

Induction of Cyclopamine Resistance in Desmoplastic Medulloblastoma Cells Increases their Cancer Stem-like Cell Compartment and their in vitro Tumorigenic Potential

Javier de la Rosa, Amaia Paredes, Mehdi H. Shahi, Bárbara Meléndez, Juan A. Rey, Miguel A. Idoate, Javier S. Castresana

Javier de la Rosa, Amaia Paredes, Javier S. Castresana, Department of Biochemistry and Genetics, University of Navarra School of Sciences, Pamplona, Spain

Mehdi H. Shahi, Interdisciplinary Brain Research Centre, Faculty of Medicine, Aligarh Muslim University, Aligarh, India

Bárbara Meléndez, Molecular Pathology Research Unit, Virgen de la Salud Hospital, Toledo, Spain

Juan A. Rey, IdiPaz Research Unit, La Paz University Hospital, Madrid, Spain

Miguel A. Idoate, Department of Pathology, University of Navarra Clinic, Pamplona, Spain

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Correspondence to: Javier S. Castresana, Department of Biochemistry and Genetics, University of Navarra School of Sciences, Irunlarrea 1, 31008 Pamplona, Spain

Email: jscastresana@unav.es

Telephone: +34-948-425600 (ext. 806486)

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ABSTRACT

Current treatment against medulloblastoma, one of the most frequent tumors in childhood and early adolescence, is not effective enough, presenting long time relapses in addition to side effects in growth and intellectual outcome of patients. Among the different types of

medulloblastoma, we focused on the sonic hedgehog (Shh) type of this disease. Cyclopamine inhibits the Shh pathway. Medulloblastoma very often presents chemotherapy resistance. We tried, in this work to understand the mechanism of cyclopamine resistance in Shh medulloblastoma. In our study we induced cyclopamine resistance to Daoy desmoplastic Shh type medulloblastoma cells by treating them with this drug for a long period of time. Cellular assays (proliferation, clonogenicity, and migration), together with qRT-PCR molecular experiments allowed us to verify the different behaviour of the cells after each cyclopamine exposure. The results highlight the evidence that cells became resistant because they were able to grow in soft agar without attachment, to migrate and to overexpress genes related to cancer stem-like cell phenotypes. The resistance induced seems to have a cyclic behaviour, as according with the results Daoy cells might sustain a subgroup of cancer stem-like cells in the tumor that never disappear despite cyclopamine treatments. Induction of cyclopamine resistance in desmoplastic Shh medulloblastoma cells increases their cancer stem-like cell compartment and their in vitro tumorigenic potential. Further studies are required to properly understand the mechanisms of cyclopamine resistance and the possible ways to overcome it.

Key words: Medulloblastoma; DAOY; Cyclopamine resistance; Sonic hedgehog

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INTRODUCTION

Medulloblastoma^[1,2] represents 15-30% of brain tumors in childhood. The current treatment against medulloblastoma (surgical resection, radiation and chemotherapy) is not good enough, with a survival rate of 60% of the patients. Most patients suffer from long time side effects, like growth and intellectual problems, derived from the ag-

gressiveness of radiation^[3]. Due to treatment limitations, a deeper knowledge in the molecular pathways implicated in medulloblastoma might help us find specific targets and consequently, better, more specific, treatments against this disease.

We focused our research in the sonic hedgehog (Shh) medulloblastoma group^[4-6]. The Shh pathway^[7] has two transmembrane receptors, Ptch and Smo. In absence of Shh (a ligand of the Ptch receptor), Ptch acts as an inhibitor of Smo. On the contrary, when Shh binds Ptch, it drives cell proliferation as Smo gets active to release Gli from Sufu in the cytoplasm. This action activates Gli1 and Gli2 transcription factors, and inactivates the transcriptional repressor Gli3. Among Gli1 target genes we have Gli1, Ptch, N-Myc, Cyclin D2, Bmi1, and Bcl-2. 25% of medulloblastomas have some type of mutations in Shh genes, above all Ptch, Smo and Sufu, that make the pathway to be constantly activated^[8,9].

