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Culture methods of diffuse intrinsic pontine glioma cells determine response to targeted therapies

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ABSTRACT

Diffuse intrinsic pontine glioma (DIPG) is an aggressive type of brainstem cancer occurring mainly in children, for which there currently is no effective therapy. Current efforts to develop novel therapeutics for this tumor make use of primary cultures of DIPG cells, maintained either as adherent monolayer in serum containing medium, or as neurospheres in serum-free medium. In this manuscript, we demonstrate that the response of DIPG cells to targeted therapies *in vitro* is mainly determined by the culture conditions. We show that particular culture conditions induce the activation of different receptor tyrosine kinases and signal transduction pathways, as well as major changes in gene expression profiles of DIPG cells in culture. These differences correlate strongly with the observed discrepancies in response to targeted therapies of DIPG cells cultured as either adherent monolayers or neurospheres. With this research, we provide an argument for the concurrent use of both culture conditions to avoid false positive and false negative results due to the chosen method.

1. Introduction

Despite major advances in the understanding of the biology of diffuse intrinsic pontine glioma (DIPG) in the past decade, the dismal prognosis of children with this aggressive brain tumor has remained unchanged [1,2]. Major efforts to elucidate the molecular basis for this disease have led to the discovery of mutations in genes encoding histone 3 proteins [3–6] and *ACVR1* [6–8] as potential oncogenic driver events. Also, as in many other types of cancer, mutations or aberrations in *TP53*, *ATM*, *ATRX*, *PPM1D*, *PIK3CA*, *PIK3CD*, *PTEN*, *MYCN* and *NF1* are frequently found in DIPG [5,6,9–12]. Additionally, amplifications and mutations in the *EGFR* and *PDGFRA* gene are commonly reported and believed to be of potential therapeutic value [11,13,14]. Based on this increased understanding of the molecular background of DIPG and the availability of relevant primary, patient-derived cell lines, several groups have attempted to identify effective targeted therapies for this disease by *in vitro* drug testing.

Whereas traditionally glioma cells have been cultured as adherent monolayers in the presence of fetal bovine serum (FBS), nowadays 3-dimensional serum-free culture methods, producing so-called neurospheres, have become increasingly popular tools for *in vitro* drug screening approaches. Although it is commonly assumed that these neurospheres are enriched for brain tumor stem cells (BTSC) [15] and consequently better represent the original tumor [16–18], this has recently been questioned [19]. In preclinical DIPG research, most groups have used either neurosphere [9,20–24] or adherent monolayer patient-derived cell cultures [25–29] for the *in vitro* testing of novel drugs. However, the influence of the different culture methods on the responsiveness of DIPG cells to drugs, and the underlying mechanisms causing these differences, remains elusive. This lack of knowledge may lead to the execution of animal studies, or even clinical trials, based on apparent efficacy of drugs, while this observed efficacy is actually induced by the specific culture method. Here we provide the first direct comparison of the response of genetically identical primary DIPG

Abbreviations: BTSC, Brain Tumor Stem Cell(s); DIPG, Diffuse Intrinsic Pontine Glioma; EGF, Epidermal Growth Factor; FBS, Fetal Bovine Serum; FGF, Fibroblast Growth Factor; PAGE, Parametric Analysis of Geneset Enrichment; PCA, Principle Component Analysis; PDGF, Platelet Derived Growth Factor; RTK, Receptor Tyrosine Kinase; TSM, Tumor Stem Medium

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cultures to targeted therapies, using different culture methods. For this purpose, we made a selection of small molecule inhibitors that target commonly activated pathways in DIPG and are in clinical or late preclinical development for brain tumor treatment. Furthermore, we describe how different culture conditions induce alterations in the activation of important signaling pathways that help explain the discrepancies in drug response. Our aim is to provide an argument for the concurrent use of both methods in drug screening and functional *in vitro* studies, to avoid unnecessary animal and clinical studies involving compounds whose apparent efficacy is merely caused by the chosen cell culture system.

