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ORIGINAL ARTICLE

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PAK3 is a key signature gene of the glioma proneural subtype and affects its proliferation, differentiation and growth

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⁹ Abstract

Purpose Gliomas are the most lethal adult primary brain cancers. Recent advances in their molecular characterization have contributed to a better understanding of their pathophysiology, but there is still a need to identify key genes controlling glioma cell proliferation and differentiation. The p21-activated kinases PAK1 and PAK2 play essential roles in cell division and brain development and are well-known oncogenes. In contrast, the role of PAK3 in cancer is poorly understood. It is how we how one of the pronouvel subture

- ¹⁴ known, however that this gene is involved in brain ontogenesis and has been identified as a gene of the proneural subtype
- ¹⁵ signature in glioblastomas.
- ¹⁶ Methods To better understand the role of PAK kinases in the pathophysiology of gliomas, we conducted expression analy-
- ¹⁷ ses by querying multiple gene expression databases and analyzing primary human glioma samples. We next studied *PAK3*
- expression upon differentiation in patient-derived cell lines (PDCLs) and the effects of *PAK3* inhibition by lentiviral-mediated shRNA on glioma cell proliferation, differentiation and tupor growth
- ¹⁹ shRNA on glioma cell proliferation, differentiation and tumor growth.
 ²⁰ **Populte** We show that contrary to *PAK1* and *PAK2*, bick *PAK2* average.
- ²⁰ **Results** We show that contrary to *PAK1* and *PAK2*, high *PAK3* expression positively correlates with a longer survival of $\frac{21}{10000}$ glioma patients. We also found that *PAK3* displays differential expression patterns between glioma sub-groups with a higher
- ²¹ glioma patients. We also found that *PAK3* displays differential expression patterns between glioma sub-groups with a higher ²² expression in 1p/10a codeleted eligodendrogliomas and is highly expressed in tumors and **PDCI** is of the proneural subture
- expression in 1p/19q-codeleted oligodendrogliomas, and is highly expressed in tumors and PDCLs of the proneural subtype.
- ²³ In PDCLs, high *PAK3* expression negatively correlated with proliferation and positively correlated with neuronal differentia-
- ²⁴ tion. Inhibition of *PAK3* expression increased PDCL proliferation and glioma tumor growth in nude mice.
- ²⁵ Conclusions Our results indicate that *PAK3* plays a unique role among PAKs in glioma development and may represent a
 ²⁶ potential therapeutic target.
- Keywords Gliomas · Proneural signature · p21-activated kinase · Patient-derived cell line · Proliferation and neuronal
 differentiation · Glioma tumor growth
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1 Introduction

Diffuse gliomas, including glioblastomas (GBMs), are the most common malignant primary brain tumors and contain diverse populations of cells ranging from highly tumorigenic stem-like cells to more differentiated cells [1, 2]. Molecular studies have related genetic alterations, gene expression profiles, and DNA methylation signatures to distinct glioma subtypes with prognostic value [3–7]. Those studies have also led to the deciphering of key signaling pathways involved in glioma biology, and inlightened the altered differentiation and multipotency of the tumor cells [8]. They also helped to characterize potential therapeutic targets relevant for each subtype of

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glioma. p21-activated kinases (PAKs) play important func-42 tional roles in neuronal differentiation and cancer biology. 43 The three group I PAKs (PAK1, PAK2 and PAK3) form a 44 small gene family encoding the main effectors of the Rac 45 and Cdc42 GTPases, which play crucial roles in cell pro-46 liferation and migration [9]. These kinases are expressed 47 in the developing and adult brain where they have unique 48 functions during brain development [10]. PAK1 regulates 49 cortical development via the control of progenitor cell pro-50 liferation and neuronal differentiation [11]. PAK2 is an 51 effector of the Rheb/TSC complexes in benign tuberous 52 sclerosis in neuronal tissues [12]. 53

Within this family, PAK3 may have unique signaling 54 properties since its mutations have been found to be respon-55 sible for several neurological disorders including intellec-56 tual disability [10]. PAK3 is involved in brain development, 57 regulating cell cycle exit of neuronal precursors during pri-58 mary neurogenesis, and in neurite outgrowth and neuronal 59 60 differentiation during telencephalon ontogenesis [13–15]. PAK3 also regulates oligodendroglial lineage rate specifi-61 cation [16] PAK1 and PAK2 are oncogenes often found to 62 be overexpressed or activated in different cancers such as 63 breast and colon cancer [17, 18]. Their over-activation and 64 over-expression favour cell proliferation, inhibit apoptosis, 65 enhance invasion and metastasis, and sustain angiogenesis, 66 all cell functions that trigger oncogenesis [17]. In contrast, 67 PAK3 overexpression or mutation have only been associated 68 with adrenocorticotropic hormone-secreting tumors [19]. It 69 has also been found, however, that this gene belongs to the 70 proneural transcriptomic signature in glioblastomas (GBMs) 71 72 [6, 20].

