Snail and Sonic Hedgehog activation in neuroendocrine tumors of the ileum

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Abstract

The transcription factor Snail represses E-cadherin and induces epithelial–mesenchymal transition, a process also exploited by invasive cancer cells. Aberrant Hedgehog (Hh) signaling was recently observed in a variety of epithelial cancers and it has been shown that the Hh target gene *Gli1* induces expression of Snail. In this study, we examined whether Snail and Sonic Hedgehog (SHH) are expressed in neuroendocrine tumors (NETs) of the ileum. Using immunohistochemistry, we found expression of Snail in 22 out of 37 (59%) of evaluated NET samples, but not in adjacent normal tissues. Snail expression was mostly restricted to the invasive front of the tumors. Six of seven liver metastases analyzed were positive for Snail. Intratumoral expression of SHH was detected in 27 out of 37 (73%) tumors. As opposed to Snail, cells expressing SHH were found to be distributed more randomly throughout the tumors. Out of 30 primary NETs, 16 (53%) showed both Snail and SHH expression. Furthermore, we found downregulation of E-cadherin in Snail-expressing cells by immunofluorescence. Real-time RT-PCR revealed conservation of the Hh target genes *Gli1*, *Gli2*, and *Ptch* in the pancreatic carcinoid cell line BON-1, which were downregulated upon Hh inhibition with cyclopamine. Moreover, Hh inhibition attenuated *in vitro* cell growth in a dose-dependent manner. In conclusion, we describe for the first time that Snail and SHH are overexpressed in a large subset of NETs of the ileum. Aberrant activation of these pathways might be involved in invasion and metastatic spread in NETs.

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Introduction

Neuroendocrine tumors (NETs) of the gastrointestinal (GI) tract are a poorly understood group of lesions that encompass a broad category of neoplasms derived from neuroendocrine cells of the GI mucosa. As a result of increasing incidence over the past 25 years, NETs are approximately as common as esophageal and gastric cancer, occurring in an estimated 38.5 per million in the US population (Maggard *et al.* 2004). In this heterogeneous group, the 5-year survival rate lies around 60% for NETs of the ileum which comprise the majority of the tumors (Rorstad 2005). The presence of metastases, especially to the liver, is associated with a reduced 5-year survival.

The increased motility and invasiveness of cancer cells in the first phase of metastasis are reminiscent of epithelial–mesenchymal transition

(EMT) during embryonic development. In EMT, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility (Zhou & Hung 2005). Numerous observations support the idea that EMT has a central role in tumor progression. During progression to metastatic competence, carcinoma cells acquire mesenchymal gene-expression patterns and properties. This results in changed adhesive properties and the activation of proteolysis and motility, which allows the tumor cells to metastasize and establish secondary tumors at distant sites (Thiery & Sleeman 2006). During EMT, the E-cadherin promoter is frequently repressed by specific transcriptional repressors, including Snail, Slug, and Twist and by subsequent promoter hypermethylation. Of the transcriptional repressors, the

best studied is Snail, a highly unstable protein. It is rapidly phosphorylated by glycogen synthase kinase-3 β (GSK-3 β) and subsequently degraded by the ubiquitin–proteasome pathway. Conversely, inhibition of GSK-3 β function results in upregulation of Snail by an NF- κ B-dependent pathway, loss of E-cadherin expression, and EMT. Additional protein modification further stabilizes Snail protein and promotes EMT and tumor invasion (Christofori 2006). Expression of Snail in epithelial tumors increases their aggressiveness, as seen in experimentally induced breast tumors, where high Snail expression correlates with an increased risk of tumor relapse and poor survival rates in human breast cancer (Moody et al. 2005).

Recently, aberrant activation of the Hedgehog (Hh) pathway has been found in the majority of upper aerodigestive tract epithelial cancers of endodermal lineage (Berman et al. 2003). In a recent study, Louro et al. (2002) showed that the Hh target gene *Gli1* can rapidly and directly induce expression of Snail.

