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Reprogramming glioma cell cultures with retinoic acid: Additional arguments for reappraising the potential of retinoic acid in the context of personalized glioma therapy

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Abstract

Background: Glioma, notably glioblastoma multiforme, is characterized by extensive inter-and intra-tumoral heterogeneity. Surprisingly, the potential for differentiation of glioma cells has not been systematically analyzed and included in patient stratification methods. In the current study, retinoic acid (RA), a neuronal differentiation agent, was assessed for the pro-differentiative and anti-proliferative effects on glioma cells. **Methods:** Using RA-responsive glioma culture as an experimental paradigm, we analyzed the differentiation process both by videomicroscopy and at the mRNA (RNA-seq and reverse transcription-quantitative-polymerase chain reaction) and proteomic levels. **Results:** Glioma cells can differentiate into neurons in response to RA by (i) extending ultra-long cytoplasmic extensions, (ii) using these extensions to move from cell to cell either by perikaryal translocation or in a “spider-flight” like process, (iii) slowing their cell cycling, (iv) acquiring several neuronal differentiation markers such as MAPT, GAP43, DCX, NRCAM, NeuroD2, NeuroG2, and NeuN, and (v) decreasing the expression of several genes associated with glioma aggressiveness. **Conclusion:** These results indicate the existence of a subgroup of patients harboring RA-responsive glioma cells amenable to differentiation therapy, and stratifying such patients with a functional test is easily achievable. This provides the first step to reassess the potential of RA in the context of personalized medicine.

Keywords: Differentiation therapy, glioma, personalized medicine, retinoic acid

INTRODUCTION

Gliomas are one of the most deadly primary cancers. These primary brain tumors almost invariably relapse despite multimodal treatments combining surgery, radiotherapy, and chemotherapy. Surgery and chemo/radiotherapy can significantly increase the survival rate.^[1] However, glioma still remains a deadly disease. Promising results from early Phase II clinical trials are rarely confirmed in Phase III trials, and innovative targeted therapies have failed to make major breakthroughs to date. This situation again emphasizes the importance of stratifying the patients included in Phase III clinical trials on the basis of biological or molecular markers in relation to the therapeutic target of the trial.

The remarkable degree of plasticity of glioma cells is of major therapeutic concern as it contributes to treatment resistance. However, the differentiation/dedifferentiation potential of cancer cells can be also viewed as a therapeutic opportunity. For instance, inhibiting cancer stem cell or more generally cancer cell renewal by promoting their differentiation toward a postmitotic phenotype would limit cancer progression and tumor growth. This is the basis for

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the concept of reprogramming and differentiation therapy.^[2] One of the most remarkable results of differentiation therapy was achieved approximately three decades ago with the use of retinoic acid (RA) to treat acute promyelocytic leukemia harboring the fusion protein PML-RAR α .^[3] However, therapeutic breakthroughs due to differentiation therapy, either with RA or with other differentiating agents, remain an exception. RA is a Vitamin A metabolite that induces the differentiation of numerous cell types *in vivo*, both during embryogenesis and regenerative processes. For example, RA plays a critical role in the development and maintenance of the nervous system.^[4] Regarding stem cell differentiation, the capacity of RA to induce a neural phenotype is extensively documented both *in vivo* and *in vitro*. RA induces neuronal differentiation of mouse and human embryonic stem cells, neural stem cells, bone marrow hematopoietic stem cells,^[4] and cancer cells, such as teratocarcinoma and neuroblastoma cell lines.^[5-7]

The therapeutic potential of RA in the treatment of glioma was evaluated two decades ago.^[8,9] Although some promising effects were initially reported,^[10] these attempts were considered not conclusive.^[8,9] Consequently, the interest of RA for treating glioma waned, and RA was no longer considered a therapeutic option for primary brain tumors.

Regarding *in vitro* studies, a common observation is that some glioma cells may respond to RA by initiating a differentiation process through the elongation of cell protrusions.^[11,12] In one study, the predominant phenotype within stem-like glioma cells (SLGCs) responding to RA treatment was noted to express an augmented glial staining pattern.^[11] Another study conducted with six different SLGC cultures provided limited evidence that RA could shift one of the six cultures toward neural-like phenotype.^[12] These studies did not focus on the capacity of RA to induce a neural phenotype, and the therapeutic potential of this finding was not further documented. However, understanding and controlling glioma cell plasticity is a prerequisite to devise new strategies to circumvent the problem of therapeutic resistance. Unfortunately, research on the neuronal-like differentiation of glioma cell is currently hampered by the absence of well-defined experimental models. The possibility that RA can induce a neural cell fate in the patient-derived glioma cultures warrants further investigations. This finding suggests that a subgroup of patients might either harbor SLGC or transit amplifying cancer cells with the capacity to differentiate toward a neuron-like phenotype in response to RA. This notion would be consistent with the observation that although retinoid treatment generally failed to improve survival in clinical trials, rarely some patients respond to this therapy.^[13-15] If this is the case, it is necessary to consider the pro-differentiative and anti-proliferative potentials of RA in the stratification of patients to identify the cohort of patients amenable to RA therapy.

MATERIALS AND METHODS

Cell culture and cell cycle analysis

Cells were isolated from freshly resected gliomas and routinely cultured in a serum-free medium consisting of DMEM/F12 (1:1) containing 0.5 N2 and 0.5 B-27 supplements, 2 μ g/mL heparin (StemCell Technologies, Grenoble, France), 30 ng/mL basic fibroblast growth factor, and 30 ng/mL epidermal growth factor (Peprotech, Neuilly Sur Seine, France) as previously described.^[16] All samples were obtained from patients who provided consent to use tissue in accordance with the Institutional Review Board. All glioma samples were from patients with primary glioma undergoing surgery as the first-line therapy. Neuropathological classification of the primary tumor was glioblastoma for the Glio16, 66, 76, and 96 cell cultures and anaplastic oligoastrocytoma class 2 for Glio56. Except Glio96, these cells have already been used in prior publications.^[16-18] Note that in these previous publications, Glio66 was named Glio6. With respect to self-renewal and tumorigenicity, all cells were tumorigenic when implanted in the brain of nude mice [Supplementary Figure 1].^[18] Glio66 (or Glio6) has also been used to induce brain tumor in pig model.^[19] Glio16, 66, 76, and 96 cultures have been passaged at least 30 times, and Glio56 has been maintained in culture for at least 25 passages. All cells grow as floating and/