Recently, it has been proved that small molecules might have an important role against medulloblastoma^[10]. These molecules can be synthetic or natural and act as modulators of Smo activity. Cyclopamine is a plant-derived alkaloid that down-regulates Smo. Therefore, in cases in which the pathway is active due to Smo activation, cyclopamine can block Shh signaling^[11]. It has been demonstrated that a single exposure of cyclopamine to the drug can inhibit medulloblastoma cell migration and cell growth^[12]. But sometimes, the tumor does not disappear after long treatments with this drug, which make us consider the possibility that tumor cells have become resistant to the drug. To prove whether this resistance is actually happening, we treated the Daoy desmoplastic medulloblastoma cell line, a representative cell line of the Shh type of medulloblastoma^[4-6], with consecutive exposures to cyclopamine, and further we studied the changes suffered by the cells at the cellular and molecular levels: cell viability, tumorigenic capacity and gene expression deregulation were included in our research.

MATERIALS AND METHODS

1. Cell line

We used DAOY, a desmoplastic Shh type medulloblastoma cell line, from the American Type Culture Collection, Manassas, VA, USA. It 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% CO₂ to expand the cells. Cells were harvested to subculture them after 80% confluence with trypsin/EDTA. We used a different culture medium when the cells were exposed to cyclopamine. In this case, RPMI L-glutamax medium was supplemented with 2.5% fetal bovine serum instead of being supplemented with 10% serum. This change in the medium is to avoid the inactivation of cyclopamine by a high serum concentration. The rest of the supplements added were the same ones for both conditions.

2. MTT assay and cyclopamine treatment

After culturing the cells, 5000 were plated out in each well in a dilution of 200 µl with different concentrations of drug. We studied the viability of the cells at different times; 0, 24 and 48 h. Color was measured at 570 nm in a micro-titer plate reader (Perkin-Elmer, Boston, MA). 10 mM cyclopamine dissolved in ethanol was added to 70% confluent cells cultured in 75 cm² flasks with medium containing a low percentage of serum (2.5% FBS), for the next 24 h. Cells were treated in the same way in each exposure up to six subcultures. The control group was not treated with cyclopamine.

3. Colony formation assay

We evaluated the ability of the cells to create colonies in Petri dishes. After each exposure of cyclopamine, 1000 cells were cultured in 10 ml of growth medium for 10 days. Then, the cells were fixed with 4% paraformaldehyde for 30 min, and stained with 1% crystal violet (Sigma Aldrich, UK) for 15 min. The colonies of each dish were counted. The experiment was performed three independent times.

4. Soft agar clonogenic assay

By this assay the ability of the cells to form colonies without attachment can be assessed. Cells were mixed with 0.4% agar, and 5000 cells per well were plated onto 6-well plates that had solidified bottom layers of 1% agar. When the top layer of agar containing the cells was solidified, 2 ml of growth medium were added onto it. After 20 days, colonies were formed, and the medium was discarded. It was not necessary to fix the colonies because they were into the agar. They were stained with 250 µl of 2.5% crystal violet (Sigma Aldrich, UK) for 5 min, washed 4-5 times with PBS, and counted.

5. Scratching or wound healing assay

After each exposure to cyclopamine, the control and treated cells were harvested with trypsin/EDTA (Gibco), and 700000 cells in 2 ml were seeded into the wells of 6-well plates. After 12 h of incubation the cells got attached to the surface. A linear wound was produced in each well by scratching the monolayer formed by the cells. The ability of migration was analysed microscopically at different times: 6, 12, 30 and 48 h.

6. RNA extraction, retrotranscription and qRT-PCR

RNA was extracted by the AllPrep DNA/RNA/Protein MiniKit (Qiagen). DNA and proteins were kept at -20°C for other studies. We used nanodrop to determine the purity and concentration of total RNA. One mg was required for retrotranscription in a final volume of 12 µl, with 1 µl of random primers and 2 µl of the mix of the four dNTPs. The whole mix was incubated at 65°C for 5 min; then, synthesis buffer and DTT were added. After incubation at lower temperature for 2 min, the SuperScript Reverse Transcriptase was added for cDNA synthesis. qRT-PCR was performed for the following genes: GAPDH, Gli1, Gli3, Sufu, Bax, Bcl-2 and CD133 (Table 1). The amplification reactions were carried out in the IQ5 Multicolor Real-Time PCR Detection System (BioRad, Hercules, CA, USA). 2.5 µg of template DNA were added to 25 µl of reaction mix that contained 12.5 µl 2X IQ™ SYBR Green Supermix and 0.5 µg of forward and reverse primers. For PCR we included a hot start step of the enzyme (95°C for 10 min), followed by 40 cycles of amplification. Temperature changed between 92°C, 72°C and the corresponding annealing temperature for each gene (Table 1). The samples were studied in triplicate. In addition, all genes were normalized to human GAPDH present in the samples.