2. Methods and materials

2.1. Cell lines and culture protocols

The HSJD-DIPG-007 [30] and HSJD-DIPG-012 cell lines were kindly provided by Dr. Montero Carcaboso (Hospital San Joan de Déu, Barcelona, Spain). The JHH-DIPG-01 cell line [24] was a kind gift from Dr. Raabe (Johns Hopkins Hospital, Baltimore, USA). The VUmc-DIPG-11 cell culture was derived from the autopsy of a 15-year old boy. Autopsy was performed within 2 h of the patient's death. Tumor pieces were collected in Hibernate-A (Thermo Fisher, Waltham, MA, USA) and transferred to the laboratory, where they were dissociated using the Miltenyi Brain Tumor Dissociation Kit (T)(Miltenyi Biotec, Cologne, Germany) according to the manufacturer's instructions. For neurosphere cultures, single cells were cultured in modified Tumor Stem Medium, consisting of 48% DMEM/F12, 48% Neurobasal-A medium, 1% HEPES 1 M, 1% MEM Non-essential amino acids, 1% 100 mM Sodium Pyruvate, 1% GlutaMAX (TSM Base, all components from Thermo Fisher, Waltham, MA, USA), supplemented with 2% B27 (Thermo Fisher, Waltham, MA, USA), 1% N2 (Thermo Fisher, Waltham, MA, USA), 20 ng/mL human EGF, 20 ng/mL human bFGF, 10 ng/mL human PDGF-AA, 10 ng/mL human PDGF-BB (all from Peprotech, London, UK) and 5IU/mL heparin (VUmc hospital pharmacy). To generate genetically identical cell lines growing as an adherent monolayer, small-sized neurospheres were transferred to new flasks and cultured in TSM Base supplemented with 10% heat-inactivated FBS. After several days, the spheres started to adhere to the flask and cells migrated eccentrically to form an adherent monolayer. These monolayers were cultured for a minimum of 3 passages before being used for experiments, to enable them to adjust to the new conditions.

All cell lines were routinely subjected to mycoplasm testing and only used for experiments when confirmed negative. Additionally, routine short tandem repeat (STR) analysis was performed to ensure cell line identity (Supplementary Table S1). All cell lines possessed the K27M mutation in the H3F3A gene that is typically associated with DIPG. Additionally, the HSJD-DIPG-07 cells contained a R206H mutation in the ACVR1 gene [30].

2.2. Drug screens

DIPG neurospheres were dissociated by incubation in Accutase (Thermo Fisher, Waltham, MA, USA) for 5 min at 37 °C, followed by mild mechanical dissociation by pipetting. Cells were then washed in PBS and filtered through a 100 μm cell strainer to generate a single cell suspension. Afterwards, cells were centrifuged for 5 min at 200*g*, manually counted under a microscope, and resuspended in Tumor Stem Medium at 30,000 cells/mL. Finally, cells were seeded at 3000 cells/well in 96-well plates with cell-repellent coating (Greiner Bio-One, #650970, Alphen aan den Rijn, NL).

Adherent monolayers were washed once with PBS and incubated with trypsin and EDTA (Thermo Fisher, Waltham, MA, USA) for 5 min at 37 C. Cells were then collected in PBS, centrifuged for 5 min at 200*g*, resuspended in TSM Base with 10% heat-inactivated FBS, filtered through a 100 μm cell strainer, manually counted under a microscope, and brought to a final concentration of 15,000 cells/mL. Finally, cells were seeded at 1500 cells/well in conventional 96-well cell culture plates (Greiner Bio-One, Alphen aan den Rijn, NL). After 24 h, drugs were added at various concentrations using a Tecan D300e picoliter dispenser (Tecan Group Ltd, Switzerland). After being exposed to drugs for 96 h, relative cell numbers were determined using Cell Titer Glo (Promega, WI, USA) according to the manufacturer's instructions. Luminescent signals were measured on a Tecan Infinite 200 plate reader (Tecan Group Ltd, Switzerland). All screening experiments were carried out in quintuplo. LDN214117 [31] was a gift from Dr. Yu (Harvard Medical school, Boston, MA, USA) and marizomib was a gift from Prof. Moore (Scripps Institution of Oceanography & Skaggs School of Pharmacy and Pharmaceutical Sciences, UC San Diego, CA, USA). All other drugs were purchased from Axon Medchem (Groningen, The Netherlands).