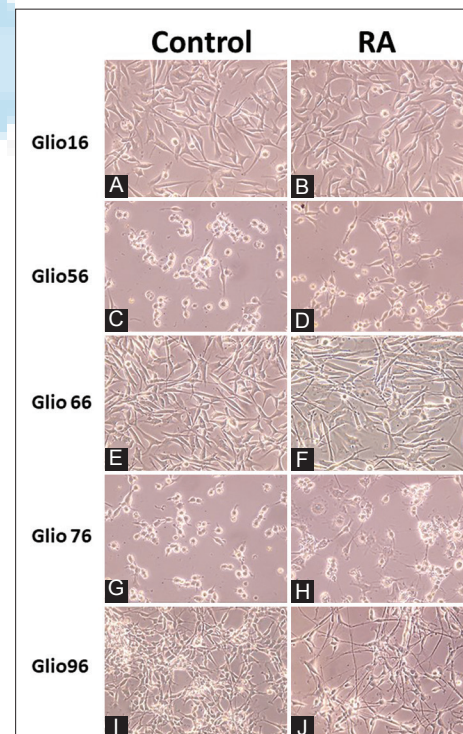


Figure 1: Morphological analysis of the retinoic acid-induced differentiation in glioma cell cultures. Five cell cultures were tested for retinoic acid-induced differentiation. A marked retinoic acid-induced morphological differentiation was observed in Glio96 cells after 7 days of retinoic acid-exposure (J) compared with control (I), and differentiation was absent or weaker in the other retinoic acid-treated cultures (B, D, F and H) against their controls (respectively A, C, E and G)

or semi-adherent cultures. For the differentiation assays and to allow a uniform cell spreading, culture plates were precoated with a mixture of poly-L-lysine and poly-L-ornithine (10 $\mu\text{g}/\text{mL}$ each) (Sigma-Aldrich, Saint Quentin Fallavier, France) and then coated with laminin (2 $\mu\text{g}/\text{mL}$) (StemCell Technologies). For experiments, a B27 supplement without retinol was used.

For cell cycle analysis, cultured cells were collected, washed with phosphate-buffered saline (PBS) and fixed by drop-wise addition of cold 70% ethanol. Cells were then pelleted, resuspended in PBS containing 0.1 mg/mL RNase A and 50 $\mu\text{g}/\text{mL}$ propidium iodide, and incubated for 1 h at room temperature. Then, cells were analyzed by flow cytometry with a BD Accuri flow cytometer (Becton Dickinson Bioscience, Pont de Claix, France). Videomicroscopy experiments were performed using a $\times 10$ objective lens on an Olympus IX81 microscope (Olympus, Hamburg, Germany) equipped with a motorized stage and a digital color DP72 camera (Olympus,

Hamburg, Deutschland). Acquisitions were planed every 20 min for each field of view and were reconstructed as a video with the CellSense software (Olympus, Hamburg, Germany).

Chromosomal microarray analysis

Glio96 cells were analyzed with a 60,000-oligonucleotide microarray (Human Genome CGH Microarray Kit 60K, Agilent Technologies, Santa Clara, CA, USA) as per the manufacturer's instructions. Posthybridization washes, the arrays were scanned using a Microarray Scanner (Agilent Technologies), and the spot intensities were measured by "Feature Extraction Software" (version 10.10.11, Agilent Technologies, Santa Clara, CA, USA). Analyses and visualization were performed with Genomic Workbench, standard edition 6.5 (Agilent) using the following parameters: aberration algorithm ADM-2, threshold 6.0, centralization, and average moving window 2 kb. Aberrant signals including three or more adjacent probes were considered as genomic copy

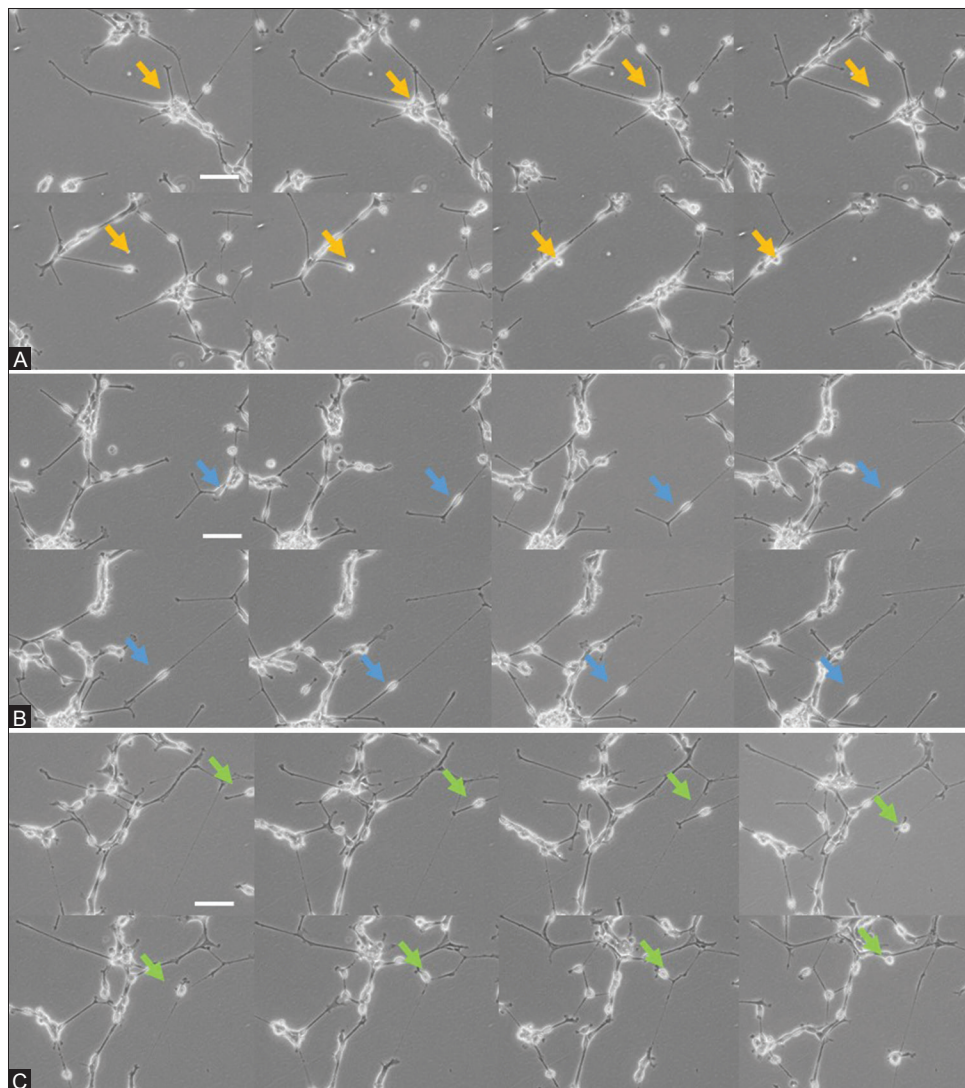


Figure 2: Videomicroscopic analysis of Gli096 cell culture treated with retinoic acid. Cells used their cytoplasmic extension to move via two distinct processes. Migration occurred via soma translocation along the cytoplasmic processes (A; blue arrows) or by swinging in a "spider-flight" or brachiation-like manner (B and C; green and orange arrows). See also supplementary Video 1. Acquisitions were obtained every 20 min; scale bar 50 μm

number variation. Genomic positions are based on the UCSC GRCh37/hg19 assembly.

