Non-canonical Hedgehog signaling activation in ovarian borderline tumors and ovarian carcinomas

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Abstract. Hedgehog signaling pathway has been implicated in the pathology of ovarian cancer, and Survivin (BIRC5) has been suggested as a novel target of this pathway. Herein we investigated the role of Hedgehog signaling pathway and Survivin in ovarian carcinoma and borderline tumor samples. We aimed to determine possible ways of pathway modulation on primary ovarian cancer cells and an established cell line. RNA was extracted from fresh tumors and control tissues and gene expression was examined using qRT-PCR. Pathway activity in cell lines was examined after treatment with cyclopamine, SHH protein, GANT-61 or lithium chloride using qRT-PCR, western blot and confocal microscopy. The difference between control tissue, borderline tumors and carcinomas can be seen in GLII and SUFU gene expression, which is significantly higher in borderline tumors compared to carcinomas. SUFU also shows lower expression levels in higher FIGO stages relative to lower stages. BIRC5 is expressed in all tumors and in healthy ovarian tissues compared to our control tissue, healthy fallopian tube samples. Primary cells developed from ovarian carcinoma tissue respond to cyclopamine treatment with a short-term decrease in cell proliferation, downregulation of Hedgehog pathway genes, including BIRC5, and changes in protein dynamics. Stimulation with SHH protein results in increased cell migration, while GLI1 transfection or PTCH1 silencing demonstrate pathway upregulation. The pathway activity can be modulated by LiCl at the GSK3β-SUFU-GLI level, suggesting at least partial non-canonical activation.

Downregulation of the pathway with GANT-61 has proved to be more effective than cyclopamine. GLI inhibitors may be a superior treatment option in ovarian cancer compared to SMO inhibitors.

Introduction

Ovarian cancer is the eighth most frequent tumor type in women worldwide, with 225,500 new cases and 140,200 deaths each year. There is almost no difference in mortality between developed and developing countries (1). Epithelial ovarian cancer comprises the majority of malignant ovarian tumors. Borderline epithelial tumors have been classified as a separate category, although their origin is still unclear. Their clinicopathological features are between the benign cystadenomas and malignant cystadenocarcinomas (2). Borderline tumors and low-grade serous carcinomas are associated with KRAS mutations. High-grade serous carcinomas are associated with loss of BRCA1 or BRCA2, and with TP53 mutations, but no BRAF and rarely KRAS mutations. This suggests that low-grade and high-grade serous carcinoma arise via different genetic pathways (3). High BIRC5 gene expression (Survivin) is often detected in various tumors, and is correlated with poor outcome. Survivin protein expression correlates with progression of epithelial ovarian carcinomas (4). Epithelial ovarian cancer shows a diverse pattern of genomic rearrangements which lead to altered gene expression and upregulation of cancer pathways, including Hedgehog signaling (5).

Hedgehog signaling pathway is a developmental pathway involved in formation of various tissues and organs. In mammals, the signal transduction is triggered by binding of the ligand Hedgehog (HH) to the transmembrane receptor Patched (PTCH). This leads to internalization of PTCH and exposure of the protein Smoothened (SMO) on the cell surface. SMO triggers a cytoplasmic phosphorylation cascade leading to the release of transcription factor GLI (GLI1-3) from Suppressor of Fused (SUFU) and translocation of GLI to the nucleus, where it triggers transcription of genes involved in cell proliferation. In the absence of the HH signal, the cascade is inactive and GLI proteins are retained in the cytoplasm and subsequently degraded in the proteasome (6). GLI proteins are regulated by SUFU, protein kinase A (PKA), glycogen synthase kinase-3β (GSK3β) and casein kinase 1 (CK1). The...
role of GSK3β in the Hedgehog signaling pathway is 2-fold: it phosphorylates GLI proteins, tagging them for degradation and processing into repressor forms, but it can also elicit a positive effect on the signal transduction in ligand-stimulated cells, binding to SUFU and enabling the release of GLI from the complex (7). Genes activated by the Hedgehog pathway are involved in cell cycle regulation, proliferation, adhesion, epithelial-mesenchymal transition, self-renewal and pathway autoregulation. A recent study by Brun et al implicated Survivin in Hedgehog-driven medulloblastoma (8), and a study by Vlčković et al demonstrated a direct link between Survivin expression and Hedgehog signaling, where expression of Survivin was directly regulated by GLI2 and GLI3 proteins (9). Therefore, Survivin is considered a novel Hedgehog pathway target in a large panel of tumor cell lines (9).