2.3. Phospho-protein arrays

Neurospheres and monolayers were collected during exponential growth. Spheres were centrifuged at 200*g* for 5 min, washed once in ice-cold PBS and flash frozen in liquid nitrogen. Adherent monolayers were washed once with ice-cold PBS, scraped from the flask and centrifuged for 5 min at 200*g*, after which cell pellets were flash frozen in liquid nitrogen. Human phospho-kinase and phospho-RTK antibody arrays (R & D, MN, USA) were performed according to manufacturer's instructions, using 1 mg of protein per membrane. Images were made using the UVITEC Alliance 4.7 chemiluminescence imager (UVITEC, Cambridge, UK).

2.4. RNA-sequencing and data analysis

Neurospheres and monolayers were prepared in the same way as for the phospho-protein arrays. RNA was isolated using the miRvana miRNA isolation kit without phenol (Cat#AM1561, Ambion/Life Technologies, Thermo Fisher, Waltham, MA, USA), supplemented with Acid-Phenol:Chloroform, pH 4.5 (with IAA, 125:24:1) (Cat# AM9720, Ambion/Life Technologies, Thermo Fisher, Waltham, MA, USA). RNA quality was analysed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States) using the Agilent RNA 6000 Nano Kit (Cat#5067-1511, Agilent Technologies, Santa Clara, CA United States) according to the manufacturer's instructions.

The NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420L, New England Biolabs, UK) was used to process the samples according to the manufacturer's instructions. Briefly, mRNA was isolated from total RNA using the oligo-dT magnetic beads. After fragmentation of the mRNA, a cDNA synthesis was performed. This was used for ligation with the sequencing adapters and PCR amplification of the resulting product. The quality and yield after sample preparation was measured using the Fragment Analyzer. The size of the resulting products was consistent with the expected size distribution (broad peak between 300 and 500 bp). Clustering and DNA sequencing (at a concentration of 1.6 pM DNA) using the Illumina cBot and HiSeq. 2500 was performed according to the manufacturer's protocols. Nextseq control software v2.0.2 was used. Image analysis, base calling and quality check was performed with the Illumina data analysis pipeline RTA v2.4.11 and Bcl2fastq v2.17. Fastq files were uploaded to the R2 platform (http://r2.amc.nl) for further analysis and statistics, including the Parametric Analysis of Geneset Enrichment (PAGE) and Principle Component Analysis (PCA). Results from these analysis were considered significant at $p < 0,05$ after correcting for false discovery rates (FDR). Z-scores of expression of single genes were calculated using R2, significance of differences in z-scores was tested in a two-tailed fashion.