Proteomic experiments

Proteomic analysis

Cell lysis was performed in Laemmli buffer (2.5×). Samples were deposited on a NuPAGE Bis-Tris Gel 4%–12% acrylamide. Electrophoretic migration was performed in order to fractionate each protein sample into six gel bands. The gels were stained with R-250 Coomassie blue. Protein bands were then excised. Gel slices were washed by three incubations of 20 min in 25 mM ammonium bicarbonate at 37°C, followed by three incubations in 50%/50% (v/v) of 25 mM ammonium bicarbonate and acetonitrile. Gel pieces were then processed and nano-liquid chromatography-mass spectrometry (MS/MS) analyses and analysis of proteomic data were performed as recently described.^[20]

Bioinformatic analysis

The analysis of data was performed using JMP v. 13.0.0 software (SAS Institute JMP, Brie Comte Robert, France). Criteria used to classify the proteins were the Welch *t*-test difference (difference between the two compared conditions of the mean value for triplicate MS/MS analyzes) and the fold change between the two compared conditions.

RNA-sequencing and reverse transcription-quantitative polymerase chain reaction analyses

Total RNA was isolated from cells with the MirVana isolation kit™ (Ambion, Applied Biosystems, Foster City, CA, USA). The quality and quantity of RNA were assessed

with an Agilent Eukaryote Total RNA Nano assay in a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA sequencing was performed with 2 µg of RNA from Gli096 cells treated with RA (10⁻⁷M) or untreated (vehicle alone) for 12 days, using a TruSeq RNA Sample Prep Kit and a HiSeq 2500 platform (Illumina, San Diego, CA, USA) by IntegraGen (Paris, France).

After demultiplexing and conversion of bcl files to fastq files, reads were aligned on the UCSC Genome Browser hg19 genome (NCBI build 37.1). The alignment was performed using TopHat2 software¹, including Bowtie2 (John Hopkins University, Washington, USA).

For quantitative reverse transcription-quantitative polymerase chain reaction (RT-qPCR), 500 ng of total RNA was transcribed into cDNA with random primers using RT reagents (iScript RT Supermix kit) from BioRad Laboratories.

Then, 1/100 of the RT reaction was assayed in duplicate for each gene on a CFX96 Touch™ Real-Time PCR Detection System (BioRad) using the SsoAdvanced™ SYBR Green Supermix kit (BioRad) with 0.25 µM primers in the reaction. RT-qPCR data were normalized using the reference genes, actin beta, ribosomal protein L6 (RPL6), and RPL27 using the 2^{-ΔΔCt} method [Supplementary Table 7]. The primers for quantitative PCR were designed with software (<https://www.roche-applied-science.com/sis/rtqpcr/upl/ezhome.html>).

RESULTS

RA induces long cytoplasmic extensions and reduces cell cycle progression in the Gli096 cell line

First, we analyzed the potential of all-trans-RA to induce differentiation in five in-house glioma cell cultures. A rapid and robust morphological differentiation with a significant sprouting of lengthy and long-lived cytoplasmic extensions

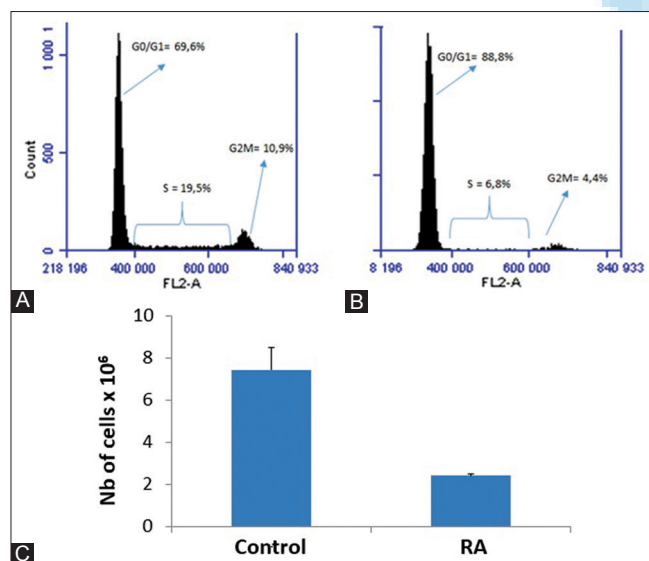


Figure 3: Control (A) or retinoic acid-treated Gli096 cells (B) were stained with propidium iodide and analyzed by flow cytometry. The percentage of cells in G0/G1 was increased in retinoic acid-treated cells (B) compared with control (A), and the percentage of cells in G2M is increased in control compared with retinoic acid-treated cells. (C) Consistent with these results, the cell number was reduced in retinoic acid-treated cultures (Student's *t*-test, *P* < 0.001)

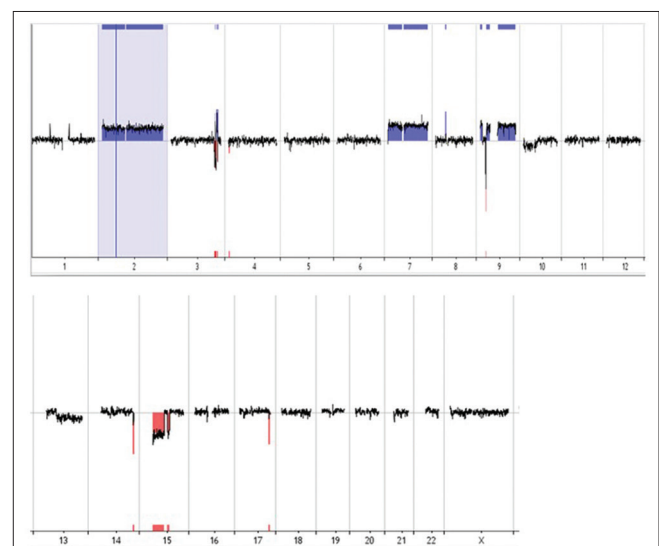


Figure 4: DNA copy number alterations in Gli096 cells. Blue = Gains, red = Losses

was observed in one of the five cell cultures tested, named Glio96 [Figure 1I and J]. Therefore, this cell line was selected for further studies. The differentiation was observed with RA concentrations ranging from 10^6 M to at least 10^{-10} M (data not shown) and the concentration of 10^{-7} M was therefore used in all experiments. Videomicroscopic analysis of Glio96 cells after 7 days of treatment with RA revealed that these ultra-long cytoplasmic protrusions were highly dynamic and that Glio96 cells can use the protrusions to migrate by perikaryal translocation, or in a “spider-flight” like manner [Figure 2 and Supplementary Video 1]. The effect of RA on the cell cycle profile of Glio96 cells was next examined after 11 days of treatment. As expected for a differentiation process, RA-treated

cell cultures exhibited reductions in the cell number and percentage of cells in S and G2/M phases [Figure 3].

Glio96 cytogenetic characteristics

Chromosomal microarray analysis of Glio96 cells revealed the presence of two typical genetically altered regions of primary GBM: gain of chromosome 7 with amplification of the *EGFR* locus and partial loss of 9p including highly recurrent *CDKN2A/B* homozygous deletions. In addition, other well-known amplified regions in GBMs were detected, such as 7q21 (*CDK6*) and amplification of chromosome 2 including *MYCN*. Aberrations enriched in G96 cells are shown in Figure 4.