7. Statistical analysis

Statistical analysis was performed with the SPSS statistical software (Chicago, IL, USA). A correlation study was chosen to compare the expression pattern of each gene during the different exposures to the drug, and the correlation of each gene with cell phenotype (colony formation assay and soft agar clonogenic assay data). Student's t test was performed to compare the number of colonies formed after each exposure to cyclopamine versus controls. Results are presented as mean ± SD. Differences were considered statistically significant when p value was less than 0.05, and very significant when it was less than 0.01.

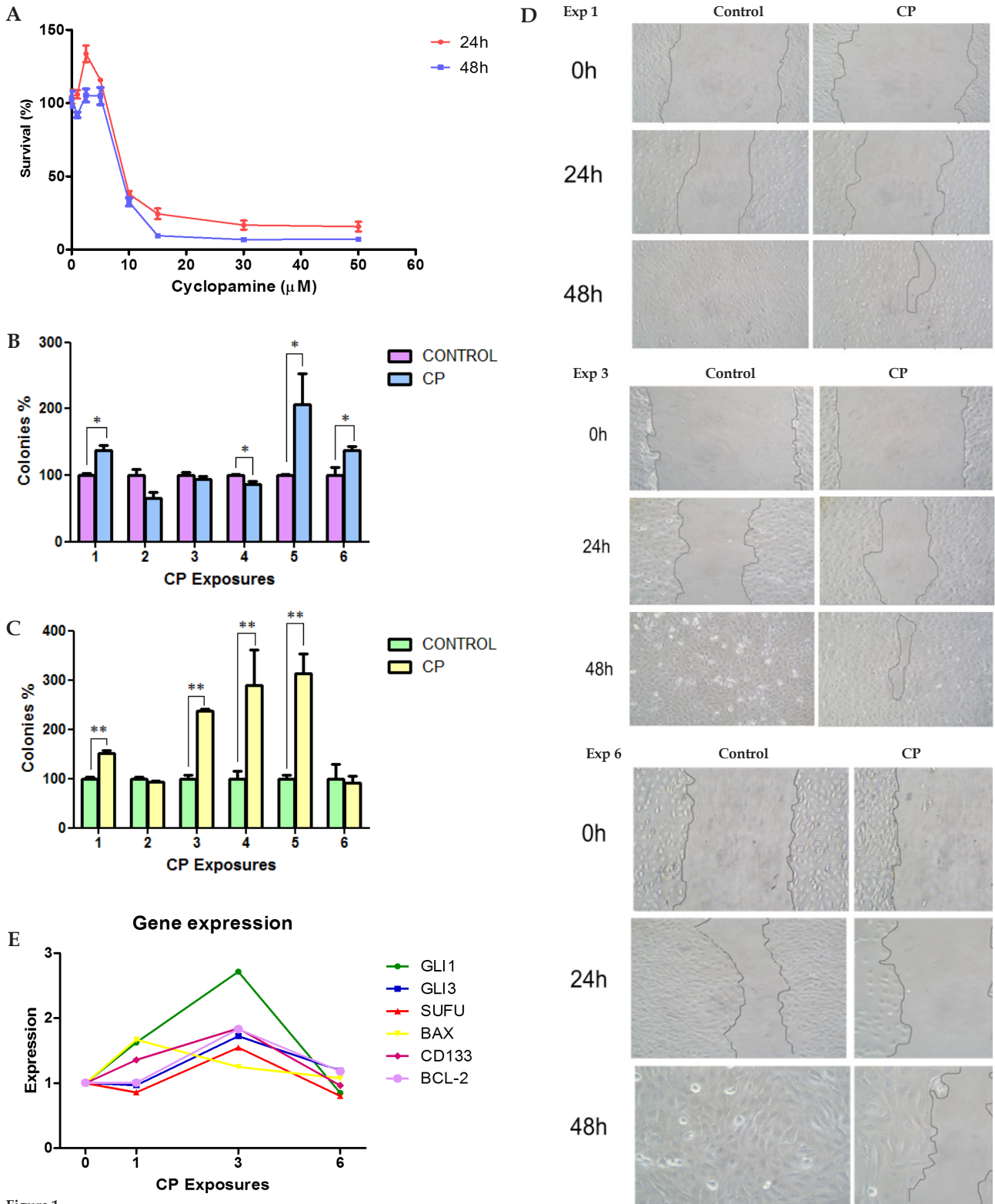


Figure 1
A: Cyclopamine inhibits Daoy cell viability. The effects of different concentrations of the drug (1-50 μM) dissolved in DMSO were studied by MTT. X-axis: cyclopamine concentration; y-axis: percentage of cell survival. The graph shows the effects at 24 and 48 h, which were similar at a concentration of 10 μM. Data reflect the means ± SD.
B: Effect of cyclopamine (CP) treatment on the ability to form colonies by Daoy cells after each of the six exposures of CP. Number of grown colonies was visually counted after staining them. Treated cells were able to form more colonies than control groups only in the last exposures. Significance: $p < 0.05$ (*). X-axis: cyclopamine exposures; y-axis: percentage of colonies grown from treated and control cells. Data reflect the means ± SD of triplicate experiments.
C: Effect of cyclopamine (CP) treatment on the ability to form colonies in soft agar by Daoy cells after each of the six exposures of CP. The most significant differences appeared in the 3rd, 4th and 5th exposures. Significance: $p < 0.01$ (**). X-axis: cyclopamine exposures; y-axis: percentage of colonies grown from treated and control cells. Data reflect the means ± SD of triplicate experiments.
D: Cell migration assay of Daoy cells after one, three and six cyclopamine (CP) exposures versus control at three different times (0, 24, and 48 h). The expected CP migration inhibition happens only after the sixth exposure of the cells to CP.
E: mRNA expression assayed by qRT-PCR after each cyclopamine exposure of Daoy cells. X-axis: cyclopamine exposures; y-axis: relative expression of each gene compared with the control group.

RESULTS AND DISCUSSION

Previous studies of our group proved that a single exposure of Daoy cells to cyclopamine could inhibit cell proliferation^[12]. One of the main clinical problems in medulloblastoma is that relapses usually appear after long term treatments. We subjected Daoy desmoplastic Shh type medulloblastoma cell line to repetitive doses of cyclopamine to obtain resistant cells to this drug. Once the resistance was established, we studied the cellular and molecular changes suffered by the cells.

1. Cellular effects during a repetitive cyclopamine treatment

1.1 Cyclopamine reduces DAOY cell viability

A MTT assay was performed by treating the cells with different concentrations of cyclopamine: 1, 2.5, 5, 10, 15, 30 and 50 mM. The drug was dissolved in DMSO, but never exceeding 1% of the total reaction volume. The IC₅₀ corresponded to 10 mM (Figure 1A), which produced the same effect at 24 h or 48 h.