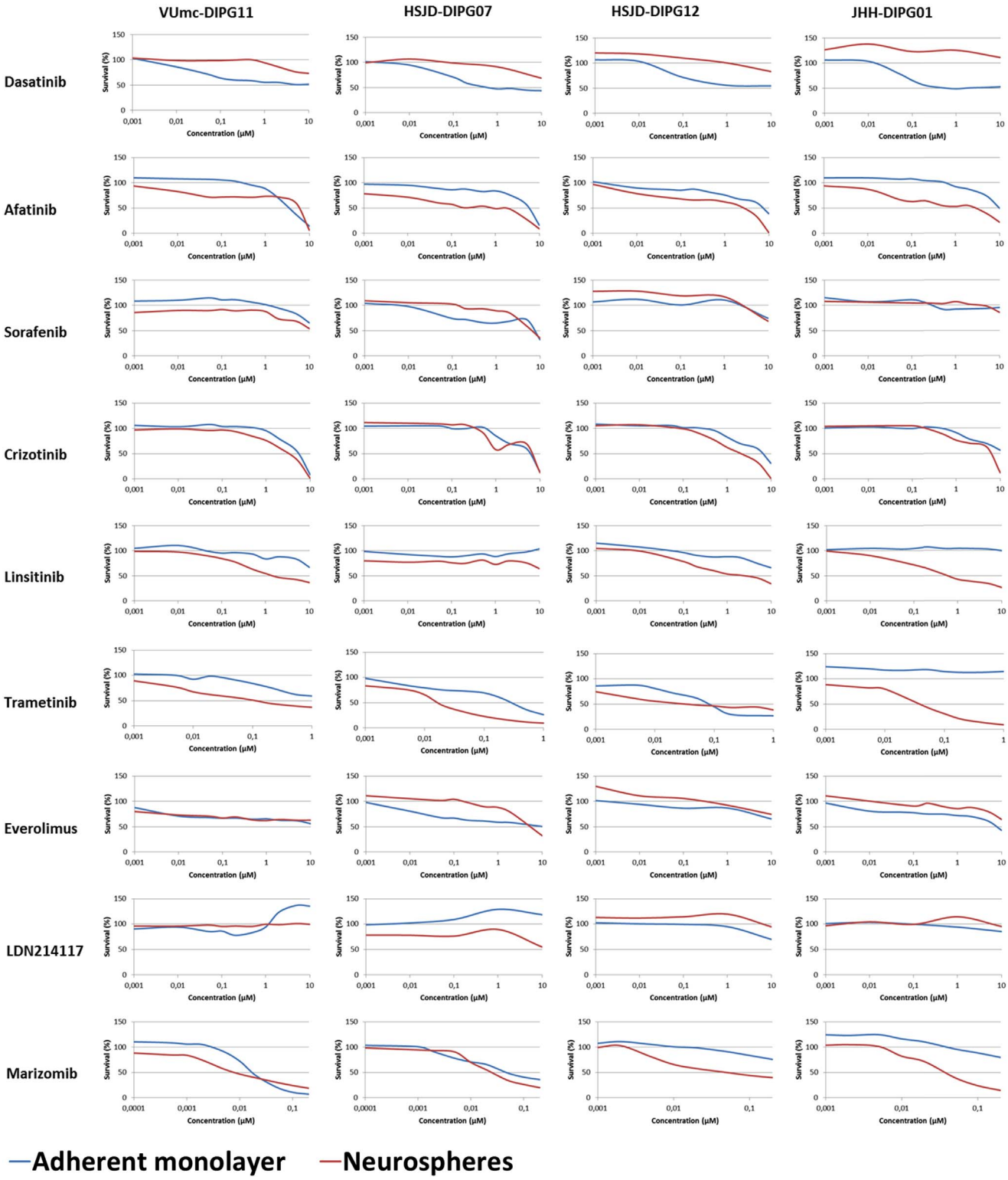

Fig. 1. Dose-response curves of primary DIPG cultures treated with various small molecules, either as neurospheres under serum-free conditions or as an adherent monolayer in the presence of FBS.

3. Results

3.1. Culture methods determine sensitivity of primary DIPG cells to small molecular inhibitors

We first determined the influence of culture conditions on the response of primary DIPG cells to various targeted therapies. Four primary DIPG cultures, VUMC-DIPG-11, HSJD-DIPG-07 and -12 and JHH-DIPG-01, were cultured in both serum-free TSM and TSM containing FBS. Dose-response curves of these cultures were generated for nine different small molecules (Fig. 1). Major differences in cell survival between culture conditions, in at least one primary DIPG culture, were observed for seven out of nine inhibitors; only the c-MET/ALK inhibitor crizotinib and the multi-kinase inhibitor sorafenib showed comparable

cytotoxicity to adherent monolayers and neurospheres in all four cultures (Fig. 1). Dasatinib, an inhibitor of PDGFR which has been reported to have potent antitumor activity in DIPG cell lines [27], effectively inhibited proliferation of adherent monolayers of our DIPG cultures at concentrations between 100 nM and 1 μM, but had almost no, or even a cell growth inducing, effect on neurosphere cultures at concentrations up to 10 μM (Fig. 1). Likewise, everolimus, a commonly used mTOR inhibitor, was more potent in reducing proliferation of DIPG cell monolayers compared to neurospheres in three out of four cell lines (Fig. 1). In contrast, the EGFR inhibitor afatinib demonstrated greater antiproliferative activity on neurosphere cultures than on adherent monolayers of DIPG cells (Fig. 1). Similar selective antiproliferative efficacy to neurospheres was observed for the INSR/IGF1R inhibitor linsitinib, the proteasome inhibitor marizomib [32] and the MEK1/2 inhibitor trametinib (Fig. 1). The ACVR1 inhibitor LDN214117 [31] was effective in reducing proliferation of HSJD-DIPG-07 neurospheres, which was the only culture possessing an activating ACVR1 mutation in our panel (Fig. 1), but did not reduce growth of HSJD-DIPG-07 adherent monolayers, and even slightly induced proliferation of these cells.

