Comparative expression analysis reveals lineage relationships between human and murine gliomas and a dominance of glial signatures during tumour propagation in vitro

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Running title

Comparative analysis of human and murine brain tumours
Abstract

Brain tumours are thought to originate from stem/progenitor cell populations that acquire specific genetic mutations. While current preclinical models have relevance to human pathogenesis, most do not recapitulate the histogenesis of the human disease. Recently, a large series of human gliomas and medulloblastomas were analysed for genetic signatures of prognosis and therapeutic response. Using a mouse model system that generates three distinct types of intrinsic brain tumours, we correlated RNA and protein expression levels with human brain tumours. A combination of genetic mutations and cellular environment during tumor propagation defined the incidence and phenotype of intrinsic murine tumours. Importantly, in vitro passage of cancer stem cells uniformly promoted a glial expression profile in culture and in brain tumours. Gene expression profiling revealed that experimental gliomas corresponded to distinct subclasses of human glioblastoma, while experimental supratentorial primitive neuroectodermal tumours (sPNET) correspond to atypical teratoid/rhabdoid tumor (AT/RT), a rare childhood tumor.

Précis:

Brain tumor microenvironment strongly modifies tumor genetics that initiate disease, ultimately directing the pathway for growth dynamics, histology and therapeutic responsiveness of brain tumors, with implications for understanding how to properly model and treat disease more effectively.
Introduction

Stem and progenitor cells in the subventricular zone (SVZ) are the likely origin of many primary intrinsic brain tumours, in particular of gliomas (1-4). The most common intrinsic adult human malignant brain tumour is the glioblastoma (GBM), which is characterised by moderate-to-high mitotic activity, vascular endothelial proliferation and/or tumour cell necrosis. GBM’s are diagnostically uncontroversial and there is a broad consensus regarding their diagnostic criteria. They can include an oligodendroglial component or show elements resembling human supratentorial primitive neuro-ectodermal tumours (sPNET). Other common glial tumours in adults are oligodendrogliomas, oligoastrocytomas and astrocytomas which often show characteristic molecular signatures, such as loss of heterozygosity (LOH) of chromosomes 1p and 19q and an IDH gene mutation. However, unlike GBM, the morphological boundaries of these tumours are subject to considerable inter-observer variability. Molecular tests that correlate with prognosis, e.g. loss of heterozygosity (LOH) at various genomic loci (1p/19q; PTEN, NF1), IDH mutation status, MGMT methylation and others have improved the stratification of diagnostic and prognostic accuracy (5-7). Genomic and expression studies on large series of gliomas identified tumour signatures correlated to outcome and/or treatment response (8-10). Glial tumours can be experimentally generated by inactivation of tumour suppressor genes, such as p53 together with loss of Nf1, Pten, Rb, in astrocytes, neural stem- and progenitor cells (1-4, 11, 12), by adenovirus-mediated cre expression in the stem/progenitor cells of the SVZ (2, 3), constitutive cre expression in all stem/progenitor cells using GFAP-cre transgenic mice (3, 4, 12), or by tamoxifen inducible cre expression (11). The cre expression system influences the cell population that undergoes recombination and thus the type of tumours that originate from a given combination of genetic mutations. In fact, recombination of floxed Rb, p53 and Pten alleles by tamoxifen-inducible GFAP-cre activation in parenchymal astrocytes as well as SVZ stem/progenitor cells yielded exclusively high grade gliomas (11), while we observed also sPNET when the same combination of genetic mutations was induced by intraventricular Adeno-Cre or Adeno-GFAP-cre injection (2). Here, we have analysed the spectrum of intrinsic brain tumours arising from the neurogenic zone of the SVZ or from cancer stem cells that
were generated and propagated \textit{in vitro} and introduced into the brain by orthotopic allografting. We analysed the relationship of the combination of mutations, the morphological tumour phenotype and the expression profile. Finally, to validate our model system, correlations with the expression and the phenotype of their human counterparts were analysed.
Materials and Methods

Transgenic mice

Combinations of the conditional mouse mutants Rb$^{loxP/loxp}$, p53$^{loxP/loxp}$ and Pten$^{loxP/loxp}$, all in a ROSA26$^{loxP/loxp}$ background were used as described before (2). All mice were in a mixed background of C57 BL/6 and FVB. Further description of the mouse strains and primers for genotyping are given in (13), Rb$^{loxP/loxp}$ and p53$^{loxP/loxp}$ mice in (14) and R26R$^{loxp/loxp}$ reporter mice are described in (15). Animals were kept according to institutional and UK Home Office guidelines (Project licences 70/5540 and 70/6603).