As yet, the role of *PAKs* in gliomas is scarcely described 73 in the literature [21]. To investigate this further, we ana-74 lyzed group PAK1-3 expression according to histological 75 and molecular criteria in a well-characterized patient cohort 76 and in data deposited in public cancer databases. Contrary 77 to PAK1 and PAK2, high PAK3 expression was found to 78 be positively correlated with a longer patient survival. We 79 also found that PAK3 was differentially expressed between 80 glioma subgroups, with a higher expression in 1p/19q-code-81 leted oligodendrogliomas. Comparison of PAK expression in 82 GBM patient-derived cell lines (PDCLs) and in their paren-83 84 tal tumors indicated that PAK3 expression may serve as one of the more reliable markers of the proneural subtype. In 85 PDCLs, increasing PAK3 expression upon a set of differ-86 87 entiation paradigms was correlated with those of neuronal markers. Interestingly, short hairpin RNA-mediated inhibi-88 tion of PAK3 expression increased PDCL proliferation and 89 astrocytic marker expression, and also accelerated in vivo 90 tumor growth. Together, these data indicate that PAK1-2 and 91 PAK3 play different roles in the pathophysiology of gliomas. 92 PAK3 exhibits an inhibitory role in glioma tumor growth and 93 may be of potential interest for glioma therapy. 94

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2 Materials and methods

2.1 In silico tumor database analysis

RNA-seq data were obtained from the transcriptomic data-97 base of Zhang et al. [22] and are expressed in FPKM (Frag-98 ments Per Kilobase of exon per Million reads mapped) val-99 ues. RNAs were extracted from different cell types purified 100 from the postnatal mouse cerebral cortex (http://web.stanf 101 ord.edu/group/barres lab/brain rnaseq.html). Gene expres-102 sion analyses from primary tumors were performed employ-103 ing the Academic Medical Center (AMC) database, using 104 the R2 genomics analysis and visualization platform (http:// 105 r2.amc.nl) and across different tumor datasets for each ana-106 lyzed gene [23]. Kaplan-Meier survival curves of patients 107 were derived from the Tumor Glioma-French-284 -data-108 set, composed of 276 glioma samples of all histologies and 109 8 control samples [24]. Samples were sorted according to 110 the expression of each PAK and divided into two groups on 111 the basis of a cutoff expression value giving the higher prob-112 ability of confidence, using the log-rank test with Bonferroni 113 correction for multiple comparisons. The clinical and molec-114 ular data of lower grade gliomas (LGGs) and GBM samples 115 for this study were downloaded from The Cancer Genome 116 Atlas database (https://tcga-data.nci.nih.gov/docs/publicatio 117 ns/tcga/) generated by the TCGA Research Network (http:// 118 cancergenome.nih.gov/), firstly in 2014 and subsequently in 119 February 2016 (cancer genome). mRNA expression levels 120 of PAK1 and PAK3 were obtained from RNAseq analyses 121 using Illumina TruSeq Kit Paired-end Sequencing on an 122 Illumina HiSeq2000. Detection of somatic variants (IDH-123 mut) from TCGA whole-exome sequencing and RNAseq 124 data was performed using RADIA [25]. The mRNA levels 125 were represented as "normalized count" corresponding to a 126 transformation of the "raw_count". For each gene of interest, 127 all "raw_count" values were divided by the 75th percentile 128 of the column patient (after removing zeros) and multiplied 129 by 1000. 130

2.2 Glioma sample analyses

Patient samples (from the OncoNeuroTek tissue bank) and 132 informed consent, RNA extraction and gene expression 133 analyses have been described before [26]. 134

2.3 GBM cell culture, shRNA and lentiviral infection 135

Primary sphere cultures were initially derived from patient 136 tumors as described before [27]. Glioblastoma-derived 137 spheres were cultured in DMEM/F12 medium supplemented 138 with penicillin/streptomycin and B27 (Life Technologies), 139

referred to as basal medium, supplemented with EGF (20 ng/ 140 ml; Peprotech) and FGF (20 ng/ml; Miltenyi), referred to as 141 proliferative medium. Spheres were dissociated using Stem-142 Pro Accutase (Life technologies) when they reached a size 143 of 100-200 µm. For cell growth analysis, dissociated cells 144 were seeded at 5×10^4 cells/ml in triplicates, and on day 1, 145 4 and 7, live cells from dissociated spheres were counted. 146 Viability was tested by Trypan blue exclusion. For differ-147 entiation experiments, single-cell suspensions were seeded 148 on a laminin substrate (10 µg/ml; Life technologies) in the 149 proliferative medium, as described before [28]. Twenty-four 150 hours later, the medium was replaced with basal medium 151 containing 0.5% fetal calf serum, or in some experiments, 152 with basal medium containing Bone Morphological Pro-153 tein-4 (BMP-4, 10 ng/ml, Peprotech) or all-trans-retinoic 154 acid (ATRA, 10 µM, Sigma). For PAK3 expression inhibi-155 tion, viral particles containing TRC human PAK3 shRNA 156 (Thermo Scientific) cloned in the pLKO.1 lentiviral vector 157 were produced at the ICM Vectorology platform and used to 158 transduce PDCLs. After puromycin selection, the efficiency 159 of PAK3 expression inhibition was analyzed in proliferative 160 and differentiative conditions by qRT-PCR. 161

162 2.4 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using TRIzol Reagent 163 (Life Technologies) according to the manufacturer's proto-164 col. RNA quality analysis was performed using a Bio-Rad's 165 Experion system and nanodrop, and cDNAs were generated 166 from 0.5 µg mRNAs using an iScript Reverse Transcrip-167 tion Supermix (Biorad). Real-time PCR was performed on 168 a CFX96 Touch Real-Time PCR Detection System (Bio-169 rad) using a SsoAdvanced Universal SYBRGreen Supermix 170 (Biorad), Primers were designed by retrieving nucleotide 171 sequences from the NCBI gene database using the Primer-172 3Plus program (http://www.bioinformatics.nl/cgi-bin/prime 173 r3plus/primer3plus.cgi/). Gene-specific primers were syn-174 thesized by Eurofins Genomics and their sequences are listed 175 in Supplementary Materials. Samples were amplified in 176 triplicate and relative copy numbers were determined using 177 the comparative $\Delta\Delta$ Ct method, with *PPIA* and *GAPDH* as 178 normalization genes, and expression in a human embryonic 179 brain mRNA sample as exogenous reference (Clinisciences). 180

181 2.5 Western blotting

For Western blot analysis, protein samples (10 μg) were
separated by 10% SDS-PAGE and transferred to PVDF
membranes (Millipore), as previously described [29].
Immunoblot analyses were performed using antibodies
described in Supplementary Materials. Immunodetection
was performed using a Luminata Crescendo Western HRP

substrate (Merck Millipore). Quantification of chemiluminescence signals was performed using NIH ImageJ software. 188

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2.6 Immunocytochemistry

Monolayer PDCL cultures were PFA-fixed and immunolabeled for native proteins using antibodies described in Supplementary Materials. Images were acquired in a sequential mode, using suitable filter cubes on a Leica DMI6000 microscope controlled by SimplePCI Software (Compix-Hamamatsu).

