Journal of Cancer Research & Therapy

García-López R et al., J Cancer Res Ther 2013, 1(1): 11–23
http://dx.doi.org/10.14312/2052-4994.2013-3

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Sonic hedgehog inhibition reduces *in vitro* tumorigenesis and alters expression of Gli1-target genes in a desmoplastic medulloblastoma cell line

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Abstract

Medulloblastoma is one of the most frequent and aggressive tumors of childhood. The Sonic hedgehog (Shh) pathway, related to human development, is altered in most medulloblastomas: genes like Ptch, Smo, or Sufu suffer mutations in 15% to 25% of these tumors. We tested Shh inhibition in the Daoy medulloblastoma cell line by two methods: a molecular one, direct Gli1 siRNA inhibition; and a pharmacological inhibition of Smo, upstream of Gli1, by cyclopamine. Afterwards, a comparison of cellular and molecular responses was done. In general, we proved that cell viability, cell migration and cell colony formation decreased after Shh inhibition, which might confer a less tumorigenic status to Daoy cells. Moreover, we assessed the expression of different Gli1 target genes and other genes and found that Shh shows a crosstalk with oncogenes and tumor suppressor genes that have been described in numerous tumors. All these experiments give an overview of the Shh pathway in medulloblastoma, together with the demonstration of the efficacy of cyclopamine and Gli1 siRNA Shh inhibition *in vitro*.

Keywords: sonic hedgehog; cyclopamine; siRNA; Gli1; Ptch; Smo; *in vitro* tumorigenesis; medulloblastoma; Daoy

Introduction

Medulloblastoma (MB) is the most common tumor of the posterior fossa in childhood, representing 30% to 40% of tumors in this age group and one of the most common pediatric solid tumors (15% to 25%) with an annual incidence of about 6.5 cases per million population in Europe [1]. Incidence is significantly higher in boys than in girls (approximately 60% in boys). Although its world incidence is lower than in other tumors, the treatment of these tumors that includes surgical resection, craniospinal radiation and chemotherapy only cures 60% of the affected children and most suffer from long-term side effects derived from the aggressiveness of the treatment [2, 3]. Due to limitations of the current treatment, the understanding of signaling pathways involved in medulloblastoma pathogenesis might lead to the design of new targeted treatments against medulloblastoma.

The most important signaling pathways that correlate with medulloblastoma development are those that regulate the formation of granule cell neurons and neural stem and precursor cell proliferation in the central nervous system (CNS), allowing a medulloblastoma classification that could also serve as a guide for successful anti-neoplastic therapy. According to the current consensus nomenclature [4], Wingless (Wnt) signaling pathway gives the name to a subgroup of medulloblastomas, as does the Sonic hedgehog (Shh) pathway. Alterations in both affect 30% to 40% of medulloblastomas [5, 6]. Mutations activating the Wnt pathway member beta-catenin are more common than adenomatous polyposis coli (APC) loss in sporadic medulloblastoma and are found in 5% to 10% of cases

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Received 2 November 2012 *Revised* 5 January 2013 *Accepted* 13 January 2013 *Published* 20 January 2013

Citation: García-López R, Vera-Cano B, Vacas-Oleas A, de la Rosa J, Gallo-Oller G, Alonso MM, Rey JA, Castresana JS (2013) Sonic hedgehog inhibition reduces *in vitro* tumorigenesis and alters expression of Gli1-target genes in a desmoplastic medulloblastoma cell line. J Cancer Res Ther 1: 11–23. doi:10.14312/2052-4994.2013-3

[7, 8]. The two remaining subgroups of medulloblastoma classification (groups 3 and 4) are based on a clustering algorithm [9] and are different in terms of demographics, histology, DNA copy-number aberrations and clinical outcome [10]. A number of other genes important in cerebellar stem and progenitor cells are deregulated in medulloblastoma. These genes include the oncogene N-myc, Bmi-1 and Otx2, bone morphogenic proteins (Bmp), the Notch cell signaling pathway and others.

The Shh signaling pathway is a major mitogenic regulator of granule neuron precursor cells (GNPCs) [11]. The mitogen Sonic hedgehog (Shh) drives proliferation of GNPCs by binding to the 12 transmembrane receptor Patched (Ptch) [12]. In the absence of Shh, Ptch represses the function of Smoothened (Smo), a seven transmembrane G-protein-coupled receptor-like protein that activates the Gli1 and Gli2 transcription factors and inactivates the transcriptional repressor Gli3 that together regulate the transcriptional program in the cell nucleus triggering transcription of several Shh target genes including Gli1, Ptch1, Hh interacting protein (Hhip), Cyclin D2, N-Myc, Bmi-1 and Bcl-2 [11, 13]. In addition, transfection of Gli1 results in decreased expression of Plakoglobin, Embigin and TSC-22 [14]. The transduction cascade from Smo to Gli is regulated at several different levels by activators such as Dyrk1, IFTs, Ras and Akt and inhibitors such as Sufu, Ren, PKA and GSK3β (Figure 1).

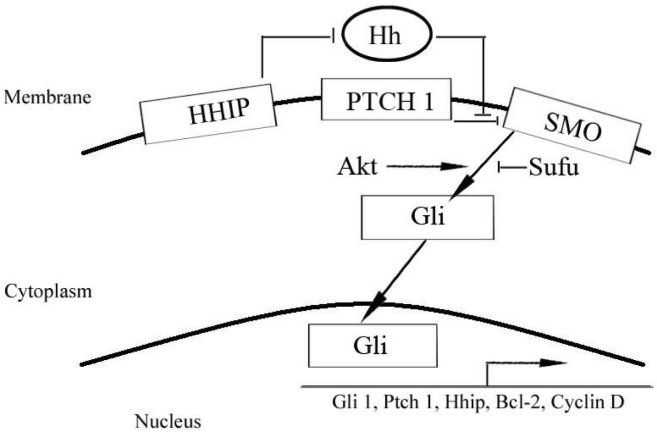


Figure 1 The sonic hedgehog pathway regulators.

Mutations in Shh pathway genes are found in approximately 25% of medulloblastomas [15]. Particularly, somatic mutations in the Ptch receptor, which result in activation of the Shh pathway, have been identified in 15% of cases. The Shh pathway genes Smo and Sufu, suppressor of fused (protein of Gli Drosophila analog complex), are also occasionally mutated in human medulloblastoma [16, 17].

Moreover, there are certain homeobox transcription factors such as NK2 homeobox 2 (Nkx2.2) and paired box gene 6 (Pax6) that are related to Shh. Nkx2.2 is induced by Shh signaling during neuronal development [18], while Pax6 is inhibited by Shh signaling during neuronal embryonic development. Other associated molecules are secreted frizzled-related protein 1 (SFRP1), downregulated by Shh signaling [19], Bmi-1 that shows overexpression in human medulloblastoma [20] and hedgehog interacting protein (HHIP) that functions as an endogenous antagonist for Shh [21].

Medulloblastoma associates with an inappropriate Shh-Gli activity [22]. Therefore, it may be sensitive to treatments with Shh pathway inhibitors such as cyclopamine, a plant-derived alkaloid that antagonizes this pathway by blocking Smo which triggers Gli to bind DNA and express its target genes [23]. From a genetic approach the pathway can be inhibited by siRNA against Gli1 resulting in a high and specific knockdown.