The purpose of the present study is to examine the *in vivo* expression of Snail and the Hh ligand Sonic Hedgehog (SHH) in NETs of the ileum and to clarify whether the Hh pathway is activated in BON-1 human carcinoid cells.

Patients and methods

Subjects

A series of 37 NETs of the ileum, including seven liver metastases, were obtained from the tissue bank of the Department of Pathology, Philipps-University of Marburg, Germany. Histological diagnoses were confirmed by an experienced pathologist (A R). Histopathological diagnosis and grading were done in accordance to Klöppel (2004). The immunohistochemistry results for Snail1 and SHH were scored as follows: negative, <5% of cells positive; +, <30% of tumor cells positive; ++, >30% of tumor cells positive.

The study protocol conformed to the guidelines of the local ethics committee.

Immunostaining

For immunolabeling, formalin-fixed and paraffin-embedded archived tumor samples and corresponding normal tissues were stained as previously described (Esni et al. 2004). Concentrations and sources of primary antibodies are listed in Table 1. Briefly, slides from archived NETs were heated to 60 °C for 1 h, deparaffinized using xylene, and hydrated by a graded series of ethanol washes. Antigen retrieval was accomplished by microwave heating in 10 mM sodium citrate buffer of pH 6.0 for 10 min. For immunohistochemistry, endogenous peroxidase activity was quenched by 10-min incubation in 3% H₂O₂. Non-specific binding was blocked with 10% serum. Sections were then probed with primary antibodies overnight at 4 °C. For immunohistochemistry, bound antibodies were detected using the avidin–biotin complex (ABC) peroxidase method (ABC Elite Kit, Vector Labs, Burlingame, CA, USA). Final staining was developed with the Sigma FAST DAB peroxidase substrate kit (Sigma). For immunofluorescence, bound antibodies were developed with the secondary antibody (Jackson Immuno Research, West Grove, PA, USA) and corresponding Cy3-, Cy2-, and Cy5-conjugated antibodies (Jackson Immuno Research).

Mammary carcinoma samples from our tissue bank, which had previously been shown to express high levels of Snail, were used as positive controls along with each batch of Snail IHC stainings. SHH expression in crypts of adjacent normal ileum tissues, which has been described previously (van den Brink et al. 2002), was used as internal positive control for SHH immunohistochemistry.

Fluorescence microscopy

Fluorescence confocal microscopy was performed with a confocal laser scanning microscope (410LSM, Zeiss) using 40 $\times$ (NA 1.2) C-apochromat or 100 $\times$ (NA 1.4) objectives. The sections were scanned for Cy3-tagged (excitation 543 nm) and FITC-tagged (excitation 488 nm) markers. Sections (ten sections with images 512 $\times$ 512 pixels) were scanned at a pixel size of 0.07 mm and step size of 1 mm. Image analysis was

Table 1 Working dilutions of antibodies used for immunohistochemistry and immunofluorescence

Antibody	Species	Working dilution	Source
α -E-cadherin	Rat	1:200	Zymed, San Francisco, CA, USA
α -SHH	Goat	1:50	R&D Systems, Minneapolis, MN, USA
α - β -Catenin	Rabbit	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
α -Snail	Goat	1:100	Santa Cruz Biotechnology
Chromogranin A	Mouse	1:100	Santa Cruz Biotechnology

performed using MetaMorph series 5.0 software (Universal Imaging Corp). For specimens not requiring confocal analysis, images were obtained on an inverted motorized microscope (Axiovert 200M, Zeiss) out-fitted with Cy5, Cy3, FITC, and DAPI filters. Three- and four-color images were captured using a Zeiss Axiocam, and pseudocolors assigned using Zeiss Axiovision software.