2.7 Animal experimentation

All animal experiments were conducted in accordance 199 with the European Communities Council Directive for 200 animal care and experimentation (Directive 2010/63/ 201 UE), and following the guidelines of the animal facil-202 ity in Orsay (France) approved by the Ethical Commit-203 tee (France, CEEA 59) and the Direction of veterinary 204 services (France, agreement D91-471–104). 1×10^{6} dis-205 sociated cells were subcutaneously injected into the left 206 flank of 8-weeks-old female nude mice. Tumor growth was 207 monitored regularly and measured twice a week using a 208 caliper as soon as tumors reach 25 mm³. 209

2.8 Statistical analysis

Patient survival was defined as the time between histo-211 logical diagnosis and either death or the last follow-up. 212 Differences between Kaplan-Meier survival curves were 213 assessed using the log-rank test. For samples from the 214 Pitié-Salpêtrière Hospital's cohort, gene expression lev-215 els were measured by qRT-PCR and compared between 216 different molecular subgroups of patients using a one-way 217 analysis of variance (ANOVA) and Mann-Whitney test 218 (two-tailed) included in GraphPad Prism software. In the 219 TCGA datasets, gene expression was compared between 220 glioma subgroups with ANOVA and Tukey's comparison 221 tests using R software. For cellular and molecular assays, 222 experiments were performed in triplicates and repeated 223 independently at least three times. Whenever relevant, 224 Kruskal-Wallis or Mann-Whitney Wilcoxon tests were 225 performed using R software. Differences were considered 226 significant below *p < 0.05, **p < 0.01 and ***p < 0.001227 thresholds. 228

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229 3 Results

3.1 A higher *PAK3* expression in gliomas is associated with a longer patient survival

We first analyzed PAK1-3 expressions in postnatal brain 232 cell types by interrogating the database from the Barres 233 group [22]. We found that *PAK1* is highly expressed in 234 neurons, oligodendrocyte lineage cells, microglia and to a 235 lesser extent in astrocytes (Fig. 1a). PAK2 was expressed 236 in all cell types and represented the main *PAK* isoform in 237 astrocytes and endothelial cells. PAK3 expression, overall 238 lower than that of the two other PAK genes, was restricted 239 to neurons and cells of the oligodendroglial lineage. We 240 next compared the expression levels of each PAK isoform 241 in tumors from the nervous system and other organs, by 242 mining the Academic Medical Center (AMC) database 243 (Fig. 1b) [23]. PAK1 was almost equally expressed in all 244 tumor types, but at a slightly lower level in gliomas com-245 pared with other tumors, and less than in normal brain 246 tissue. PAK2 was expressed at higher levels in all tumor 247 types including gliomas, compared with normal brain tis-248 sue. Interestingly, PAK3 levels were higher in gliomas 249 and neuroblastomas compared with other cancers, with 250 a heterogeneous distribution in gliomas. Interestingly, we 251 found that PAK3 displayed the same pattern of expression. 252 as *DCX* in neural cell types and also in tumors [19, 22] 253 (Fig. 1a,b; Supplementary Fig. S1). The relatively lower 254 expression of PAK1 and PAK3 in gliomas compared to 255 normal brain tissue is likely due to the high proportion 256 of astrocyte-like cells, which express the lowest levels of 257 PAK1 and PAK3. 258

We next investigated the correlation between PAKs 259 expression and patient overall survival in a large cohort 260 of glioma samples of diverse histological types and grades 261 described by French and colleagues [24] (Fig. 1c). As we 262 found that PAK2 expression was not significantly corre-263 lated with survival (p = 0.084), we decided to focus on 264 PAK1 and PAK3. Interestingly, high PAK1 expression was 265 found to be associated with a shorter survival and, con-266 versely, high PAK3 expression with a longer survival. For 267 PAK3, similar results were obtained from the Kawaguchi 268 and REMBRANT glioma databases, underscoring a cor-269 relation between PAK3 expression and patient survival 270 (Supplementary Fig. S2). For PAK1 expression, similar 271 results were obtained from most databases but not all, sug-272 gesting that the relationship between PAK1 expression and 273 tumor progression is weaker, or may depend on the dif-274 ferent histological subtypes composing the cohort (data 275 not shown). Using R2 software, we found that the PAK1 276 and PAK3 expression levels did not correlate with each 277 other (r = 0.54), supporting an independent expression 278

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3.2 *PAK3* is highly expressed in *IDH*-mutant 282 and 1p/19q-codeleted oligodendrogliomas 283

for each gene during cancer progression. Together, these

data point to distinct roles for PAK1 and PAK3 in glioma

development.