Stereotaxic injection of Adenovirus

The cre adenovirus vector was constructed and propagated as described (16). Viral infection of SVZ progenitors was achieved by unilateral stereotaxic injections of $10^9$ plaque-forming-units of Adenovirus expressing cre recombinase (in short Adeno-cre) with a 26 G needle attached to a 10μl gastight Hamilton syringe (Model 1701 RN#80030) in 5 μl PBS, into the left ventricle of compound mutant mice as described and characterised in detail previously (2).

Isolation, propagation and stereotaxic injection of tumour-spheres.

Neurospheres were isolated from young adult mouse brains and propagated in serum free medium based on DMEM/HamF12 (#D8437, Sigma), and supplemented with B27 (1:50; #17504-044, Invitrogen), EGF (20ng/ml; # 315-09, PeproTech), bFGF (20ng/ml #100-18B, PeproTech), as described previously (2). Tumour spheres were generated after one passage in vitro by infecting neurospheres with Adeno-Cre (MOI of $\geq$5) and passaged 3-5 times to produce quantities sufficient for orthotopic allografting. 5 μl of the neurosphere suspension was injected into the left striatum of adult mice (bregma, 1.5 mm lateral, 2 mm deep), with a 22G needle attached to a 25μl Hamilton syringe (Model 1702 RN#80230).

Histological examination and immunostaining
Brains were fixed in 10% formalin, embedded in paraffin, cut into 3μm sections and stained with haematoxylin-eosin (H&E). A list of all antibodies and antisera is given in the supplementary methods section. Immunostaining was done on Ventana Discovery automated staining machines (Ventana Medical Systems) following the manufacturer’s guidelines, using horseradish peroxidase-conjugated streptavidin complex and diaminobenzidine as a chromogen.

**Image capturing and analysis:**

Histological slides were digitised on a LEICA SCN400 scanner (LEICA UK) at 40x magnification and 65% image compression setting. Digital image analysis was performed using a Definiens Tissue Studio Library and Developer XD2 (Definiens, Munich, Germany). Full details of the image analysis algorithms and software settings are given in the supplementary methods section.

**Tumour sampling and use of human tissue**

Mice that developed clinical signs of intracranial pressure were injected with BrdU two hours before culling. Brains were taken and assessed for tumour content, and tumours were separated from normal brain under a dissection microscope. One part was processed for formalin fixation and paraffin embedding and the other part was snap-frozen. Tumour identity and content were confirmed on histological sections. The use of human tissue was ethically approved (Reg. Number NHNN/08/H0716/16) and the storage of human tissue was according to the Human Tissue act (UK) (Human Tissue Authority License for UCL/ION 12054s).

**RNA extraction and micro array hybridisation**

RNA was extracted from frozen tumour fragments using Trizol (Invitrogen). RNA analysis was performed using Affymetrix Mouse Exon 1.0ST microarrays in the UCL/ICH genomics core facility. Samples were processed following the Affymetrix GeneChip Whole Transcript (WT) Sense Target Labelling Assay Manual (P/N 701880 Rev.4). Following ribosomal RNA removal (Invitrogen, cat# K1550-02) samples were amplified, fragmented and labelled using the GeneChip WT Sense Target Labelling and Control reagent kit (Affymetrix, cat# 900652), and hybridised to the arrays. Chips were
stained using the GeneChip Fluidics Station 450 and scanned with the GeneChip Scanner 3000 7G. For ATRT data, Illumina HT12_v3 beadchip data generated from mixed brain tumour samples and controls was exported un-normalised from Genespring software and imported into Partek GS 6.6 as described in (17). The array data are available in GEO Ref. GSE42515.

**p53 sequencing**

RNA was extracted from tissues or cells, reverse transcribed and subjected to PCR using three p53-specific primer sets, followed by gel-extraction of the PCR products and sequencing with the appropriate primers (see supplementary materials). Fluorescent sequence traces were analysed, assembled and compared to NCBI reference sequences using the CLC Main Workbench 6.

**Statistical and data analysis**

Statistics internal to R, GSEA and Partek applications were used for microarray analysis. Array data were obtained from own hybridisation experiments or downloaded from GEO and other locations. Subsequent data analysis, including HC and PCA and generation of tumour-specific gene sets, were done with Partek GS workflows with GC-RMA quantile normalisation. In cases where expression differences were extreme, i.e. when comparing *in vitro* and *in vivo* samples, quantile normalisation was not used. Pearson correlation plots and tab-delimited annotated expression sets for gene set enrichment assays (GSEA) were generated using Affymetrix Expression Console with GC-RMA quantile normalisation. Unless otherwise stated all p-values and false-discovery rates are multiple-testing corrected according to Benjamini-Hochberg (18). GSEA was performed using software downloaded from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp (19, 20)). Single sample GSEA (ssGSEA) (21) was performed using the ssGSEA projection module at the Broad Institute (http://www.broadinstitute.org/cancer/software/genepattern, (22)).

