ROLE OF HEDGEHOG SIGNALING IN MALIGNANT PLEURAL MESOTHELIOMA

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TRANSLATIONAL RELEVANCE

Malignant pleural mesothelioma (MPM) are particularly resistant to current chemotherapy. In this study, we observed hedgehog signaling pathway activation in MPM and we show that inhibition of this pathway decreases cell proliferation in cell culture and inhibits the growth of MPM in vivo in a xenograft model. These findings suggest that hedgehog signaling inhibitors may have therapeutic potential for MPM.

ABSTRACT

Purpose. The aim of this study was to assess the activity of hedgehog (HH) signaling pathway in malignant pleural mesothelioma (MPM).

Experimental Design. The expression of HH signaling components was assessed by q-PCR and in situ hybridization in 45 clinical samples. Primary MPM cultures were developed in serum-free condition in 3% oxygen and were used to investigate the effects of Smoothened (SMO) inhibitors or GLI1 silencing on cell growth and HH signaling. In vivo effects of SMO antagonists were determined in a MPM xenograft growing in nude mice.

Results. A significant increase in GLI1, sonic hedgehog, and human hedgehog interacting protein gene expression was observed in MPM tumors compared to non tumoral pleural tissue. SMO antagonists inhibited GLI1 expression and cell growth in sensitive primary cultures. This effect was mimicked by GLI1 silencing. Reduced survivin and YAP protein levels were also observed. Survivin protein levels were rescued by overexpression of GLI1 or constitutively active YAP. Treatment of tumor-bearing mice with the SMO inhibitor HhAntag led to a significant inhibition of tumor growth in vivo accompanied by decreased Ki-67 and nuclear YAP immunostaining and a significant difference in selected gene expression profile in tumors.

Conclusions. An aberrant HH signaling is present in MPM and inhibition of HH signaling decreases tumor growth indicating potential new therapeutic approach.
Introduction

Malignant pleural mesothelioma (MPM) is associated with asbestos exposure. Chronic tissue inflammation and tissue repair have been postulated to be the central mechanism leading to tumorigenesis (1, 2). Tissue repair involves the activation of stem cells and the expression of stem cell renewal genes. Activated stem cell signaling has already been suggested in MPM with the presence of an 11-gene signature, correlating with a stem-cell-like expression profile, which is associated with a poor prognosis in patients with MPM (3). Cells staining positive for nuclear β-catenin, a marker for Wnt signaling activation have been reported in a few studies (4-6). A significant transcriptional downregulation of the secreted frizzled-related proteins (sFRPs) glycoproteins, which are negative modulators of the Wnt signal transduction pathway, has been observed in MPM primary tissues and cell lines (7). More recently the downregulation of several miRNA antagonizing Wnt signaling have been described in MPM(8). Another stem cell signaling pathway that has been investigated in vitro is Notch, whereby Notch1 has been found to control PTEN expression in MPM lines (9). Concerning Bone Morphogenetic Proteins (BMPs), which are members of the transforming growth factor-β (TGF-β) superfamily and are critical mediators of early embryonic patterning, methylation of BMP3b and BMP6 promoters has been observed in MPM (10). In addition, in a case of biphasic malignant mesothelioma with osseous and cartilaginous differentiation expression of BMP2 has been observed (11).

Our study now identifies aberration in Hedgehog (HH) signaling in MPM. HH signaling has a key role for normal organ development and is dysregulated in several types of cancer (12). We recently observed increased expression of PTCH1 (patched, the receptor binding Hedgehog ligands) in mesothelioma side population-derived tumors which exhibited a tendency to have increased tumor initiating properties and developed tumors with precursor phenotype similar to tumors in patients with relapse after chemotherapy (13). This prompted
us to investigate whether HH pathway is activated in MPM and the effect of its inhibition in primary mesothelioma cell cultures and in a xenograft.

**Material and Methods**

*Tissue samples*

Forty-five tumor specimens were collected at the time of surgery and were immediately processed for primary culture or total RNA extraction using Qiagen RNAeasy®. In addition, parts of tumor specimens were embedded in Tissue-Tek® O.C.T™ Compound (Sakura, Alphen aan den Rijn, The Netherlands) and immediately frozen. Non-tumoral pleural tissue was received from ten patients undergoing mesothelioma unrelated thoracic surgery. The study was approved by the Institutional Review Board of Zurich University Hospital and a written informed consent was obtained for each patient.