To better understand the roles of PAK1 and PAK3 in the 284 pathophysiology of gliomas, we next set out to investigate 285 more precisely their expression levels in glioma subtypes, 286 using qRT-PCR in 223 tumor samples from the OncoNeu-287 roTek bank (Fig. 2 a-c). By doing so, we did not observe 288 any significant difference in PAK1 expression between 289 grade II/III gliomas (lower grade gliomas: LGGs) and 290 GBMs, whereas PAK3 was significantly higher expressed 291 in LGGs than in GBMs (Fig. 2a). More specifically, PAK3 292 expression was higher in oligodendrogliomas compared 293 with astrocytomas or oligoastrocytomas. In contrast, PAK1 294 expression was not significantly different among the LGG 295 subtypes (Fig. 2b). In view of the new molecular classifi-296 cation for gliomas, we examined PAK1 and PAK3 expres-297 sion according to the major alterations IDH1/2 and 1/19q298 codeletion. Analysis of PAK expression in IDH-WT and 299 IDH-mutant LGGs revealed opposite results for each iso-300 form, with a tendency towards increased PAK1 expression 301 in *IDH*-WT-LGGs and a significantly higher expression of 302 PAK3 in IDH-mutant LGGs (Fig. 2c). Moreover, in contrast 303 to PAK1, PAK3 expression was significantly higher in glio-304 mas harboring the 1p/19q codeletion compared with non-305 codeleted tumors. To validate these results, we performed a 306 meta-analysis of data from the TCGA including 582 human 307 glioma samples (Fig. 2 d-f). For PAK3, we detected a lower 308 expression in GBMs than in LGGs, a higher expression in 309 oligodendrogliomas than in other histological types, and 310 a higher expression in IDH-mutant LGGs compared with 311 non-mutant LGGs. In line with the PSH data, the TCGA 312 analysis revealed a higher expression of PAK1 in oligoden-313 drogliomas and a lower expression in IDH-mutant LGGs. 314 Together, these results indicate a higher PAK3 expression 315 in less aggressive gliomas. 316

3.3 High *PAK3* expression is a reliable marker for proneural subtypes of tumors and PDCLs 318

GBMs can be classified in molecular subtypes according to 319 their gene expression profiles. Interestingly, PAK3 was found 320 to be enriched in the proneural glioma subtype signature 321 (Fig. S3) [6, 20]. We next examined this relationship using 322 the transcriptional data obtained from tumors and matched 323 cell lines described by Rosenberg [30]. We focused on a 324 panel of selected genes that define the proneural signature, 325 as established by Crisman [20]. We were able to confirm 326 the higher expression of PAK3 in samples classified as 327



Fig. 1 *PAK expression in CNS cellular population and correlation with survival of glioma patients. a*, RNA-seq analysis of PAK expression in the postnatal mouse cerebral cortex shows specific patterns in different cell types. Data are mined from the transcriptomic database of Zhang et al. [22] and expressed in FPKM (Fragments Per Kilobase of exon per Million reads mapped) values. Astrocyte: As; neuron: Ne; oligodendrocyte progenitor cell: OP; newly formed oligodendrocyte: NF; myelinating oligodendrocyte: MO; microglia: Mi; endothelial cell: En. **b**, *PAK1* is similarly expressed in all types of tumors, whereas *PAK3* is highly expressed in gliomas and neuroblastomas. Box plots show log2 gene expression levels in tumors from the Affymetrix u133p2 microarray, and from R2 platform analysis-

Academic Medical Center (AMC). The data are derived from the following cohorts: normal brain (NB, Berchtold, n=172); glioma (Gl, French, n=284); neuroblastoma (Ne, Versteeg, n=88); bladder (Bl, Riester, n=93); colon (Co, Sieber Smith, n=355); lung (Lu, Expo, n=114); breast (Br, Bos, n=204); kidney (Ki, Expo, n=261). **c**, Contrary to *PAK1*, high *PAK3* expression in gliomas is associated with a longer patient survival. Kaplan–Meier survival plot splitted according to *PAK* expression level, AMC, French database. For *PAK1*, high expression n=185, low expression n=88; for *PAK2*, high expression n=113, low expression n=160; for *PAK3*, high expression n=186, low expression n=87





Fig. 2 *PAK3 is highly expressed in IDH-mutant and 1p/19q-codeleted glioma subtypes. a-c: PAK1* and PAK3 gene expression levels were analyzed by qRT-PCR in glioma samples from the OncoNeuroTek bank. *PAK1* and *PAK3* mRNA expression levels are presented as $\Delta\Delta$ Cq; **a**, in lower grade gliomas (LGGs, n = 122) versus glioblastomas (GBMs, n = 101); **b**, in the various histological classes of LGG (A: astrocytoma, n = 15; O: oligodendroglioma, n = 54; OA: oligoastrocytoma, n = 53) and including II and III tumor grades; **c**, accord-

ing to IDH status and to 1p/19q-codeletion (*IDH* wild type: WT, n=28; *IDH* mutant: mut; no codeletion 1p/19q: -, n=34; codeletion 1p/19q: +, n=47). **d-f**, *PAK1* and *PAK3* gene expression analyzed from the TCGA database; **d**, in lower grade gliomas (LGGs, n=417) versus glioblastomas (GBMs, n=165); **e**, in the various histological classes of LGG (A, n=149; O, n=159; OA, n=109) and including II and III tumor grades; **f**, according to IDH status (wild type: WT, n=98; mutant: mut, n=434)