Therefore, our working hypothesis is that directly targeting Smo and Gli1 will enable us to understand better the core pathway its regulation and role in desmoplastic medulloblastomas.

In general, the aim of this work was to analyse the role of the Sonic hedgehog signaling pathway and its connections with other genes which regulate the proliferation and tumor development of medulloblastoma. An adhesive cell line as an initial study was used to be treated with down stream inhibitor (siRNA against Gli1) and also to prove the effect of a pharmacological treatment by an upstream antagonist (cyclopamine). In this sense, the two treatments were compared performing molecular and cellular trials using a desmoplastic medulloblastoma cell line, including assessment of cell proliferation, *in vitro* colony forming tumorigenic capacity, viability, and gene expression. All these experiments will give an overview of the Shh pathway in medulloblastoma, together with the possibility to demonstrate the efficacy of cyclopamine versus Gli1 siRNA and whether it could be a good strategy against human medulloblastoma in the near future.

Materials and methods

Cell line

We used a desmoplastic medulloblastoma cell line: Daoy. This was purchased from the American Type Culture Collection, Manassas, VA, USA. The cell line was cultured in RPMI L-glutamax medium (Gibco-BRL, Gaithersburg MD, USA), supplemented with 10% fetal bovine serum, 4% non-essential amino acids, 1% penicillin, and 0.1% amphotericin B and maintained in an incubator at 37°C in an atmosphere with 5% CO_2 to expand the cells. Subculture of cells was performed after 80% confluence with the help of trypsin/EDTA (Gibco).

RNA extraction and retrotranscription

RNA was extracted using the RNAeasy KIT (with DNAsa treatment included) (Qiagen) following manufacturer's instructions. Purity and quantity of total RNA was determined by nanodrop measurement. Next, 2 µg RNA were retrotranscribed in a final volume of 20 µL. RNA was preincubated at 72°C for 10 min with 250 µg random

primers to eliminate secondary structures. After chilling on ice, 1x RT-buffer synthesis buffer was added, 0.01 M DTT and 0.5 mM of each dNTP. The reaction mixture was heated for 2 min at 42°C for random primer annealing. Then, 1 U SuperScriptTM II Reverse Transcriptase was added. cDNA was synthesized at 42°C for 40 min and stored at -20°C until use.

Quantitative RT-PCR

For qRT-PCR of HPRT, Gli1, Ptch1, Smo, Sufu, Gli3, cyclin D2, plakoglobin, Nkx2.2, Bmi-1, Pax6, N-myc, Notch1 and Notch2, the amplification reactions were carried out in an IQ5 multicolor real-time PCR detection system (BioRad, Hercules, CA, USA). 75 ng of template DNA were used in a total volume of 25 µL, the reaction mix contained 12.5 µL 2X IQTM SYBR Green Supermix and 12.5 pmol forward and reverse primers (primer sequences are shown in Table 1). An initial denaturation step at 95°C for 10 min was followed by 40 cycles of amplification alternating between 95°C for 30 s, the corresponding annealing temperature for each gene for 30 s and 72°C for 30 s. Each sample was assayed in triplicate. The relative amount of target transcripts was normalized to the number of human HPRT transcripts found in the same sample. They were quantified using an efficiency corrected quantification model to obtain the derivative ratio values:

$$ratio = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}}(control-sample)}}{(E_{\text{ref}})^{\Delta CT_{\text{ref}}(control-sample)}}$$

Table 1 Sequences and annealing temperatures of primers used for the qRT-PCR expression assay

Gene	*Primer (5′-3′)*		*Ta [1] (°C)*	*Bp[2]*
	Forward	*Reverse*		
HPRT	ATGGTGGGTTGTGCTTTCC	AGTTGTGTTTGTGCTTTCTGCT	60	123
Gli1	CAGTGTGGGGACAGAAGGA	CGGGGAGAAGAAAAGAGTGG	57.4	132
Gli3	ATGGACCCCAGGAATGGT	CAACCTTCTTGCTCACACATGTAAG	64	167
Ptch1	CTTCGCTCTGGAGCAGATTT	CAGGACATTAGCACCTTCT	55	354
Cyclin D2	GAAGGACATCCAACCCTACA	TCTTCTTCGCACTTCTGTTCC	58.5	81
Plakoglobin	ACGCTCAAGAAAACCACCAC	GTCCTCGCCTGACACACC	58	132
Nkx2.2	TCTACGACAGCAGCGACAAC	CGCCTTGGAGAAAAGCACTC	60	221
Smo	AAGGCTGCACGAATGAGGT	GGGTTCTGGCACTGGATG	57	133
Sufu	CCTCCAGATCGTTGGTGTCT	CCCCTCCGCATGTCAGTT	65	128
Pax6	ATGCCTCATAAAGGGGGAAG	AGTTGTGTTTGTGCTTTCTGCT	60	112
Bmi-1	ATGCCTCATAAAGGGGGAAG	AGTTGTGTTTGTGCTTTCTGCT	60	195
Notch1	ATTGTTCGTTACCTGGAGACC	GGCAGCATCAGCAGAAGG	64	243
Notch2	GAGGCGACCGAGAAGATG	CCTGTGCCATTGTGGTAGG	63.7	338
Myc-N	CCCTGAGCGATTCAGATGAT	AATGTGGTGACAGCCTTGGT	64.3	158

[1] Annealing temperatures.
[2] Base pairs of the amplified product of primers.

MTT assay and cyclopamine treatment

MTT assay was performed as follows. After preparing serial dilutions of cells in culture medium 5 × 10^3 cells were plated out, in triplicate, in 200 μl of the dilutions (medium and cyclopamine in DMSO) into wells of a micro-titer plate. Eight control wells of medium alone were included to provide the blanks for absorbance readings. Cells were incubated under growing conditions with 15 μM cyclopamine (Sigma Aldrich, UK) dissolved in DMSO for 24 and 48 h. Fifty μl of 3-(4, 5-dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide (MTT) reagent were added to each well, including controls. Then, plates were further incubated for 2–4 h. When the purple precipitate was clearly visible under the microscope, we removed the medium from each well and added 100 μl of detergent reagent (DMSO) to all wells, including controls. After gently swirling (not shaking), we left plates for 15-30 min at room temperature. The absorbance was measured in each well, including the blanks, at 570 nm in a micro-titer plate reader (Perkin-Elmer, Boston, MA). The experiment was performed for three independent times. In our experiment model for cyclopamine treatment,

cells cultured in normal medium were seeded in 6-well plates at a density of 250,000 cells/well. After 24 h, the medium was changed to low serum condition (2.5% FBS). Then, the cells were treated with 15 μM of cyclopamine dissolved in DMSO for 48 h. Cells were also treated with DMSO alone as control. The amount of DMSO never exceeded the 1% of the total volume as 1% DMSO alone showed no cytotoxicity.

Gli1 siRNA knockout

For the transfection study, we took the Daoy medulloblastoma cell line grown in supplemented RPMI media with 2% fetal bovine serum at 37^{0}C with 5% CO_2. Moreover, we obtained a pool of siRNAs against Gli1 gene from Stealth™ (Invitrogen, Carlsbad, CA) and mixed them: Gli1 siRNA:

GCACAUACCUGCUUCGGGCAAGAUAU (GLI-HSS104170),
AUAUCUUGCCCGAAGCAGGUAGUGC (GLI-HSS104170),
CAGCUUGGACGAGGGACCUUGCAUU (GLI-HSS178441),
AAUGCAAGGUCCCUCGUCCAAGCUG (GLI-HSS178441),
GGGAUGGCUGCAGCCAGGAAUUUGA (GLI-HSS178442),
UCAAAUUCCUGGCUGCAGCCAUCCC (GLI-HSS178442).