*Gene expression analysis*

Selected gene expression analysis was performed as previously described(14). Additional primers are listed in the Supplementary Table I. In order to compare the profile of HH pathway gene expression in clinical samples vs. primary cultures the ΔΔCt method was used were all ΔCt were normalized to 12.2, being the lowest ΔCt determined. The heatmap of genes expressed in the xenografts was produced as previously described on ΔCt raw data (13) (14) and only tumors with the same levels of human housekeeping normalizer gene (GAPDH) were considered.

*Primary malignant pleural mesothelioma cultures*

Primary malignant pleural mesothelioma cultures were established from surgical specimens as previously described (13) except that at the end of enzymatic digestion cells
were resuspended in culture medium (DMEM: F12, 0.4 μg/ml hydrocortisone, 10 ng/ml EGF, 20 ng/ml bFGF, 10 μg/ml insulin, 5.5 μg/ml transferrin, 6.7 μg/ml selenium, 1 mM sodium pyruvate, 100 μM beta-mercaptoethanol) supplemented with non-essential amino acids and 30% conditioned medium (15), and incubated in 3% oxygen. The paired cultures of SDM61, SDM62, SDM74 and SDM76 grown in the presence of serum have been previously described (16). All cultures used in this study were authenticated by DNA fingerprinting (Microsynth, Balgach, Switzerland). Primary cultures were used between passage 3 and 20.

**SMO inhibition and measurement of cell growth**

Cells were treated either with cyclopamine (Toronto Research Chemicals, Toronto, Canada), HhAntag (Genentech), both being specific antagonists of SMO, or with tomatidine (Sigma–Aldrich, Buchs SG, Switzerland), a structurally similar compound with non-specific inhibition of Hh signaling. Cell growth was determined as previously described (17).

**Western blot analysis**

Primary cultures were characterized for mesothelioma marker expression as described elsewhere (16) and expression of apoptosis or survival markers was achieved using rabbit polyclonal antibody anti PARP (polyclonal, 1:1000 dilution, Cell Signaling), anti-survivin (polyclonal, 1:1000 dilution, R&D), anti-caspase-3 (polyclonal, 1:1000, Cell Signaling), anti-phospho histone (polyclonal, 1:1000, Millipore), anti Gli-1 (polyclonal, 1:1000 dilution, Cell Signaling), anti- YAP (polyclonal 1:1000 Cell Signaling), and anti-P-YAP (polyclonal 1:1000, Cell Signaling).

**In situ hybridization and immunohistochemistry**

Tissue-Tek® O.C.T™ embedded tumors were used to prepare 12 μm thick sections which were processed for in situ hybridization with digitonin-labeled sense and anti-sense
riboprobes as described (18). Human *PTCH1*, *GLI1* and *Sonic hedgehog* (*SHH*) encoding plasmids (kindly provided by Dr. Ruiz I Altaba) were linearized with XbaI (sense) and XhoI (antisense *PATCH1*) and HindIII (antisense *GLI1*, *SHH*), respectively. Human *desert hedgehog* (*DHH*) encoding plasmid (kindly provided by Dr. McMahon) was linearized with Ndel (antisense) and XhoI (sense).

Immunohistochemistry was performed on paraffin-embedded sections using polyclonal anti YAP (1: 25), Ki-67 (Mib-1, clone B126.1, 1:50, Abcam) and HHIP (clone M01, 1:100, Abnova) as detailed in Supplementary Methods.

**Transfection of *GLI1* or constitutively active YAP**

ZL55SPT cells plated at a density of 3500 cells/cm² and were transfected with either pcDNA3.1 encoding human GLI1 (19)(kindly provided by Dr Bert Vogelstein and Dr. Sasaki) or pcDNA3.1 using DMRIE-C (Invitrogen, Basel, Switzerland) according to the manufacturer’s instructions. 48h after transfection G418 400 μg/ml (Roche Applied Biosciences, Rotkreuz, Switzerland) was applied for selection. For constitutively active YAP expression, transient transfection of pcDNA3.1 encoding human YAP with the five LATS phosphorylation sites (Ser61, Ser109, Ser127, Ser164, and Ser397 (20)) (kindly provided by Dr. X. Yang) was used applying the same protocol.