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Fig. 3 High PAK3 expression in primary tumors and their PDCLs correlates with proneural subtypes. Data were extracted from a transcriptome analysis performed by Rosenberg et al. [30]. a, Expression of Crisman proneural signature genes in proneural or

non proneural subtypes (16 samples corresponding to 8 tumors and 8 PDCLs); **b**, *PAK1 and PAK3* expression levels in tumors and their corresponding PDCLs. **c**, expression levels of proneural signature genes in paired tumors and corresponding PDCLs

proneural, regardless of sample type (tumors or PDCLs) 328 (Fig. 3a). PDCLs have been developed both for analysing 329 the biology of human tumors and as preclinical models for 330 screening potential therapeutic agents. The analysis of phe-331 notype transition may be relevant with regard to tumor evo-332 lution towards a more aggressive mesenchymal phenotype 333 [3]. Although in most cases PDCLs maintain the molecu-334 lar subgroup signature of the parental tumor, some PDCLs 335 acquire characteristics of other phenotypic groups [30]. We 336 asked whether the PAK3 expression level may represent a 337 marker for the proneural subtype during tumor/PDCL phe-338 notypic transition. Previously, GBM subtypes were found to 339 be maintained in 5/9 of the paired tumors/PDCLs, whereas 340 4/9 cases exhibited subtype changes [30]. Interestingly, 341 when we analysed these cases, we consistently observed 342 higher PAK3 levels in the proneural samples, regardless of 343 the kind of change and tissue (Fig. 3b,c). Note that PAK1 344 expression was always lower in PDCLs compared to tumors, 345 without subtype correlation (Fig. 3b). In case the subtype 346 changes during cell line establishment, increased expres-347 sion of PAK3 was observed in PDCLs which acquire this 348 proneural subtype and, conversely, its expression was found 349 to decrease when the PDCLs lost this proneural subtype. 350 Expression comparisons of the other proneural genes, such 351 as ASCL1, DCX, MAP2, NKX2.2, OLIG2, PDGFRA and 352 SOX2 selected from the Vehraak list, and BCAN, CDC25A, 353 CHD7, CLAPS2, DCX, DDX42, GSK3, PPM1D, WASF1 354 and ZEB2 selected from the shortlist defined by Crisman, we 355 found that tumors and cell samples attributed to the proneu-356 ral subtype compared to other subtypes clearly indicated that 357 among the genes of the proneural signature, only PAK3 and 358 DCX displayed a higher expression in proneural tumors and 359 PDCLs (Fig. 3c). Thus, these data indicate that PAK3 serves 360 as a reliable marker for the proneural characterization of 361 tumors and PDCLs. 362

363 3.4 High *PAK3* expression negatively relates 364 to PDCL proliferation

In order to better characterize the association between PAK3 365 expression and glioma cell biology, we analyzed PAK3 366 expression in 3 PDCL cultures named 3731a, 3731b and 367 4371 [27] that are growing as spheroids in EGF-FGF defined 368 medium (Fig. 4a). These PDCLs harbor EGFR amplifica-369 tions and 3731a and b, derived from the same initial 3731 370 cell line, express the mutated oncogene EGFRvIII [27]. We 371 found that the two 3731 PDCLs displayed a transcriptomic 372 profil with a more prounnonced mesenchymal/classical 373 signature compared to the 4371 PDCL, which displays a 374 proneural expression phenotype, as established by qRT-PCR 375 analysis of genes representative of the four Verhaak GBM 376 subtypes (heatmap: Fig. 4b, Fig. S4). 377

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In cultured spheroids, PAK3 expression was significantly 378 higher in 4371 cells compared to the two other cell lines. 379 as measured by qRT-PCR (Fig. 4c). Cell growth analysis 380 showed that 3731a and 3731b exhibit faster proliferation 381 rates than 4371, with fold increases after one week, of 16, 382 17 and 12, respectively (Fig. 4d). Accordingly, the expres-383 sion levels of CCND1 and of CDKN1B were the lowest and 384 highest, respectively, in the slow proliferative 4371 cells 385 compared with the 3731a/b cells (Fig. 4e). We conclude 386 that PAK3 expression is higher in slower growing PDCLs. 387

3.5 High *PAK3* expression positively associates with neuronal differentiation

We next analyzed PAK3 expression during PDCL differen-390 tiation induced after growth factor withdrawal in monolayer 391 cultures as previously described [31]. In these conditions, no 392 cell death was observed in the three PDCL cultures (data not 393 shown). PAK3 expression was analyzed after 3 days (t3) and 394 8 days (t8), compared with proliferative conditions (t0). We 395 found that in the two 3731 PDCL cultures PAK3 expression 396 was strongly induced at both the mRNA and protein levels. 397 in contrast to that in 4371 cells (Fig. 5a-b, Fig. S5a). In pro-398 liferative conditions (t0), the proportion of KI67⁺ cycling 399 cells varied between PDCL cultures, from 90% (3731b) to 400 60% (4371) (Fig. 5c and S5b, first column), in accordance 401 with the rates observed in sphere cultures (Fig. 4b). After 402 growth factor withdrawal, the proportion of KI67⁺ cells was 403 largely reduced for 3731a and 4371 (t8, 22%), and to a lesser 404 extent for 3731b, which remained highly proliferative (t8, 405 65%). Accordingly, p27-KIP1 expression increased after 406 induction of growth arrest in the three PDCL lines (Fig. 5d). 407 We next analyzed GFAP and DCX expression in monolayer 408 cultures by immunolabeling and mRNA and protein quanti-409 fication (Fig. S5c, Fig. 5e-f). For 3731b cells, growth factor 410 withdrawal had little effect on the expression of the cell dif-411 ferentiation markers DCX and GFAP. In contrast, we found 412 that the 3731a and 4371 cell lines displayed a reduced pro-413 liferation with an increased expression of both GFAP and 414 DCX. 415

Next, PDCL cultures were exposed to bone morpho-416 genetic proteins 4 (BMP4) and all-trans retinoic acid 417 (ATRA) in order to induce astroglial and neuronal differ-418 entiation, respectively [32, 33]. We found that BMP4 or 419 ATRA treatment did not modify the proportion of prolif-420 erating cells compared to growth factor withdrawal alone 421 (Fig. S6a). Cell lineage differentiation was assessed using 422 qRT-PCR expression analysis of DCX GFAP, OLIG2, 423 NG2, PDGFRa, S100beta and TUBB3 (Fig. 5, Fig. S6b). 424 BMP4 treatment had a relatively little effect on *PAK3* 425 expression in the three PDCL cultures, except for a strong 426 GFAP transcriptional activation in 3731b cells and mod-427 erate in 3731a cells, suggesting astroglial differentiation 428