The Hedgehog signaling pathway is involved in embryonic development and maturation of Drosophila ovaries. In the adult fly it is involved in division of somatic stem cells which give rise to follicle cells surrounding the gamete. Inactivation of the pathway causes a reduction in follicle production, while hyperactivation causes proliferation of somatic cells between egg chambers (10). In mice, activation of the pathway by Sonic Hedgehog (SHH) protein increases growth and proliferation in cultured granulosa cells, and cyclopamine was shown to prevent this induction. Russell et al claim that many of the Hedgehog pathway genes are expressed in immature and adult murine ovaries, granulosa cells and corpora luteum: HHIP are expressed in all ovarian tissues (11). Another more recent study by Huang et al indicates that Hedgehog signaling is inactive in fetal, but active in the adult mouse ovary (12).

A paracrine model of signaling was suggested in the ovary: Hedgehog target genes GLI1 and PTCH1 are primarily expressed in theca cells, while the ligand is produced in the granulosa cells (13,14).

According to most authors Hedgehog pathway is activated in ovarian carcinomas, where the protein or gene expression is higher than in healthy tissue or benign tumors (15-18). Other authors report Hedgehog pathway protein expression in a subset of tumors, but claim it is not a frequent event in ovarian cancer (19). The authors mostly focus on the four most frequently examined components of the pathway: SHH, PTCH1, SMO and GLI1. In healthy human ovaries, the Hedgehog pathway genes/proteins are usually not expressed (15,17). Expression of GLI1 and PTCH1 correlates with poor clinical outcome of ovarian carcinoma patients (16,20). Cell culture experiments on ovarian cancer cell lines show that inhibition of Hedgehog signaling leads to inhibition of proliferation, migration and apoptosis (15-17,21,22). It has also been demonstrated that inhibition of Hedgehog signaling on the level of GLI1 inhibits the growth of ovarian cancer xenografts (17,23,24), and growth of ovarian cancer spheroids (25).

We and others have shown that loss of heterozygosity (LOH) of the PTCH1 gene is a frequent event in various malignant and benign tumors of the ovary (26-30). Benign tumors, such as dermoids and fibromas, also show methylation of the PTCH1 promoter (31), but this does not seem to be the case for ovarian carcinoma (32).

In this study, we confirm that the Hedgehog signaling pathway is active in ovarian tumors, and that GLI1 and SUFU are associated with tumor type and FIGO stage. It seems that the activation occurs downstream of the membrane components of the pathway, suggesting non-canonical activation. We also demonstrate the activity in a primary tumor cell line developed from the ovarian tumor sample, which seems to be a superior model for study of the pathway compared to the established lines.

Materials and methods

Sample collection. Twenty-three samples of ovarian tumors [16 carcinomas (CA), 7 atypical proliferative (borderline) tumors (BDL)] and tissue from 9 healthy ovaries (OV) and 9 healthy fallopian tubes (FT) excised for reasons other than malignant transformation, were collected at the Department of Obstetrics and Gynaecology, University Hospital Centre Zagreb, School of Medicine, University of Zagreb. Of the 16 carcinoma samples, 3 were diagnosed at stage I, 2 at stage II, 7 at stage III and 4 at stage IV according to the International Federation of Gynecology and Obstetrics (FIGO) classification. Of 16 carcinomas, 4 were grade 1 and 12 were grade 3. By type, 12 were serous adenocarcinoma and 4 were clear cell carcinoma. Of the 7 borderline tumors, 1 was diagnosed at stage III and the remaining 6 were at stage I of FIGO classification. All patients gave their informed consent before the samples were taken, and samples were collected with the approval of the hospital's ethics committee. All tissue samples taken during surgery were immediately placed in a vial containing 1 ml RNALater solution (Invitrogen), kept at 4°C overnight, and RNA was extracted using TRIzol reagent (Invitrogen) the following day. RNA concentration was determined spectrophotometrically, and quality was checked on 1% agarose gel.