Daoy cells were exposed to 10 mM cyclopamine for six consecutive times. After each of the exposures, cells were subcultured for colony formation determination. The two types of experiments of colony formation (in dishes and in soft agar), although seeming quite similar, study two different characteristics of tumor cells. The colony formation assay in dishes allows the cells to be attached to the surface of the dish, as it usually happens with well differentiated cells. Quite the contrary, the soft agar clonogenic assay studies the possibility of cells to grow without attachment to a solid surface. They grow in suspension in soft agar, and form colonies. This is a better experiment to show the tumorigenic potential of cancer stem-like cells, as these cells have the ability of growing three-dimensionally. Therefore, the clonogenic assay in soft agar is more similar to *in vivo* tumorigenesis than the colony formation assay, as the agar assay may manifest the existence of cancer stem-like cells in the tumor.

1.2 Colony formation assay

For the colony formation assay, we cultured cyclopamine treated cells, as well as the non-treated cells, in Corning dishes, in three independent experiments. After 10 days, cells got attached to the surface and we could count different number of colonies in each dish (Figure 1B). The more significant differences appeared after the 5th and 6th exposures to the drug, with more colonies counted than in controls, probably due to induced resistance to cyclopamine. After lesser numbers of exposures to cyclopamine, the treated cells formed fewer colonies than the controls, which make us think those cells were still sensitive to cyclopamine. In conclusion, the more exposures to cyclopamine, the easier to induce cyclopamine resistance, at least when we refer to the colony formation assay.

1.3 Soft agar clonogenic assay

The clonogenic assay in soft agar requires the presence of a first layer of this material, which does not allow the cells to be attached

to the surface of the plates. Therefore, the cells can grow three-dimensionally. Control and treated cells were cultured for 20 days in 6 well plates, and then stained with 0.5% crystal violet solution. Colonies were counted with a magnifying glass. Treated cells were able to form more colonies than control cells after most of the exposures; specifically in the 3rd, 4th and 5th ones. Nevertheless, just the opposite happened after the 6th exposure: treated cells formed fewer colonies than control cells. Therefore, we could interpret these results by assuming that Daoy cells got resistant to cyclopamine from the 3rd to the 5th exposure, but finally Daoy cells got sensitive to cyclopamine after the 6th exposure. The significance ($p < 0.05$) obtained in the colony formation assay was not as high as the significance obtained in the soft agar assay ($p < 0.01$) (Figure 1B and 1C), maybe because the formation of colonies in a dish is easy to any type of cells, differentiated and non-differentiated, as colony formation is based on the possibility of cell attachment to the dish.