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Fig. 4 High PAK3 expression negatively correlates with PDCL proliferation. a, Representative images showing spheroids grown for 7 days. **b**, Heat map representation of subtype signature gene expression in 3731a, 3731b and 4371 cells. Expression is shown relatively to the mean (red: higher expression level; green: lower expression; white for the mean). Genes are organized according to gene expression signatures: Mesenchymal (MES), Classical (CL), Proneural

(PN) and Neural (NL). **c**, qRT-PCR analysis of *PAK3* expression in PDCL spheres. Expression levels are presented as $\Delta\Delta$ Cq, relatively to human fetal brain as external reference. **d**, Fold increase in cell number over time, day1 to 7 (d1, d4 d7). **e**, qRT-PCR analysis of cell cycle genes. Expression levels are presented as $\Delta\Delta$ Cq, relatively to human fetal brain as external reference. For all analyses, data represent at least three independent experiments

(Fig. 5g). ATRA treatment induced a strong transcriptional activation of *PAK3* in 4371 cells and, to a lesser extent, in 3731a cells, associated with expression of the neuronal markers *DCX* (Fig. 5h) or *TUBB3* (Fig. S6b). In summary, we found that growth factor withdrawal induced an increase in *PAK3* expression associated with *GFAP* and *DCX* expression in mesenchymal/classical 3731 cell lines, whereas ATRA treatment induced PAK3 and DCX436up-regulation mainly in the proneural PDCLs (Fig. 5i).437In contrast, BPM4 induced glial differentiation in mesen-
chymal cell lines without affecting PAK3 expression. This438highlights a link between PAK3 expression and neuronal
differentiation.440

Fig. 5 High PAK3 expression is positively correlated to neuronal differentiation. a-f: PDCLs were grown as monolayers in proliferative medium (t0) or without growth factors in 0.5% FCS for 3 (t3) or 8 (t8) days. a, qRT-PCR analysis of PAK3 expression during the time course after growth factor withdrawal, expressed relatively to that in normal human fetal brain as external reference. b, Western blot analysis of PAK3 protein expression with actin as loading control. c, Percentage of Ki67-positive cells relative to DAPI staining. d, qRT-PCR analysis of p27 expression relative to that in normal human fetal brain as external control. e, qRT-PCR analysis of DCX and GFAP expression relative to that in normal human fetal brain as external reference. f, Western blot analysis of DCX and GFAP expression with actin as control, in PDCLs over time. g-i: Cells were grown as monolayers in proliferative medium and then without growth factors in the absence of FBS (0%), or in the presence of BMP4, DMSO or all trans retinoic acid (ATRA) for 3 (t3) or 8 (t8) days. g, h, qRT-PCR analysis of gene expression at t3 and t8, expressed relative to the t0 value of each PDCL culture, in the absence (0%) or presence of BMP4 (g), and in the presence of DMSO as a control or ATRA (h). i, Diagram showing the variations in gene expression levels after growth factor withdrawal, BMP4 or ATRA treatment. For all these qRT-PCR (expressed as $\Delta\Delta$ Cq) or Western blot analyses, data represent at least three independent experiments



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Fig. 6 ShRNA-mediated inhibition of PAK3 expression modifies glioma cell proliferation, differentiation and tumor growth. a-c: 3731a PDCLs stably transduced with Control-shRNA (Ctrl) or *PAK3*-shRNA (03A, 04A) lentiviral vectors were grown as monolayers in proliferative medium (t0) or three days after growth factor removal and in 0.5% FCS (t3). a, qRT-PCR analysis of *PAK3* expression relative to that in normal human fetal brain (n=3). b, Percentage of Ki67⁻positive cells. c, qRT-PCR analysis of *GFAP* and *DCX* expression relatively to that in normal human fetal brain (n=3). d, e: Mice

were subcutaneously injected with *PAK3*-shRNA-03A-transduced 3731a cells (03A) (n=9) or the Control-shRNA-transduced 3731a cells as control (Ctrl) (n=8). **d**, Tumor growth over time analyzed after tumors reached 25 mm³; mean curves were obtained by LOESS method. **e**, Mean doubling time \pm SD of Control and Sh03A tumor growth. The doubling time parameter was extracted after exponential growth curve modeling. Each individual data point represents one mouse

442 3.6 *PAK3* knock-down alters glioma cell 443 proliferation, differentiation and tumor growth

To more precisely define the role of PAK3 in glioma physiopathology, we knocked down PAK3 expression using *PAK3*-specific shRNAs. We selected two shRNAs (03A and 04) expressed from lentiviral vectors in COS7-infected cells, based on their efficiency in inducing *PAK3* expression decrease. The greatest efficacy of *PAK3* expression

inhibition was obtained in 3731a cells with Sh-03A, and 450 to a lesser extent with Sh-04A, in proliferative conditions 451 (t0) and after growth factor withdrawal (t3), compared to 452 the Control-shRNA (Fig. 6a). In proliferative conditions 453 (t0), inhibition of PAK3 expression by the two shRNAs had 454 no effect on cell proliferation, as revealed by Ki67 analysis 455 (Fig. 6b). However, after three days without growth fac-456 tors (t3), the proportion of Ki67-positive cells increased in 457 the PAK3-knock down compared to control cells. We next 458

