The role of proto-oncogene *GLI1* in pituitary adenoma formation and cell survival regulation

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Abstract

The Hedgehog (Hh) pathway is an important regulator of early tissue patterning and stem cell propagation. It was found to be aberrantly activated in numerous types of human cancer and might be relevant in cancer stem cells. The identification of adult stem cells in the pituitary raised the question if tumor-initiating cells and Hh signaling are involved in pituitary adenoma formation. The present study aimed at the evaluation of Hh signaling in relation to stem cell and cell cycle markers in 30 human pituitary adenomas and in cultured murine adenoma cells. Therefore, expression levels of components of the Hh pathway, stem cell marker SOX2, cell cycle regulator tumor-protein 53 (TP53), proliferation marker Ki67 (MKI67) and superoxide dismutase 1 (SOD1) were evaluated in 30 human pituitary adenomas in comparison to control tissue. Modulation of cell function and target gene expression by the inhibition and activation of the Hh pathway were studied in murine adenoma cells. We show that transcription factor glioma-associated oncogene 1 (GLI1) is overexpressed in 87% of all pituitary adenomas. The expression of GLI1 significantly correlated with that of SOX2, TP53, MKI67 and SOD1. Inhibition of GLI1 resulted in the downregulation of the above genes and severe cell death in mouse adenoma cells. On the other hand, activation of the Hh pathway increased cell viability and target gene expression. In conclusion, our findings point toward an alternative, ligand-independent Hh pathway activation with GLI1 playing a major role in the cell survival of pituitary adenoma cells.

Key Words

- ▶ Hedgehog
- ▶ GLI1
- ▶ pituitary adenoma
- ▶ stem cells

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Introduction

The anterior pituitary gland is a key regulator of the endocrine system. During the life-span it undergoes extensive remodeling responding to metabolic changes and is therefore prone to tumor formation. Pituitary adenomas are the most common type of tumors arising in the sellar region, and although usually displaying benign growth behavior, they frequently cause severe

morbidity in affected individuals. Functioning adenomas are associated with the uncontrolled secretion of pituitary hormones, whereas nonfunctioning adenomas can expand into functionally important adjacent structures (Ezzat *et al.* 2004, Melmed 2011).

High-throughput technologies made it apparent that tumors are highly heterogeneous resulting in the

distinction of several clinical and molecular subtypes within one tumor entity (De Sousa *et al.* 2013). This heterogeneity is currently explained by two alternative but not necessarily mutually exclusive concepts of tumor formation. According to the clonal evolution model, adaptation to the tumor microenvironment as well as mutations and epigenetic modifications select for superior clones with similar proliferation and differentiation potential. In contrast, the cancer stem cell (CSC) model is based on the hypothesis that tumor initiation, maintenance and recurrence are driven by tumor-initiating CSCs. This rare population of tumor-initiating cells seems to share common pathways with embryonic and tissue stem/progenitor cells and can therefore be identified through the expression of specific marker genes (Frank *et al.* 2010).

The recent characterization of stem cells in the adult pituitary gland raised the question if the CSC model could also be relevant in the pathogenesis of pituitary adenomas (Chen *et al.* 2005, Garcia-Lavandeira *et al.* 2009). Xu *et al.* (2009) reported the isolation of putative tumor-initiating cells from human pituitary adenomas. These clones were able to self-renew, had a differentiation potential for multiple pituitary cell lineages, expressed several stem/progenitor-associated marker genes and initiated pituitary tumor formation after transplantation into immunodeficient mice. Further studies indicated that the stem cell regulator pathway Wnt/ β -catenin, stem/progenitor cell transcription factor Sox2, chemokine receptor CXCR4 and stem cell factor BMI1 as well as the progenitor cell marker Pax7 could play a potential role in human and mouse pituitary adenoma formation (Vankelecom & Chen 2014). Such assumption is strengthened by the detection of a side population in human pituitary adenomas that could represent tumor-initiating cells due to the expression of multidrug transporters, oncogenes and components of several stem cell pathways (Vankelecom & Gremeaux 2010). Moreover, craniopharyngiomas arising from embryonic pituitary tissue were found to express the stem cell markers SOX2, SOX9, OCT4, KLF4 and β -catenin (Gaston-Massuet *et al.* 2011, Andoniadou *et al.* 2012, Garcia-Lavandeira *et al.* 2012).