Cell culture

BON-1 pancreatic carcinoid cells (Parekh *et al.* 1994) were grown in DMEM/FK-12 (Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen) and $1 \times$ Pen/Strep (Biofluids, Camarillo, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO₂. The cell line was routinely tested for mycoplasma infection using the MycoSensor PCR Assay Kit (Stratagene, La Jolla, CA, USA).

Proliferation assays

Cells were seeded into 96-well plates at a density of 2000 cells in 200 µl full medium/well and incubated overnight. Next, they were starved in low-serum medium (0.5% FBS) for 24 h. Then, medium was replaced for 100 µl low-serum medium supplemented with vehicle, 1.5, 3, or 6 µM cyclopamine, respectively, for 6 days. Untreated cells served as controls and wells containing medium only were used for background correction. To determine viable cell mass, 20 µl of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide reagent (Promega) were added per well. Plates were incubated at 37 °C for another 60 min and optical density read at 490 nm on a Wallac 1420 plate reader (Perkin–Elmer, Boston, MA, USA). All experiments were done in triplicate.

RNA extraction and real-time quantitative RT-PCR

Cells in the log growth phase were serum starved in 0.5% FCS for 1 day and then incubated with cyclopamine (6 µM) or ethanol for another 4 days. Next, cells were lysed in the culture dish with 600 µl buffer RLT and whole RNA extracted using the RNeasy kit (Qiagen) with on-column DNA digestion following the standard protocol provided by the manufacturer.

Human tissue samples were homogenized with a rotor stator homogenizer (Polytron PT1200C, Kinematica, Newark, NJ, USA) in 600 µl lysis buffer RLT (Qiagen). Total RNA was then extracted using the RNeasy kit (Qiagen) following the standard protocol.

The mRNA was reverse transcribed into cDNA with oligo-dT primers using the Superscript First

Strand System for RT-PCR (Invitrogen) at 42 °C for 50 min. For amplification of cDNA specific for Pth1, Gli1, Gli2, Gli3, SHH, and PGK-1 Assays-on-Demand (Applied Biosystems, Foster City, CA, USA) were used together with the QuantiTect Probe PCR kit (Qiagen). All PCRs were carried out on a 7300 Real-Time PCR System (Applied Biosystems) over 40 cycles, with denaturation for 15 s at 95 °C and combined annealing/extension at 60 °C for 1 min. Determination of Snail mRNA was performed over 40 cycles with 15 s of denaturation at 95 °C, annealing at 61 °C for 20 s, extension at 72 °C for 30 s and data acquisition at 78 °C using the Quantitect SYBR Green PCR kit (Qiagen); primer sequences were forward: 5'-gggtctctctgegtactgtct-3' and reverse: 5'-tagggctgctggaaggtaaa-3'. Relative fold mRNA expression levels were determined using the $2(-\Delta\Delta C_t)$ method (Livak & Schmittgen 2001) with PGK1 (phosphoglycerate kinase 1) as housekeeping control.

Statistical analysis

Kruskal–Wallis analysis was performed using SPSS version 14.0 for Microsoft Windows. Two-tailed *t*-test was performed using GraphPad Prism for Windows version 4.0. *P* < 0.05 was regarded as statistically significant. Prism was also used to generate boxplots in Figs 1E and 2E.

Results

Patients

Eighteen males and nineteen females with a median age of 63 years (range 34–91 years) at the time of surgery were included in this study. All NETs were histologically well-differentiated and tested positive for chromogranin A by IHC (not shown). Clinical characteristics are listed in Table 2.