We delivered siRNAs against Gli1 (100 nM/well) and its negative control to the Daoy cell line using Lipofectamine™ 2000 as a transfection reagent and Opti-MEM® I reduced serum media. The transfections were conducted on six well plates. RNA was extracted from transfected, negatively transfected and untransfected cells after 48 h. The extracted RNA was used for further analysis of expression levels of the downstream target genes of the Shh pathway. The experiment was performed in triplicate.

Scratching assay for cell migration

The Daoy monolayers from untransfected or untreated, DMSO or Lipofectamine 2000 and cyclopamine or siRNA-treated dishes were suspended by treatment with trypsin/EDTA (Gibco) and approximately 2.5 × 105 cells in 2 ml of medium were seeded into wells of a 6-well plate and grown for 24 h to about 90% confluent in medium containing 10% FBS. Next, cell migration was assayed by evaluating closure of a linear wound produced by scratching the cell monolayer on a 6-well plate in 2.5% fetal bovine serum plus medium. The open gap was then inspected microscopically over time as the cells move in and fill the damaged area. To analyse the cell migration we took pictures at 0, 6, 12, 24 and 48 h and the cells were kept in the incubator at 37^{0}C in an atmosphere with 5% CO_2 while waiting between pictures. Therefore, we evaluated cell migration in pre-treated cells.

Colony formation assay in culture

This assay was performed to evaluate the ability of pre-treated Daoy cells (at the same conditions as before) to create a colony in monolayer cultures. Given that Daoy cells grow adhered to a layer, the test would take less time than in soft agar. 2.5 × 10^2 Daoy cells per well of both conditions were cultured in a 6-well plate for 10 days. After that, we discarded the medium, cells were fixed 30 min with 4% paraformaldehyde. They were then stained with 1% Crystal Violet (Sigma Aldrich, UK) for 15 min and counted. The experiment was performed in triplicate.

Colony formation assay in soft agar

Pre-treated Daoy cells were mixed with 0.4% Noble agar (in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum) and plated at 5000 cells/well into 6-well plates containing a solidified bottom layer (1% Noble agar in the same growth medium). After 20 days, colonies were stained with 250 μL of 2.5% Crystal Violet (Sigma Aldrich, UK) for 5 min, washed 4-5 times with PBS to remove excess of dye and finally photographed. The experiment was performed for 3 independent times for the cell line cultured in medium with or without cyclopamine in DMSO or siRNA in Lipofectamine 2000.

Statistical analysis

Statistical analysis was performed using SPSS Statistical Software (Chicago, IL, USA). The data represent mean ± SEM. One-way analysis of variance (ANOVA) was used to compare the controls and treated or transfected group of three independent soft agar clonogenic assays. The same analysis was performed in MTT assay comparing cyclopamine treatment with untreated cells. Differences were considered statistically significant when the p value was less than 0.05 and highly significant when the p value was less than 0.01. Remaining studies were performed in triplicate.

Results

Cyclopamine and Gli1 siRNA reduce Daoy cell viability

We investigated the concentration of cyclopamine that could inhibit cell growth in these cells. The MTT cell proliferation assay was performed to measure the cell proliferation rate when Daoy cells were treated with cyclopamine at different concentrations (from 1 to 50 μM). This assay allowed us to obtain the IC_{50} of cyclopamine to be used for next cellular and molecular studies. We took 15 μM as cyclopamine IC_{50} as it was the closest and less

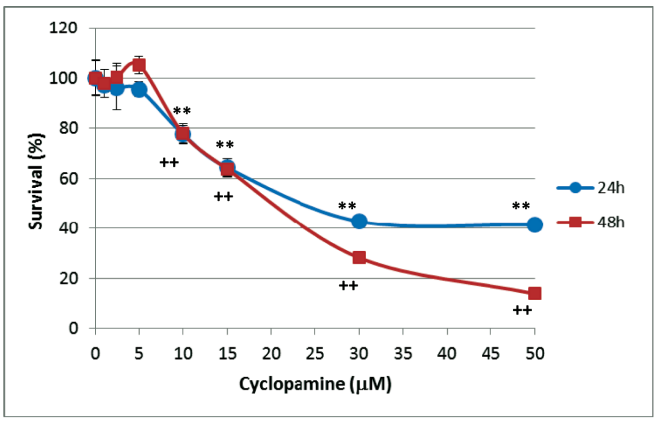


Figure 2 Cyclopamine inhibits growth of Daoy cell line. Sensitivity of Daoy cells to different concentrations of cyclopamine (1; 2.5; 5; 10; 15; 30 and 50 μM) dissolved in DMSO compared to controls (none or DMSO-treated cells) was assessed by the MTT assay. Data reflect the means ± SEM of three independent experiments. **P < 0.01 for 24 h and $^{++}$P < 0.01 for 48 h.

concentrated value to the real IC_{50} (Figure 2). In addition, this concentration showed the same effect at 24 or 48 h. The differences observed from 10 to 50 μM were highly significant. The amount of DMSO never exceeded 1% of the total reaction volume.

Downregulation of Gli1 expression in Daoy by siRNA or cyclopamine

We evaluated the effects of direct silencing of Gli1 expression on cell behavior by transfecting cells with a three-pool Gli1 siRNA or a control siRNA for 48 h (Figure 3A). In addition, we compared Gli1 siRNA with a direct inhibitor of Smo receptor (cyclopamine) that acts upstream of Gli1 (23). Surprisingly, the chemical approach, 15 μM of cyclopamine, resulted in 87% reduction of Gli1 mRNA expression in Daoy cells compared to untreated cells and around 70% reduction compared to DMSO (Figure 3B). Gli1 siRNA decreased 94% of Gli1 mRNA levels (Figure 3A).