The Hedgehog (Hh) pathway is involved in embryogenesis and tissue repair as a regulator of developmental processes, stem cell maintenance and cell proliferation (Briscoe & Therond 2013). The Hh ligand sonic Hh (SHH) was shown to be mandatory for normal pituitary development because downregulation of the Hh pathway in mice results in pituitary hypoplasia (Treier *et al.* 2001). Similar effects could be seen in zebrafish in which attenuation of the Hh signaling severely disrupts pituitary

organogenesis (Herzog *et al.* 2003, Sbrogna *et al.* 2003). The Hh pathway is frequently activated in several human cancer types and assumed to contribute to tumor formation, progression, recurrence and therapy resistance by maintaining the CSC character (Xie *et al.* 2013). The downstream mediators of the Hh signaling are the GLI family of transcription factors that alter target gene expression. The main activator in tumor pathogenesis is thought to be glioma-associated oncogene 1 (GLI1).

Because there is only limited knowledge on a potential role of the Hh signaling in pituitary oncogenesis, the present study evaluated the expression of key players of the Hh pathway as well as other stem cell and proliferation markers in 30 human pituitary adenomas. Moreover, the Hh pathway was manipulated in the mouse pituitary adenoma cell line AtT-20 to study effects on target gene expression, cell proliferation and cell survival.

Material and methods

Patient samples

Thirty human pituitary adenomas were surgically removed at the Department of Neurosurgery, Medical University of Vienna, Austria, and immediately snap-frozen in liquid nitrogen. All patients gave written consent prior to the operation. Patient and tumor details are summarized in Table 1. Three human postmortem pituitaries and healthy pituitary tissue obtained from three patients who underwent surgery due to a pituitary cyst were used as control tissue. The study was approved by the ethics committee of the Medical University of Vienna, Austria (EK No. 018/2004).

Tumor volume determination

Magnetic resonance imaging (MRI) and computed tomography (CT) scans obtained the day before surgery were available from 23 patients. CT scans were performed on two patients due to various contraindications for MRI. Seven patients had scans from outside institutions and were not available for volume determination. Tumor volume was calculated using OsiriX imaging processing software (Pixmeo, Geneva, Switzerland) at the Department of Radiology, Medical University of Vienna, Austria.

Cell culture

Mouse corticotropinoma cells from the cell line AtT-20 (American Type Culture Collection; passages 28–32) were maintained in 75 cm² cell culture flasks in DMEM, 10% fetal bovine serum, 2 mM glutamine and 10⁵ U/l

Table 1 Patient and tumor details analyzed in the present study

No.	Sex	Age (onset)	Symptoms	TU volume (cm ³)	Expression	Relapse	GLI1 expression
1	F	73	Aphasia, difficulty in concentration	7.07	FSH	N	4
2	M	41	Acromegaly	2.28	GH, PRL	N	4
3	F	42	Nausea	7.74	None	N	3
4	F	63	Vertigo, bitemporal hemianopsia	20.4	LH	N	3
5	M	68	Progressive vision impairment	16.39	None	N	3
6	F	32	None	3.99	FSH, TSH, PRL	N	3
7	M	66	Acromegaly	1.06	GH, FSH, α -chains	N	4
8	M	48	Vision impairment	4.29	FSH, α -chains	N	3
9	F	42	Cushing's disease	50	ACTH, FSH, α -chains	Y	4
10	F	16	Amenorrhea, galactorrhea	0.61	PRL	N	3
11	F	30	Vertigo, hazy vision, amenorrhea, galactorrhea	15.57	None	N	4
12	M	52	Vision impairment, bitemporal hemianopsia	27.74	None	N	4
13	M	68	Bitemporal hemianopsia	20.71	None	N	4
14	M	49	Vertigo, double vision, constant headache	9.32	None	N	3
15	F	29	Amenorrhea, galactorrhea, weight gain	NA	PRL	N	2
16	M	51	Vision impairment, bitemporal hemianopsia	3.26	None	Y	3
17	F	62	Hazy vision	3.36	None	N	4
18	M	33	Acromegaly	3.06	GH	N	1
19	M	51	Vision impairment	9.63	ACTH	N	3
20	M	69	Vision impairment	NA	None	N	3
21	M	56	Emesis, damaged vestibular nerve	7.65	None	N	3
22	M	42	Acromegaly	NA	GH	N	3
23	F	37	Acromegaly	0.78	GH, α -chains	N	2
24	M	50	Oculomotor nerve palsy	50.08	ACTH	Y	1
25	F	45	NA	NA	None	NA	4
26	M	44	Acromegaly, hypertension	NA	GH, ACTH	N	3
27	F	NA	NA	NA	None	NA	4
28	F	25	Cushing's disease	0.99	ACTH	Y	1
29	M	47	Fatigue, weakness, headaches	15.63	None	Y	3
30	F	52	Acromegaly	NA	GH, α -chains	N	1

