Fibroblast growth factor receptor 4 promotes glioblastoma progression: a central role of integrin-mediated cell invasiveness

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Abstract

Glioblastoma (GBM) is characterized by a particularly invasive phenotype, supported by oncogenic signals from the fibroblast growth factor (FGF)/FGF receptor (FGFR) network. However, a possible role of FGFR4 remained elusive so far. Several transcriptomic glioma datasets were analyzed. An extended panel of primary surgical specimen-derived and immortalized GBM (stem)cell models and original tumor tissues were screened for FGFR4 expression. GBM models engineered for wild-type and dominant-negative FGFR4 overexpression were investigated regarding aggressiveness and xenograft formation. Gene set enrichment analyses of FGFR4-modulated GBM models were compared to patient-derived datasets. Despite widely absent in adult brain, FGFR4 mRNA was distinctly expressed in embryonic neural stem cells and significantly upregulated in glioblastoma. Pronounced FGFR4 overexpression defined a distinct GBM patient subgroup with dismal prognosis. Expression levels of FGFR4 and its specific ligands FGF19/FGF23 correlated both in vitro and in vivo and were progressively upregulated in the vast majority of recurrent tumors. Based on overexpression/blockade experiments in respective GBM models, a central pro-oncogenic function of FGFR4 concerning viability, adhesion, migration, and clonogenicity was identified. Expression of dominant-negative FGFR4 resulted in diminished (subcutaneous) or blocked (orthotopic) GBM xenograft formation in the mouse and reduced invasiveness in zebrafish xenotransplantation models. In vitro and in vivo data consistently revealed distinct FGFR4 and integrin/extracellular matrix interactions. Accordingly, FGFR4 blockade profoundly sensitized FGFR4-overexpressing GBM models towards integrin/focal adhesion kinase inhibitors. Collectively, FGFR4 overexpression contributes to the malignant phenotype of a highly aggressive GBM subgroup and is associated with integrin-related therapeutic vulnerabilities.

Keywords: Glioblastoma, FGFR4, Integrins, Invasiveness, FAK, FGF19

Introduction

Glioblastoma (GBM) represents the most common malignant brain tumor in adults [1]. First-line therapy comprising surgical debulking followed by concomitant radio-chemotherapy with temozolomide [2] results...
in a five-year survival rate of only ~6.8% [1]. Besides high invasiveness, GBM is generally characterized by pronounced heterogeneity and cell plasticity, explaining why frequently neither surgical nor pharmacological intervention results in patient cure [3]. Hence, there is an urgent need to dissect oncogenic GBM programs allowing development of novel, integrative treatment perspectives.

The fibroblast growth factor receptor (FGFR) family comprises four highly conserved receptor homologs named FGFR1-4. Out of the 18 known fibroblast growth factors (FGFs) in humans, members of the FGFR subfamily, including FGFR9, FGFR21, and FGFR23, bind with higher affinity to FGFR4 than to other family members [4]. FGFRs have emerged as potential cancer targets, as overexpression and hyperactivation have been described in a wide array of cancer types [5, 6]. Regarding FGFR3, and in rare cases also FGFR1, oncogenic chromosomal fusions to transforming acidic coiled-coil (TACC) 3 or TACC1, respectively, have been identified in ~3% of GBM [7] and related to tumor initiation and progression [8–10]. Compared to other FGFR family members, genetic aberrations of FGFR4 are rather rare [4, 11]. Instead, a single nucleotide polymorphism (SNP) at codon 388 of FGFR4 (rs351855), leading to a glycine to arginine conversion (G388R), has been connected to enhanced tumor susceptibility and aggressiveness in different tumor entities [12]. Concerning GBM, one earlier study did not find an impact of the G388R SNP on GBM patient prognosis, despite enhanced FGFR4 gene expression in case of the 388Arg allele [13]. Nevertheless, a driving role of FGFR4 in astrocytoma malignancy has been suggested, supported by a correlation of FGFR4 protein expression with malignant progression [14]. However, functional studies on FGFR4 in GBM are missing yet.

Here, we identify FGFR4 overexpression in a highly aggressive GBM subgroup and discover a key contribution to the malignant phenotype, especially concerning cell adhesion and migration via an integrin-mediated mechanism. This warrants further investigations of FGFR4 as therapeutic target in this disease, for which currently no targeted agents have reached worldwide clinical approval.

## Materials and methods

### Availability of transcriptomic datasets

FGFR4 expression in non-malignant tissues was analyzed in the GTEx dataset and the Allen brain atlas (brain-map.org). Data on glioma were derived from the REMBRANDT and TCGA-GBM datasets. Detailed information on data processing and visualization are given in the supplementary materials.

mRNA expression microarray and array comparative genomic hybridization (aCGH)

Analyses of whole genome gene expression (4 × 44 K microarrays, Agilent Technologies, Santa Clara, USA) and copy number changes by aCGH (4 × 44 K human whole genome oligonucleotide-based arrays, Agilent Technologies) were performed and data were extracted as previously described [15, 16] (compare supplementary materials).