**RNA interference**

For down-regulation of *GLI1* with small interfering RNAs (siRNA), ZL55SPT cells were transfected with ON-TARGET plus SMARTpool siRNAs targeted to *GLI1* or control non targeting (NT) siRNA (Thermo Scientific Dharmacon), according to the manufacturer’s reverse transfection protocol. Cells were then plated at 7000 cells/cm² or 700 cells/cm² to
extract RNA or determine effect on cell growth, respectively. RNA was extracted after 48 and 72h. Cell growth was investigated by crystal violet staining after 12 days.

**Assay for HH pathway activation**

NIH3T3 cells were plated into 12 well at the density of 80’000 cells/w. The next day cells were transfected with 8xGli Bswt-luc reporter, GLI binding site mutated 8xGlimut-luc reporter (21) provided by Dr. Sasaki and Renilla luciferase (pRL-TK) at 50:1 ratio using DMRIE-c. After 9 hr ZL55SPT conditioned medium with or without 300 nM HhAntag was added and cells were incubated for another 40 hr before luminometric detection (Promega dual luciferase assay). Results are expressed as firefly luciferase activity normalized to Renilla luciferase activity. The presence of DHH in the conditioned medium was determined by ELISA as detailed in Supplementary methods.

**Animal studies**

Human mesothelioma ZL55 cells (10^6 per animal) were subcutaneously injected under general anaesthesia into the left flank side of eight-week-old CD1 nude mice (from Charles River and Harlan). Treatments started when the tumor volumes reached 31±8mm^3. Mice were treated with HhAntag 38 mg/Kg body weight or vehicle alone, by oral gavage twice per day 5d/week during 2 week. Tumor volume was measured by calliper and calculated with the formula: Width^2×Length/2. All animal experiments were performed in accordance with the ethical principles and guidelines for experiments on animals of Swiss Academy of Medical Sciences.

**Results**
HH pathway expression in mesothelioma

To address the activation of HH signaling in MPM we examined fresh frozen tumor tissues from 39 patients. There were 34 males and 5 females, with a median age of 62 (range 42-77). In 6 patients tumor samples were obtained before and after neoadjuvant chemotherapy or at progression giving a total of 45 tumor samples. Additionally, 4 samples of chronic inflammation of the pleura were analyzed as well as 6 samples of normal pleura as control (16). The histopathology showed 25 tumors (64%) being of epithelioid type, 13 (33 %) of the biphasic type and one sarcomatoid type. SHH gene expression could be detected in tumor tissue but not in non-tumoral pleural samples (Figure1A). Downstream HH targets GLI1 and human hedgehog interacting protein (HHIP) mRNA levels were 2-fold and 6-fold higher, respectively, in MPM tissue compared to non-tumoral pleural tissue. No significant expression difference was observed for PTCH1 (Figure1A), Indian hedgehog, SMO, and GLI2 expression (Supplementary Figure1). In situ hybridization was performed in tumors from ten patients. It confirmed that expression of GLI1, PTCH1, SHH and DHH (Fig. 1 B) was mostly associated with tumor cells and correlated with q-PCR data. Data on overall survival was available for 23 patients that had received chemotherapy. In an exploratory analysis we looked for a possible association of high GLI1 expression with overall survival. There was a significant association of high GLI1 expression with poor survival (p=0.042, supplementary Figure 1B).

HH pathway expression and activity in mesothelioma cultures

Although only one fifth of MPM primary culture grows in the absence of serum compared to the ones that grow in medium containing serum, growth in the presence of their own conditioned medium, and 3% instead of 20% oxygen culture conditions allowed increased expression of “stemness” genes compared to serum-free medium at 20% oxygen.
(Supplementary Figure 2). In primary cultures established in these conditions we observed a profile of relative expression of the components of HH pathway (Supplementary Figure 3A) similar to the one determined in tumors, thereby allowing functional studies. DHH was the only ligand expressed and levels were low (Supplementary Figure 3A). Although the expression of HHIP was highly variable in the tumor tissue, HHIP protein basal levels were similar in the three primary cultures tested (Supplementary Figure 3B).