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evaluated GFAP and DCX expression in PAK3-shRNA-459 3731a cells, in proliferative conditions (t0) and after growth 460 factor withdrawal (t3), compared to Control-shRNA express-461 ing cells. Inhibition of PAK3 expression had no effect on 462 GFAP expression in proliferative medium (t0) whereas the 463 GFAP level increased notably after growth factor withdrawal 464 (t3) (Fig. 6c). Furthermore, no significative effect was 465 observed on DCX expression under the same experimental 466 conditions. These results indicate that inhibition of PAK3 467 expression in GBM cells induces astroglial differentiation. 468

Finally, to analyze the effect of PAK3 inhibition on 469 glioma cell tumorigenicity in vivo, we decided to choose 470 the 3731a PDCL that forms subcutaneous tumors in mice, 471 whereas 4371 does not (data not shown). Sh-03A or Control-472 shRNA transduced 3731a cells were injected subcutaneously 473 in nude mice. Monitoring of tumor sizes clearly indicated 474 that tumors grew faster in the Sh-03A-cells injected mice 475 compared to the Control-shRNA injected mice (Fig. 6d). 476 Doubling times were extracted from the exponential growth 477 curves. Compared to the control $(15.38 \pm 3.23 \text{ days})$, the 478 doubling time for the Sh-03A-cells injected mice was shorter 479 $(6.43 \pm 1.64 \text{ days})$, indicating an increase in tumor growth 480 (Fig. 6e). PAK3 expression levels were found to be lower 481 in tumors isolated from the four mice injected with PAK3-482 shRNA transduced cells, compared with tumors isolated 483 from mice injected with Control-shRNA-cells, indicating 484 that Sh-mediated PAK3 silencing is maintained over time 485 (Fig. S7). This allows us to confirm the correlation between 486 PAK3 expression in GBM cells and tumour growth. How-487 ever, we did not observe any increase in the expression of 488 glial markers, such as GFAP or SOX9, in tumors derived 489 from cells transduced with PAK3-shRNA, compared to Con-490 trol-shRNA tumors (Fig. S7). These results strongly suggest 491 that inhibition of PAK3 expression in GBM cells impacts 492 their differentiation fate towards an astroglial phenotype 493 in vitro and promotes their tumor growth in vivo. 494

495 **4 Discussion**

We here report correlations between PAK expression in glio-496 mas and patient survival that are specific of each isoform. 497 We found that *PAK1* expression in tumors is negatively 498 correlated to patient survival, suggesting that PAK1 may 499 favor glioma tumor growth, in accordance with its reported 500 oncogenic functions in other cancers [17, 18]. We found 501 that the PAK2 expression level is not related to survival but 502 that, in contrast, a high PAK3 expression is associated with 503 a better survival. These results clearly indicate distinct and 504 unique roles for each *PAK* isoform in the pathophysiology of 505 gliomas. Interestingly, we found a higher PAK3 expression 506 level in 1p/19q-codeleted tumors, which are tumors with 507 oligodendrocyte characteristics associated with a better 508

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prognosis, raising the question whether PAK3 acts as tumor 509 suppressor or simply as a marker of the differentiated status 510 of these tumors. Indeed, during brain development PAK3 511 possesses differentiating functions, being involved in neu-512 ronal cell specification, neuroblasts differentiation and oligo-513 dendrocyte differentiation [13-16]. These functions may act 514 during tumor development and favor neuronal differentiation 515 of glioma cells. 516

To address this question, we performed a comparative 517 study on several PDCLs. The different cell cultures used dis-518 played distinct characteristics, particularly regarding PAK3 519 expression. The two 3731 PDCL clones grew fast, exhibited 520 classical/mesenchymal transcriptomic signatures, formed 521 tumors after subcutaneous engraftment and displayed a low 522 PAK3 expression, compared to the 4371 PDCL. Following 523 growth factor withdrawal or ATRA treatment the 3731a 524 subclone differentiated towards an immature neuronal state 525 associated with an increase in PAK3 expression, whereas the 526 3731b subclone showed upregulated GFAP expression upon 527 BMP4 treatment, without any change in PAK3 expression. 528 This suggests that these cells undergo an astroglial differ-529 entiation or acquire stem cell-like properties, independently 530 of PAK3 expression. The 4371 PDCL culture possesses 531 opposite characteristics: it grew more slowly, expressed 532 a more proneural transcriptomic profile and did not form 533 tumors after xenografting. Interestingly, it expressed a rela-534 tively higher level of PAK3 and neuroglial differentiation 535 markers. Growth factor withdrawal and BMP4 treatment 536 induced only a small increase in neuroglial differentiation, 537 whereas treatment with ATRA induced a strong increase in 538 the expression of neuronal markers such as DCX, as well 539 as PAK3 expression. These results are in agreement with 540 distinct differentiation characteristics of PDCLs upon dif-541 ferent differentiation-inducing regimens [34]. However, our 542 data clearly show that an increased PAK3 expression was 543 associated with neuronal differentiation in two PDCL cul-544 tures, suggesting that PAK3 favors neuronal differentiation 545 and decreased proliferation of glioma cells, as PAK3 does 546 during normal brain development. 547

We found that PAK3 expression knockdown resulted in an 548 increase in PDCL proliferation associated with an increase 549 in GFAP expression in vitro and an increase in tumor growth 550 rate in engrafted mice. These results strongly suggest that 551 PAK3 can promote neuronal differentiation and/or inhibit 552 astroglial differentiation, leading to slow tumor growth. In 553 our setting, we did not observe any increase in astrocytic 554 markers in PAK3-shRNA tumors. Among several possible 555 explanations, the most likely is that the analysis of the cel-556 lular phenotype was carried out relatively late, i.e., mice 557 were euthanized after having reached a tumor stage at which 558 cellular regulations and phenotypes are radically modified. 559 Some brain-specific factors present in culture media of 560 PDCLs, but absent in the xenograft environment, could be 561

necessary for inducing an astrocytic phenotype shift. Astroglial cell fate inhibition may be due to a direct inhibition of
astrocyte differentiation or an indirect effect of the activation of neuronal differentiation triggered at the expense of
astrocytic differentiation, as observed earlier during Xenopus embryogenesis [13].