Overexpression of Snail in NETs of the ileum

Immunohistochemical staining revealed expression of Snail in 22 out of 37 (59%) of NETs tested (Table 2). In tumors, in which Snail was found to be expressed, Snail-positive cells were not distributed homogeneously throughout the whole tumor. Instead, Snail expression was mostly restricted to peripheral areas rendering what appeared to be the invasive front of the tumors, i.e., where malignant cells invade bordering non-malignant tissue (Fig. 1A and B). Fifteen tumors did not show any Snail-positive cells (1C). Six of seven (86%) liver metastases analyzed were positive for Snail in IHC (Fig. 1D). In normal intestinal epithelium, Snail staining was absent in

Figure 1 IHC staining for Snail in tissue samples from human GI-NETs. (A) Representative example of Snail expressing carcinoid and adjacent normal tissue lacking Snail expression (lower half of image). (B) In Snail-positive samples, Snail was not expressed homogeneously throughout the whole tumor, but was mostly limited to the invasive front. (C) In some of the examined carcinoid samples, no Snail expression could be found in the tumor, but only in mesenchymal cells. (D) All liver metastases showed Snail expression. (E) Steady-state Snail mRNA levels were determined by real-time RT-PCR in a subset of 19 samples. In each group, the upper and lower hinges of the boxes mark the 25th and 75th percentiles respectively; whiskers denote the ranges.

all samples. Correlation of intratumoral Snail overexpression with clinicopathological features was not analyzed due to the small sample number.

These results were confirmed at the mRNA level on a subset of 19 samples using quantitative real-time RT-PCR. We found increased steady-state Snail mRNA levels in samples that were classified as ‘+’ ($P=0.0043$) or ‘++’ ($P=0.0007$) at the protein level, respectively, when compared with samples with negative IHC staining (Fig. 1E).

Overexpression of SHH

Intratumoral expression of the Hh ligand SHH was detected in 27 out of 37 (73%) tumors studied (Table 2). As opposed to Snail, cells expressing SHH were found to be distributed more randomly throughout larger areas of the tumors (Fig. 2A). A total of ten tumors did not show any SHH-positive cells (Fig. 2C). Three of the seven (43%) liver metastases also expressed SHH (Fig. 2D). As expected, SHH expression was also seen in normal intestinal epithelium (Fig. 2B).

SHH mRNA levels tended to be higher in samples that were classified as positive by IHC, but these differences did not reach statistical significance, possibly due to the

small sample number and considerable variation between different cases, as well as SHH expression in crypts of non-malignant, normal intestine (Fig. 2E).

Out of 30 primary NETs, 16 (53%) showed both Snail and SHH expressions, whereas six (20%) were negative for both markers. Eight (27%) primary NETs which expressed SHH showed no Snail staining, but none of the primaries expressed Snail in the absence of SHH.

Three of six liver metastases, which expressed Snail, lacked expression of SHH as seen in IHC (Table 2). Of four pairs of primary tumors with matched liver metastasis tissue samples from the same patients included in this series, Snail expression was found in all four metastases, but only in two of four primaries. In case number 30, the primary tumor was classified as ‘+’ for snail by IHC, whereas the matched metastasis is ‘++’.

Neither Snail nor SHH protein expression was detected in non-malignant liver tissue adjacent to metastatic foci.

Suppression of E-cadherin expression by Snail in NETs

To examine the influence of Snail on E-cadherin expression in NETs, immunofluorescence analysis for

Figure 2 IHC staining for SHH in tissue samples from human GI-NETs. Marked overexpression of SHH was seen in the majority of studied samples (A), whereas some completely lacked expression of SHH (B). SHH expression was also detected in normal intestinal epithelium (C). Two of four liver metastases also expressed SHH (D). (E) Steady-state SHH mRNA levels as revealed by real-time RT-PCR. Results are presented as boxplots as described above.

Snail, E-cadherin, and β -catenin was performed in a subset of eight randomly selected Snail-positive NETs of the ileum (Fig. 3). E-cadherin was found to be downregulated on Snail-expressing cells, while high levels of E-cadherin were expressed abundantly on the surfaces of cells lacking Snail expression (Fig. 3A and C). β -Catenin was expressed on the membranes of all cells irrespective of presence or absence of Snail protein and was used as control counterstaining in areas with suppressed E-cadherin expression (Fig. 3B).