Treatment of mesothelioma primary cultures with cyclopamine resulted in a significant downregulation of GLI1 expression in four out of six primary cultures tested (Figure 2A), while tomatidine, which was used as control for specificity, had no or little effect. Response to cyclopamine correlated to higher basal levels of GLI1 and was accompanied by downregulation of HHIP (data not shown). In addition we observed that conditioned medium from a primary mesothelioma culture specifically increased an HH pathway specific reporter (21) in NIH3T3 mouse embryonic fibroblasts cells (Figure 2B). Conditioned medium activity was abolished in the presence of HhAntag, a recently developed potent synthetic SMO inhibitor (22), indicating that biologically active HH ligands are present in the conditioned medium from mesothelioma culture as it had been shown in another model system (23). The presence of DHH in the conditioned medium (10.5±4.5 pg/ml, n=5) was confirmed by ELISA. Finally, we determined that in MPM xenografts (13) the expression of human DHH correlated with human GLI1 and PTCH1 expression (r²=0.77, p= 0.004; r²=0.54, p= 0.037, respectively) while no correlation was observed with murine GLI1 or PTCH1; similarly no correlation was observed between the expression of mouse DHH and human GLI1 or PTCH1.

Altogether these data suggest that HH signaling has an autocrine activity in MPM.

Inhibition of HH signaling decreases MPM growth

The growth of primary MPM cultures without serum was very slow and long term cultures were obtained only with ZL55SPT and SDM103T2 which were originated from MPM.
xenografts in mice. Therefore, most of the following studies were performed with these two lines.

Treatment with HhAntag resulted in a dose-dependent decrease of cell survival, indicating a role for HH signaling in mesothelioma growth (Figure 3A). The involvement of HH signaling was confirmed by HhAntag induced-downregulation of GLI1 target HHIIP (Supplementary Figure 4). To gather a better understanding of the mechanisms leading to decreased cell survival we investigated apoptosis markers PARP and caspase-3, mitotic marker phospho-histone and survivin, the latter being essential for mesothelioma survival (24), in cells treated during 48h with either 5 μM cyclopamine or 5 μM HhAntag (Figure 3B). Although no PARP cleavage or caspase-3 decrease could be detected, we observed a significant decrease of phospho-histone mitotic marker and survivin levels in cells treated with HhAntag. Furthermore, a significant decrease in survivin mRNA expression was observed (Figure 3C).

In order to investigate adequate targeting of HhAntag we tested the effect of the treatment in ZL55SPT cells transfected with GLI1 or control vector. Western blot analysis of GLI1 expression (Figure 4A) using a commercial antibody against the region surrounding amino acids 420 recognized the diverse forms of GLI1: full length, the partially active 130 KDa, the weak repressor 100 KDa (25) and an additional 70 KDa band not yet identified, which were most visible in the transfected cells. The 100 KDa inactive form was the most abundant consistent with the notion that it is the more stable GLI1 form (25), nevertheless increased GLI1 activity was confirmed by increased HHIIP expression (Supplementary Figure 5). GLI1 transfection rescued survivin decreasing effects of HhAntag (Figure 4A). In addition it rescued the expression of HH target SOX2 (Figure 4B) which is expressed in ZL55SPT (13) and was downregulated by HhAntag.
The role of HH signaling in MPM growth was further confirmed in ZL55SPT cells by downregulation of GLI1 expression using small interfering RNA (Figure 4C). Silencing GLI1 was already observed after 48h (data not shown) but was more efficient after 72h (Figure 4C) and resulted in decreased clonal cell growth comparable to the effect of cyclopamine. In addition decreased levels of HHIP, survivin and SOX2 expression compared to non-targeting siRNA were observed.

_HhAntag suppression of survivin expression is associated with decreased YAP_

Survivin is not described as a direct target downstream HH pathway. Hence, we sought for other transcription activators known to be expressed in MPM and to regulate survivin expression and the most obvious was YAP. YAP is a transcriptional co-activator which localizes in the nucleus unless it is inactivated by phosphorylation by LATS kinase (26) downstream of NF2 signaling. YAP is constitutively active in more than 70% of primary MPM (27), it has been originally described to be involved in size control paralleled by a 30-fold increase in survivin expression (28) and a recent study has showed that it controls survivin expression in MPM (29). We confirmed nuclear expression of YAP in MPM (Figure 5A) and observed that HhAntag reduced YAP protein levels (Figure 5B). No obvious change in YAP mRNA or in phosphorylation (YAP-ser127) level was detected consistent with both ZL55SPT and SDM103T2 cells being NF2 protein deficient (Supplementary Figure 6) due to gene deletion (Hoda and Berger, unpublished data). Transient transfection of a constitutively active YAP (20) rescued HhAntag-dependent survivin decrease (Figure 5C), confirming the interaction between Hedgehog and YAP signaling.