According with the results, cancer stem-like cells (growing three-dimensionally and forming colonies in soft agar) might get resistant to cyclopamine earlier than differentiated tumor cells. This makes us think that the effect of cyclopamine is probably lower on cancer stem-like cells than on well differentiated tumor cells. A second hypothesis would lead us to consider that there might be a group of cancer stem-like cells in the tumor that would never completely disappear with the treatment.

1.4 Scratching or wound healing assay

Our last *in vitro* experiment measured the ability of treated cells to migrate. Thus, cells were seeded in 6-well plates, and a wound was made in the surface of each well. The cells were controlled under the microscope during the next 48 h. After the 1st exposure to cyclopamine, the effect of the drug was not very relevant when compared with its control (Figure 1D). But after the 3rd exposure to cyclopamine, treated cells migrated faster than the control group at 24 h. However, at 48 h both of them almost closed the whole wound. After the last exposure the effect of cyclopamine was, by far, more important than in the previous ones, being able to reduce the migration capacity of the treated cells when compared with control cells.

This might mean that cyclopamine is not presenting any blocking effect on cell migration, until the very end of the treatment, something compatible with Daoy cells having acquired resistance to cyclopamine after the third exposure to the drug. This statement is supported by molecular experiments, as further presented in this work.

2. Molecular alterations during a repetitive cyclopamine treatment

The expression of Gli1, Gli3, Sufu (Shh pathway genes), Bax (pro-apoptotic gene), Bcl-2 (anti-apoptotic gene), and CD133 (cancer stem cell related gene) was determined by qRT-PCR in Daoy cells after every exposure of the cells to cyclopamine (1st, 3rd, 6th), as well as in

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

GENES	FORWARD	REVERSE	T ^a
GAPDH	AACGTGTCAGTGGTGGACCTG	AGTGGGTGTCGCTGTGAAGT	57.8°C
Gli-1	CCAGTGCACGTTTGAAGGCT	CAACCTTCTGCTCACACATGT	64.1°C
Gli-3	ATGGACCCCAAGGAATGGT	AATGGAGGAATCGGAGATGG	63.3°C
Sufu	CCTCCAGATCGTTGGTGCT	CCCCTCCGATGTCAGTT	58°C
Bax	ATGGAGCTGCAGAGGATGAT	CCCAAAGTAGGAGAGGAGGC	64.9°C
Bcl-2	GATCCAGGATAACGGAGGCT	GTTGACTTCACTTGTGGCCC	64.2°C
CD133	CAGAGTACAACGCCAAACCA	AAATCACGATGAGGGTCAGC	62.5°C

the control groups (Figure 1E). Gli1 was one of the genes that first responded to the drug and increased its expression at the beginning of the treatment while it went down at the end. Gli3 and Sufu changes appeared after 3 exposures to cyclopamine, and in addition, their expression was not so much increased. At the end of the treatment their expression fell down. Bax increased its expression after the early exposures, and then, descended. However, Bcl-2 increased its expression after the third exposure, at the same time as Bax started to decrease. CD133 had its highest expression level after the third exposure, to decrease at the end.

In summary, the Shh pathway genes (Gli1, Gli3 and Sufu), Bcl-2, and CD133 correlate in expression along the cyclopamine treatments (Table 2), just the opposite to Bax expression, the pro-apoptotic gene. It seems that the Shh pathway gets inactivated (decreased in its expression) by cyclopamine inhibition of Smo only at the final point of the treatment. Before, along the middle of the treatment, Daoy cells might present resistance to cyclopamine, as also inferred by the cellular experiments.

So, taking into account the molecular results, Gli1 has the maximum expression in the third exposure (Figure 1E) because at that moment Daoy cells are becoming resistant. Gli1 expression presents a very strong correlation with CD133, Sufu and Gli3 (Table 2A). CD133 is a cancer stem-like cell related gene, and as it has been explained before, cancer stem cells might be enriched in the tumor at the middle of the treatment.