F, female; M, male; Age, the age of symptom onset; TU, tumor; NA, scans from outside institutions were not available, patients lost during follow-up; ACTH, adrenocorticotropin; GH, growth hormone; PRL, prolactin; TSH, thyroid stimulating hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone. Hormone expression was routinely analyzed and stated in the patient's medical records. *GLI1* expression extent was compared to healthy pituitaries and is listed in groups: 1, expression $\leq$ control; 2, one- to tenfold increase; 3, ten- to 100-fold increase; 4, > 100-fold increase.

penicillin-streptomycin (Invitrogen) at 37 °C and 5% CO₂. Cells were passaged at 80% confluency and seeded into 12-well plates at 150 000 cells/well as previously described (Reiter *et al.* 2011). Cells were treated with Hh agonist purmorphamine (Cayman Chemical, Ann Arbor, MI, USA, Cat. No. 10009634), Smoothed antagonist GDC-0449 (*Vismodegib*; Selleckchem, Houston, TX, USA, Cat. No. S1082), GLI antagonist GANT61 (Sigma-Aldrich, Cat. No. G9048) or an equivalent amount of DMSO as solvent control.

RNA isolation

For total RNA isolation from pituitary adenomas, 100–200 mg frozen tissue were homogenized in 1 ml QIAzol Lysis Reagent (Qiagen, Cat. No. 79306) using the Precellys 24 homogenizer (Peqlab, Erlangen, Germany,

Cat. No. 91-PCS24). For cell culture, 0.5 ml of QIAzol Lysis Reagent was added to each well (12-well plate) and cells were scraped off manually. RNA isolation was then continued according to the manufacture's protocol, and 100 ng of RNA were reverse transcribed using Superscript II (Invitrogen, Cat. No. 18064) and random primers.

Quantitative real-time PCR

RT-qPCR was performed using commercially available TaqMan Gene Expression Assays (Applied Biosystems, Cat. No. 4331182; Assay IDs: Hs02758991_g1, Hs01034249_m1, Hs01032443_m1, Hs00533490_m1, Hs01053049_s1, Hs00179843_m1, Hs01110766_m1). The relative standard curve method was used to determine the gene expression extent of the following genes:

GLI1, sex-determining region Y-box 2 (*SOX2*), superoxide dismutase 1 (*SOD1*), *SHH*, tumor-protein 53 (*TP53*), marker of proliferation Ki67 (*MKI67*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for human samples. The qPCR gene product was ligated into a pGEM-T Easy Vector (Promega, Cat. No. A1360), transformed into 50 µl One Shot Top 10 Chemical Competent cells (Invitrogen, Cat. No. C4040-03) and grown over night at 37 °C on Lysogeny Broth (LB) agar plates supplemented with 100 µg/ml of ampicillin. On the next day, single cultures were inoculated in LB medium containing 50 µg/ml ampicillin over night at 37 °C. Plasmid DNA was purified (Pure Link Quick Plasmid Miniprep Kit, Invitrogen, Cat. No. K210011) and successful integration of the target gene was determined with RT qPCR. Plasmid DNA was also run on a 1% agarose gel to check for RNA contamination. Standard curves for target genes and endogenous control in a 1-log dilution range were included on every RT-qPCR plate. Relative gene expression was normalized to the endogenous control *GAPDH* and analyzed in duplicates.

For cell culture experiments, cells were maintained for 24 h and treated with GANT61 (5, 10, 15 and 20 µM) and Purmorphamine (0.5, 1, 2 and 4 µM). Gene expression was measured using the $2^{-\Delta\Delta C_t}$ method and the DMSO-treated samples as reference with *Ubiquitin C* as endogenous control. TaqMan Assay IDs for the target genes are as follows: Mm99999915_g1, Mm01731290_g1, Mm01278617_m1, Mm01344233_g1, Mm03053810_s1, Mm00494654_m1 and Mm02525934_g1. RT-qPCR was performed in quadruplets.