The PAK3 gene belongs to a group of genes character-568 istic of a proneural signature. This was established in the 569 princeps study of Verhaak and collaborators and also in the 570 shortlist of 48 genes for this same subtype, established by 571 different algorithms in the study of Crisman and colleagues 572 [6, 20]. More recently, scRNA-seq allowed a delineation of 573 the transcriptomes of single glioblastoma cells, their intra-574 tumoral heterogeneity and their phenotypic plasticity. The 575 main identified subgroups of cells are characteristic of neu-576 ronal and glial lineages or their progenitors. Interestingly, 577 the PAK3 gene belongs to the top 30 genes that define neural 578 progenitor cells (NPCs) specifically involved in the neuronal 579 lineage (NPC2) [35]. Here, we confirmed a key status of the 580 PAK3 gene for a proneuronal signature in tumors as well as 581 in PDCLs. This underlines its weight as a subgroup indica-582 tor, but at the same time raises the question of its role in 583 the biology of these tumors. In particular, it remains to be 584 determined how PAK3 could have opposite roles to those 585 of PAK1 in the biology of gliomas. A first possibility may 586 be that PAK3 has its own substrates. Currently, most PAK 587 substrates involved in cancer have been identified for PAK1, 588 but these have not been validated for other group I kinases 589 [19, 36]. A hypothesis may be that PAK3 uses one or more 590 yet unknown substrates that are not phosphorylated by PAK1 591 and are involved in the inhibition of cell division, or in the 592 initiation of neuronal differentiation. Another hypothesis 593 may be that PAK3 belongs to a signaling pathway involved 594 in a negative regulation of tumor growth. We have previ-595 ously shown that PAK3 signaling differs from that of PAK1 596 at several points, PAK3 being activated by the Rho GTPase 597 Cdc42 and preferentially binding to the Nck2/Grb4 adapter 598 [37, 38]. These pathways are known to act in tumor pro-599 liferation, differentiation and progression, but their role in 600 the biology of gliomas is still poorly understood. Another 601 possibility may be that PAK3 can act on cell proliferation 602 by inhibiting PAK1. Indeed, the PAK kinases have been 603 reported to form dimers that allow their trans-inhibition 604 [39, 40]. However, we have previously shown that the PAK3 605 protein prefers to form heterodimers with PAK1 rather than 606 homodimers, leading to inhibition of PAK1 kinase activity 607 [29]. Thus, a transcriptional increase of PAK3 in gliomas 608 could lead to a decrease in PAK1 activity. 609

Although *PAK1* and *PAK2* are well characterized oncogenes, our data strongly suggest that *PAK3* acts as a tumor suppressor gene [17, 36, 41]. *PAK* genes are rarely mutated in cancer and the analysis of mutation databases does not alow to discriminate mutational patterns between *PAK1* and PAK3 (CBioPortal) [42]. On the other hand, PAK1/2 615 genes are often overexpressed in cancer, as recently reported 616 for breast cancer, in contrast to PAK3 [43]. PAK1 is mainly 617 involved in cancer development through functional activa-618 tion, due to transcriptional or post-translational processes. 619 Here, our transcriptomic analysis showed a strong cor-620 relation between PAK3 expression and patient survival, 621 and the functional experimens we performed indicate that 622 PAK3 may indeed be considered as a tumor suppressor. One 623 consequence of our results relates to possible therapeutic 624 approaches. They stress the need to analyze, in different 625 tumor types, the expression and activation of oncogenic 626 PAK kinases such as PAK1 or PAK2, but also that of PAK3. 627 Indeed, the use of non-selective PAK kinase inhibitors could 628 have deleterious effects by inhibiting the PAK3 tumor sup-629 pressor gene. To date, there are no specific PAK inhibitors 630 that do not inhibit PAK3 [44]. Our data do suggest, however, 631 that differential therapies may be developed depending on 632 PAK3 expression level. A therapeutic strategy for tumors 633 with a low PAK3 level could be to target the transcription of 634 PAK3 in order to force post-mitotic cell differentiation by 635 targeting the proneural Notch/Neurogenin/NeuroD pathway 636 previously identified in Xenopus [8, 13, 45]. Differentiation 637 strategies have already been considered for GBM cancer 638 stem cells [8, 34, 46]. However, this approach is complex 639 and requires a more precise description of the function of the 640 gene in different cellular contexts. This was recently illus-641 trated by data showing that the effects of overexpression of 642 ASCLI, which acts as a proneural gene during embryonic 643 development and belongs to the proneural signature gene 644 set that includes *PAK3*, depends on the GBM subtype [47]. 645 Increasing PAK3 expression may be a therapeutic goal in 646 poorly differentiated tumors, since several factors such as 647 neurogenin positively regulate its transcription [48]. 648

We have provided omics and experimental data suggest-649 ing a unique role of PAK3 in the pathophysiology of glio-650 mas. These data need to be consolidated by other approaches 651 such as orthotopic grafts of different PDCLs, in which the 652 expression of PAK3 and its kinase activity can be experi-653 mentally modified, in order to analyze tumor growth within 654 the brain environment. Our results strongly indicate that 655 each group I-PAK plays a different role in cancer progres-656 sion, in particular in breast and brain cancer, and that PAK3 657 acts as a tumor suppressor in gliomas and, as such, may 658 represent a potential therapeutic target. 659

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Declarations 684

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