Table 1: Genes and proteins associated with glioma cell differentiation and up-regulated by Retinoic Acid in both transcriptomic and proteomic analyses

Gene Name	Protein name	Function (http://www.genecards.org/)	# peptides	Fold change RNAseq	Fold change protein level
GFRA1	GDNF Family Receptor Alpha 1	neuron survival and differentiation	4	6.2	14.5
EPHB2	EPH Receptor B2	functions in axon guidance	14	4.5	7.4
INA	Interneuxin Neuronal Intermediate Filament Protein Alpha	neuronal intermediate neurofilament	19	4.4	3.9
MAPT	Microtubule-associated protein	Establishment/maintenance neuronal polarity	7	2.4	3.6
NRP2	Neuropilin 2		3	4.1	3.1
NRCAM	Neural Cell Adhesion Molecule	role in neurite outgrowth	6	525	3
SNAP25	Synaptosome Associated Protein 25	regulation of neurotransmitter release	5	2.8	2.9
CNTNAP1	Contactin Associated Protein 1		5	2.2	2.8
GAP43	Growth Associated Protein 43	role in axonal and dendritic filopodia induction	8	2.7	2.7
RAB3A	RAB3A Gene	regulation of synaptic vesicle fusion	5	4.6	2.6
HPCAL1	Hippocalcin Like 1	neuron-specific calcium-binding protein	3	3.9	2.5
EPB41L1	Erythrocyte Membrane Protein Band 4.1 Like 1	binds and stabilizes D2 and D3 dopamine receptors	7	2.8	2.5
DPYSL4	Dihydropyrimidinase Like 4	axon guidance, neuronal growth cone collapse	22	0.2	2.5
DCX	Doublecortin	neuronal migration	8	3.9	2.4
NFASC	Neurofascin	neurite outgrowth	4	6.6	2.3
MAP1B	Microtubule Associated Protein 1B	neurogenesis	93	2.2	2.1
LPPR1	Phospholipid Phosphatase Related 1	mediate lipid phosphatase activity in neurons, involved in neuronal plasticity	2	9.9	CTRL ND
CEND1	Cell Cycle Exit And Neuronal Differentiation	neuron specific protein, enhances neuroblastoma cell differentiation	2	2.5	CTRL ND
CNTN2	Contactin 2	role in proliferation, migration and axon guidance of neurons	6	14.9	CTRL ND
STX1B	Syntaxin 1B	role in exocytosis of synaptic vesicles	2	3.2	CTRL ND
TOR1A	Torsin Family 1 Member A	involved in the regulation of synaptic vesicle recycling	3	2	CTRL ND
LSAMP	Limbic System-Associated Membrane Protein	neuron surface glycoprotein	2	2.5	CTRL ND
ARHGEF11	Rho Guanine Nucleotide Exchange Factor 11	involved in neurotrophin-induced neurite outgrowth	3	2.2	CTRL ND
CADM3	Cell adhesion Molecule 3	axon and radial glial cell adhesion molecule	4	3.7	CTRL ND
KALRN	Kalirin	regulate neuronal shape, growth and plasticity	4	2.8	CTRL ND
GDAP1L1	Ganglioside Induced Differentiation Associated Protein 1 Like 1	involved in cell differentiation with neurite sprouting	3	4.4	CTRL ND
NRXN1	Neurexin1	neuronal cell surface protein	3	3.2	CTRL ND
SGK223	Related Kinase	regulates neurite outgrowth	4	3.1	CTRL ND
PEAK1					
PLXNA3	Plexin A3	axon guidance	6	3.2	CTRL ND
SDK1	Sidekick Cell Adhesion Molecule 1	synaptic connectivity	2	7.4	CTRL ND

Table 2: Genes and proteins associated to glioma cell differentiation or cell cycle progression and down-regulated by Retinoic Acid in both transcriptomic and proteomic analyses

Gene Name	Protein Name	Function (http://www.genecards.org/)	# peptides	Fold change RNAseq	Fold change protein level
CNTN1	Contactin 1	cell surface interaction during nervous system development	19	0.3	0.5
SMC2	Structural Maintenance of Chromosomes 2	required for mitotic-like condense chromosome	32	0.5	0.5
RRM1	Ribonucleotide Reductase Catalytic Subunit M1	essential for the conversion of ribonucleotides into deoxyribonucleotides	15	0.5	0.5
WDHD1	WD Repeat and HMG-Box DNA binding Protein	involved in chromatin assembly, transcription and replication	10	0.4	0.5
NDC80	NDC80 Kinetochore Complex Component	required for proper chromosome segregation	3	0.4	0.5
RRM2	Ribonuclease Reductase Regulatory Subunit M2	catalyzes the formation of deoxyribonucleotides from ribonucleotides	5	0.02	0.4
MCM2	Minichromosome Maintenance Complex Component 2	initiation of eukaryotic genome replication	22	0.4	0.4
MCM3	Minichromosome Maintenance Complex Component 3	initiation of eukaryotic genome replication	25	0.4	0.4
MCM4	Minichromosome Maintenance Complex Component 4	initiation of eukaryotic genome replication	21	0.4	0.4
MCM6	Minichromosome Maintenance Complex Component 6	initiation of eukaryotic genome replication	24	0.1	0.4
MCM7	Minichromosome Maintenance Complex Component 7	initiation of eukaryotic genome replication	18	0.2	0.4
CHRAC1	Chromatin Accessibility Complex 1	DNA transcription, replication and packaging	4	0.5	0.4
OLIG2	Oligodendrocyte Lineage Transcription Factor 2		3	0.4	0.4
CCND2	Cyclin D2	regulates the cell cycle G1/S transition	4	0.1	0.4
KIF4A	Kinesin Family Member 4A	interdigitates spindle microtubules during mitosis; role in cytokinesis	8	0.5	0.4
KIF4B	Kinesin Family Member 4B	role in anaphase spindle dynamics and cytokinesis	8	0.5	0.4
GINS4	GINS Complex Subunit 4	essential role in the initiation of DNA replication	3	0.1	0.4
TYMS	Thymidylate Synthetase	maintenance of the dTMP pool critical for DNA replication and repair	4	0.3	0.3
DUT	Deoxyuridine Triphosphatase	provides a precursor for thymine nucleotides needed for DNA replication	4	0.2	0.2
UHRF1	Ubiquitin Like With PHD and Ring Finger Domains 1	major role in G1/S transition	13	0.3	0.1
TONSL	Tonsoku-Like DNA repair Protein	maintains genome integrity during DNA replication	2	0.3	RA ND
WDR62	WD repeat Domain 62	role in neuronal proliferation and migration, and in centriole duplication	2	0.2	RA ND
HAUS8	Haus Augmin Like Complex Subunit 8	involved in microtubule generation within the mitotic spindle	2	0.5	RA ND
GLI2	GLI Family Zinc Finger 2		2	0.2	RA ND
TLK1	Tousled Like Kinase 1	regulates processes involved in chromatin assembly	2	0.5	RA ND
SMC5	Structural Maintenance Of Chromosome 5	required for sister chromatid cohesion during mitotic progression	2	0.1	RA ND
TCF3	Transcription Factor 3	axonal growth inhibitor	2	0.5	RA ND
GINS3	GINS Complex Subunit 3	essential for the initiation of DNA replication	3	0.4	RA ND
CENPV	Centromere Protein V	required for centrosome organization and chromosome alignment	2	0.1	RA ND
CEP112	Centrosomal Protein 12	component of the human centrosome	2	0.4	RA ND
ELMO1	Engulfment And Cell Motility	promotes glioma cell invasion	4	0.5	RA ND
GADD45GIP1	GADD45G Interacting Protein 1	inhibits G1 to S phase progression	5	0.5	RA ND
EML1	Echinoderm Microtubule Associated Protein Like 1	required for normal proliferation of neuronal progenitor cells	5	0.5	RA ND