Immunofluorescence stainings

Cryosections (6–8 µm) of the pituitary adenomas were brought to room temperature and fixed in acetone for 10 min. Sections were stained with primary antibodies for GLI1 (Abcam, Cambridge, GB, Cat. No. ab49314; 1:100), *SOX2* (Abcam, Cat. No. ab75485; 1:100) and *SOD1* (Abcam, Cat. No. ab20926; 1:100) and incubated at 4 °C in a wet chamber overnight. After PBS washes, sections were incubated with secondary antibodies (Alexa Fluor 488 goat anti-rabbit, Invitrogen, Cat. No. A11070; 1:200) and TRITC goat anti-mouse (Sigma, Cat. No. T5393; 1:200) at room temperature for 1 h and washed in PBS. Cell nuclei were stained using 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) and slides were mounted with Vectashield (Vector Laboratories, Servion, Switzerland, Cat. No. H-1000). Matched isotype antisera were used instead of primary antibodies as control. Images were acquired using

the LSM 700 laser scanning microscope and ZEN 2012 imaging software (both from Zeiss, Oberkochen, Germany).

Immunohistochemistry stainings

Formalin-fixed, paraffin-embedded postmortem pituitary and tumor tissue were cut into 5 µm thick sections and pretreated with citrate buffer (pH 6) for 1 h in a steamer and then stained with anti-GLI1 antibody (rabbit polyclonal, Abcam, Cat. No. ab151796; 1:750) followed by the appropriate secondary system (Envision Dako cytometry) and DAB. For nuclear staining, Mayer's hemalum solution (Merck, Cat. No. 109249) was used. Pictures were taken with a Nikon Eclipse E600 microscope using ProgRes C5 (Jenoptik L.O.S) software.

Cell viability analysis

AtT-20 cells were maintained as described above and treated with Purmorphamine (0.5, 1, 2 and 4 µM), GANT61 (5, 10, 15 and 20 µM), GDC-0449 (5, 10, 20 and 25 µM), caspase-8 inhibitor (70 µM; BD Biosciences, San Jose, CA, USA, Cat. No. 550380) or DMSO for 24 h. Cells were stained with trypan blue (Invitrogen, Cat. No. 15250-061) and counted using Nikon's Eclipse Ti inverted microscope.

Western blotting

AtT-20 cells were harvested after 48 h and proteins were isolated using radioimmunoprecipitation assay (RIPA) lysis buffer containing protease and phosphatase inhibitors (cOmplete, Cat. No. 04693124001 and PhosSTOP, Cat. No. 04906845001, both from Roche). Precast tris glycine gels were loaded with 20 µg of proteins and transferred to a PVDF membrane. The membrane was blocked with 1% BSA, and anti-GLI1 antibody (rabbit polyclonal, Abcam, Cat. No. ab151796; 1:700) was incubated over night at 4 °C. Secondary antibody (rabbit polyclonal, Dako, Glostrup, Denmark, Cat. No. P0448; 1:4000) was incubated for 1 h at RT. For *GAPDH*, anti-*GAPDH* primary antibody (rabbit polyclonal, Santa Cruz; Cat. No. sc-25778; 1:3000) and the above secondary antibody were used.

Statistical analysis

For tumor gene expression, correlation analyses were performed using Spearman's rank correlation coefficient. For cell culture analyses, matched one-way ANOVA with a subsequent Dunnett's multiple comparisons test was

performed. Results are expressed as mean $\pm$ s.e.m. and considered statistically significant at a P value ≤ 0.05 . Statistical significance is reported as following: not significant (NS) $P > 0.05$; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$. GraphPad Prism 6 software was used for data analyses.

Results

GLI1 is expressed in human pituitary adenomas

The expression of *GLI1* was analyzed in 30 human pituitary adenomas by RT-qPCR. Detailed histological reports were available from all tumors, dividing them into 57% hormone-positive and 43% nonfunctioning pituitary adenomas. Patient and tumor details are summarized in Table 1. As indicated, all tested adenomas were found to express *GLI1* although to various extents. Compared to normal pituitary tissue, 26 out of the 30 adenomas (87%) showed overexpression of *GLI1*, whereas *GLI1* expression was only detectable in three out of six pituitaries of healthy subjects. *GLI1* expression was also confirmed on the protein level. The localization of *GLI1* in healthy post-mortem pituitary tissue is depicted in Fig. 1. In comparison, Fig. 2 shows an adenoma with only little *GLI1* expression on the RNA level and another adenoma highly expressing *GLI1*. Immunohistochemistry (IHC) stainings confirmed our previous RT-qPCR results regarding *GLI1* expression extent. *GLI1* expression did not correlate with tumor volume, relapse rate or hormonal status.