**Results:**

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Experimental primary CNS tumours correspond to known human tumour entities and express stem and progenitor cell markers.

In order to generate primary brain tumours, we injected Adeno-cre into the lateral ventricle of mice carrying “floxed” double mutant (in short: p53/Pten, p53/Rb, Pten/Rb) or triple mutant (p53/PTEN/Rb) alleles. The lineage relationships to neural stem/progenitors was studied with a panel of markers against GFAP (B-type stem cell; astrocyte (23)), PDGFRA (radial glia-like cell; B-Stem cell (24)), Nestin (radial glia and transient amplifying cells, C Type cell (25)), Sox2 (26), Olig2 (27) and Doublecortin (28) (transient amplifying and neuroblast, C; A-cell). CD15 (29), MAP2, Synaptophysin and NeuN are expressed in maturing neurons. p53/Pten mice developed a spectrum of gliomas including anaplastic astrocytomas, oligodendrogliomas and most commonly, anaplastic oligoastrocytomas, but no glioblastoma with palisading necrosis or microvascular proliferation. These gliomas expressed the stemness markers GFAP, PDGFRA, Nestin, and the transient amplifying markers Sox2, Olig2, Doublecortin and CD15, while markers of neuronal differentiation, such as synaptophysin and NeuN, were not expressed (Fig 1A). This expression pattern is similar to that of human anaplastic oligo-astrocytomas (Fig 1D). The quantification of marker expression is summarised in Fig 2. Additional deletion of the Rb gene (p53/PTEN/Rb, Fig. S1) or deletion of p53/Rb only (Fig S2) causes predominantly poorly differentiated, well circumscribed neoplasms, with a morphological similarity to human supratentorial primitive neuroectodermal tumours (sPNET). sPNETs in p53/Rb (Fig 2A, S2A) or in p53/PTEN/Rb mutants (Fig 2A, S1A) were negative for stemness (GFAP, PDGFRA, Nestin) or transient amplifying markers (Sox2, Olig2, and Doublecortin), in keeping with their primitive neuroectodermal phenotype. CD15 was expressed in a subset of these tumour cells, in keeping with previous reports in medulloblastoma (29), while MAP2 and synaptophysin were consistently strongly expressed in all sPNETs (Fig S1A, S2B), in keeping with the profile of human counterparts (Fig S2D). Mice with the underlying combination of mutations Rb and Pten developed no tumours (0 tumours in 20 mice after up to 440 days, Fig S3, Fig S4).

The growth factor-rich environment of tumour spheres modulates the tumour phenotype.
SVZ derived stem/progenitor cells isolated from double and triple mutant mice were recombined in vitro to form tumour spheres and were subsequently allografted to generate tumour grafts. To understand the role of the growth factor-enriched medium on the modulation of the tumour phenotype, we analysed the profiles of tumour spheres and tumour grafts. Unlike the distinctive genotype–phenotype correlation of SVZ derived primary tumours, spheres of all four genotypes (i.e. p53/Pten (Fig 1B), p53/Pten/Rb (Fig S1B), p53/Rb (Fig S2B) and Pten/Rb (Fig. S3B), as well as non-recombined controls expressed GFAP, PDGFRA, Nestin, Olig2 and Sox2 and Doublecortin at high levels, while markers of mature neurons, synaptophysin and NeuN, were only rarely expressed. This indicates that in vitro recombined tumour spheres favour an expansion of progenitor cells akin to stem/transient amplifying cells (Fig 2A) and suggests that the culture of spheres in growth factor-enriched medium overrides the effect of the genetic mutations (Fig 2A), possibly by the preferential growth of a particular cell type. Grafted p53/Pten spheres recapitulate the primary tumour phenotype, forming infiltrative oligoastrocytomas with an expression profile indistinguishable from primary p53/Pten gliomas and similar to that of tumour spheres (Fig 1A, C). p53/Rb and triple mutant p53/Pten/Rb tumour grafts show dual differentiation, with a stem/progenitor cell profile (Sox⁺, Olig2⁺, Dcx⁺ and GFAP⁺) (Fig S1C,D) adjacent to primitive neuroectodermal areas with little glial differentiation but strong MAP2, synaptophysin and moderate NeuN expression (FigS1D).