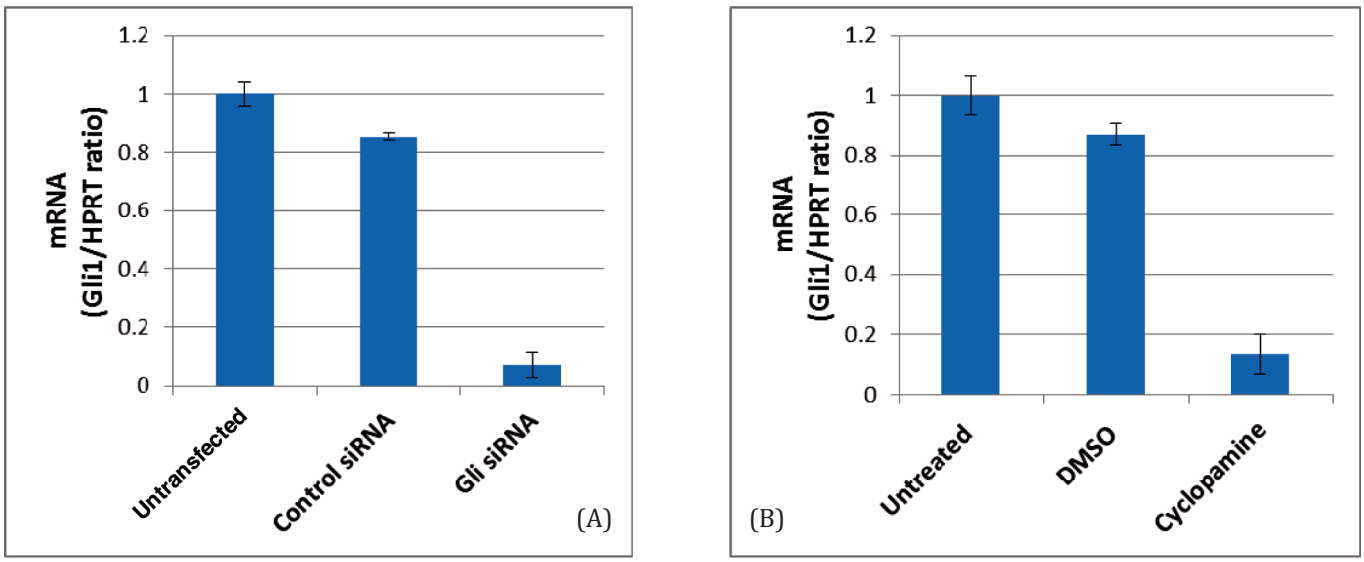


Figure 3 Knockdown of Gli1. (A) Gli1 siRNA highly reduces Gli1 in Daoy cell line. (B) Cyclopamine inhibition produces a Gli1 expression which is two-fold Gli1 mRNA levels managed by Gli1 siRNA as determined by quantitative RT-PCR (representative of triplicate experiments). Values represent the mean levels of Gli1 mRNA ± SEM of untransfected/untreated and Gli1 siRNA/cyclopamine in DMSO-treated cells related to control siRNA/DMSO (cyclopamine solvent)-treated Daoy cells. The derivative ratio values describe the relative expression change of the target gene relative to the HPRT reference gene expression.

Shh inhibition reduces in vitro tumorigenesis of Daoy cells

We cultured non-transfected and siRNA transfected Daoy cells, either control or Gli1, in 6-well plates where they anchored. After 10 days, we detected less number of colonies (Figure 4A) that corresponded to a 70% decrease in colony formation (Figure 4B, left panel). Likewise, cells treated with cyclopamine or with DMSO showed similar results (Figure 4B, right panel).

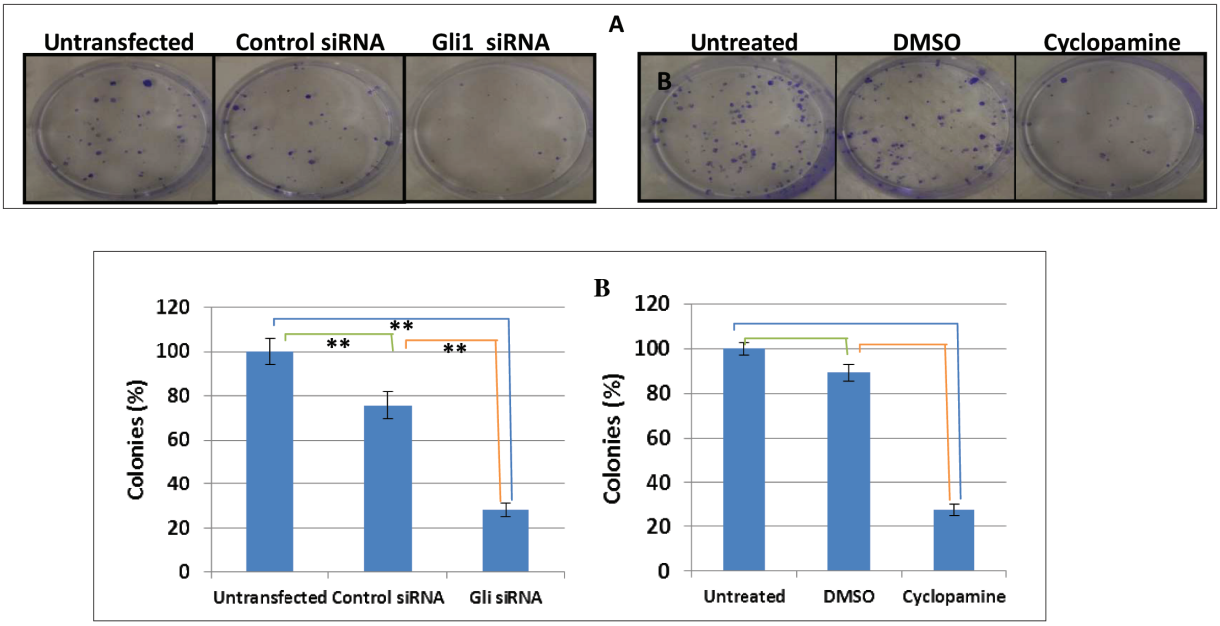


Figure 4 Effects of Gli1 downregulation on the ability to form colonies. (A) The colonies formed after 10 days were stained with 0.5% crystal violet solution (left and right panels). (B) Number of grown colonies of all conditions was counted visually (left and right panels). Data reflect the means ± SEM of triplicate experiments (*$p < 0.05$; **$p < 0.01$).

In the same way, we sought to determine the tumor formation ability using an *in vitro* 3-dimensional colony formation assay in soft agar media (Figure 5) to better simulate the process of *in vivo* carcinogenesis.

This analysis demonstrated that cells underexpressing Gli1 (by either siRNA or cyclopamine) formed distinct microscopic three-dimensional colonies embedded in different layers of soft agar (Figure 5A). The number of colonies was counted under a microscope and plotted in Figure 4B. Interestingly, there were very few small colonies in the plates containing untransfected or untreated cells as well as control siRNA and DMSO treatment and almost no colonies were observed in the Gli1 siRNA transfected or cyclopamine treated Daoy cells (Figure 5A-B). These observations demonstrated that Gli1 downregulation reduced *in vitro* tumorigenesis of Daoy cells. The differences found between groups were highly significant. The reduction in number of colonies and sizes of the 3D-growth demonstrated potential roles of Gli1 in 3D-colony formation and tumorigenesis.