GLI1 expression correlates with stem cell and cell cycle markers

To further evaluate the role of *GLI1* expression in pituitary adenoma pathogenesis, the expression status of stem cell

Figure 1
Localization of *GLI1* in a postmortem healthy pituitary. *GLI1* can be seen throughout the cytoplasm and in some nuclei. (A) 200 $\times$ magnification; (B) 400 $\times$ magnification. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0109>.

Figure 2
GLI1 expression in (A) normal pituitary, (B) pituitary adenoma not expressing *GLI1* and (C) pituitary adenoma overexpressing *GLI1*. All magnifications are 100 $\times$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0109>.

marker *SOX2*, tumor suppressor *TP53*, proliferation marker *MKI67* and scavenger enzyme *SOD1* was analyzed. We found that *GLI1* expression correlates with *SOX2* ($P \leq 0.001$, $r = 0.581$), *TP53* ($P \leq 0.001$, $r = 0.611$), *MKI67* ($P \leq 0.05$, $r = 0.380$) and *SOD1* ($P \leq 0.001$, $r = 0.589$) expression. Out of the 30 adenomas tested, eight overexpressed *SOX2* (27%), 25 (83%) *TP53*, 4 (13%) *MKI67* and 13 (43%) *SOD1*. Furthermore, *SOX2*, *TP53*, *MKI67* and *SOD1* also exhibit significant correlations among each other. *SOX2* correlated with *TP53* ($P \leq 0.001$, $r = 0.556$), *SOD1* ($P \leq 0.001$, $r = 0.627$) and *MKI67* ($P \leq 0.05$, $r = 0.402$). *TP53* and *SOD1* expressions also correlated significantly ($P \leq 0.0001$, $r = 0.833$). *MKI67* expression did not correlate with *TP53* and *SOD1* respectively. The Hh ligand *SHH* was found to be overexpressed in 18 (60%) of the tumors but did not correlate with any other analyzed genes including *GLI1*. Only 50% of the control pituitaries expressed *SHH*. Expression of Hh receptors *PTCH1/2* and *SHH* was described previously (Vila et al. 2005a).

Because we were particularly interested in the coexpression of *GLI1*, *SOX2* and *SOD1*, further analyses of their interactions were performed using immunofluorescence stainings. On activation, *GLI1* translocates into the cell nucleus where it induces the expression of its target genes. Immunofluorescence staining of cryosections of human pituitary adenomas confirmed the expression of *GLI1* in these tumors. We found that *GLI1* was mainly located in the cell nucleus rather than in the cytoplasm, representing its active state. The expression of *GLI1* was not restricted to

Figure 3

Immunofluorescence stainings of two nonfunctioning human pituitary adenomas. GLI1 is mainly located in the cell nucleus. (A) Co-localization of GLI1 and SOX2 in the cell nucleus spread across the tumor.

(B) Co-localization of GLI1 and SOD1; cropped image, images are shown in 400× magnification. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0109>.

specific zones within the adenoma but seemed to follow a diffuse, homogenous expression pattern across the tumor (Fig. 3). By double staining SOX2 and GLI1, we could see that the SOX2 pattern closely resembled that of GLI1. SOD1, however, was expressed in the cytoplasm and co-localized with GLI1 in close proximity to the nucleus.

Inhibition of GLI reduces cell viability *in vitro*

Due to the overexpression of *GLI1* seen in this study, we further evaluated the role of the Hh pathway in the mouse pituitary adenoma cell line AtT-20. GLI1 expression in AtT-20 cells was confirmed on the RNA and protein level (Fig. 4). We decided to downregulate Hh signaling at two different levels. Smoothed inhibitor GDC-0449 acts at the beginning of the cascade, while GANT61 inhibits activation of the transcription factors GLI1/2. A significant reduction in cell viability was already apparent after 24 h of treatment with GANT61 and GDC-0449 respectively (Fig. 5). Inhibiting the Gli proteins seemed to have a much stronger effect on cell viability than inhibiting the smoothed. That effect was clearly dose dependent for GANT61, as 5 μM GANT61 reduced cell viability to about 30%, whereas not a single viable cell was found at a dose of 20 μM (Fig. 5A). GDC-0449 gradually led to decreased cell viability but with significant effects only seen at a dose of 25 μM (Fig. 5B).