Importantly, in vitro propagation allowed growth of tumours from Pten/Rb tumour spheres (n=12 of 17 mice (70%); Fig S3C, D, Fig S7), with a morphology resembling human GBM with pseudopalisading necrosis, vascular endothelial proliferations, and expression of stem/progenitor and glioma markers (GFAP⁺, Nestin⁺, Doublecortin⁺, Sox2⁺ and Olig2⁺ and MAP2⁺) (Fig S3C, D). This genotype is distinguished from the other tumours by the presence of intact p53, confirmed by sequencing (Fig S8), suggesting that p53 may exert a tumour suppressive function in a Pten/Rb mutant background in the context of an intact SVZ, which can be overcome in an in vitro environment with growth factor-rich conditions. Our data suggest that in vitro passage may select for a cell population with stem/progenitor properties, which continues to be prevalent in tumour cells expanding in a mature CNS environment.
mRNA Expression profiling of primary brain tumours, in vitro tumour spheres and tumour grafts suggests a critical role of in vitro culture on tumour phenotypes.

The predominance of a glial phenotype of grafts derived from tumour spheres prompted us to compare the transcriptome of primary tumours, in vitro growing tumour spheres and tumour grafts using the Affymetrix Mouse Exon 1.0ST microarrays (Fig 3A). We initially compared the global expression profiles of all samples to each other, including publicly available control samples including normal brain which had been hybridised to the same array platform (Fig 3A). A Pearson correlation plot shows a marked segregation of expression profiles of cultured spheres compared to any of the in vivo tumours (Fig 3A), indicating that cultured cells express genes that are very different from experimental tumours and controls, but shows striking similarity between all cell cultures independent of the underlying genetic mutation, with differences that did not exceed an adjusted p-value/false discovery rate (FDR) of 0.05. Further comparison within the profiles of individual cell cultures showed that cultures and tumours showed more than 50% of genes were expressed differentially (FDR <0.05; Fig. 3B). We analysed the top 2000+ changed genes (442 “upregulated” in vivo; 1689 “downregulated” in vivo, Table S1) for enrichment in gene ontology (GO) terms using the online tool DAVID (30) and found that the most significantly enriched GO terms belonged to cell culture-related pathways, i.e. growth-factor induced cell-cycle genes and oxidative stress. This indicates that differences in oxygen concentration and abundance of growth factors in the cultures were the factors most likely to cause this differential gene expression, overriding any mutation-specific effect. A saturation of these pathways in cultured cells could explain why the cultures did not express any mutation-specific signatures to achieve genome-wide significance when compared to each other, regardless of the combination of genetic mutations. It is also unlikely that host-derived vascular endothelium or stromal cells significantly contribute to these differences between tumours and spheres, as we have previously shown that tumour preparations are largely devoid of contaminating cells (2). Comparison of primary and secondary tumours shows a close match with a genome-wide difference of less than 10% across all genotypes (Fig 3B). Even the difference within
matched genotypes was reduced to below 1-3% depending on the genotype. This highlights the importance of in vitro cultures in selecting for a specific cell type or by modulating expression in a given cell population.

Hierarchical clustering of global expression profiles corresponds to tumour morphology and suggests common patterns of experimental high grade gliomas, overriding initiating mutations.

Given the predominance of glial signatures upon in vitro propagation, we next analysed whether histological appearance or tumour-initiating genotype correlated better to the transcriptome. We analysed oligoastrocytomas, sPNETs and the ‘dual-differentiated’ tumours by hierarchical clustering our samples and control brains (Fig 3C). Normal brain (Fig 3C, NB, right column) differed markedly from all tumour samples, and spheres of all genotypes (column 4 in Fig 3C, orange box) clustered together as assessed by the Pearson’s analysis (Fig 3A, B). The yellow box within the sphere cluster highlights non-recombined control spheres, confirming that the cell culture environment overrides any other differences in the transcriptome (Fig 3C). Comparison of the histological tumour entities by unsupervised hierarchical clustering segregates sPNETs away from glial tumours, and segregates the latter into oligoastrocytomas and glioblastomas. Importantly, the three p53/Rb grafts fall within the glioma cluster (top of Fig 3C, large black box), showing that their morphological “glioma” phenotype corresponds to an expression pattern akin to p53/Pten gliomas rather than to the genetically matched, but morphologically distinct p53/Rb sPNET. Strikingly, a similar expression pattern was also found in a rare SVZ-derived p53/Rb glioma (top of Fig 3C, small black box). PCA on the 36 arrays (Fig 3D) shows segregation into a cluster with tumour spheres (Fig 3, orange spheres), and solid tumours (purple, red and blue spheres). Separate analysis of primary and grafted tumours clustered them according to their histological appearance, overriding their different genotypes. Tumours with dual differentiation (all grafted) clustered closer to the gliomas (Fig 3E). These PCAs (Fig 3D, E) underpin the effects of in vitro propagation which overrides the influence of the primary genetic mutation, and further indicate that the histological morphology more closely corresponds to the global expression pattern of the tumour than its initiating genotype, as also suggested from human tumours, where for
example the 1p/19q status of oligodendrogliomas or the IDH mutation status of diffuse astrocytomas does not impact on their histological appearance.