3.2. Culture conditions determine signal transduction pathway activation in cultured DIPG cells

To explain the influence of the different culture conditions on the drug response of primary DIPG cultures, we performed phospho-receptor tyrosine kinase (RTK) and phospho-kinase arrays on paired samples of these DIPG cultures. Compared to adherent monolayers, higher levels of p-EGFR and p-INSR were detected in neurospheres. Conversely, adherent monolayers harbored higher levels of p-ERBB4, p-PDGFRα and p-PDGFRβ (Fig. 2 and Supplementary Fig. S1). Differences between adherent monolayers and neurospheres of DIPG cells were even more prominent, albeit heterogeneous, for downstream kinases (Fig. 3 and Supplementary Fig. S2). For example, VUMC-DIPG-11 and HSJD-DIPG-12 cells showed lower levels of p-Akt^{S473} when cultured as adherent monolayers, whereas the opposite was seen in HSJD-DIPG-07 cells. Further complicating the matter, the phosphorylation of downstream targets of Akt, PRAS40 and CREB, did not change concordantly. Levels of p-PRAS40^{T246} decreased paradoxically in HSJD-DIPG-07 cells cultured as adherent monolayers, despite increased Akt activation. Likewise, despite showing less phosphorylation of Akt than their neurosphere-counterpart, VUMC-DIPG-11 monolayers possessed slightly higher levels of p-CREB^{S133}. Similar ambiguous changes in phosphorylation of downstream kinases could be observed in other signaling pathways, including the MAPK pathways (p-p38α$^{T180/Y182}$, p-ERK1/2$^{T202/Y204;T185/Y187}$), JAK/STAT pathway (p-STAT3^{S727}) and heat shock proteins (HSP27$^{S78/S82}$ and HSP60). More consistent changes were seen in phosphorylation levels of WNK1^{T60} and p53$^{S15/S46/S392}$, which either decreased or remained stable in adherent monolayers, as compared to related neurosphere cultures.

3.3. Culture conditions induce major changes in gene expression of DIPG cells

To further investigate the changes induced by the different culture conditions in the biological behavior of DIPG cells, we performed RNA sequencing on primary DIPG cultures growing exponentially in either serum-free TSM or TSM with FBS. Principal component analysis (PCA) of gene expression patterns of all four primary DIPG cultures shows a preferential clustering by culture condition, rather than biological background, for at least one component (PC2, Fig. 4a), supporting the hypothesis that the biological behavior of cultured DIPG cells is determined for a large part by the specific culture conditions. Parametric Analysis of Geneset Enrichment on all four primary DIPG cultures reveals that culture conditions induce significant changes (FDR $p < 0,05$) in gene expression in multiple KEGG genesets involved in signal transduction (Fig. 4b). As expected, a significant increase in expression of genes involved in adhesion and extracellular matrix interaction was seen in DIPG cells cultured as adherent monolayers in the presence of FBS. Moreover, in line with the differences observed in STAT3 phosphorylation, enrichment of the JAK/STAT signaling pathway geneset was observed in DIPG cells cultured as adherent monolayers. This was accompanied by an enrichment of genesets involved in cytokine-receptor and neuroactive ligand-receptor interaction, which may be responsible for the increase in expression of genes associated with JAK/STAT signaling. Furthermore, supporting the results from the phospho-RTK arrays, an enrichment of the ErbB signaling pathway geneset was present in the DIPG cells cultured as neurospheres. Finally, PAGE revealed an enrichment in genesets involved in proteasome function and cell cycle progression in DIPG neurospheres, providing further insight in their selective sensitivity to proteasome inhibition and several kinase inhibitors.