_Effect of HhAntag as a Single Agent on MPM Xenografts in SCID Mice_
Finally we tested the effect of HhAntag in vivo in ZL55 xenografts. Tumor bearing mice were randomized in two groups receiving either solvent or HhAntag. The in vivo HhAntag treatment dosage (38 mg/kg bw, administered twice daily by oral gavage, 5d/week) was chosen based on therapeutic range reported in the literature (22). HhAntag led to a significant (p<0.05, t-test) 35% decrease of the tumor volume after the two weeks of treatment (Figures 6A). At the end of dosing regimen, animals were euthanized in order to collect tumor tissue for RNA extraction and immunohistochemical analysis. Indeed, in order to get further insight into the mechanism of decreased tumor growth observed in HhAntag treated mice, gene expression analysis was performed investigating the relative expression of mesothelioma markers calretinin, podoplanin and mesothelin (14); HH pathway components GLI1, PTCH1, DHH and HHIP; ABC transporters ABCG2 and ABCC1; stem cell markers nestin, OCT4A, CD90, HES1, osteoblastic differentiation markers BMP2, runx2; hypoxia controlled CAIX and Wisp2 and matrix remodelling Slug, Twist and PAI-1. Some mouse genes (mGLI1, mPTCH1, mSca-1, mABCG2 and mABCC1) were also included to take into account mouse stromal components. We observed (Figure 6B) a treatment-induced significant two-fold increase in nestin, human ABCC1 (p<0.001 for both) and HHIP (p<0.005) expression levels. The latter was accompanied by increased HHIP immunoreactivity in samples from HhAntag treated mice (Supplementary Figure 7). An almost two-fold increase expression was also observed for runx2, human PTCH1 (p<0.01 for both) and Bmp-2 (p<0.05), while the increase of CAIX, twist, and podoplanin was less extended (p< 0.05). The highest increase was observed for mouse GLI1 (p<0.01) where a three-fold increase was determined. The effect of HhAntag on tumor volumes was also accompanied by a significant (p<0.05, Mann-Whitney U test) 43% decrease in Ki-67 labelling index (Figure 6C). Furthermore, consistent with in vitro experiments, we observed a significant (p<0.05, Mann-Whitney U test) 32% decrease in nuclear YAP immunostaining in HhAntag treated tumors (Figure 6C).
All in all these data support a role for HH signaling in MPM growth.

**Discussion**

HH signaling has been implicated in several cancers (reviewed in (30)), however it is the first time that it is documented to have a role in mesothelioma cell growth. We observed a significant increased expression of *GLI1* in tumor tissue indicating the presence of an active pathway. Levels of both, *SHH* ligand and *HHIP*, which is a negative regulator by binding all ligands with nanomolar affinity (31), were also significantly upregulated in mesothelioma tumors. Since expression levels of *HHIP* were in some samples hundred-fold higher compared to the other pathway components, this may indicate a differential negative feedback mechanism. A high variation of *HHIP* expression compared to non-tumoral tissue has already been observed in lung tumors (32) but whether it corresponds to modulation of HH signaling in the tumor has not been investigated yet, although it is known that *HHIP* is essential for normal lung development (33).

HH signaling in tumors can be ligand independent and driven by mutations in signal transducers as observed in basal cell carcinoma, medulloblastoma and rhabdomyosarcoma, while in several cancers ligand dependent HH autocrine activity has been demonstrated (reviewed in (34)). On the other hand a tumor-promoting activity via a paracrine effect of HH ligands secreted from the tumor on stroma (35) or vice versa has been observed (36). We have three lines of evidence suggesting that an autocrine activity is present in some mesothelioma: SMO inhibitors could decrease cell growth and *GLI1* expression, conditioned medium could stimulate a GLI1-reporter activity and human sonic hedgehog ligand expression was correlated with human *GLI1* and *PTCH1* but not with murine *GLI1* or *PTCH1* expression in tumor xenografts.
Sensitivity to SMO inhibitors was not the same in all primary cultures tested and this did not seem to be related to HHIP expression which was similar in the three cultures tested which had differential sensitivity. One other possibility could be the differential expression of glypican-3 (GPC-3), a proteoglycan expressed at the surface of the cell, which is frequently inactivated by promoter methylation in mesothelioma (37). GPC-3 is known to inhibit HH signaling (38) and its overexpression has been shown to inhibit cell growth in mesothelioma (39). Alternatively, another member of glypican family, Glypican-5, which has been recently demonstrated to activate HH signaling (40) is maybe expressed in MPM and at different levels, thereby controlling HH signaling. Finally, it is possible that the negative regulator of HH signaling, Suppressor of fused (41, 42), is differentially operational in each primary culture. Further studies will address this question.