*The expression profiles of experimental gliomas resemble those of a subset of human GBM.*

Next, we determined whether the distinct experimental tumour types corresponded to a glioma subclass of published gene datasets. Using gene set enrichment analysis (GSEA) (19) we compared the expression profiles of our experimental tumours to the four GBM subtypes (9) of The Cancer Genome Atlas (TCGA) and three glioma subtypes identified by Philips et al (8) (Fig 4A). We ensured maximal reproducibility by using a previously published expression data set (kindly provided by C. Qu and S. Baker) (11). Comparison of “oligoastrocytomas” to the remaining tumour phenotypes (i.e. PNET+GBM; labelled “The Rest” in Fig 3A), showed that experimental oligoastrocytomas did not significantly resemble any of the human GBM subtypes (all FDR>0.25; Figure 4A, column 1), but there was a statistically non-significant resemblance to the Phillips Proneural (PN) and TCGA Classical GBM. Instead, PNET+GBM showed a significant correlation to the Phillips Proliferative subtype (FDR 0.08, see below). GBM (orange headers in Fig 4A) are more similar to oligoastrocytomas than to sPNET. The “dual-differentiation” of grafted p53/Pten/Rb neurospheres is also evident from their heatmap profile (yellow box in Fig 4A, column 1). By grouping the tumours by genotype (Fig 4A, column 2), p53/Pten tumours significantly resemble TCGA Classical, ‘Phillips’ Proneural (FDR 0.11) and TCGA Neural (FDR0.12). Finally, by comparing murine sPNET to the other tumours, they show a significant correlation of murine sPNET to the Phillips Proliferative subtype (FDR <0.05, Fig 4A, column 3). Again the heatmap shows that all grafted samples, including murine GBM, show marked resemblance to the expression profile of the p53/Pten mutant tumours (oligo-astrocytomas). There was no obvious change in the gene order compared to the analysis according to the histological grouping performed above (Table S3).

*The expression profiles of experimental gliomas are dissimilar to human oligodendrogliomas.*
As our p53/Pten mutant gliomas resemble a spectrum from astrocytomas to oligodendrogliomas, we compared their expression profile to human oligodendrogliomas, using Partek quantile normalised and RMA background corrected array data from GSE9385 (31). Expression profiles of oligodendroglioma were compared to those of control brain and other glioma samples within the dataset and an expression set comprising genes with at least a two-fold expression difference in (human) oligodendroglioma vs. control brain or “other glioma” was identified (Table S4). GSEA analysis containing 454 samples from the Rembrandt project (32) showed that the oligodendroglial gene set shows a highly significant correlation with the oligodendroglial samples (FDR 0.01) (Fig S5), while our experimental murine gliomas did not show significant overlap with the oligodendroglial expression set.

*The expression profiles of experimental sPNETs resemble human atypical rhabdoid/teratoid tumour but not medulloblastoma or sPNET.*

Our experimental supratentorial PNET appear histologically similar to human sPNET, a heterogeneous group of aggressive supratentorial tumours of children and adolescents, composed of undifferentiated neuroepithelial cells which may express synaptophysin (33) and NeuN and CD15 (29). We compared our experimental sPNET to experimental medulloblastomas arising from Ptc1–/– external granule layer (EGL) neuron precursor cells (GSE17702 (34)) by hierarchical clustering (Fig. 4B). Neither our cultures (Figure 4B, black box), nor our sPNET (Figure 4B, green box) resembled experimental medulloblastomas or granule neuron precursor cells (34) (Figure 4B, purple boxes), indicating that experimental sPNET are distinct from morphologically similar experimental medulloblastoma. Next, we compared the murine sPNET to a small set of histologically similar human sPNET (Fig S2D) prepared on the Affymetrix HG-U133+2 arrays encompassing 10 human sPNET, 1 pineoblastoma and 1 atypical teratoid /rhabdoid tumour (ATRT). A sPNET-specific gene set was derived by comparing the human sPNET with datasets (GSE21687) of ependymomas (35) and GSE15824 (gliomas including GBM and normal brain) (36). Differentially expressed genes with >2-fold expression difference were identified, yielding a sPNET-specific expression set containing 1143
genes (Table S2). The gene set was successfully tested by performing a PCA and hierarchical clustering of ependymal samples, gliomas and our samples (Figure S5A, B). A recent study (37) analysed 51 paediatric sPNET expression profiles and found three clusters, one of which corresponded closely to their previously published sPNET with amplified C19MC locus (38). The other two groups (‘oligoneural’, ‘group 2’ and ‘mesenchymal’, ‘group 3’) overlapped with each other. We derived three gene sets from the study, encompassing fewer than 15 genes each and used these for a GSEA of our sPNET. The experimental sPNET correlated best (FDR 0.15) with the mesenchymal subclass (Group 3) of sPNET. All other comparisons were non-significant.