Sufu retains Gli1 in the cytoplasm, reducing Gli1 activation in the Shh pathway^[7]. Therefore, we would expect that if Gli1 were augmented, Sufu would be decreased. But Sufu expression correlated to Gli1 expression in our results. Maybe the increase in Sufu expression might be due to a cell response to the high levels of Gli1. The cells, by other mechanisms, could increase the expression

of Sufu to try to avoid the activation of the Shh pathway as a result of cyclopamine resistance. The same reasoning might be applied to Gli3, as this protein mainly executes a repressive function in the Shh pathway^[7].

Respect to the genes related with apoptosis, Bax is a pro-apoptotic gene and as we would expect is not correlated at all with any other gene studied (Table 2A) as apoptosis gets inactive along cyclopamine resistance. Loss of Bax expression contributes to the maintenance of neurogenesis and the establishment of medulloblastoma^[13]. On the other hand, Bcl-2 is an anti-apoptotic gene and is highly correlated with Gli1, CD133, Sufu and Gli3 (Table 2A), which supports the idea of cells becoming resistant to cyclopamine.

Trying to put together the cellular and molecular studies obtained in our work, we can affirm that cyclopamine resistance appears in the third exposure to the drug. But after that, molecular and cellular assays do not seem to match a lot: while cells treated with more doses of cyclopamine are able to form more colonies (which means they seem to keep the resistance), the expression of genes affected by the resistance goes down (which means the resistant molecular profile does not prevail) (Table 2B). For explaining this cellular-molecular dissociation we must take into account that alterations in the phenotype appear later in the cell than the molecular ones. That is why cells are able to form colonies after the 5th and 6th exposure to cyclopamine, while gene expression goes down.

Finally, this loss of expression could be explained because cells might be subjected to a cyclic behavior. It might occur that a group of cancer stem-like cells never disappear from tissue culture, even in the presence of cyclopamine. Then, when the cell line forms colonies of well differentiated cells, which are sensitive to the drug, cyclopamine inhibits their growth reducing the number of colonies formed. But the group of cancer stem-like cells remains and has the ability of

Table 2 A Correlation between each pair of the genes tested for expression. R corresponds to the value of Pearson's correlation.

		GLI1	GLI3	SUFU	BAX	BCL-2	CD133
GLI1	r value	1	0,826*	0,848*	0.497	0,869*	0,994**
	p value		0.043	0.033	0.316	0.025	0
GLI3	r value	0,826*	1	0,854*	0.062	0,997**	0,814*
	p value	0.043		0.03	0.907	0	0.049
SUFU	r value	0,848*	0,854*	1	-0.023	0,871*	0.8
	p value	0.033	0.03		0.965	0.24	0.056
BAX	r value	0.497	0.062	-0.023	1	0.125	0.554
	p value	0.316	0.907	0.965		0.813	0.254
BCL-2	r value	0,869*	0,997**	0,871*	0.125	1	0,858*
	p value	0.025	0	0.24	0.813		0.029
CD133	r value	0,994**	0,814*	0.8	0.554	0,858*	1
	p value	0	0.049	0.056	0.254	0.029	

(*): the correlation is significant when $p < 0.05$. (**): the correlation is highly significant when $p < 0.01$. Gli1 expression correlates with Gli3 and Sufu, as they are part of the altered pathway, but also with Bcl-2, an anti-apoptotic gene. The correlation between Gli1 and CD133 is very high. Gli3 correlates similarly to Gli1. Sufu, compared to Gli1 and Gli3, only differs in its correlation with CD133, which is not very significant. Following this trend, Bcl-2 expression also correlates with CD133 expression. On the contrary, Bax (pro-apoptotic) does not correlate with any other gene.

Table 2 B Correlation between gene expression and the number of colonies grown in vitro. R corresponds to the value of Pearson's correlation.

		GLI1	GLI3	SUFU	BAX	BCL-2	CD133	AGAR	C.F.A.
AGAR	r value	1,000**	0.752	0.936	0.191	0.81	0,999*	1	-0.912
	p value	0.001	0.458	0.228	0.878	0.399	0.023		0.269
C.F.A.	r value	-0.912	-0.956	-0,998*	0.229	-0.979	-0.896	-0.912	1
	p value	0.27	0.189	0.041	0.853	0.13	0.292	0.269	

(*): the correlation is significant when $p < 0.05$. (**): the correlation is highly significant when $p < 0.01$. The number of colonies in soft agar correlates with CD133, and with Gli1 expression levels. This does not happen in the colony formation assay, in which colonies correlate with Sufu expression.

forming new colonies and starting the cycle again. This explanation has to be proved with larger experiments and a deeper study of the mechanisms of the resistance.

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