Quantitative real-time PCR (qRT-PCR). RNA was reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems), and qRT-PCR was performed on a CFX96 machine (Bio-Rad) using EVAGreen dye (Bio-Rad). Primers and conditions used were described previously (33,34). Expression levels for each gene were first calculated as ΔCt relative to the housekeeping gene RPLP0. Some samples showed no expression of a specific target after 40 cycles of qRT-PCR, but for purposes of statistical tests the Ct for these samples was set to 40 (35). Expression relative to the healthy fallopian tube tissue (ΔΔCt) was then calculated using the 2−ΔΔCt formula.

Cell culture experiments. Primary culture was established from a high-grade ovarian tumor sample (FIGO IIIc) as previously described (33) and maintained in DMEM+10%FBS, as was the human ovarian adenocarcinoma cell line SKOV-3. Both cultures were mycoplasma-free. Cells were treated with 7.5 μM cyclopamine (Toronto Research Chemicals), 3 ng/μl SHH protein (a kind gift from Professor A. Kenney, USA) for 96 h, 2.5 and 5 μM GANT-61 (Selleckchem, S8075) or 5 and 10 mM lithium chloride (LiCl, Kemika) for 48 h. For MTT assay, primary cells or SKOV-3 cells were plated in 96-well plates, and treated the following day. Cell proliferation was measured after 24, 48 and 72 h.

For transfection experiments, SKOV-3 cells were seeded in 6-well plates and transfected the following...
day with pcDNA4nLSmGli1 plasmid (a kind gift from Professor F. Aberger, Austria) using Lipofectamine reagent (Invitrogen). RNA was extracted 24 h post-transfection and analyzed using qRT-PCR.

For silencing experiments, cells were seeded in 6-well plates and transfected with siPTCH1 (Ambion, Silencer Select s11442) using siPORT reagent (Ambion) in DMEM without serum. After 24 h, the transfection was repeated, and 24 h later medium was changed to DMEM+10% FBS. For negative control, cells were transfected with negative control siRNA (Ambion, Silencer Select Negative Control #1 siRNA) in the same way. Cells were left to recover for additional 24 h and then RNA was extracted.

For migration analysis, cells were starved for 24 h in media without serum, and plated in Transwell migration chambers with or without cyclopamine (7.5 μM) or SHH protein (3 ng/µl). Serum-containing media (10% FBS) was used as chemoattractant in the lower chamber. Cells were fixed and stained with crystal violet 24 h (for the primary cells) or 4 h (for SKOV-3) after plating, since SKOV-3 were too migratory after 24 h and all plated cells migrated across the membrane. Minimum of 5 optical fields at x400 magnification were counted for each experiment.

Western blotting. Total proteins were extracted using standard methods and 50 µg was loaded on polyacrylamide gel. Primary antibodies were rabbit polyclonal anti-Gli1 (ProteinTech, 19949-1-AP, 1:600), rabbit polyclonal anti-Gli2 (Aviva Systems Biology, ARP31885_T100, 1:1000), rabbit polyclonal anti-PTCH1 (ProteinTech, 17520-1-AP, 1:600), mouse monoclonal anti-GSK3β (Cell Signaling Technology, #9832, 1:1000), rabbit monoclonal anti-phospho-Ser9 GSK3β (Cell Signaling Technology, #5558, 1:1000) and mouse monoclonal anti-β actin (ProteinTech, 60008-1-lg, 1:2000). Secondary antibodies were HRP-conjugated anti-rabbit (Santa Cruz Biotechnology, sc-2370, 1:2000) and anti-mouse (GE Healthcare, NA931V, 1:2000), and the signal was detected using SuperSignal West Pico and Femto (Thermo Fisher Scientific).