One further form of primitive brain tumours is the atypical teratoid rhabdoid tumour (ATRT). We collected in independent dataset of tumours that had been profiled on Illumina HT12_v3 beadchip arrays, including 5 ATRT, control brain samples, 17 pilocytic astrocytomas and 15 gliomas (17). An ATRT-specific set of 345 genes was derived and GSEA on all experimental tumours or selectively on primary tumours was performed using sPNET (Fig S5) and ATRT (Fig S7) expression sets. Tumour-type specific gene sets were derived by contrasting each tumour type against “other brain tumours” and against “normal tissue” array samples. There was a strong correlation (FDR 0.04) of experimental sPNET with ATRT (Fig S5C), which was reduced when tumour grafts were included (FDR 0.09), whilst no correlation with sPNET was seen. The tumour suppressor SMARCB1/INI1, a gene specifically downregulated in ATRT, is underexpressed in all human ATRT samples as well as in human sPNET, compared to controls, while human gliomas tend to overexpress INI1. Instead, experimental sPNET express normal levels of this transcript, probably due to a different molecular pathogenesis of these entities.

Discussion

In this study we have analysed the relationship of primary murine intrinsic brain tumours, anaplastic oligoastrocytomas, glioblastoma and primitive neuroectodermal tumours with their human counterparts. We have identified similarities between the transcriptomes of distinct human brain tumour entities, specifically glioblastomas of the TCGA classical subtype, and oligodendrogial
tumours on the one hand, and Phillips proliferative gliomas and atypical teratoid/rhabdoid tumours (ATRT) on the other hand. Our key findings are (i) that the genotype and can be strongly modified in vitro by growth factors, characterised by a shift towards a stem/transient amplifying phenotype in vitro, (ii) the correlation of experimental gliomas with the TCGA classical subtype of human glioblastomas (Fig 4A); (iii) an experimental correlate of human glioblastomas with PNET component (Fig S1E, F), and (iv) a distinct expression profile shared by experimental primitive neuroectodermal tumours and human ATRT (Fig S2A, D; Fig 4C).

As reported previously, there is a strong correlation between the initial genetic mutation and the tumour phenotype. The different efficacies of tumourigenesis (Fig S7) can be explained by a differential susceptibility of the targeted SVZ stem/progenitor population by growth-promoting signals. *p53/Rb* inactivation in the SVZ is relatively ineffective to generate tumours whilst very effective when occurring during granule cell amplification in the developing cerebellum (14). Other cell types, e.g. cortical astrocytes and neurones are not susceptible to neoplastic transformation at all. (2). Identical primary mutations in the SVZ stem cell compartment can give rise to a spectrum of gliomas, ranging from anaplastic astrocytic, to oligodendroglial tumours. There is recent evidence that a defined mutation can give rise to a spectrum of gliomas also in humans, often causing diagnostic ambiguity (39, 40). Tumours caused by a mutation in the Isocitrate dehydrogenase (IDH) gene encompass a spectrum from astrocytomas to oligodendrogliomas (41). At the same time, there is increasing evidence that a morphological subclassification of gliomas lacks clinical or prognostic relevance (42), and there is growing support for an approach combining morphological assessment and molecular profiling of gliomas (31).

*In vitro* cell cultures derived from primary tumours remain a mainstay in human brain tumour studies and are used to study signalling pathways or drug responses (43, 44). In order to generate xenografts, primary tumours are commonly subjected to a number of *in vitro* passages which has been shown to render them to a potential selection bias (45), whilst for example MGMT methylation was found to be similar in primary tumours and glioma spheres (46). We compared the transcriptome and protein expression in corresponding genotypes in genetically defined cells, and a controlled and reproducible
environment. While primary tumours show a robust relationship between initiating mutations and tumour phenotype, propagation of CNS stem/progenitor cells in growth factor-enriched conditions overrides genetic mutations and promotes a glial signature (Fig 2, 3C, D). These data can be interpreted as follows: (i) in vitro cultured cells are likely to be saturated by growth factors essential for propagation of these cells, (ii) genomic alterations are not predictive for in vitro transcriptome expression profiles; and (iii) the in vitro conditions are selective for a subgroup of tumour stem cells that express a stem/progenitor transcriptome, and share features with gliomas (Fig 1, 2A). Expansion of in vitro recombined cancer progenitor cells in an environment similar to that of primary brain tumours partly reverts this phenotype, but still with a significant shift towards a stem/progenitor or glioma phenotype (Fig 2, Fig3B, D), possibly by a reversal to a mature CNS environment where different populations of cancer stem cells have a growth advantage. An important finding is the inability to generate brain tumours by inactivating Pten and Rb alone in SVZ progenitor cells. This is a contradiction to previous studies (47, 48) where gliomas were generated by inactivation of Rb family members in mice expressing GFAP-T121, a truncated SV40 Large T antigen under the control of the GFAP promoter in a cre-inducible fashion and where additional Pten but not p53 alleles (47) were deleted. The discrepancy with our findings can be explained by an activation of the Rb pathway in a wider range of cells or by inactivation of several Rb family members in the GFAP-T121 model (47-49). The vast majority of studies with genetically modified mice demonstrated that p53 suppression is required for brain tumour initiation and propagation (1, 3, 4, 11, 12, 50). No p53 mutations are detectable in Pten/Rb cells or grafted tumours (Fig S8). Pten/Rb tumour grafts strongly express olig2, offering a possible explanation for a selective downregulation of p53 function in the absence of a genetic mutation (51).