### Cell culture and tissues

Immortalized GBM cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and kept in their respective media (Sigma-Aldrich, St. Louis, MO, USA). Primo-cell cultures were established and RNA extracted from isocitrate dehydrogenase (IDH) wild-type GBM surgical specimens in our laboratories (Medical University Vienna, Kepler University Hospital Linz) as described [17, 18]. NCH644 and NCH421K glioma stem cell-like (GSC) models were derived from CLS Cell Lines Service GmbH (Eppelheim, Germany: MTA to FE). Cell culture conditions are outlined in the supplementary materials.

### Protein expression analyses

Western blotting and immunohistochemistry (IHC) were performed as described previously [18] and in the supplementary materials. For Western blotting, the hepatocellular carcinoma model Hep3B, exhibiting high FGFR4 levels [19], was used as positive control. Additional File 3: Table S1 lists all used antibodies and working concentrations. β-actin served as loading control.

### Generation of viral constructs and transduction

Initial plasmids encoding wild-type FGFR4-388Gly were kindly provided by Prof. S. Ezzat, M.D. (University Health Network, University of Toronto, Toronto, Ontario, Canada). An FGFR4 kinase domain-mutated (K504M) vector encoding an inactivated FGFR4-kinase dead (KD) gene was generated by site-directed mutagenesis [20]. For transient FGFR4 inactivation, cells were incubated with adenoviruses encoding for a CFP-tagged truncated FGFR4 (tFGFR4) [21]. Transduction success and receptor localization were analyzed by flow cytometry, confocal microscopy, Western blots, and qRT-PCR. All details are given in the supplementary materials section.

### qRT-PCR

4 × 10⁵ cells were seeded into 6-well plates. After 24 h, RNA was isolated, reverse transcription performed,
and qRT-PCR run [18]. For SYBR-PCR, RPL-41 served as housekeeping gene. FGFR4 mRNA levels were measured using Taqman probes (Thermo Fisher Scientific) with FAM/ROX qPCR Mastermix (Thermo Fisher Scientific), and ACTB served as housekeeping gene. Hep3B [19] was used as positive control in screening approaches. All RNAs were isolated three times. Additional File 4: Table S2 lists primers and Taqman probes.

**Annexin / PI staining**

*tFGFR4- and GFP-transduced GBM cells were stained with Annexin V and PI and measured by flow cytometry (BD LSR Fortessa X-20 Flow Cytometer) as described in the supplementary materials.**

**Clonogenicity and proliferation assays**

Clonogenicity was analyzed as discussed previously [18]. *tFGFR4 or GFP* adenoviruses were added one day after seeding and cells were incubated for seven days. Plates were photographed, pictures binarized, and black pixels counted by R scripting. To test proliferation, 3 × 10⁴ cells/ml were seeded in 500 µl in 24-well plates. Cells were trypsinized and counted using CASY® cell counter. All experiments were performed at least three times in duplicates.

**Migration assays**

Filter-migration assays were performed by Boyden-chambers via a nutrient gradient and cells were incubated for 48 h. For scratch assays, cells were seeded as monolayers and wound-healing capacity was followed, as described in the supplementary materials.

**Adhesion and invasion assays**

Adhesion assays towards several coatings were performed in a time-dependent manner. Integrin-mediated cell adhesion was tested according to manufacturer’s manuals (ECM532: Merck, Darmstadt, Germany). A monolayer of m-cherry-tagged endothelial cells was used to test tumor cells’ trans-endothelial invasion. Details are described in the supplementary materials.

**Re-differentiation assay**

GBM neurospheres were re-plated in serum-supplemented medium and differentiation plasticity was tested after five days. For details see the supplementary materials.

**Cell-viability assay (MTT)**

Ponatinib, BLU554, cilegintide, and defactinib were purchased from Selleck Chemicals (Houston, TX, USA). MTT assays (EZ4U, Biomedica, Vienna, Austria) were performed as previously published [18]. All experiments were performed at least three times. Combination index (CI) values were calculated [22] using CalcuSyn software. CI values < 0.9 were considered synergistic, 0.9–1.2 additive, and >1.2 antagonistic.

**Xenograft formation experiments**

CB-17 severe combined immune-deficient (SCID) mice were subcutaneously injected with 1 × 10⁶ GBM cells. NOD-scid IL2Rnull (NSG) mice were orthotopically implanted with 5 × 10⁵ tumor cells. The tumor growth and wellbeing of the animals was followed over-time as described in the supplementary materials section. For zebrafish xenografts, see the supplementary materials section. All subcutaneous animal experiments were controlled by the Ethics Committee for the Care and Use of Laboratory Animals at the Medical University Vienna (proposal numbers: BMWF-66.009/0157-1/10b/2008, BMWF-66.009/0157-V/3b/2019). Orthotopic animal experiments were performed in an AAALAC accredited animal facility under the permit G18/78 of the Regierungspräsidium Freiburg im Breisgau, Germany.