Immunofluorescent staining. Cells were grown on cover slides in DMEM complemented with 10% FBS and treated with 7.5 µM cyclopamine for 24 h. Cells were paraformaldehyde-fixed, permeabilized with methanol and heated in epitope-retrieval solution at 85°C for 10 min. The primary antibodies diluted 1:100 were: rabbit polyclonal anti-Gli1 (Santa Cruz Biotechnology, sc-20687), goat polyclonal anti-SUFU (Santa Cruz Biotechnology, sc-10933), rabbit polyclonal anti-HH (Santa Cruz Biotechnology, sc-9024) and goat polyclonal anti-PTCH1 (Santa Cruz Biotechnology, sc-6147). Secondary antibodies, at 1:100 dilution, were mouse anti-goat-FITC (Santa Cruz Biotechnology, sc-2356) and donkey anti-rabbit-TR (Santa Cruz Biotechnology, sc-2784). Nuclei were stained with DAPI. Negative control slides were treated in the same way omitting the primary antibody. Co-localization coefficient was determined using the ImageJ software v.1.45e with the Co-localization test plugin.

Statistical analysis. D’Agostino-Pearson test was used for testing the normality of the data distribution. Since data did not show a Gaussian distribution, non-parametric tests were used for statistical analyses. Kruskal-Wallis test with a post-hoc test for pairwise comparison of subgroups according to Conover were used to determine the association between gene expression (Ct values) and tumor type (ovarian carcinoma or borderline), FIGO stages (I-IV) or grade. Correlations between gene expression levels were assessed by Spearman’s rank correlation coefficient ρ and ranked according to Colton (36). Mann-Whitney test was used for comparing co-localization coefficients, while Kruskal-Wallis test was used to compare treated and non-treated values for the migration assay. Two-tailed P-values <0.05 were considered to indicate statistically significant differences. Statistical analyses were performed using MedCalc for Windows v.15.8 (MedCalc Software bvba).

Results

Gene expression of Hedgehog pathway genes (PtcH1, SMO, GLI1, GLI2, GLI3, SHH, SUFU) and tumor survival marker Survivin (BIRC5), was examined in tumor samples (CA or BDL), healthy ovary (OV) and healthy fallopian tube (FT). Hedgehog pathway genes were found expressed in the majority of ovarian tumor samples: PtcH1 (23/23, 100%), SMO (23/23, 100%), GLI1 (17/23, 73.9%), GLI2 (17/23, 73.9%), GLI3 (23/23, 100%).
SHH (21/23, 91.3%) and SUFU (22/23, 95.7%). GLI1 was not detected in OV or FT, while all the other genes were detected in both control tissues. BIRC5 expression was used as a tool for deciding on appropriate control tissue, since it is differentially expressed in healthy tissue compared to tumor tissue (4) and its downregulation has potential application in ovarian tumor therapy (37). BIRC5 was upregulated in both tumor types (22/23 samples, 95.7%), as well as in healthy ovaries, compared to the fallopian tube tissue where it was not detected. Therefore we decided that fallopian tube represents a better control tissue for ovarian carcinomas, which also corresponds to recent literature data (38).

GLI1 and SUFU were the only differentially expressed genes. Expression of GLI1 was significantly higher in BD l compared to CA (P=0.042) (Fig. 1A). The same was observed for SUFU (P=0.025) (Fig. 1B). When ovarian tumors were classified according to FIGO, we showed that higher FIGO stages had lower expression of SUFU (P=0.044) (Fig. 1C). Other tested Hedgehog pathway genes were not associated with either tumor type or FIGO, but similarly expressed between the categories. There was no difference in gene expression levels in carcinoma samples regarding the grade (data not shown).