Conservation of Hh pathway in BON-1 cells

The pancreatic carcinoid cell line BON-1 was used for *in vitro* studies. Quantitative real-time RT-PCR analysis revealed expression of the Hh pathway-related genes *Gli1*, *Gli2*, and *Ptch*. Upon Hh inhibition with the small molecule-smoothed antagonist cyclopamine, all of these target genes were found to be downregulated, in line with preexisting aberrant activation of the Hh pathway in this cell line. Interestingly, expression of the inhibitory transcription factor Gli3 was not altered upon Hh inhibition (Fig. 4A).

Furthermore, we found that Hh inhibition with cyclopamine reduced BON1 cell growth *in vitro* in a dose-dependent manner (Fig. 4B), further supporting our hypothesis of aberrant Hh activation in this tumor entity.

Discussion

The GI tract is the predominant site of origin for NETs, which were formerly called carcinoids, although they can occur throughout the body. NETs are generally slow growing but frequently metastasize to the liver, ranking second to colorectal carcinoma as a source of isolated liver metastases (Chen *et al.* 1998). Molecular data on NETs of the GI tract have been accumulating in recent years, but the genetic basis of endocrine tumor development and progression is still poorly understood.

EMT occurs during embryonic morphogenesis in multicellular organisms, in which embryonic mesenchymal cells are formed and become motile following the loss of epithelial cell polarity. In recent years, EMT has also been recognized as a potential mechanism for cancer progression (Thiery 2002). Cancer cells undergoing EMT have lost specific target recognition and are usually equipped with autocrine loops of growth signals, mechanisms to evade apoptosis, and the potential to elicit angiogenesis for independent nutrient supply (Gotzmann *et al.* 2004). A central event in EMT is downregulation of E-cadherin that leads to the loss of cell–cell contact and the consecutive progression of the cells toward a malignant phenotype. The transcription factor Snail is one major suppressor of E-cadherin and a strong inducer of EMT. Snail downregulates

Table 2 Clinical characteristics and results of Snail and Sonic Hedgehog (SHH) immunohistochemistry in 33 patients with neuroendocrine tumors of the ileum

Patient no.	Age (Years)	Sex	NET localization	Histology	Ki-67 (%) ^a	LN Mtx ^b	Liver Mtx ^c	Snail1 expression	SHH expression
1	57	M	Liver metastasis		ND	ND	ND	Negative	Negative
2	57	F	Ileum carcinoid	Well-differentiated	ND	ND	ND	++	++
3	73	M	Liver metastasis		ND	ND	ND	++	++
4	61	M	Ileum carcinoid	Well-differentiated	ND	+	+	Negative	Negative
5	77	F	Ileum carcinoid	Well-differentiated	ND	ND	ND	++	++
6	56	M	Ileum carcinoid	Well-differentiated	30	+	+	+	+
7	72	M	Ileum carcinoid	Well-differentiated	3	+	+	++	++
8	52	M	Ileum carcinoid	Well-differentiated	1	+	–	++	++
9	63	F	Ileum carcinoid	Well-differentiated	20	+	+	+	++
10	42	M	Ileum carcinoid	Well-differentiated	1	+	+	Negative	+
11	68	M	Ileum carcinoid	Well-differentiated	1	+	–	++	+
12	91	M	Ileum carcinoid	Well-differentiated	1	+	+	Negative	+
13	60	F	Ileum carcinoid	Well-differentiated	5	+	+	++	+
14	55	F	Ileum carcinoid	Well-differentiated	5	+	+	+	++
15	52	F	Ileum carcinoid	Well-differentiated	1	+	+	Negative	+
16	66	F	Liver metastasis		1	+	+	+	Negative
17	57	M	Ileum carcinoid	Well-differentiated	5	+	–	Negative	++
18	66	M	Ileum carcinoid	Well-differentiated	3	+	+	+	+
19	67	F	Ileum carcinoid	Well-differentiated	1	+	+	Negative	Negative
20	66	F	Ileum carcinoid	Well-differentiated	2	+	+	Negative	++
			Liver metastasis		ND			+	Negative
21	62	M	Ileum carcinoid	Well-differentiated	17	+	+	Negative	Negative
22	44	M	Ileum carcinoid	Well-differentiated	5	+	+	Negative	Negative
23	43	F	Ileum carcinoid	Well-differentiated	5	+	+	+	+
			Liver metastasis		ND			+	+
24	39	M	Ileum carcinoid	Well-differentiated	2	+	+	++	++
25	79	M	Ileum carcinoid	Well-differentiated	1	+	–	+	++
26	67	F	Ileum carcinoid	Well-differentiated	5	+	–	Negative	++
27	70	M	Ileum carcinoid	Well-differentiated	1	+	–	++	++
28	65	F	Ileum carcinoid	Well-differentiated	1	+	+	+	++
29	74	F	Ileum carcinoid	Well-differentiated	ND	ND	ND	Negative	++
30	40	M	Ileum carcinoid	Well-differentiated	4	+	+	+	+
			Liver metastasis		ND			++	Negative
31	75	F	Ileum carcinoid	Well-differentiated	1	+	–	Negative	Negative
32	91	M	Ileum carcinoid	Well-differentiated	10	+	+	Negative	Negative
33	34	F	Ileum carcinoid	Well-differentiated	1	+	+	Negative	+
			Liver metastasis		ND			+	+