The genome-wide expression data confirm that experimental gliomas resemble human GBM subtypes. Two large studies (8, 9) used overlapping terminology for distinct profiles. Thus, clustering of human GBMs using either gene set will often place a given GBM in a cluster with a different name. Thus, the murine glioma fall in the “Classical” cluster as defined by Verhaak (9), whilst also resembling the “Mesenchymal” cluster as defined by Phillips (8). A morphologically distinct glioma
phenotype with a biphasic pattern with glial (GFAP+, Nestin+, PDGFRα+, Doublecortin+, Sox2+, and Olig2+) and separate, PNET-like component with synaptophysin and NeuN expression was observed in p53/Rb and p53/Pten/Rb secondary tumours, suggesting dual differentiation with glial and PNET components (Fig S1C, D). This heterogeneity can be explained by the grafting of tumour spheres that share an initial mutation and then diversified during separate clonal expansions in vitro. Such PNET-like components in the context of glial tumours have been increasingly recognised in human high grade gliomas, in particular those that arise through progression from lower grade astrocytomas (52) and could be confirmed in a series of 5 GBM-PNET from our archive (Fig S1E,F). In this context, our model system supports the notion of the PNET component as part of an expansion of a de-differentiated sub-clone in a secondary GBM.

While there is little resemblance of experimental and human sPNET expression profiles, there are significant similarities to human ATRT (Fig S5), even though the experimental tumours do not replicate the loss of SMARC/INI1. Thus, our model suggests that both primitive primary CNS tumours and gliomas are likely to originate from the same stem/progenitor compartment in the CNS. A recent study of profiling supratentorial childhood sPNET (37) reveals three subgroups, group 1 being enriched for markers of embryonic or neural stem cells, group 2 tumours showing an upregulation of markers of “oligoneural” differentiation (53) and Group 3 tumours showing upregulation of epithelial and mesenchymal differentiation genes. In this context it is important to note that despite a number of parallels between our model system and human gliomas, there a number of limitations of this and similar other model systems. To date, there is no robust data to suggest from which cell type, and importantly, from how many cells a human brain tumour arises. Instead, all genetically engineered mice (GEM) have in common that they require a “critical mass” of recombined cells, usually with initial mutations of multiple genes, to develop tumours. Despite significant advances in refining the population of cells, there are also limitations to the specificity of targeting stem progenitor cells in GEM (54, 55). Due to the simultaneous recombination of cells with identical mutations, GEM may not reflect all aspects of the biology of human gliomas, which are known to show significant intratumoural heterogeneity.
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Author contribution:

Nico V. Henriques: Generated and analysed most data. Contributed to writing manuscript.

Tim Forshew, Ruth Tatevossian, Hazel Rogers, Kerra Pearce: Generated and analysed data.

Matthew Ellis, Pablo Garcia Reitboeck: Histological and digital image analysis.

Angela Richard-Loendt: Performed experiments

Richard Grundy, Denise Sheer, Thomas S. Jacques: Contributed and analysed material

Sebastian Brandner: Idea and concept, obtained funding, analysed data, wrote manuscript.

Conflict of Interests:

The authors declare that they have no conflict of interest.
References


Figure legends:

Figure 1: Expression patterns of tumours generated in a p53/PTEN background. A) primary tumours with morphological features of oligoastrocytomas, expressing markers of “stemness” and of “transient amplifying” cells. B) SVZ-derived, in vitro recombined, neurospheres express a similar marker profile. C) Tumour graft with a morphology and expression pattern nearly identical to the primary tumour. D) Human anaplastic oligo-astrocytoma with morphology and expression pattern similar to the primary and grafted tumour of the mouse model. Scale bar 4mm (overview, first column) and 200μm (all other images).