4. Discussion

Since the publication in 2006 of an influential paper [33] claiming that glioma neurospheres, cultured in serum-free media, resemble the original tumor better than adherent monolayers cultured with FBS, many research groups have started to use these neurospheres in investigating new therapeutics for the treatment of glioma, assuming a higher predictive value for clinical efficacy of this culture method. However, although differences in drug sensitivity between adherent monolayers and neurospheres have been described, direct and comparative evidence for the assumed higher predictive value of drug screens on neurospheres is lacking. Furthermore, the assumption that neurospheres correctly represent the (totality of the) original tumor has

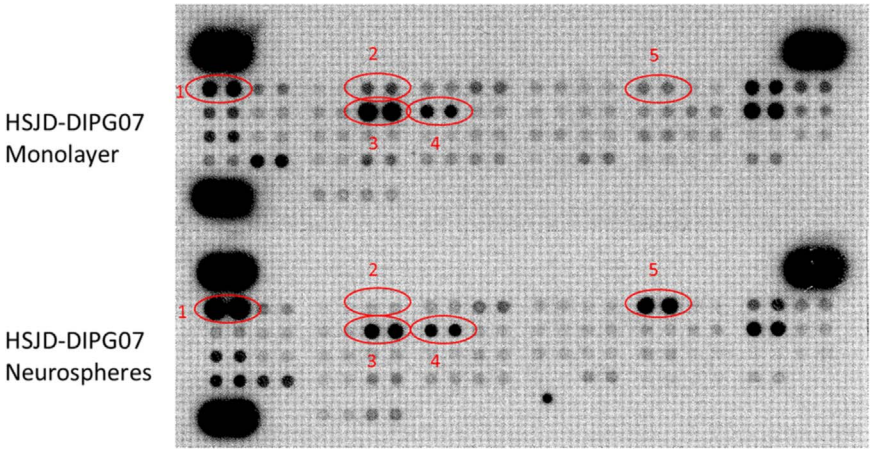

Fig. 2. Phospho-Receptor tyrosine kinase antibody array of HSJD-DIPG07 cells showing differential RTK signaling between neurospheres and adherent monolayers. 1 = p-EGFR; 2 = p-ERBB4, 3 = p-PDGFRα, 4 = p-PDGFRβ, 5 = p-INSR. Representative for 2 primary DIPG cultures (Supplementary Fig. S1).

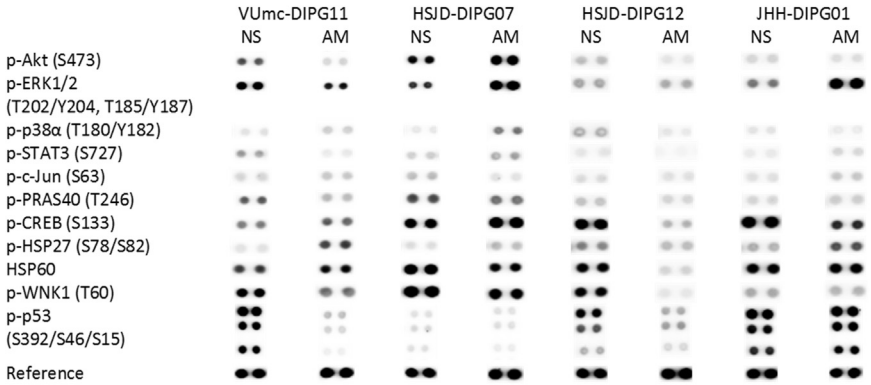

Fig. 3. Phospho-kinase antibody array results depicting differences in signal transduction between neurospheres and adherent monolayers of primary DIPG cultures.