Expression of *Gli1*, *Mki67*, *Sox2*, *Trp53* and *Sod1* is downregulated by GANT61

Correlation analyses in human pituitary adenomas revealed a possible coexpression of *GLI1* with *SOX2*, *MKI67*, *SOD1* and *TP53*. Therefore, we treated AtT-20 cells with GANT61 for 24 h to test whether the inhibition of Gli actually affects expression of the above-mentioned genes. In line with our hypothesis, GANT61 dose-dependently reduced gene expression of *Gli1*, *Mki67*, *Sox2*, *Sod1* and *Trp53* as summarized in Fig. 6. *Gli1* expression was reduced by 50% at a dose of 5 and 10 μM respectively (Fig. 6A). Expression of the proliferation marker *Mki67* was reduced to 60% at 5 μM GANT61 and <10% at a dose of 20 μM (Fig. 6B), and *Sox2* expression

Figure 4

To confirm GLI1 expression in AtT20 cells, western blot analysis of untreated samples was performed (lanes 1–3). The predicted molecular weight of GLI1 is 118 kD. GAPDH was used as housekeeping control (37 kD).

Figure 5

Cell viability analysis of AtT-20 mouse pituitary adenoma cells. Cells were treated with Gli antagonist GANT61 and smoothened antagonist GDC-0449 for 24 h. Cell viability was determined with trypan blue. (A) Dose-dependent reduction of cell viability using GANT61. (B) GDC-0449 significantly reduced cell viability at a dose of 25 μM. Calculations are based on the results of two separate experiments ($n=2$). Each experiment was performed in quadruplets. Results are presented as mean $\pm$ S.E.M. NS = $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$.

was downregulated to 35% (5 μM) and <10% (20 μM) (Fig. 6C). *Sod1* expression was reduced to 60 and 30% by 5 and 20 μM GANT61 respectively (Fig. 6D), whereas *Trp53* was diminished by 50% at 5 μM and by 75% at 20 μM GANT61 (Fig. 6E). These data indicate that inhibition of Gli is responsible for reduced cell viability of pituitary adenoma cells and that targeting of Gli also regulates *Sox2*, *Mki67*, *Sod1* and *Trp53* gene expression.

Enhancing Hh signaling results in increased cell viability

To further confirm the involvement of GLI1 signaling in the pathogenesis of pituitary adenomas, we activated the Hh pathway in AtT-20 cells with smoothened-agonist purmorphamine. Consistent with our previous data, purmorphamine increased cell viability in mouse adenoma cells. This effect seemed to be dose independent and already apparent at the low dose of 0.5 μM. Cell viability could be increased to 1.75- to 1.9-fold compared to DMSO-treated control samples (Fig. 7A). We further wanted to test if the GANT61-induced cell death could be partly reversed by purmorphamine; 10 μM GANT61 induced cell

death in 80% of the cells. Interestingly, 1 μM purmorphamine combined with 10 μM GANT61 increased cell viability by only 10%, but 2 μM purmorphamine given together with 10 μM GANT61 enhanced cell viability by 30%. Whereas purmorphamine alone could not dose-dependently increase cell viability, antagonizing GANT61 is more effective at higher doses of purmorphamine. The effect of GANT61 could also be partly reversed by caspase-8 inhibitor suggesting a caspase-dependent induction of apoptosis through GANT61 (Fig. 7B).

Purmorphamine induces expression of *Gli1*, *Mki67*, *Sox2*, *Trp53* and *Sod1*

Gene expression analyses of purmorphamine-treated mouse adenoma cells confirmed our results. Purmorphamine dose-dependently activated the gene expression of *Gli1*, *Mki67*, *Sox2*, *Trp53* and *Sod1* (Fig. 8). Minor induction of gene expression was already seen at a dose of 0.5 μM purmorphamine. At 1 μM, *Gli1*, *Mki67* and *Sod1* expression were significantly enhanced by 1.5-fold. All genes except *Sox2* were highly induced at 2 μM purmorphamine. At a concentration of 4 μM purmorphamine, *Gli1*, *Mki67* and *Sod1* expression increased by more than 100% and *Trp53* expression was even three times higher than in DMSO-treated control samples (Fig. 8A, B, D and E). *Sox2* expression was stimulated to a lesser extent because an increase of expression was only significant at a concentration of 4 μM purmorphamine (Fig. 8C). Taken together, activation of *Gli1* resulted in an increased gene expression of *Mki67*, *Sox2*, *Trp53* and *Sod1* in mouse adenoma cells.