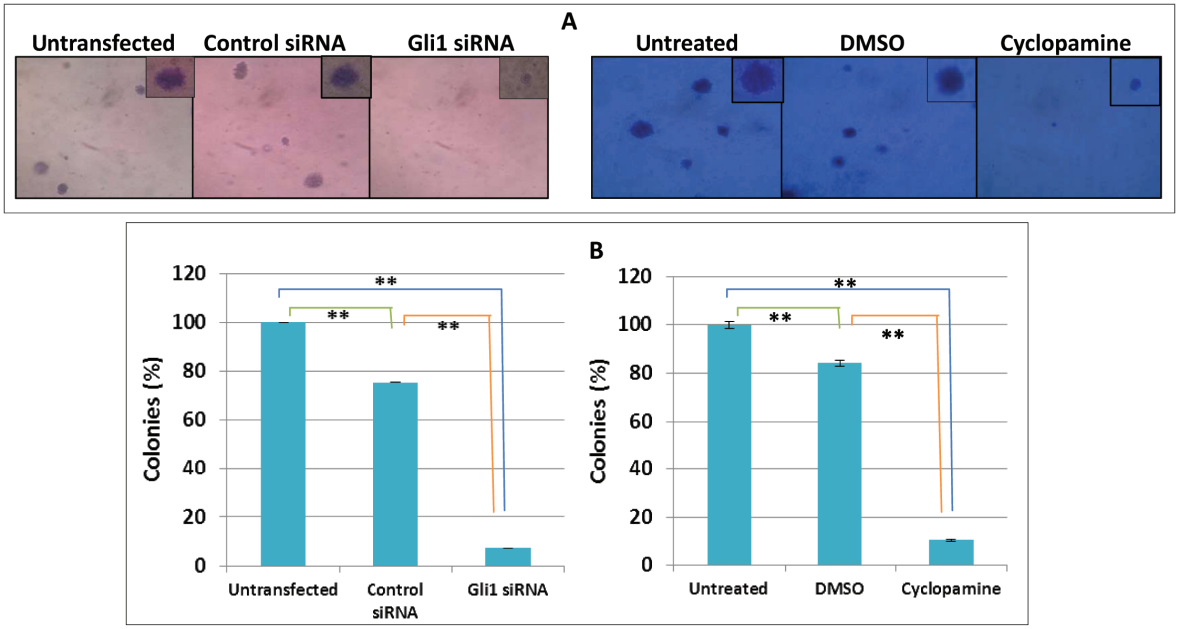


Figure 5 Cyclopamine and siRNA highly prevent tumorigenic growth. (A) Microscopic view of colonies with a magnification of x100 (big images) and x250 (their expanded pictures after 20 days). (B) Percentages of colonies were obtained after counting five-microscopic fields of each condition and compared with untransfected/treated control. Data reflect the means ± SEM of triplicate experiments. **p < 0.01.

Gli1 silencing impairs Daoy cell migration

Since targeting Gli1 inhibited cell viability and decreased tumorigenic growth in Daoy cells, we investigated the effect of Gli1 silencing on the migration potential of Daoy cells using an *in vitro* wound-healing assay, frequently used as a simple assay to mimic and assess migration [24]. In this assay, an artificial wound is created by scratching a confluent monolayer of cells, while the ability of the cells to move into and close the wound is thought to predict their migration ability *in vivo* [24]. Therefore, after culturing Daoy cells from all six conditions (untransfected; control siRNA; Gli1 siRNA and untreated; DMSO; cyclopamine in DMSO) for 24 h they were scratched. From that moment (0 h) we evaluated Daoy migration ability for 48 h. Here, we can see a huge different behavior between controls and siRNA or cyclopamine treatments supporting our hypothesis (Figure 6). Surprisingly, cyclopamine did not only decrease cell migration, but also diminished initial confluence (Figure 6, right panel).

qRT-PCR expression of Shh pathway components and related genes

After transfection with a three-pool siRNA against Gli1 and treatment with cyclopamine, we first validated the success on downregulating Gli1 by qRT-PCR. Next, we attempted to determine the expression of Gli1 target genes, including Ptch1, Cyclin D2, Plakoglobin, Pax6, Nkx2.2 and Bmi-1. Furthermore, we analysed components of Shh signaling pathway such as Smo, Sufu and Gli3. Focusing on Shh pathway crosstalks that have been reported recently to be related with N-myc, Notch1 and Notch2 (25, 26). Here, we found that Gli1 inhibition by siRNA or Smo antagonist produced downregulation of Ptch1, Cyclin D2, Plakoglobin, Nkx2.2, Bmi-1, Smo and N-myc (Figure 7A, B, C, D, E, G and J respectively). With connection with Sufu and Gli3, they showed parallel results, where Gli1 siRNA did neither decrease nor increase expression of both genes, whereas cyclopamine reduced them in 15-25% (Figure 7H and I). Pax6 and Notch1 mRNA levels were up-regulated by either Gli1 siRNA or cyclopamine (Figure 7F and 7K). However, Notch2 was decreased by Gli1 siRNA and increased by cyclopamine (Figure 7L). Percentage data on expression changes after Gli1 knock-down appear on Table 2.

Discussion

Sonic hedgehog signals through a cascade of downstream effectors, most of which are still unknown. Yet, to our

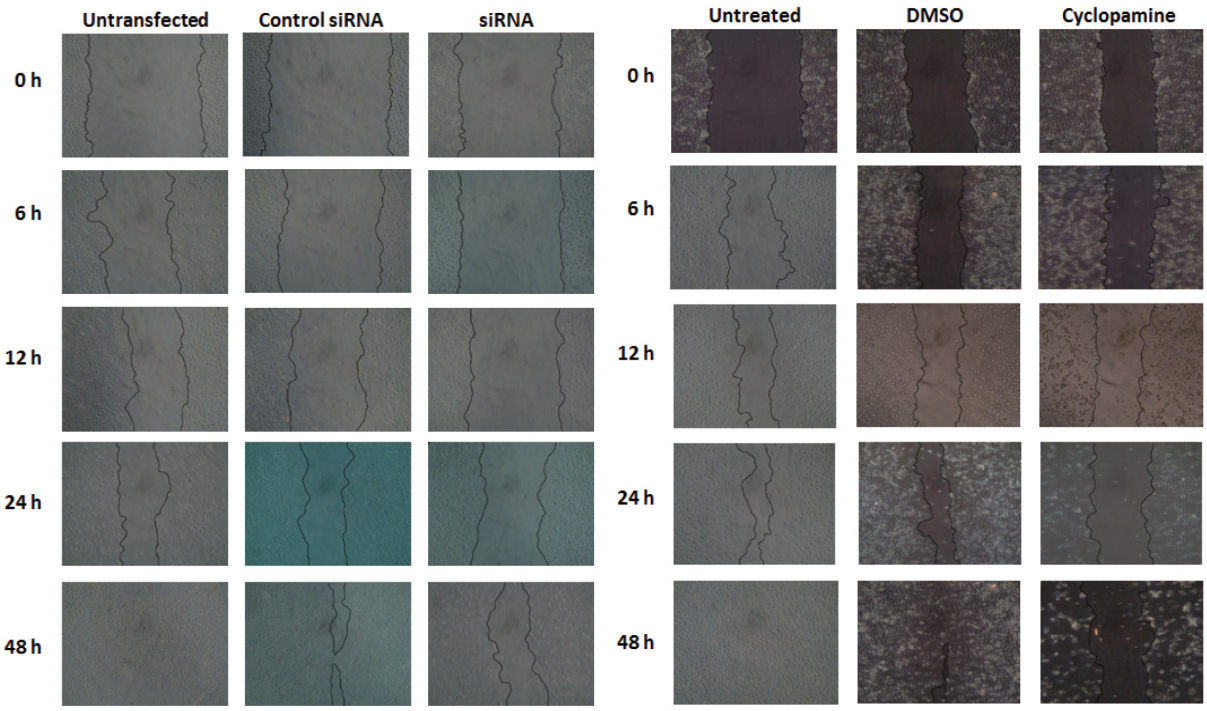


Figure 6 Scratch wound assay for cell migration determination. Both, 100 nM of Gli1 siRNA (left panel) and 15 μM cyclopamine (right panel) decrease cell migration. Cells were untransfected or transfected by control siRNA and Gli1 siRNA (left panel), while right panel shows untreated and DMSO (vehicle) or cyclopamine in DMSO-treated Daoy cells. Similar results were obtained in triplicate.