Potential ligands present in the conditioned medium are either DHH which was expressed in primary cultures or oxysterols, which can be derived from endogenous cellular biosynthesis and are efficient stimulators of HH signaling (43). DHH is known to be expressed in gonads, including Sertoli cells of testis and granulosa cells of ovaries (reviewed in (44) and there are no obvious reasons why it is expressed in mesothelioma. However, homogenous increase of DHH, but not of SHH, has been recently described in osteosarcoma cell lines (45) suggesting that DHH expression is aberrantly activated in cancer.

Decreased survivin expression upon inhibition of HH signaling has been described in at least one study in colon cancer cells in culture (46). The fact that we could antagonize the SMO inhibitor effect by GLI1 overexpression and that this phenomenon was also observed after GLI1 silencing indicates that this effect is specific. Many signals control survivin expression (47), the one relevant to mesothelioma and linked to HH signaling is active YAP. Indeed, YAP is constitutively active in more than 70% of primary MPM (27) and we
confirmed YAP activation in this study. The observation that HhAntag decreases YAP protein is consistent with the role of HH in maintaining YAP protein stability (48).

In addition to a decrease in survivin by HhAntag, we observed a decrease in the expression of the stem cell marker SOX2. The latter is controlled by HH signaling in neural stem cells (49) but YAP has also been described to directly positively regulate SOX2 expression (50). Functional studies are necessary to identify whether GLI transcription factors or YAP-dependent transcription are involved in SOX2 expression in mesothelioma.

In the xenograft model the inhibition of HH was accompanied not only by a decrease of nuclear YAP but also by a significant change in gene expression. The increase in HHIP and PTCH1 might be relevant for a negative signaling regulation, while the increase in ABCC1 transporter expression might be linked to drug-induced adaptation, assuming that HhAntag is effluxed by ABCC1. The selected gene expression analysis included genes (runx2 and Bmp-2) along mesenchymal stem cell (MSC) differentiation toward osteoblast (51, 52). This is due to the fact that we recently observed that mesothelioma primary cultures express MSC markers CD90, CD105 and CD73 (13), therefore if HH signaling is involved in maintaining stemness, its inhibition should result in promoting differentiation as it has been recently observed in chronic myeloid leukemia (53). The increase in runx2 and Bmp-2 expression upon HhAntag treatment is consistent with knowledge about HH control of osteoblastic differentiation (54) and with defects in bone structure observed in young mice treated with this agent (55). This result is also consistent with the recent observation that YAP reduces the expression of Bmp-2 (20).

The increase in nestin and mouse GLI1 expression are more intriguing. An unexpected increased vasculature and decreased stroma have been observed in a mouse model of pancreatic ductal carcinoma treated with a HH inhibitor for the same length of time (56) but no such changes were identified by histopathology analysis in our study.
In conclusion, upregulation of HH signaling was observed in malignant pleural mesothelioma tumors and SMO inhibitors decreased cell growth both \textit{in vitro} and \textit{in vivo} in sensitive mesothelioma. Growth control was associated with down-regulation of YAP and its target survivin. Further studies identifying factors associated with response will allow defining patient who may potentially benefit from HH antagonist therapy.

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**References**


LEGENDS TO THE FIGURES

Fig. 1. Expression of HH pathway components in non-tumoral pleural tissue and mesothelioma tumors. (A) Quantitative real-time PCR analysis of HH pathway gene expression in non-tumoral pleural tissue (NT) and tumor (T), * p< 0.05, ** p<0.005. (B) SHH, PTCH1, GLI1 and DHH transcripts (blue as positive) were detected by in situ hybridization (ISH) in frozen sections of mesothelioma tumors (left panel), and the middle panel pictures are their controls with respective sense probes. The right panel shows corresponding H&E staining (ISH for SHH and DHH are shown for the same patient). Bar indicates 20 μm.