Discussion

The Hh pathway is a crucial regulator of fetal development but is also found to be reactivated in several types of human cancer. Hh signaling seems to contribute to

Figure 6

Gene expression levels after 24-h treatment with GANT61. (A) *Gli1* expression could only be determined at 5 and 10 μM GANT61 concentration. (B, C, D and E) The downregulation of the genes *Mki67*, *Sox2*, *Sod1* and *Trp53* at doses of 5 and 20 μM GANT61. Calculations are based on the

results of two separate experiments ($n=2$). Each experiment was performed in quadruplet. Results are presented as mean $\pm$ S.E.M. NS = $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 7

(A) AtT-20 cells were treated for 24 h with smoothened agonist purmorphamine, which resulted in a dose-independent increase in cell viability. (B) GANT61-induced reduction of cell viability could be partly reversed by purmorphamine (PM) and caspase-8 inhibitor (C.Inh). Calculations are based on the results of two separate experiments ($n=2$). Each experiment was performed in quadruplets. Results are presented as mean $\pm$ S.E.M. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

oncogenesis through maintaining the stem cell character of stem cell-like cancer cells, which is in line with the CSC paradigm. The discovery of adult stem cells in the pituitary gland raised the question if tumor-initiating cells are also involved in the pathogenesis of pituitary adenomas. To provide another piece of evidence, we evaluated the expression of Hh pathway members, stem cell markers and cell cycle regulators in 30 human pituitary adenomas.

In the present paper we show that *GLI1*, the terminal transcription factor of the Hh pathway, is expressed in normal human pituitary tissue and pituitary adenomas. Only 50% of the pituitaries of healthy subjects express *GLI1*, whereas *GLI1* expression is clearly upregulated in adenomas. This is in line with the aberrant activation of the Hh pathway found in several cancer types (Brechtel et al. 2014). The expression of *GLI1* shows great variability not just between healthy and tumor tissue but also within both groups. However, there was no tumor not expressing *GLI1*. We could confirm actual activation of *GLI1* in pituitary adenomas due to its almost exclusive location in the cell nucleus.

In mammals, the main activator of the canonical Hh cascade is SHH, but several alternative, ligand-independent

activating mechanisms have been identified in human cancers (Scales & de Sauvage 2009). We hypothesize that this might also be the case in pituitary adenomas, as *GLI1* expression is found in almost all tumors studied, whereas *SHH* expression is present in only 60% of the adenomas in our study, and even to a lesser extent in other studies (Vila et al. 2005a). *GLI1* but not *SHH* expression also significantly correlated with stem cell marker *SOX2*, cell cycle regulator *TP53*, proliferation marker *MKI67* and *SOD1*. Furthermore, inhibition of the Hh pathway in mouse adenoma cells was more effective on the level of Gli rather than upstream of the cascade. These findings could be explained by SHH signaling from adjacent structures (Treier et al. 2001) as well as SHH-independent activation of Gli1. As *GLI1* signaling increases pituitary hormone secretion (Vila et al. 2005a), hypophysiotrophic hormones and cytokines might be possible *GLI1* activators. Indeed, corticotropin-releasing hormone (CRH) upregulates *Gli1* transcription in AtT-20 cells (Vila et al. 2005b). The SHH-independent *GLI1* expression in pituitary tumors supports the hypothesis that *GLI1* activation plays an important role in tumorigenesis, while the reduction in SHH expression is highly likely a compensatory mechanism.

This hypothesis was also confirmed by our functional experiments, as inhibition of endogenous *GLI1* signaling in mouse pituitary adenoma cells resulted in a dose-dependent reduction of cell viability and *Sox2*, *Trp53*, *Mki67* and *Sod1* gene expression. On the other hand, inhibition of smoothened, which indirectly activates Gli1 signaling, increased cell viability and target gene expression. These results confirm that increased endogenous Gli1 activity in pituitary adenoma cells is linked to increased cell viability.