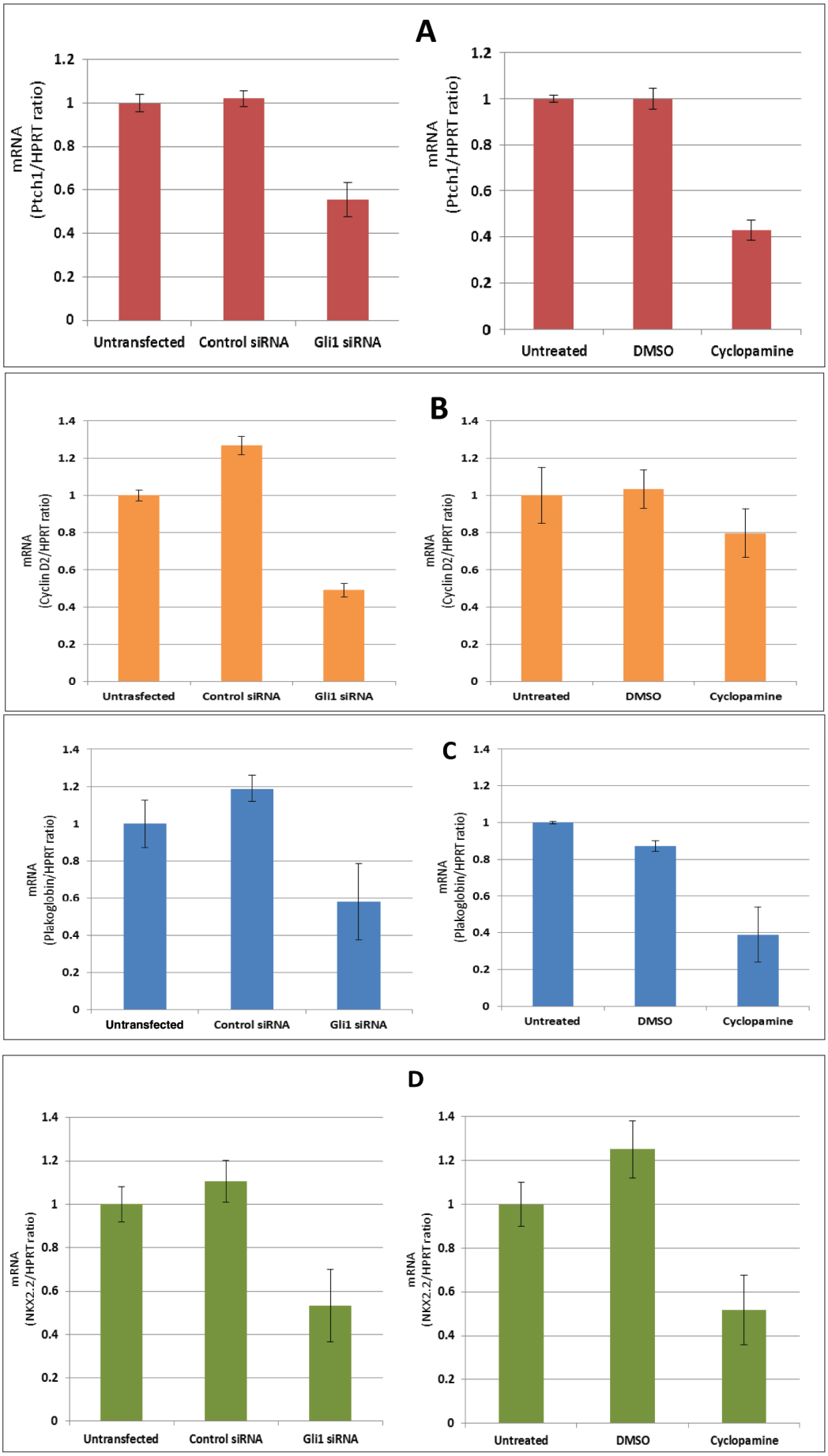
Table 2 Percentage of change in gene expression after GLI1 knock-down

Gene	*GLI1 knock-down after siRNA treatment or cyclopamine1 (%)*	
	Gli1 siRNA	*Cyclopamine*
Ptch1	- 43	- 58
Cyclin D2	- 50	- 20
Plakoglobin	- 40	- 60
Nkx2.2	- 50	- 50
Bmi-1	- 75	- 40
Pax6	+ 20	+ 40
Smo	- 50	- 60
Sufu	+ 5	- 25
Gli3	+ 5	- 15
Myc-N	- 45	- 85
Notch1	+ 56	+ 65
Notch2	- 18	+ 10

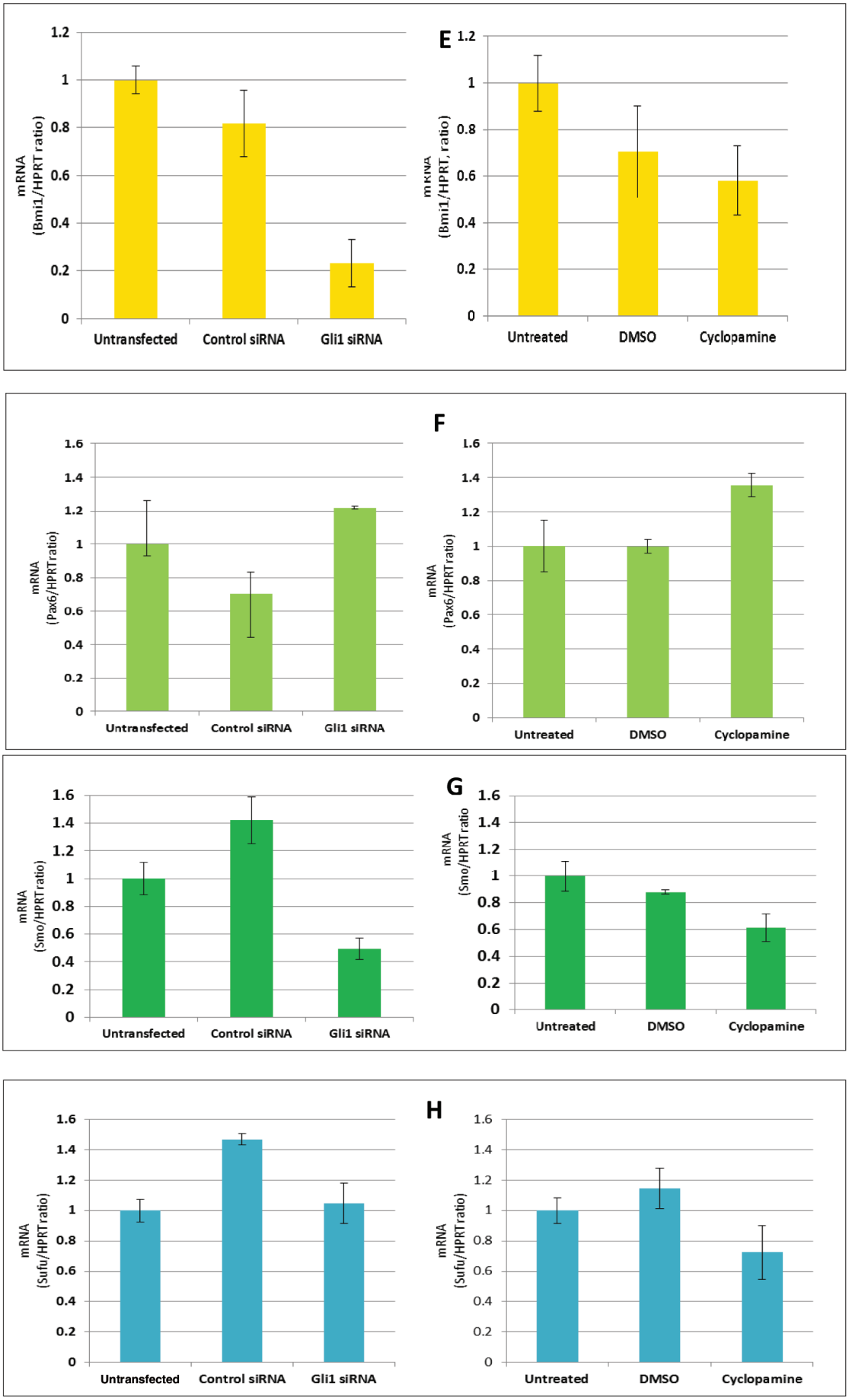
[1] (+) Increase in the expression of each gene after Gli1 siRNA or cyclopamine treatment.
(-) Decrease in expression respect to controls.