ND, no data available.

^aKi-67 proliferation index in percent.^bPresence (+) or absence (–) of lymph node metastases at time of diagnosis.^cPresence (+) or absence (–) of liver metastases at time of diagnosis.

Figure 3 Immunofluorescence staining for (A) E-cadherin, (B) β -catenin, (C) Snail, and (D) merge. E-cadherin expression was downregulated on cells, in which Snail was expressed (arrow), whereas E-cadherin expression was seen on the surface of cells that did not stain for Snail (arrowhead). β -Catenin was expressed on surfaces of both Snail-positive and -negative cells. The figure shows results from one representative of eight performed experiments.

E-cadherin in different types of tumors, e.g., hepatocellular carcinomas (Jiao *et al.* 2002), carcinomas from the esophagus, cardia, stomach (Rosivatz *et al.* 2006), and colorectal carcinomas (Roy *et al.* 2005).

Our study is the first to show that Snail is expressed in NETs of the ileum, and to the best of our knowledge this is also the first report of Snail overexpression in an endocrine related cancer at all. We found significant expression of Snail in more than 50% of the NETs we investigated. Our results are in line with a meta-analysis from Zikusoka *et al.* (2005) of comparative genomic hybridization data, which found that in up to 50% of all carcinoids there is a gain on chromosome 20q13, the location of Snail.

As seen in tumors induced in the skin of mice (Cano *et al.* 2000), Snail-expressing cells were mostly found at the invasive front of the NETs. At this site, tumor cells migrate into and invade the surrounding tissue either as single cells or in collective clusters. Figure 1 shows the Snail-positive invasive front of a NET of the ileum, with no Snail staining detectable in normal intestinal epithelium. The presence of hepatic metastases in GI NETs has obvious implications in terms of patients' quality of life and overall prognosis. The 5-year survival rate in patients with hepatic metastases from midgut carcinoids is 0–50% as opposed to 75–99% in patients without liver metastases (Rorstad

2005). Six of seven (86%) liver metastases analyzed in our study were positive for Snail, possibly supporting our hypothesis that Snail might be involved in the development of metastases by promoting a more invasive phenotype through EMT.

Our observation of lack of SHH and Snail expression in non-malignant normal liver tissue is in line with previous reports by others (Sugimachi *et al.* 2003, Sicklick *et al.* 2006), although the latter group detected Snail mRNA transcripts in adult hepatocytes by *in situ* hybridization.