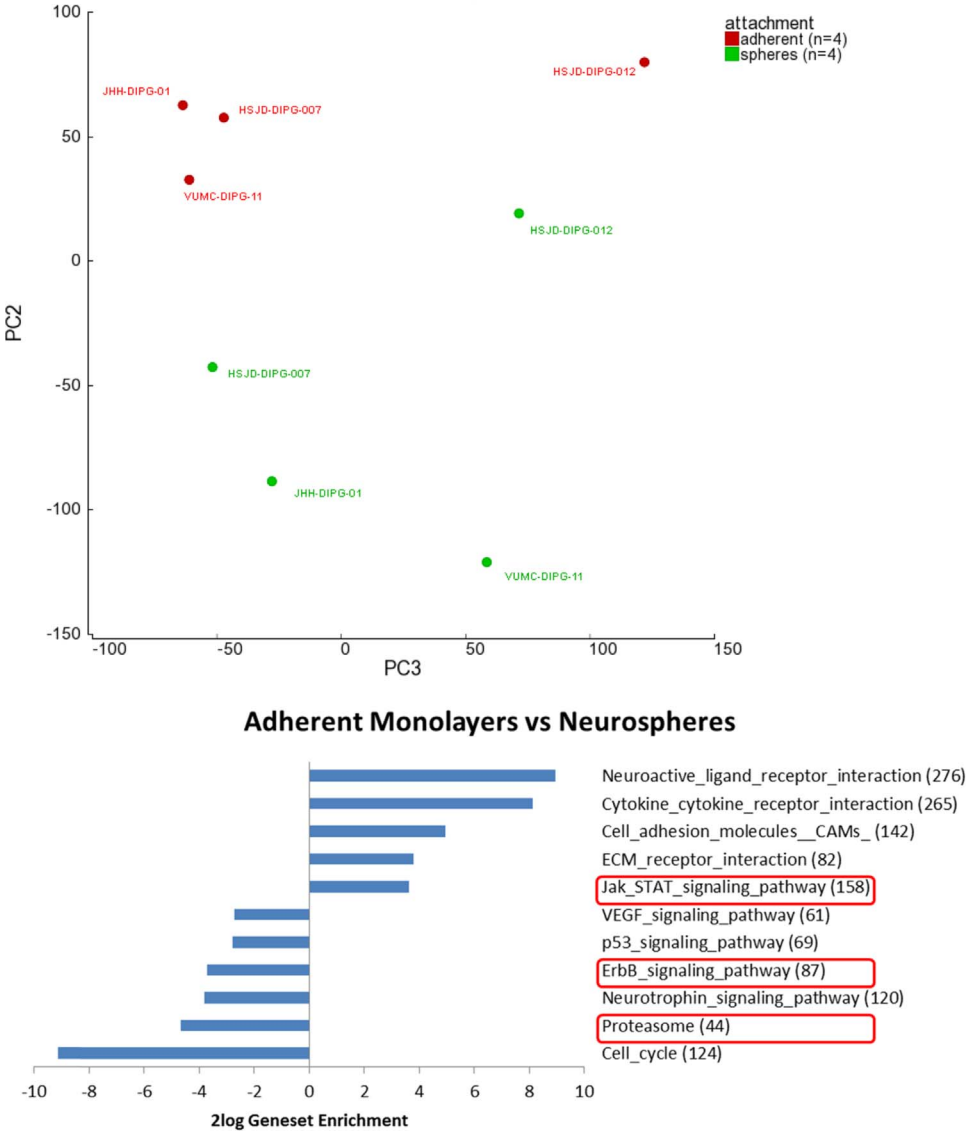

Fig. 4. RNA sequencing of genetically identical primary DIPG cultures reveals culture condition-dependent changes. a: Principle Component Analysis (PCA) reveals preferential clustering of primary DIPG cultures based on culture condition, compared to culture identity, for at least 1 component (PC2). b: Parametric Analysis of Geneset Enrichment (PAGE) showing differential expression between culture conditions of genes involved in signal transduction pathways. Analysis was performed using RNA expression data from all four primary DIPG cultures used in this study. Only genesets with FDR p-value < 0,05 involved in signal transduction are shown.

recently been questioned [19]. The influence of culture conditions on the response of adult glioma cells to chemotherapeutics was highlighted already in 1993 in a study showing the relative resistance of glioma neurospheres over adherent monolayers to the antifolate methotrexate [34]. Although direct comparisons like this are scarce, there are several other studies exemplifying this influence of culture conditions on drug response. Recently, the selective toxicity of Hedgehog pathway inhibition to neurospheres was studied, as compared to genetically identical adherent cells cultured in the presence of FBS [35]. Another recent study showed differential activation of the Akt pathway, and consequent differences in response to inhibition of this pathway, in glioma neurospheres as compared to adherent glioma monolayers [36]. Furthermore, glioma cells cultured as neurospheres were found to display a different metabolic profile compared to identical cell lines grown as monolayers in serum-containing media [37]. Many of these differences are likely caused by the biological actions of components of FBS, as a

2015 study demonstrated no biological or functional differences between glioma cells grown in serum-free medium as neurospheres or as adherent cells on laminin [38].