Significant correlation was detected between the tested genes in the dataset of ovarian carcinoma samples. Weak or moderate correlation was detected between many components of the Hedgehog signaling, with excellent correlation between PTCH1 and SUFU (ρ=0.85, P<0.0001). Hedgehog signaling genes were also positively correlated with BIRC5 expression (GLI2, PTCH1 and SUFU). The correlation table also confirms the negative correlation between FIGO status and SUFU but also GLI1 and PTCH1 expression (for details see Table I).

To determine if the pathway is fully functional in ovarian tumors, we established a primary culture from one of the tumor samples (FIGO IIIC). Cyclopamine shows an inhibitory effect on primary cell growth 24 h after treatment, but cells quickly recover and achieve the proliferation equal to non-treated cells after 48-72 h (Fig. 2A). The effect on primary culture is stronger than on the ovarian tumor cell line SKOV-3, although the pattern is similar (Fig. 2B). SHH protein shows no additional proliferative effect on either primary cells or the SKOV-3 cell line (Fig. 2A and B). On gene expression level, cyclopamine treatment of the primary culture induces significant downregulation of Hedgehog pathway genes PTCH1, GLI1 and GLI2, while GLI3 remains mostly unchanged (Fig. 2C). The gene expression level for the tested genes remains unchanged in cylopamine-treated SKOV-3 cell line (Fig. 2D). SHH treatment of the primary culture shows no change in gene expression of most pathway genes, except an upregulation of GLI2 expression (Fig. 2C). The SKOV-3 cell line is also mostly unresponsive to SHH stimulation, and even shows downregulation of GLI2 and GLI3 genes (Fig. 2D).

To establish the effect of GLI1 upregulation, cells were transiently transfected with GLI1. The primary culture was difficult to transfect, and only a 20-fold increase in GLI1 expression was achieved. This proved to be too weak to demonstrate the effect of pathway activation, but a slight upregulation was detected (Fig. 2E). Therefore we tested the effect of GLI1 transfection on SKOV-3 cell line, which was easier to transfect. Apart from the expected upregulation of GLI1, other Hedgehog signaling

<table>
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<th>GLI2</th>
<th>GLI3</th>
<th>PTCH1</th>
<th>SHH</th>
<th>SMO</th>
<th>SUFU</th>
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*Spearman rank correlation coefficient ρ. Statistically significant P-values are in bold.*
Pathway genes PTCH1 and GLI2, are also upregulated after transfection with GLI1. GLI3 levels remain unaltered, showing upregulation of Hedgehog pathway activity (Fig. 2F).

PTCH protein is the negative regulator of Hedgehog signaling. Therefore, PTCH1 silencing should increase Hedgehog functional pathway activity in cells. In the primary cell line, upon PTCH1 silencing GLI1 and GLI2 are upregulated and GLI3 downregulated (Fig. 2G). For the SKOV-3 cell line, GLI1 is also upregulated upon PTCH1 silencing, but GLI2 is downregulated while GLI3 is upregulated (Fig. 2H).

Immunofluorescent staining of the primary cells shows nuclear localization of GLI1 in non-treated conditions, while SUFU is located mostly at the nuclear periphery and the cytoplasm. After treatment with cyclopamine GLI1 shows a tendency to move to the nuclear periphery and cytoplasm and co-localizes with SUF (Fig. 3A), with significant increase of the co-localization coefficient (P=0.0495) (Fig. 3C). In non-treated conditions SHH shows cytoplasmic localization, while PTCH1 localizes to the cytoplasm with slightly more intense staining in the region of SHH, suggesting their interaction.
After cyclopamine treatment PTCH1 is located in different parts of the cytoplasm and ceases to co-localize with SHH (P=0.021) (Fig. 3B and D).

Although SHH protein treatment shows no effect on the level of gene expression of Hedgehog pathway genes, its effect is noticeable in migration assays. SHH protein treatment results in significant increase in cell migration in both the primary cells and in the sKOv-3 cell line (P=0.045 and P=0.009, respectively), while cyclopamine shows no effect on cell migration (Fig. 3E and F).