knowledge, there are no highly effective approved targeted therapies to treat Shh-driven medulloblastoma. However, vismodegib, an oral inhibitor of the hedgehog pathway, is being studied in an ongoing clinical trial in adult patients with recurrent, progressive, or refractory to standard therapy medulloblastoma [27]. In addition, although it is unclear what side effects may occur in children, as permanent defects in bone growth have been seen in mouse models of Hh inhibitors [28], few clinical trials have been started in this population with recurrent or refractory medulloblastoma [29, 30]. Moreover, vismodegib became on January 30, 2012 the first drug approved by the US Food and Drug Administration (FDA) for the treatment of adults with metastatic basal cell carcinoma, or with locally advanced basal cell carcinoma that has recurred following surgery or who are not candidates for surgery and who are not candidates for radiation [31]. This situation may stem from our limited knowledge of which proteins are directly responsible for mediating Shh signaling in this tumor type and, hence, serve as effective therapeutic targets. Individuals with somatic mutations of Ptch, Smo, and Sufu, as well as amplifications of Gli1 have been found to be predisposed to sporadic medulloblastoma [32]. Moreover, it is reported that 1/200 spontaneous abortions are due to mutation in Shh and Ptch. As illustrated in this study, the role of Gli1 transcription factor is significant for tumorigenicity in Daoy cells. Hence, inhibition of Gli1 activity within a tolerated therapeutic window may provide a successful pharmacological approach to treat Shh-positive medulloblastomas. In this perspective, we report a role for the transcription factor Gli1 in the growth, survival, migration and expression of related signaling in Daoy medulloblastoma cell line.

Here, we evaluated cyclopamine inhibition as an approach to inhibit the Gli1 trigger (Smo) and compared with direct Gli1 downregulation by a three-pool siRNA. An initial study demonstrated that cyclopamine decreased Daoy cell viability in a dose-dependent manner *in vitro*. In this assay we found that IC_{50} of cyclopamine for Daoy cells



Fugure 7 A, B, C, D



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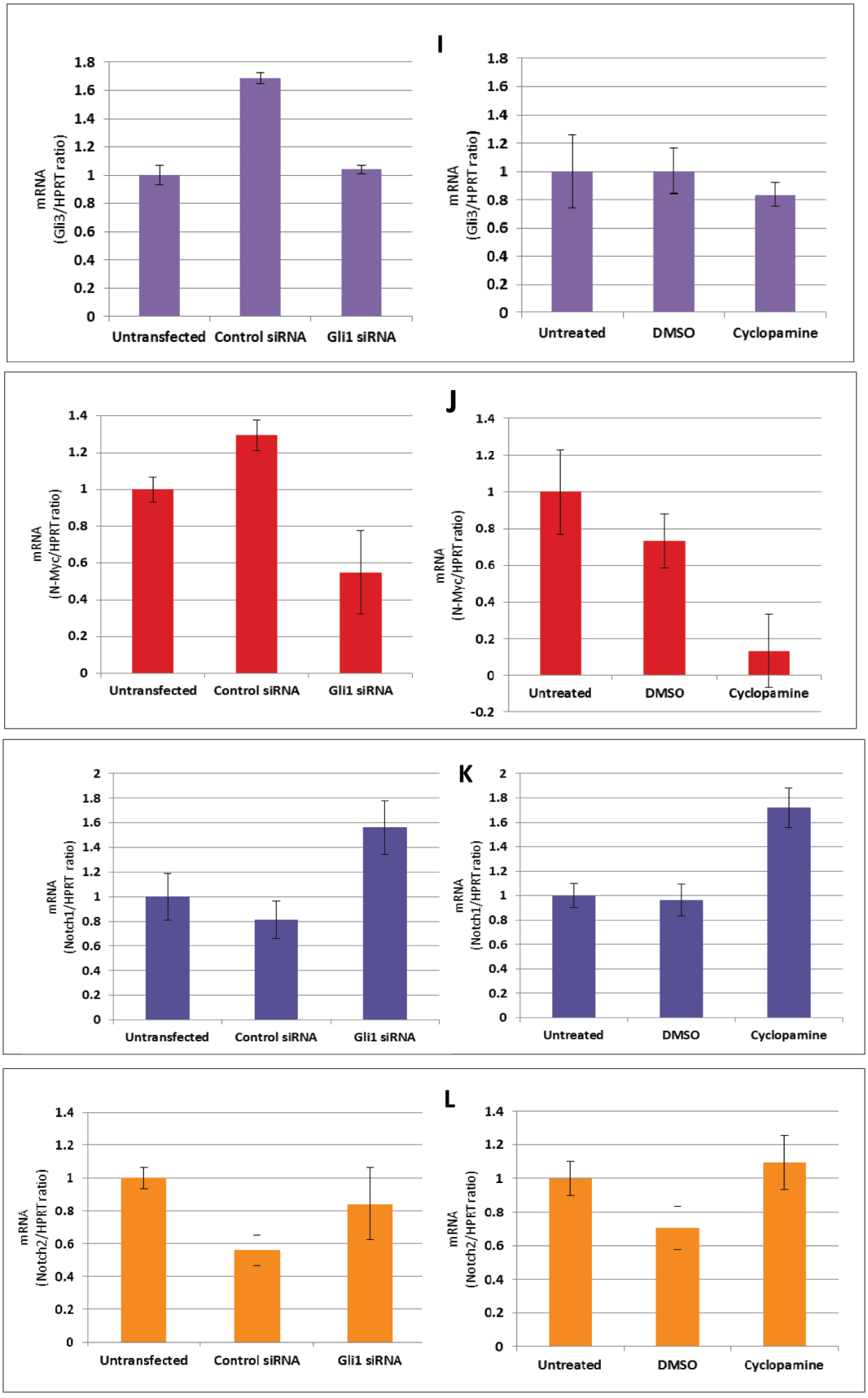

Fugure 7 I, J, K, L

Figure 7 qRT-PCR of Ptch1, Cyclin D2, Pax6, Plakoglobin, Nkx2.2, Bmi-1, Smo, Sufu, Gli3, N-myc, Notch1 and Notch2 genes in Gli1 knockdown in Daoy MB cell line. The derivative ratio values describe the relative expression change of the target gene relative to the HPRT reference gene expression. Downregulation of Ptch1, Cyclin D2, Plakoglobin, Nkx2.2, Bmi1, Smo and N-myc was detected after Shh inhibition. Sufu and Gli3 showed parallel results, where Gli1 siRNA did neither decrease nor increase expression of both genes, whereas cyclopamine reduced them in 15-25%. Pax6 mRNA levels were upregulated by either Gli1 siRNA or cyclopamine. Finally, Notch1 was upregulated after inhibition of SHH, while Notch2 showed contrasting results, as siRNA inhibition decreased its expression, while cyclopamine induced upregulation of Notch2 (See Table 2 for quantification of expression results).

was around 15 μM and we took that concentration as the closest one tested. Similar results were chosen in a study performed with PNET cell lines, including Daoy [35].