The influence of culture conditions on the sensitivity of DIPG cells to targeted therapies is especially relevant given that some of the most common genetic aberrancies in DIPG involve genes encoding receptor tyrosine kinases (RTKs). Most frequently, the platelet-derived growth factor receptor alpha (PDGFRα) and epidermal growth factor receptor (EGFR) are either amplified or mutated in DIPG to cause constitutive activation [10,11,13,14]. Based on these findings it was found that adherent monolayers of DIPG cells were sensitive to PDGFRα inhibition by dasatinib, either alone or in combination with the multi-kinase inhibitor cabozantinib [27]. When comparing efficacy of dasatinib between genetically identical DIPG neurospheres and adherent monolayers, we found that dasatinib was unable to inhibit the growth of DIPG neurospheres, whereas the adherent cells were sensitive at the same concentrations as previously described. On the contrary, EGFR inhibition by afatinib inhibited proliferation of DIPG neurospheres, but had very little effect on adherent monolayers. Recently, the multi-kinase inhibitor BMS-754807 has been proposed as another RTK-directed therapeutic option for DIPG [23]. Among its main targets are the insulin receptor (InsR) and insulin-like growth factor-1 receptor (IGF1R). We tested the efficacy of linsitinib, a more specific inhibitor of these RTKs, and found that, as with afatinib, DIPG neurospheres were significantly more vulnerable to the drug than adherent monolayers.

Although not specifically addressed in DIPG research to this date, MEK inhibition has received much attention in the field of adult neuro-oncology and pediatric low-grade gliomas [39–44]. When testing the allosteric MEK inhibitor trametinib, we found that, as for EGFR and InsR/IGF1R inhibition, DIPG neurospheres were selectively sensitive to its antiproliferative effect. Using phospho-kinase and phospho-RTK arrays we could explain the differential sensitivity to many of these compounds. For example, neurospheres possessed higher phosphorylation levels of INSR and EGFR, whereas adherent monolayers displayed higher levels of p- PDGFRα/β. This provides an explanation for the differential sensitivity of adherent monolayers of DIPG cells to dasatinib, and of DIPG neurospheres to afatinib and linsitinib. However, the differential sensitivity of DIPG neurospheres to trametinib could not be explained in this way, as adherent monolayers displayed equal or even higher levels of MAPK pathway activation. A possible explanation for this observation can be found in the gene expression profiles generated by RNA sequencing. In these profiles, an enrichment in transcription of genes involved in the JAK/STAT pathway was seen in adherent monolayers of DIPG cells, providing them with an escape mechanism to MEK inhibition. This correlates with the higher levels of p-STAT3 in adherent monolayers of the cell lines that displayed higher levels of p-ERK1/2 compared to their related neurospheres. Further analysis of RNA sequencing data also provided an explanation for the differential sensitivity of DIPG neurospheres to proteasome inhibition by marizomib, as these neurospheres displayed a significant enrichment in expression of genes involved in proteasome functioning. Finally, the differential expression of cell cycle-related genes, and the differential phosphorylation of p53, between culture conditions may influence the response of DIPG cells to many different drugs, including traditional chemotherapeutics.

One explanation for the differential drug sensitivity of DIPG cultures between culture conditions could be the induction of differentiation by exposure to serum, and thereby a depletion of cancer stem cells in these cultures. However, analysis of a panel of eight genes associated with stemness and differentiation in glioma cells revealed significant differences in the expression of these genes in only one of the four cultures (HSJD-DIPG-12, Supplementary Table S2). Therefore, it seems that serum-induced differentiation of glioma stem cells does not play a major role in the differential drug response and signal transduction between culture conditions. It is important to realize that not all primary DIPG cultures show the same differences in pathway activation or gene expression between culture conditions. This points to different ways of adaptation to a specific culture condition between DIPG cultures, which may in turn influence their sensitivity to drugs. In addition, it is important to realize that both culture methods may reflect different states of tumor cells that probably both occur in DIPG patients. As such, combinational therapies that affect both neurospheres and adherent cells will be needed to develop effective treatment strategies. Therefore, we advocate testing novel therapeutics on DIPG cells cultured using multiple methods before proceeding to animal studies and clinical trials.

Competing interests

None of the authors have any conflicts of interest to disclose.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2017.09.032.

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