To verify that in GI-NETs expression of Snail is associated with loss of E-cadherin, we performed immunofluorescence analysis for Snail, E-cadherin, and β -catenin. In fact, E-cadherin was found to be downregulated on Snail-expressing cells, while high levels of E-cadherin were expressed abundantly on the surfaces of cells lacking Snail expression (Fig. 3), a finding commonly related to Snail-induced EMT and metastasis (Huber *et al.* 2005).

Genes of the Hh pathway, commonly involved in embryonic patterning and stem cell maintenance in adult tissues, are frequently mutated in basal cell carcinomas (Athar *et al.* 2006) and medulloblastomas (Taylor *et al.* 2002). Moreover, recent studies by our own group as well as by others described aberrant activation of Hh signaling in several malignancies of

Figure 4 (A) Expression of Gli1, Gli2, Gli3, and Ptch mRNA in BON-1 cells treated with solvent only or cyclopamine. Upon treatment with cyclopamine, RT-PCR revealed downregulation of all Hh target genes tested. (B) Hh inhibition with cyclopamine lead to reduced proliferation of BON-1 cells in a dose-dependent manner.

the aerodigestive tract (Berman *et al.* 2003, Thayer *et al.* 2003), and particularly high Hh pathway activity has been found in metastatic lesions of prostate and pancreatic cancers (Karhadkar *et al.* 2004, Feldmann *et al.* 2007), suggesting that Hh signaling might play a role in metastatic tumor spread. Exogenous expression of the Hh-dependent transcription factor Gli1 in low-metastatic rodent prostate carcinoma cells caused enhanced migration *in vitro* and development of visceral metastases *in vivo*, correlating with induction of Snail expression and E-cadherin repression. All these effects were completely inhibited by treatment with the pathway inhibitor cyclopamine, showing that the Hh pathway indeed contributes to EMT (Karhadkar *et al.* 2004). Recently, Li *et al.* (2006) showed in an elegant study that Snail is rapidly

induced by Gli1 *in vitro* and in skin tumors. In line with these results, we found expression of SHH and Snail in 53% of primary NETs. Eight primary NETs expressed SHH without consecutive Snail expression, but no primary tumor expressed Snail in the absence of SHH. These results suggest that also in ileum NETs Snail is induced as a downstream target of SHH or, likewise, Gli1. Overall, we found expression of SHH in 27 out of 37 (73%) ileum-derived NET samples analyzed (Table 2). SHH-positive cells were distributed throughout larger areas of the tumors. As expected, SHH expression was also seen in normal intestinal epithelium (Nielsen *et al.* 2004).

Our *in vivo* findings are in line with the *in vitro* expression of Hh-related genes *Gli1*, *Gli2*, *Gli3*, and *Ptch* in BON-1 cells (Fig. 4). After treating this cell line with cyclopamine, we found a significant down-regulation of the Hh target genes *Gli1*, *Gli2*, and *Ptch* at the mRNA level, as demonstrated by RT-PCR (Fig. 4). Furthermore, we found that Hh inhibition with cyclopamine reduced cell growth *in vitro* in a dose-dependent manner (Fig. 4). Although to date cyclopamine cannot be chemically synthesized but has to be extracted from the corn lily *Veratrum californicum* (Cooper *et al.* 1998) and is thus costly and difficult to obtain in larger amounts for clinical use, considerable research effort is currently undertaken to pharmacologically target the Hh-signaling pathway using new small molecule inhibitors, and our results suggest that the Hh pathway might constitute a potential therapeutic target in NETs. One such synthetic drug has recently been shown to reduce medulloblastoma growth in a transgenic mouse model (Romer *et al.* 2004).

In conclusion, our data presented here show that the sister pathways Snail and Hh are activated in a considerable subset of GI-NETs, leading to down-regulation of E-cadherin in line with EMT. Hh activation is observed in BON-1 cells and Hh inhibition with cyclopamine reduces NET cell growth *in vitro*.

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