Figure 2

Summary of quantitative image analysis of immunohistochemical stainings of all primary tumours, tumour spheres and grafted tumours. A) The heat map reflects a mean of the values determined by image quantification of all samples in each group. There are major differences between primary tumours (oligo-astrocytomas vs. PNET), while tumour spheres express a similar profile of markers across all genotypes including non-recombined spheres. Tumour grafts show predominantly glial profile. Dual differentiation of p53/Pten/Rb and p53/Rb grafts is shown in two rows. B) The resultant figure is colour coded representing a range of 0-10.

Figure 3

Expression profiling primary and secondary (grafted) tumours, cultures, and control tissues. A) Pearson correlation plot of global expression profiles of all samples including control samples (3x whole brain tissue, 1x sorted neurons, 2x sorted oligodendrocytes and 2x sorted astrocytes). Marked segregation of the expression profiles of cultured spheres independent of genotypes (orange and grey), compared to tumours (blue fields), indicating that the cultured cells express very different genes from experimental tumours and from normal samples. B) Cultures differ significantly from primary and grafted tumours, with more than 50% of genes being expressed differentially (FDR <0.05), while primary and grafted tumours show only 10% difference (1733) of their genome-wide gene expression.
C) Hierarchical clustering of global expression profiles using a subset of the TCGA clustering genes. The first row of the colour codes of the cluster denote the sample genotype, the second row indicates the tissue type and the third row of colour indicates the histological diagnosis which is repeated above the heat map. Normal brain (NB, right column with green box) differs markedly from all other samples. Spheres of all genotypes (orange box) including non-recombined controls (pink box) cluster together. Among the solid tumours PNETs (purple) segregate apart from glial tumours, whilst oligoastrocytomas (blue) segregate slightly apart from glioblastomas (red). The larger black box within the colour codes shows that the p53/Rb grafts fall within the glioma cluster and a similar expression profile was also found in a rare SVZ-derived p53/Rb glioma (small black box).

D) Principal component analysis (PCA) on the 36 arrays shows segregation into a cluster with tumour spheres (orange spheres), and solid tumours (purple, red and blue spheres). E) PCA of primary and grafted tumours groups them according to their histological appearance, overriding their different genotypes. Grafted tumours with dual differentiation cluster closer to the gliomas (blue and red).

Figure 4:

Gene set enrichment analysis (GSEA) and comparison of experimental tumours to histologically corresponding human brain tumours. A) Experimental gliomas and experimental PNET resemble different subsets of human GBM: Column 1, expression profiles of the histological phenotype of experimental “oligoastrocytomas” show no significant resemblance to any of the 7 human GBM subtypes tested (all FDR>0.25). Column 2, p53/Pten samples express a profile significantly resembling TCGA Classical (FDR 0.11), ‘Phillips’ Proneural (FDR 0.11) and TCGA Neural (FDR 0.12). Column 3, Experimental PNET expression resembles the “Phillips Proliferative” human GBM subset (FDR 0.06). B) Hierarchical clustering of experimental medulloblastoma derived from the external granular layer of PTCH1/2 mice, and related cell lines shows no resemblance to our experimental PNET. From left to right: black box, neurospheres (NSC, light blue index) and tumour sphere cultures (CSph, red index), followed by normal brain (brown index). The purple boxes outlines
cerebellar granule cell precursor cells (GNP, green index) and murine medulloblastoma (MB, orange index). Green box, experimental sPNET (purple index). The remaining samples in the right columns are oligoastrocytomas (dark purple index), gliomas and GBM (blue index). Experimental PNET show a pattern distinct from all other groups. C) Expression profiles of experimental gliomas resemble human oligodendrogliomas whereas experimental PNET resemble human ATRT rather than human sPNET. Column 1, The expression profiles of experimental brain tumours were tested against a published sPNET gene set (Huang et al), and three further self-generated gene sets for ATRT, sPNET and oligodendroglial tumours. The PNET gene set shows a strong resemblance to the ATRT gene set, but not to the two sPNET-derived gene sets or the oligodendroglioma gene set. Column 2 shows an identical analysis, but with omission of grafted samples, resulting in a reduced FDR of 0.04 but still no overlap with experimental sPNET. Column 3, experimental oligoastrocytoma profiles show significant correspondence to human oligodendrogial tumours.
Figure 2

Quantitative histological imaging of tumours and \textit{in vitro} cultures

### A

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Medium intensity

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High intensity

| High intensity | 0 | 2 | 4 | 6 | 8 | 10 |

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Figure 3

**A**

Pearson correlation plot of global expression profiles

**B**

Differential gene expression

**C**

Hierarchical clustering

**D**

PCA Mapping (49.7%)

**E**

PCA Mapping (41.4%)
Comparative expression analysis reveals lineage relationships between human and murine gliomas and a dominance of glial signatures during tumour propagation in vitro

Nico V Henriquez, Tim Forshev, Ruth Tatevossian, et al.

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