC lines and did not differ between cell lines with or without inducibility of the pathway (cf. Fig. 3). Even lower levels were found in tumor tissues. Cyclopamine and SHH had no effect on the growth of TCC lines, indicating likewise that the hedgehog pathway is not a decisive determinant of TCC proliferation.

In contrast to TCC lines, normal urothelial cells show activity and responsiveness of the hedgehog pathway although to a limited extent. A weak, but consistent specific activity of the GLI-dependent promoter was seen in multiple experiments in independent cultures, which could be further induced by GLI1 co-transfection. However, neither GLI factors nor PTCH1 were found to be expressed more strongly than in non-inducible TCC lines indicating that the activity during normal culture conditions was indeed weak. Moreover, the pathway as a whole appeared to be functional, since SHH was capable of inducing reporter activity; which could not be observed in TCC lines. The source eliciting the basal promoter activity in normal urothelial cells could not be identified. Various components of the culture medium, which might conceivably influence the activity of the pathway were tested and proved without effect. Expression of SHH mRNA in urothelial cells was even lower than in TCC lines, making an autocrine effect unlikely, and neither SHH nor cyclopamine affected BrdU incorporation of the cultures.

Proliferation of normal urothelial cells is dominated by autocrine EGF-like factors, and supported by medium supplements such as EGF and the various factors contained in BPE (Southgate et al., 1994; Freeman et al., 1997). In normal urothelial cells, proliferation signals are relayed through MAP kinase pathways, which are much more active in these cells than in TCC cells (Swiatkowski et al., 2003). A possible explanation, therefore, may be that a moderate level of GLI-dependent promoter activity may be elicited through cross-talk with highly active effector pathways downstream of the EGFR. The dominant role of EGF-like factors in the proliferation of urothelial cells (Southgate et al., 1994; Freeman et al., 1997; Swiatkowski et al., 2003) may also explain why neither SHH nor cyclopamine influenced DNA synthesis in normal urothelial cells, although SHH was capable of inducing GLI-dependent promoter activity. Therefore, the hedgehog pathway is most likely intact in normal adult urothelial cells, but does not appear to play a prominent part in controlling their proliferation in vitro. This may, of course, be different in certain situations in vivo, such as extensive tissue remodeling following damage to the urothelium or chronic inflammation. Moreover, since the hedgehog pathway has pleiotropic effects, it is possible that its low activity in normal urothelial cells influences properties other than proliferation.

In summary, therefore, the data suggest that the hedgehog pathway is not regularly activated in urothelial cancers, and, if anything, is less active than in normal urothelial cells. In an analogous study (Thievessen et al., 2003), we have obtained data suggesting that the WNT/β-catenin pathway is likewise rarely activated in TCC, in accord with data by others (Stoehr et al., 2002). Interestingly, the WNT pathway as well seemed to be subject to repression in many TCC cells (Table 1). However, in contrast to the hedgehog pathway, it appears to be also inactive in normal urothelial cells. Interestingly, both pathways could be induced only in the SW1710 cell line, which may therefore

deserve further detailed investigation. Like the Hedgehog pathway, the WNT/β-catenin pathway has come to be regarded as being involved in maintaining stem cells in adult human tissues (Taipale and Beachy, 2001). Consequently, constitutive activation of these pathways in human cancers is thought to confer a tissue precursor or stem cell phenotype which permits continuous growth. It may therefore be significant that the common genetic abnormalities in TCC also displayed by the cell lines investigated here (Table 1), that is, obliteration of the p53 and RB1 control systems, resemble those in squamous cell carcinoma of the skin and in glioblastoma which are derived from more mature cells, instead of basal cell carcinoma and medulloblastoma. Indeed, TCC characteristically express markers of differentiated urothelial cells such as cytokeratin 20 and uroplakins (Wu et al., 1998; Southgate et al., 1999; Yuasa et al., 1999). If one follows this interpretation, the lack of activity of the hedgehog pathway in TCC, then, would not come as a surprise.

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