Table 3: Confirmatory quantitative reverse transcription-polymerase chain reaction on the subset of genes identified by RNA-sequence or proteomic analysis as retinoic acid regulated in Gli096 cells

Gene Symbol	Gene Name	Fold (G96 RA vs. G96 CTL) mRNA Seq	Mean Fold (G96 RA vs. G96 CTL) RT-qPCR	Wilcoxon Mann-Whitney Test (<i>n</i> =6) RT-qPCR
ACHE (NeuN)	Homo sapiens acetylcholinesterase (Cartwright blood group) (ACHE), transcript variant E4-E6, variant 4, variant 5	4	10	<i>P</i> <0.001
AURKB	Homo sapiens aurora kinase B (AURKB), transcript variant 1,2,3,4,5,6,7,8,9	0,1	0,4	<i>P</i> <0.01
CHRNA3	Homo sapiens cholinergic receptor nicotinic alpha 3 subunit (CHRNA3), transcript variant 1	5	8	<i>P</i> <0.05
CRABP1	Homo sapiens cellular retinoic acid binding protein 1 (CRABP1)	12	27	<i>P</i> <0.001
CXCR4	Homo sapiens C-X-C motif chemokine receptor 4 (CXCR4), transcript variant 2,3,4	0,3	0,1	<i>P</i> <0.01
CYP26B1	Homo sapiens cytochrome P450 family 26 subfamily B member 1 (CYP26B1), transcript variant 1	90	320	<i>P</i> <0.001
DCX	Homo sapiens doublecortin (DCX), transcript variant 2,5	3	6	<i>P</i> <0.001
GAP43	Homo sapiens growth associated protein 43	3	3	<i>P</i> <0.001
INA	Homo sapiens Internexin neuronal intermediate filament protein, alpha	4	7	<i>P</i> <0.001
ITGB3	Homo sapiens Integrin subunit beta 3	6	9	<i>P</i> <0.05
L1CAM	Homo sapiens L1 Cell Adhesion Molecule transcript variant 1,4	14	9	<i>P</i> <0.001
MAPT	Homo sapiens microtubule-associated protein tau	2	2	<i>P</i> <0.05
MCM2	Homo sapiens minichromosome maintenance complex component 2 (MCM2), transcript variant 1	0,4	0,4	<i>P</i> <0.05
MCM7	Homo sapiens minichromosome maintenance complex component 7 (MCM7), transcript variant 1,2,3	0,2	0,4	<i>P</i> <0.01
MYC	Homo sapiens V-Myc Avian Myelocytomatosis Viral Oncogene Homolog	0,5	0,4	<i>P</i> <0.05
MYT1L	Homo sapiens myelin transcription factor 1 Like	15	50	<i>P</i> <0.001
NEFL	Homo sapiens neurofilament, light polypeptide	ND	8	<i>P</i> <0.05
NEUROD2	Homo sapiens neuronal differentiation 2 (NEUROD2)	5	9	<i>P</i> <0.05
NEUROG2	Homo sapiens neurogenin 2 (NEUROG2)	23	16	<i>P</i> <0.05
NFASC	Homo sapiens neurofascin (NFASC), transcript variant 2,3,4	7	3	<i>P</i> <0.05
NRCAM	Homo sapiens neuronal cell adhesion molecule	525	10	<i>P</i> <0.05
OLIG2	Homo sapiens oligodendrocyte lineage transcription factor 2	0,4	0,4	<i>P</i> <0.001
PCNA	Homo sapiens proliferating cell nuclear antigen	0,2	0,2	<i>P</i> =0.05
RBFOX3	Homo sapiens RNA binding protein, fox-1 homolog 3 (RBFOX3), transcript variant 1,2,3	7	9	<i>P</i> <0.01

Transcriptomic and proteomic response of Gli096 cells to RA treatment

As a first step to investigate the molecular basis of the Gli096 cell response to RA, a genome-wide transcriptomic analysis by RNA sequencing (RNA-seq) was performed. Gli096 cells were treated, or not, with 10^{-7} M RA for 12 days. The dataset of genes with different expression levels between control and RA-treated cells was identified using cutoff values of ≤ 0.5 and ≥ 2 -fold change. Under these conditions, RNA-seq analysis identified a change in 8567 transcripts (4683 increased and 3884 decreased) within a total set of 26,660 transcripts [Supplementary Table 1]. These 8567 differentially regulated transcripts correspond to 8071 genes (4455 upregulated and 3616 downregulated).

To further refine the analysis, we next compared the proteome profiles of RA-differentiated cells with control cells. In total, 3721 proteins were detected and quantified [Supplementary Table 2]. Using the same cutoff values as those used for RNA seq

analysis (≤ 0.5 and ≥ 2 -fold change), we identified 815 proteins with expression that varies at least two folds following RA treatment (433 upregulated and 382 downregulated by RA treatment, respectively) [Supplementary Tables 3 and 4].

By crossing the set of the 8567 transcripts previously described as differentially expressed following RA treatment with the 815 proteins detected as up- or down-regulated by RA, we identified an overlapping set of 247 mRNA sequences (126 upregulated [Supplementary Table 5] and 121 downregulated [Supplementary Table 6]) that correspond to 215 proteins. These 215 proteins were then used for a GeneOntology enrichment analysis (<http://www.geneontology.org/>). The top four “gene biological processes” obtained with the upregulated genes/proteins are “regulation of synaptic plasticity”, “synapse organization”, “neuron projection guidance”, and “axonogenesis”. The top four gene biological processes corresponding to the list of downregulated genes/proteins are “DNA unwinding involved