SOX2 was found to be a key marker of adult stem cells in various tissues including the pituitary. *Sox2*⁺ cells in the mouse pituitary can self-renew and are multipotent, giving rise to any of the endocrine cell types of the gland (Gleiberman et al. 2008). Only recently, *Sox2*⁺ cells were

Figure 8

(A, B, C, D and E) Gene expression levels after 24 h purmorphamine treatment. Gene expression of Gli1, Mki67, Sox2, Sod1 and Trp53 could be dose-dependently stimulated by purmorphamine. Calculations are based

on the results of three separate experiments ($n=3$). Each experiment was performed in quadruplets. Results are presented as mean $\pm$ S.E.M. NS = $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

shown to contribute to pituitary tumor formation through paracrine signaling (Andoniadou et al. 2013). Similarly, Hh signaling was also found to be activated through paracrine mechanisms due to tight interactions of tumor cells with their microenvironment (Yauch et al. 2008). In line with this, synergy of SOX2+ cells and Hh signaling could be an important mechanism in the development of pituitary adenomas, possibly involving stem cell-like tumor cells.

Our data is also in line with previous findings that overexpression of p53 is commonly seen in pituitary adenomas. Whereas p53 is usually undetectable in healthy cells due to rapid degradation, mutations in the *TP53* gene can lead to overexpression of oncogenic p53 in cancer cells (Janicke et al. 2008). Because *TP53* mutations have only rarely been detected in pituitary adenomas, mutation-related p53 upregulation seems not very feasible (Suliman et al. 2001, Tanizaki et al. 2007). However, oxidative stress is a general feature of cancer cells, going along with DNA damages and apoptosis. Cellular response to oxidative stress can activate antioxidant mechanisms including the expression of p53 and scavenger enzymes like the SOD family. In return, p53 can detect reactive oxygen species (ROS)-induced DNA damage and subsequently activates the expression of further antioxidant regulators (Trachootham et al. 2009).

Although our findings of an increased *TP53* and *SOD1* expression fit well into the mentioned scenario, they do not allow any conclusions whether this upregulation occurs in adaption to oxidative stress, response to DNA damage or other mechanisms. These adaption mechanisms are hypothesized to be responsible for radiation resistance in tumor therapy that targets cancer cells through induction of oxidative stress (Huang et al. 2012). Even though the redox status of CSCs is largely unknown, it was shown that adult stem cells have a lower level of ROS than more mature cells, probably to maintain the stem cell character (Dayem et al. 2010, Huang et al. 2012). Therefore, it is assumed that CSCs have upregulated levels of antioxidant scavenger mechanisms that protect CSCs and contribute to radiation therapy resistance (Ogawa et al. 2013). Inhibition of the Hh pathway in a mouse model for ischemic stroke was shown to worsen brain damage by downregulation of *Gli1*, *Ptch1* and *Sod1* in neurons (Ji et al. 2012). Hh signaling also seems to protect neurons against oxidative stress by upregulation of SOD and glutathione peroxidase (Dai et al. 2011, Ji et al. 2012). Furthermore, SOD1-overexpressing glioma cells are less sensitive to radiation and SOD1 is upregulated in neuronal stem cells (Gao et al. 2008).

Consistent with our findings, several GLI1 target genes including proto-oncogenes *CXCR4*, *BMI1* and *BCL-2* and cyclins D1, D2 and E1 have been shown to contribute to pituitary adenoma pathogenesis. Strikingly, cyclin D positive cells also had a significantly higher expression of BCL-2 and Ki67 (Wang et al. 1996, Jordan et al. 2000, Turner et al. 2000a,b, Sanchez-Beato et al. 2006, Lee et al. 2010, Westerman et al. 2012).

In summary, our results expand the knowledge on Hh pathway members in pituitary tumorigenesis, indicating smoothened-independent activation of GLI1, which is positively linked to cell viability and correlates with *SOX2*, *MKI67*, *TP53* and *SOD1* expression. These data support the hypothesis that stem cell-like tumor cells are involved in the development of pituitary adenomas, nourishing the hope that those molecules could represent target genes for future therapeutic approaches.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K Lampichler designed and performed experiments, conducted research, analyzed data, interpreted results, wrote the manuscript and approved the final version. P Ferrer, M I Lutz and F Wolf conducted research and reviewed and approved the final manuscript. E Knosp and A Luger reviewed and approved the final manuscript. G Vila, L Wagner and S Baumgartner-Parzer reviewed the data, contributed to discussions, edited and approved the final manuscript.

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