However, response to cyclopamine varied among pancreatic adenocarcinoma cell lines with IC50 values differing over 5-fold (from ~9 to 45 μM) [36]. Kandala and Srivastava [37] reported the treatment of ovarian cancer cells with 25 μM cyclopamine, although they also used 10 μM. These differences may be due to differences in format and time assessed as one report used cyclopamine under suspension culture conditions [37].

The data from Gli1 siRNA silencing and Smo antagonizing studies suggest that the chemical approach is adequate to target Shh pathway. Notwithstanding, it was twofold increased comparing with the three-pool siRNA against Gli1 used. Other study which focused on a direct Gli1 inhibitor, GANT58, which has been reported to block Gli1-induced transcription *in vitro* showed lower capacity to diminish Gli1 expression than cyclopamine [38]. Taken together, our findings might indicate that the Shh-Gli1 pathway remains active in Daoy cell line by either a mutation in Ptch or Smo, or eventually by high expression of Shh as an autocrine signaling, and not by Sufu dysregulation.

Besides, we show that downregulation of Gli1, by any of both methods, greatly diminishes colony forming ability in adherent and nonadherent conditions in terms of both quantity and colony size in the medulloblastoma cell line. In particular, we observed a decrease of 75% in number of colonies by Gli1 siRNA compared with untransfected cells when cells were cultured attached, and even a reduction of 15% more in unattached assays using soft agar. Similar results were obtained with cyclopamine treatment. A previous study found cyclopamine more effective in prostate carcinoma xenograft models than in prostate carcinoma cell line 22Rv1 *in vitro* [33]. In consistence with that, we suggest that due to the greater difference in soft agar than in the attached assay and that independent growth assay in soft agar is assumed to be closely related to the process of *in vivo* carcinogenesis, cyclopamine might show better results in tumor growth in a xenograft assay with regard to the surrounding stroma.

Medulloblastoma has been described as an invasive and a rapidly growing tumor that, unlike most brain tumors, spreads through the cerebrospinal fluid (CSF) and frequently metastasizes to different locations in the brain and spine. Further, we observed that the migration ability of the Daoy cells when Gli1 was silenced was dramatically reduced compared with untransfected or untreated cells as assessed in conventional scratch assays. Moreover, we observed that cells treated with cyclopamine not only diminished migration, but also decreased initial confluence. Although this could be mediated also by the effect of DMSO, solvent of cyclopamine, a huge difference can be detected intra-experimentally (between DMSO and cyclopamine) and inter-experimentally (between Gli1 siRNA and cyclopamine).

In the course of this study, we sought to understand the regulation of certain downstream target genes and other genes related with the Shh pathway in the Daoy cell line. In addition, we attempted to explore any putative regulation of these genes by the major transcription factor involved in Shh signaling, Gli1. Among the Gli1 target genes studied we found that Ptch1, Cyclin D2, Plakoglobin, Nkx2.2, Bmi-1 were downregulated after both treatments. Especially, Ptch1 and Plakoglobin mRNA levels showed less expression using cyclopamine. This result does not corroborate a previous report which suggests that Gli1 downregulates Plakoglobin in Gli1 transformed epithelial cells [39]. For Cyclin D2, our finding is supported by the previous report showing similar results in Gli1 transformed epithelial cells [39]. From Nkx2.2 result, we support the conclusion of a report [40] which suggests that Gli1 up-regulates Nkx2.2. Bmi-1 is a stem cell marker gene linked to Shh [41] that is up-regulated as Nkx2.2 in medulloblastoma. But treatment of the Shh pathway makes Nkx2.2 and Bmi-1 mRNA levels diminish. Nevertheless Pax6, another target gene, presented higher mRNA level than untransfected or untreated controls in agreement with this work [42] involving the role of Gli1 in downregulating its expression. Focusing on Smo, qRT-PCR revealed an mRNA reduction between 40-50% by both approaches. Gli3 and Sufu tumor suppressors showed opposite mRNA values. While inhibition by Gli1 siRNA produced a subtle increase, the inhibition by the Smo antagonist resulted in a decrease of Gli3 and Sufu mRNA levels, result that could be explained by the difference on Gli1 mRNA levels obtained.

Finally, in relation with the Shh pathway and other cancer pathways, N-myc and Notch, we found encouraging results related with prior works. It has been found that continued N-myc expression in granule neuron progenitors is a key factor contributing to Shh-driven medulloblastoma tumorigenesis [43]. N-myc was downregulated by Gli1 or Smo targeting and they could potentially affect stem-like cells in the tumors [44]. Here, we observed that cyclopamine inhibition produced twofold N-myc mRNA levels managed by Gli1 siRNA. This makes us think that Smo plays, in normal conditions, activating N-myc expression before Gli1. In connection with Notch1 and 2, it is known that Notch1 is associated with cell cycle exit and differentiation, whereas Notch2 promotes proliferation of cerebellar granule neuron progenitors [45]. Parallel results were found in Notch1 expression when targeting Smo or Gli1, obtaining an increase in Notch1 mRNA level. In particular, we found that Notch2 showed opposite outcomes than Notch1. Gli1 siRNA produced a decrease of Notch2 mRNA unlike cyclopamine which increased Notch2 expression. Hence, we suggest that this difference in Notch2 mRNA levels can be explained by the fact that cyclopamine inhibition produced twofold Gli1 mRNA levels managed by Gli1 siRNA.

To sum up, this work represents a quick overview of the Shh pathway's role and its connections with other signals related with carcinogenesis in medulloblastoma. Two ways to inhibit Shh were tested. Inhibition led to *in vitro* tumorigenesis decrease in terms of cell viability, migration and colony formation in culture and in soft agar. Further studies remain to be done for a better understanding of the crosstalks to provide more relevant information toward the development of effective targeted therapies for the treatment of Shh-driven medulloblastoma.

Funding

This research was supported in part by grants from the Departmento de Salud del Gobierno de Navarra, Caja Navarra (project 13912), Fundación Universitaria de Navarra, Pamplona; and Fondo de Investigación Sanitaria (PI081849, to JSC; and PI-101972, to JAR), Madrid.

Conflict of interest

The authors wish to express that they have no conflict of interest.

Acknowledgements

We thank Pablo Sáez for his help and advice with the artwork.

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