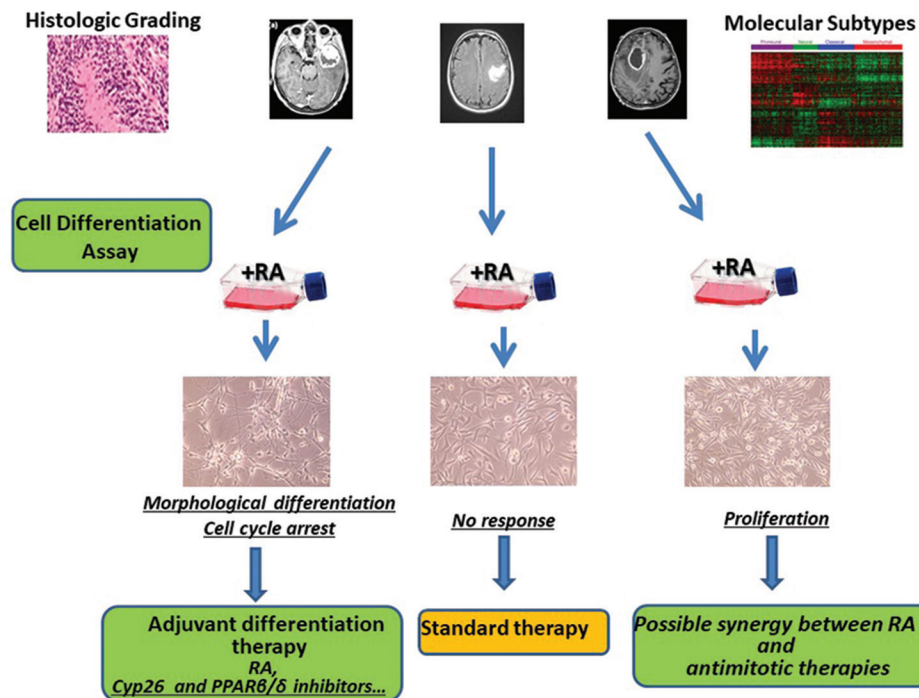


Figure 5: From a therapeutic standpoint, a classification based on a functional test makes sense. For example, this method is the basis of profiling of antibiotic sensitivity of bacterial species isolated from clinical samples. Currently, the diagnosis and treatment of gliomas are based on tumor histology and molecular classification. The existence of glioma cells with differentiation potential in a subset of patients suggests that testing this potential for differentiation could be utilized as an additional criterion for patient stratification and to define those patients amenable to differentiation therapy. Determining how the results of this functional assay overlap with currently used histologic markers or genomic or transcriptomic classifications, which is critical

in DNA replication”, “deoxyribonucleotide biosynthetic process”, “DNA replication initiation”, and “G1/S transition of the mitotic cycle”. The differentially regulated genes/proteins we identified that are relevant to these processes are listed in Tables 1 and 2.

We next confirm these results by RT-qPCR on a set of genes representative of these two biological processes, namely “neural differentiation” (*ACHE*, *CHRNA3*, *DCX*, *GAP43*, *INA*, *ITGB3*, *LICAM*, *MAPT*, *MYT1L*, *NEUROD2*, *NEUROG2*, *NFASC*, *NRCAM*, and *OLIG2*, *RFX3*) and “cell cycle progression” (*AURKB*, *MCM2*, *MCM7*, *MYC*, and *PCNA*). As controls, we also included *CRABP1* and *CYP26B1*, two prototypical genes regulated by RA. Several proteins with transcripts differentially expressed in the transcriptomic analysis were not detected in the proteomic analysis, likely due to their low levels. Therefore, our RT-qPCR confirmatory analysis also included some relevant genes such as *NeuroD2* and *NeuroG2* selected on the basis of their differential expression in the RNA-Seq dataset only [Table 3]. Regarding the set of genes downregulated and associated with DNA replication, confirmatory RT-qPCR was performed for *MMC2* and *MMC7*, two key components of the prereplication complex, and *AURKB*, the inhibition of which impairs malignant glioma growth *in vivo*.^[21] The downregulation of *Olig2*, *cMyc*, and *CXCR4* was also confirmed.

Overall, RT-qPCR results confirmed the conclusions of the morphologic, transcriptomic, and proteomic analyses, namely, that RA induces a neuronal fate associated with an inhibition of the cell cycle in Gli096 cells.

DISCUSSION

The intratumoral heterogeneity of gliomas is a major cause of therapeutic failure. However, the glioma cell plasticity can in principle be turned into a therapeutic opportunity. This notion serves as the basis of differentiation therapy. Unfortunately, glioma differentiation therapy has been an elusive goal. Due to the intertumoral heterogeneity of gliomas, the efficiency of differentiating drugs would probably differ between patients depending on whether the glioma initiates from cells in which differentiation has been arrested at the NSC stage or if the SLGCs arise from the transformation of an oligodendrocyte progenitor cell, a radial glial cell with progenitor potential, or even from the dedifferentiation of mature astrocytes or neurons.^[22] With respect to the latter, the direct generation of human neuronal cells from fetal and adult astrocytes by small molecules demonstrates the potential for retro-differentiation that exists even in adult mature human astrocytes.^[23] Note that the purpose of differentiation therapy is not the same for neurodegenerative disease and glioma treatment. Here, contrary to regenerative therapy, we do not intend to derive functional neurons from glioma cells, which is likely an unfeasible task if we consider the extent of their

chromosomal anomalies and the pathological microenvironment to which cancer cells are challenged in patients. The therapeutic purpose is rather to induce a sufficient phenotypic shift to decrease the proliferative potential of glioma cells and/or increase their sensitivity to therapy.

A key issue that limits research on the differentiation potential of glioma cells is the lack of relevant experimental cell lines. This matter should not be overlooked. Although culture systems cannot recapitulate the complexity of the *in vivo* situation, they offer the opportunity to study how differentiation agents impact the differentiation potential of glioma cells and/or increase their sensitivity to adjuvant therapies under well-defined conditions.

Few studies have analyzed the effect of RA on the neurogenic potential of glioma cells *in vitro*.^[11,12,24,25] In one study, the effect of RA on glioma cells is supported by increased GFAP synthesis, impaired secretion of angiogenic factors, decreased cell motility, and reduced tumorigenicity.^[11] However, the effect of RA on the neurogenic potential of these cancer cells was not addressed. Other reports describe either a decreased proliferative effect of RA^[24] or, conversely, an increased proliferation.^[25,26] In this later study, RA increases expression of the neuronal marker TUJ1, but this observation was not further exploited.^[25] In a more recent study using SLGC lines from six human glioblastomas and two gliosarcomas, a neuronal differentiation process assessed on the basis of increased expression of the neuronal proteins TAU and MAP2 was observed in only one of the eight tested cell lines.^[12] Again, investigations on the therapeutic potential of this observation were not advanced. Taken together, these published data demonstrate that: (i) the RA can have either proliferative or anti-proliferative effects depending on glioma cell cultures and (ii) the RA-induced neuronal-like differentiation of glioma cell cultures is far from a general feature. Apparently, such *in vitro* conflicting observations together with the disappointing results of clinical trials explain the loss of interest in RA therapy for the treatment of gliomas.

In the present study, RA rapidly induces ultra-long cytoplasmic protrusions in one of the five of our cell cultures, namely, Gli096 cell. This ratio is consistent with previous observations,^[11,12] and is not surprising if we consider the well-known intertumor heterogeneity of gliomas.