Fig. 2. HH pathway activity is maintained in MPM primary cultures maintained without serum in 3% oxygen. (A) Downregulation of Gli-1 expression by cyclopamine (5 μM). Tomatidine (5 μM) was used for control of specificity. Results are expressed relative to vehicle treated control. * p<0.05 compared to tomatidine. (B) Conditioned medium from a mesothelioma primary culture stimulated wt but not mutated Gli-luciferase reporter activity and this property was abolished by HhAntag. * p<0.001 compared to mGli.

Fig. 3 HH pathway controls MPM growth in vitro. (A) HhAntag dose-dependently inhibited MPM cell proliferation, *p <0.05, ** p<0.001, compared to vehicle control. (B) Blocking Hh pathway with HhAntag does not induce apoptosis, assessed by determination of PARP and caspase 3 cleavage, but decreases phospho-histone and survivin protein expression. (C) HhAntag significantly (p<0.05) decreases survivin mRNA expression.
Fig. 4 Exogenous GLI1 expression rescues HhAntag-induced changes on survivin protein levels (A) and SOX2 expression (B, *p<0.05) while GLI1 silencing using small interfering RNA decreases clonal cell growth (C, left panel) and HHIP, survivin and SOX2 expression levels (C, right panel).

Fig. 5 HH pathway regulates YAP expression in mesothelioma. (A) Nuclear immunostaining of YAP was observed in MPM. Bar indicates 50 μm. (B) Treatment of ZL55SPT and SDM103T2 with HhAntag resulted in decreased YAP protein expression. (C) Constitutively active YAP expression rescues HhAntag-induced decrease of survivin protein levels.

Fig. 6 HH pathway controls MPM growth in vivo. (A) ZL55 tumor growth curves in animals of vehicle treated controls vs. HhAntag (38 mg/Kg b.w., bid, 5d/wk, 2 wks, n=6/group). * p<0.05. (B) Clustering of genes regulated by HhAntag analyzed in tumors from controls (C) or HhAntag treated (T) collected at the end of treatment period. Analyzed genes include, mesothelioma markers podoplanin, mesothelin, calretinin; sonic hedgehog pathway components GLI1, PTCH1, DHH and HHIP; ABC transporters ABCG2 and ABCC1; stem cell markers nestin, OCT4A, CD90, HES1; osteoblast differentiation markers BMP2, runx2; hypoxia controlled CAIX and Wisp2 and matrix remodelling Slug, Twist and PAI-1. Matrix of relative gene expression values is shown as heatmap. Green indicates down-regulated genes; red indicates up-regulated genes. (C) HhAntag significantly (* p<0.05) decreases nuclear Yap and Ki67. Bar indicates 50 μm.
Figure 1 Shi et al

(A) mRNA expression of SHH, PATCH1, HHIP, and GLI1 in NT and T. SHH expression was significantly increased in T compared to NT (***p < 0.001). PATCH1 expression was increased in T compared to NT (p < 0.05). HHIP expression was increased in T compared to NT (*p < 0.05). GLI1 expression was increased in T compared to NT (*p < 0.05).

(B) Immunohistochemical staining of GLI1, PTCH1, SHH, and DHH in NT and T. The expression of GLI1, PTCH1, SHH, and DHH was increased in T compared to NT.
Figure 2 Shi et al

A

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Legend:
- * indicates statistical significance.

Figure 2 Shi et al
Figure 3 Shi et al
Figure 4 Shi et al

A

Control vector  GLI1 transfected

B

Control vector  GLI1 transfected

C

1: siNT  1+: siNT+cyclop  2: siGLI1

mRNA level

GLI1  HHIP  survivin  Sox-2

NT  siGli  NT  siGli  NT  siGli  NT  siGli
Figure 5 Shi et al

A

HE     control     YAP

B

Cyclopamine    HhAntag
-     +     -     -     +     -     -     +

MW  65KDa
-     +     -     -     +     -     -     +

65KDa
-     +     -     -     +     -     -     +

42KDa
-     +     -     -     +     -     -     +

ZL55SPT     103T2

C

Control vector     YAP5SA transfected

YAP

Survivin

Actin

0  0.5  1  2.5  5  10  0  0.5  1  2.5  5  10 μM HhAnt
Figure 6 Shi et al

A

B

C

control

HhAntag

% nuclear YAP

% nuclear Ki67

control HhAntag

control HhAntag

author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
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Yandong Shi, Ubiratan Moura, Isabelle Opitz, et al.

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