Since cyclopamine treatment affected both the primary and SKOV-3 cell lines only in the first 24 h of treatment, after which their proliferation rate returned to the level of non-treated cells we decided to test for potential non-canonical activation. We tested the responsiveness of cells to pathway modulation downstream of SMO. Lithium chloride is an inhibitor of GSK3β (39), a protein kinase involved in GLI protein processing into activator or repressor forms, while GANT-61 is a specific Gli inhibitor. Both LiCl and GANT-61 decrease the proliferation rates of the primary and SKOV-3 cell lines after 72 h (Fig. 4A and B). On the protein level, it can be noted that LiCl increases the Ser9 phosphorylated fraction of GSK3β in both cell lines as we expected, while there is almost no change in total GSK3β levels in either cell line (Fig. 4C). Our laboratory has previously shown that dysregulated GSK3β can cause upregulation of the Hedgehog pathway (40) and increasing the fraction of Ser9 phosphorylated GSK3β promotes GLI3 processing into the transcriptional repressor form and subsequent downregulation of the pathway. After treatment with LiCl a decrease in the levels of GLI3 activator form (GLI3A) can be noted in both cell lines. The GLI3 repressor form (GLI3R) was undetectable under these conditions in the SKOV-3 cell line, but in the primary cell line an increase in GLI3R levels can be observed after treatment with 10 mM LiCl (Fig. 4C). Moreover, levels of PTCH1, a marker of Hedgehog pathway activity, are decreased in both cell lines after LiCl treatment, indicating signaling downregulation (Fig. 4C). GLI inhibition with GANT-61 causes a decrease in GLI2 protein levels in both the primary and SKOV-3 cell line, as well as a decrease in the levels of PTCH1 protein indicating pathway downregulation. A decrease in GLI3A levels is detected in both cell lines, and a decrease in GLI3R levels in the primary cell line (Fig. 4D). GLI1 is weakly expressed in both cell lines and was undetectable on western blotting.

**Discussion**

Hedgehog pathway genes are expressed in the majority of ovarian tumor samples, with *GLI1* and *SUFU* differing between...
tumor types, and SUFU between FIGO stages. Majority of studies examining Hedgehog signaling used healthy ovaries as control tissue, which makes sense when examining the tumors immunohistochemically. However, ovarian carcinoma can arise from either the fallopian tube or the ovary itself, with each tissue giving rise to different tumor type (41). For this research we used both types of controls: healthy ovary samples and healthy fallopian tube samples. For our purposes FT controls seem a better control than the OV samples, although the differences between the two control groups were mostly not significant. The only difference between FT and OV groups was observed for BIRC5 expression, where FT has no expression of the gene, while OV expresses it. Since Survivin is a protein associated with normal function of mitotic spindle, and the ovarian tissue undergoes periodic cycles of cell division, some expression of the protein is to be expected in OV.

There is not much data on Hedgehog pathway expression in the healthy human ovary. The groups that used OV as control tissue for immunohistochemical staining report no or weak signal of Hedgehog pathway proteins (15,17,20). Studies on mouse ovaries show reactivation of the pathway in the adult ovary (12). Since tissues in the adult ovary go through periodical phases of proliferation, it is not surprising to detect Hedgehog pathway activity. Still, we did not detect the major effector of the pathway, GLI1, in the OV or FT. Given that expression of PTCH1 and SMO were detected, it is possible that in these tissues the Hedgehog signaling is poised for reactivation, and requires a specific stimulus to become active. The key proteins that seem to be involved in pathway upregulation in clinical samples are GLI1 and SUFU protein, suggesting non-canonical activation of the pathway in ovarian tumors. The expression of pathway genes GLI2, PTCH1 and SUFU is correlated with BIRC5 expression in these samples. This, taken together with the recent work by Vlčkova et al (9), suggests that Hedgehog signaling regulates BIRC5 expression in ovarian tumors as well. Our group has previously demonstrated a very similar correlation of Hedgehog signaling with BIRC5 expression in oropharyngeal squamous cell carcinoma (PTCH1 positively and SHH negatively correlated with BIRC5 expression) (34).