Regarding molecular considerations, the Gli096 cell line used in this study harbors some of the recurrent genomic abnormalities of glioma including chromosome 7 gain with amplification of EGFR and deletion of the cyclin-dependent kinase inhibitors CDKN2A and CDKN2B on chromosome 9.^[27] The duplication of chromosome 2 detected in Gli096 is not a recurrent feature of gliomas. Gains of chromosome 2 have been detected in approximately 10% of gliomas.^[28] Polysomy for chromosome 2 was also observed in a glioblastoma with neuroectodermal tumor-like components^[29] and in the HNGC-1 glioma neuro-epithelial cell line, which exhibits large cytoplasmic extensions analogous to those observed in RA-treated Gli096 cells.^[30] The possibility that this polysomy

for chromosome 2 defines a subtype of glioma accessible to differentiation therapy warrants further investigations.

By cross analyzing our transcriptomic and proteomic analyses, we demonstrated that the change in cell morphology induced by RA is accompanied by (i) the upregulation of a large number of neuronal markers [Table 1] and (ii) the downregulation of several proteins involved in DNA replication and mitosis [Table 2]. The strength of this cross-analysis is supported by the finding that the change (increase or decrease) varies in the same direction for nearly each couple RNA/protein. This trend toward a neuronal differentiation is also confirmed by RT-qPCR on a limited number of genes (*DCX*, *GAP43*, *INA*, *MAPT*, and *NRCAM*) selected for analysis on the basis that their corresponding proteins are well-known markers of the neuronal differentiation process. In addition to its role in neurogenesis, GAP43 is also used by glioma cells to extend the recently described tumor microtubes (TMs).^[31,32] We also observed a reduction in Olig2 expression at both proteomic and transcriptomic levels. Olig2 belongs to a set of transcription factors essential for glioblastoma propagation that reprogram differentiated glioblastoma cells to SLGCs.^[33] Accordingly, Olig2 deletion delays glioma growth.^[34] RT-qPCR analysis was also performed for some additional genes relevant to neuronal differentiation selected on the basis that they are upregulated by RNA-seq. This set of genes includes two transcription factors involved in neurogenesis (NeuroD2, NeuroG2), the neuronal marker RBFOX3/NeuN, the neuronal cell adhesion molecule L1CAM, the neurotransmitter hydrolyzing enzyme Ache, and the neurotransmitter receptor CHRNA3. These results confirm the conclusion of the transcriptomic analysis and are consistent with the shift toward neuronal fate in response to RA.

Our cross-analysis also provides first clues on the effects of the RA treatment on several proteins implicated in cell division. Of importance is the downregulation of proteins involved in deoxynucleotide synthesis (RRM1, RRM2, TYMS, and DUT) and in the mitotic process (WDHD1, NDC80, CHAC1, KIF4A, KIF4B, GINS4, TONSL, TLK1, SMC5, GINS3, CENPV, and CCND2) [Table 2]. An interesting feature is the downregulation of the members of the MCM family (MCM2, 3, 4, 6, and 7), which constitute the core of the replicative helicase involved in all stages of the chromosome replication cycle.^[35] Regarding cell cycle regulation and in light of the extensive list of proteins involved and the consistency of the results including cell cycle analysis and cell counting, we limited our confirmatory RT-qPCR analysis to two of these downregulated transcripts, namely, those coding for MCM2 and MCM7 [Table 3].

We also confirmed the downregulation of *cmyc*, *CXCR4*, and *Aurora B* transcripts by RT-qPCR [Table 3]. The observed downregulation of *cmyc* expression is consistent with the results of the cell cycle analysis, given that cMyc is involved in G1 cell cycle progression. CXCR4 is a cell surface chemokine receptor for CXCL-12, which is overexpressed in SLGCs compared with the corresponding differentiated

cells.^[36] As CXCR4 is the receptor of CXCL12, it mediates glioma cell invasion and proliferation in response to this inflammatory cytokine.^[36] Therefore, the observed CXCR4 downregulation in response to RA treatment is therapeutically relevant. The downregulation of Aurora B expression is also therapeutically relevant. Aurora B levels are correlated with reduced survival in glioma patients.^[37] Inhibition of Aurora kinases also enhances the chemosensitivity to temozolomide and the efficiency of radiotherapy.^[38,39] This finding raises the possibility that RA might synergize with the radiotherapy/temozolomide protocol in some patients.

Morphologically, Gli96 cells respond to RA by the extension of ultra-long cell protrusions. These structures are highly dynamic and interconnect glioma cells to create a multicellular network. Such cytoplasmic extensions are reminiscent of the neuronal growth cones observed during development and recently described TMs.^[31,32] TMs are ultra-long cytoplasmic protrusion exceeding 500 μm in length that have been recently identified in astrocytoma *in vivo*.^[31,32] These cell protrusions are used *in vivo* by astrocytoma cells to migrate and to form therapy-resistant multicellular networks of interconnected cells.^[31,32] Here, we demonstrate that Gli96 cells use their RA-induced cytoplasmic protrusions *in vitro* either as cables to migrate by perikaryal translocation in a manner similar to that observed in neuroblastoma or radial glial cells,^[40-42] or by swinging in a “spider-flight” or a brachiation-like process [Figure 2 and Supplementary Video 1]. Our *in vitro* observations are reminiscent of the role played by glioma TMs *in vivo*, given that TMs are associated with glioma cell migration.^[31] It seems unlikely that these RA-induced cell extensions evolved *de novo* only to ensure the migration of glioma cells. We would like to suggest that the physiological function of this process is to participate in neural cell migration and the formation of neuronal networks during embryogenesis. Glioma cells might reactivate and hijack this developmental program. As a result, glioma cells could migrate away from the acidic and hypoxic tumor mass and create the therapy-resistant network as recently described.^[31,32] However, in the tumor microenvironment of glioma patients and in the absence of adequate morphogenetic cues, such as RA gradients, the reactivation of this embryogenic process would be maladaptive and would contribute to cancer progression and recurrence.

Regarding the clinical interest of RA, it may seem counterintuitive to use a drug that can induce such extensions that can be used for cell movement and may also be associated with a therapy-resistant phenotype. However, we need to address several generally overlooked key points which all converge to suggest that this apparently maladaptive response could be turned into a therapeutic response if it is adequately managed.

First, although glioma cells invade the brain parenchyma, multifocal glioma is not the rule. A possible explanation to this apparent paradox is provided by the “Go-or-Grow” hypothesis which proposes that cell division and cell

migration are two temporally exclusive events.^[43-46] In cancer patients, tissue injury, inflammation, and hypoxic and acidic microenvironments, which are all associated with the tumor mass, play a central role in tumor progression and invasion.^[47-50] Therefore, the migration of glioma cells away from this protumorigenic- and cancer progression-prone microenvironments should limit the progression of glioma cells toward a more aggressive phenotype. In other words, the relative scarcity of multifocal glioma, despite the invasiveness of glioma cells in the brain parenchyma, suggests that glioma cells might acquire a dormant phenotype once they have migrated into “normal” noninjured brain parenchymal area. In this still hypothetical model, such dormant glioma cells would be reactivated when they are challenged again by the injuries induced either by the continuously growing primary tumor mass or by some therapy-triggered side effects.^[51] This issue warrants further investigations.

The other point to consider is the therapy resistance of the TM-bearing glioma cells.^[31,32] TMs are associated *in vivo* with glioma chemo- and radiotherapy resistance and tumor regrowth after surgery.^[31,32] Thus, the inhibition or disruption of TMs appears as a promising therapeutic approach.^[32] In this context, the suggestion to promote a differentiation therapy inducing TM-like cytoplasmic protrusions may seem counterintuitive. However, we must also take into account that the morphological differentiation process and the multicellular network induced *in vitro* by RA are associated with cell cycle braking, at least in Gli96 cells. If such exit from the cell cycle also occurs *in vivo*, then interconnected glioma cells would be in a relatively quiescent state, which could participate in the observed chemo- and radio-resistance.^[31] Indeed, cells arrested in their cell cycle by a differentiation therapy become resistant to therapies targeting actively proliferating cells.

It took a long time to accept that cancer treatments can have either anti-tumorigenic or pro-tumorigenic effects on the same patient depending on a space–time context.^[51-53] This notion has led to the target/anti-target concept,^[54,55] which is supported by the paradoxical responses that can be observed with therapies targeting, for example, angiogenesis, matrix metalloprotease activities, or tumor growth factor- β .^[54,56-58] From a clinical standpoint, double-edged effects of RA therapy must be anticipated. For example, we must address with the observation that RA can be either proliferative or anti-proliferative in glioma cells.^[24-26] One molecular basis for this double-edged effect of RA, either facilitating or inhibiting cell growth, is provided by the finding that RA has a dual transcriptional activity; one action is mediated by RARs and often results in growth inhibition and cell differentiation, and the other action is mediated by PPAR β/δ for cell proliferation.^[59] An achievable strategy for improving the potential of a RA-induced differentiation therapy would be to concomitantly promote the activation of RAR and to inhibit the activation of PPAR β/δ .^[60]

A limitation of this study was that the in-depth proteomic and transcriptomic analyses were focused on glioma cell

culture (namely Gli096 cells), from one patient. Of note, using personalized models should result in more accurate predictions of drug responses. This is the very principle of personalized cancer therapy. Importantly, our results are consistent and further strengthen previously published observations on the pro-differentiative and anti-proliferative effects of RA on a limited subset of cultures. The key point we needed to address in this study was that this RA responsiveness can be easily assessed on the basis of an *in vitro* functional test for patient stratification. Another limitation of the present study was that we did not investigate the molecular basis of the response of RA. Investigating the signaling pathway(s) recruited by RA is of obvious importance and warrants further studies. The response to RA is incredibly complex and not restricted to its genomic action through its nuclear RAR/RXR receptors. For example, Crabp1 has recently shown to mediate nongenomic action of RA and to modulate stem cell proliferation by activating extracellular-regulated kinase ERK1/2.^[61] Deciphering the mechanisms of action and the differences existing between responsive and nonresponsive cells was well beyond the scope of our study. It is important to underline again that our study is not dedicated to investigate how RA works but to propose a functional test to reassess the potential of RA in clinical trials after patient stratification. Our article must be viewed as an observational study. Many important therapeutic breakthroughs have originated from simple observations and were clinically applied well before their mechanisms of action were elucidated. Semmelweis discovered the principle of asepsis in 1847 three decades before Pasteur and Koch provided the corresponding mechanistic explanation with the germ theory of disease.^[54] Aspirin has been widely used well before it was shown to suppress the production of prostaglandins and thromboxanes in 1971.^[62] Fleming reported the antibacterial action of cultures of a penicillium in 1929,^[63] but the mechanism of action was elucidated in the late 1950s to early 1960s.^[64,65] Benzodiazepine was first discovered accidentally in 1955 and was made available in 1960 in the ignorance of its mechanism of action. More recently, in 1995, deep brain stimulation was reported to reduce motor disturbance in Parkinson's disease in the absence of any mechanistic explanation.^[66] How many patients would have suffered or died of their diseases if we have awaited mechanistic explanations before publishing these seminal observations? Stratifying glioma patients on the basis of a functional test to assess their responsiveness to RA would have much more modest consequence, but this approach warrants to be considered. Indeed, as regards in particular glioma, the request for mechanisms as a standard for academic publication might be debatable as it could significantly delay the therapeutic breakthroughs our patients urgently need.

We characterized the transcriptomic and proteomic responses to RA in the Gli096 cell line. In these cells, RA induces a differentiation process characterized by the extension of ultra-long cytoplasmic protrusions concomitant to cell cycle braking. Consistent with this morphological

differentiation process, the transcriptomic and proteomic analyses support a shift toward a neuronal cell fate with the induction of numerous neuronal markers and the concomitant downregulation of components of the proliferation machinery. Hence, Gli096 cells provide a useful tool for deciphering the mechanisms of glioma cell differentiation, and for studying the genesis, the development, and the function of these cytoplasmic extensions. We also confirm that the rapid acquisition of this neuronal-like differentiation process following RA treatment is not a general feature of all cultures. Current histologic grading and molecular subtype classification do not prospectively predict or even attempt to predict which patients could benefit from the neurogenic effects of RA. Assessing the neurogenic potential of glioma cells for each individual patient can be routinely performed *in vitro* and could be relevant for patient stratification [Figure 5]. Whether this stratification will overlap with already well-identified molecular or genomic alterations,^[67,68] or with one of the four subtypes (proneural, neural, classical, and mesenchymal) identified on the basis of gene expression must now be investigated.^[69] Developing an *in vitro* cell culture assay routinely assessing the RA response of glioma cells would provide the rationale to reassess the therapeutic potential of RA on the cohort of patients harboring such RA-inducible neurogenic cells. However, we must bear in mind the possible dual role played by this differentiation process in tumor growth and recurrence. In any case, RA treatment should be a sequential step in a multimodal therapeutic schedule.

However, considerable experimental work remains to be done, and several critical issues need to be addressed before any *in vivo* studies. The finding that RA can induce either proliferation, or differentiation processes in glioma cell cultures, and the complex regulation of RA signaling in glioma patients must be considered.^[25,26,59,70] It is also worth noting that any RA therapy must address the rapid and dramatic catalysis of RA by its metabolizing and inactivating enzyme CYP26B1. Consistent with this notion is the marked decline in plasma RA concentration observed in patients during continuous RA treatment.^[9] This negative regulatory loop is not unexpected, given the morphogenetic role of RA during embryogenesis, which is mostly mediated by local concentration gradients acting through paracrine signaling. Hence, the systemic delivery of RA might not be the best method to handle this drug efficiently. The pharmacokinetics of RA must be more clearly defined before any *in vivo* trial. The interest of combining RA therapy with CYP26 or PPAR β/δ inhibitors can be considered.^[71,72] Of note, the response to RA therapy might differ when glioma cells are challenged by the inflammatory and acidic/hypoxic microenvironments of the tumor mass or side effects of the conventional therapies.^[51] Addressing the therapeutic potential of RA in a differentiation therapy clinical trial is surely more complex than previously considered. Any reassessment of the potential of RA as an adjuvant therapy in the treatment of glioma will have to consider these different points to determine both the relevant cohort of patients and the optimal therapy schedule.

Supplementary Material

Supplementary material is available at Glioma online (<http://www.jglioma.com/>).

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Conflicts of interest

There are no conflicts of interest.

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