Vlčkova et al also demonstrated that the major regulator of BIRC5 expression in their luciferase model system is GLI2 protein, followed by GLI3 (9). In our samples we detect the expression of all three GLI genes, but the only one that seems to be associated with tumor progression is GLI1. However, it must be taken into account that regulation of activity of GLI proteins occurs on post-translational level, so gene expression may not be the best choice for monitoring these effects. Protein expression, on the other hand, is limited by quality of antibodies currently on the market, and their inability to distinguish between activator and repressor forms on tissue sections. Experiments on primary cells developed from ovarian carcinoma tissue demonstrate that the Hedgehog signaling pathway can be temporarily downregulated using cyclopamine, and cell proliferation is delayed but not completely inhibited. This is further supported by the retention of GLI1 and SUFU in the cytoplasm and reduced PTCH1-SHH co-localization following cyclopamine treatment. SKOV-3 cell line also shows a weak effect of cyclopamine on cell proliferation (a slight delay), but no effect on gene expression level. Exogenous SHH stimulation shows no direct effect on gene expression of Hedgehog pathway genes, but it positively affects the migratory potential of the cells. It is possible that the SHH ligand binds to an alternative target and stimulates cell migration, as we have shown recently on breast cancer (42), and this effect should be investigated further. Endogenous stimulation of the pathway by GLI1 results in upregulation of PTCH1 and GLI2/3. Silencing of PTCH1, a negative regulator of the pathway, also upregulates signaling,
Hedgehog signaling pathway was downregulated. GANT-61 both cell lines decreased after treatment with GANT-61, and the literature does not affect GLI3 (47,48). The proliferation rate of GANT-61 inhibits GLI1 and GLI2 proteins but according to inhibition with GANT-61 was very similar to the effect of LiCl. cated by decreased PTCH1 protein levels. The effect of GLI can be modulated through GSK3β downregulated Hedgehog signaling, after LiCl treatment, indicated in the primary cell line. Nevertheless, both cell lines showed decreased in both cell lines. However, the effect of increased processing of GLI3 into repressor form was only observed in the primary cell line. Nevertheless, both cell lines showed downregulated Hedgehog signaling, after LiCl treatment, indicated by decreased PTCH1 protein levels. The effect of GLI inhibition with GANT-61 was very similar to the effect of LiCl. GANT-61 inhibits GLI1 and GLI2 proteins but according to literature does not affect GLI3 (47,48). The proliferation rate of both cell lines decreased after treatment with GANT-61, and the Hedgehog signaling pathway was downregulated. GANT-61 significantly decreased GLI2 protein levels in both cell lines, which in turn caused a decrease in PTCH1 protein levels. These results indicate that the activity of Hedgehog signaling pathway can be modulated through GSK3β inhibition in ovarian cancer cells, as well as through direct GLI inhibition.

Downstream effectors play a role in regulation of GLI proteins, and may be the key to successful Hedgehog pathway modulation. For example, it has recently been shown that diindolylmethane inhibits GLI1 and leads to anoikis of ovarian cancer cells (24), and GLI proteins are becoming preferred therapy targets since they can inhibit both canonical and non-canonical Hedgehog signaling (49). All these results signify that the signaling pathway is fully functional in ovarian carcinoma primary cells and could be targeted by compounds modifying Hedgehog signaling. Finally, primary cell cultures seem to be a superior model compared to the cell line SKOV-3. It has been shown that ovarian cancer cell lines show great genomic instability, which definitely affects their reliability in cell culture experiments and explains the discrepancy in results obtained by different research groups (50). The primary culture more accurately reflects conditions in vivo, but has a limited lifespan.

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