**Statistics**

In silico analyses were performed and visualized in R statistical environment (v.4.0.0) by application of the according packages as described in the supplementary materials. GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA) software was used to analyze and visualize raw data. p-values of less than 0.05 were considered statistically significant.

**Results**

**FGFR4 overexpression is associated with shorter GBM patient survival and tumor recurrence**

Analyses of several publicly available datasets revealed the brain as an organ with relatively low FGFR4 expression (Additional File 1: Figure S1A) as compared to other FGFR family members (Additional File 1: Figure S1B), with the highest expression level in the cerebellum (Additional File 1: Figure S1A-C). Concerning GBM, elevated FGFR4 expression levels were identified in malignant tissue as compared to non-malignant brain (Fig. 1A). Unsupervised sample stratification by maximally selected rank statistics resolved a distinct GBM patient population (13%) with high FGFR4 expression that was associated with worse prognosis in two independent cohorts (Fig. 1B, Additional File 1: Fig. S2). FGFR4 showed a broad expression range in the TCGA-GBM cohort (Fig. 1C), which was well reflected in cell cultures on mRNA (Fig. 1D) as well as on protein levels (Fig. 1E). Glioblastoma stem cell (GSC) models exhibited comparably high FGFR4 mRNA levels (Fig. 1D). Again an FGFR4high subset was resolved by maximization of
the t-statistics (Fig. 1C) and confirmed in corresponding tumor tissues (shown for the BTL1376 FFPE sample in Fig. 1F). Based on the FGFR4 expression screening in GBM, we selected the immortalized cell line U251-MG as well as the primo-models BTL1529 and BTL53 as endogenously FGFR4low, and BTL1528 and BTL1376 as FGFR4high models for further analyses. High FGFR4 expression in BTL1528 and BTL1376 was not attributed to gene amplification (Additional File 1: Figure S3A). Concerning GBM progression, FGFR4 mRNA expression was significantly enhanced in recurrent compared to primary lesions (Fig. 1G, TCGA collection). Analyses of patient-matched primary and recurrent GBM tissues from our clinics corroborated this finding in the majority of cases (Fig. 1H). At this progressed disease stage, expression of FGFR4-specific ligand-coding genes FGF19 and FGF23 was distinctly enhanced in the TCGA cohort (Additional File 1: Figure S3B), and our own sample collection (Additional File 1: Figure S3C). Simultaneous upregulation of ligands and receptors is exemplified in a selected GBM patient in Additional File 1: Figure S3D.

**FGFR4 overexpression promotes GBM cell aggressiveness**

In order to evaluate the impact of FGFR4 on the malignant phenotype of GBM, we performed DESeq2 analyses of the FGFR4high versus FGFR4low subgroups of the RNA sequencing TCGA-GBM dataset (compare Fig. 1C). Accordingly, FGF19 was among the top-ranked genes in the FGFR4high versus FGFR4low GBM subset (Fig. 2A). Despite a generally higher expression of FGF1 and FGF2 as compared to FGF19, the FGFR4high GBM subgroup expressed reduced FGF1 and FGF2, but enhanced FGF19 levels (\( p = 9 \) (Fig. 2A, Additional File 1: Figure S4A). Resembling FGFR4, also expression of FGF19 was very low in non-malignant brain (Additional File 1: Figure S4B). To test the functional relevance of FGFR4 in our endogenously high- compared to low-expressing GBM models, we stimulated BTL1528 and BTL1529 cells, respectively, with the FGFR4-specific activating ligand FGF19. We observed significantly enhanced clonogenicity and wound-closure potential upon FGF19 stimulation in the FGFR4high model, while the FGFR4low model remained unaffected (Additional File 1: Figure S4C-D). Interestingly, FGF19 stimulated sphere formation capacity in both cell models, pointing towards a critical role of FGFR4 signaling in stemness and 3-dimensional growth (Additional File 1: Figure S4E). Together, these data suggest that FGFR4 exhibits a pivotal functional impact on GBM aggressiveness, and that the FGF19/FGFR4 interaction should dominate the FGFR-related signaling in this FGFR4high GBM subgroup. Using gene set enrichment analyses (GSEA) we found, besides various FGF- and FGFR-related ontologies, growth and differentiation processes including mesenchyme development (NES = 1.632), mesenchymal cell differentiation (NES = 1.454), central nervous system neuron differentiation (NES = 1.709) and epithelial cell proliferation (NES = 1.424) significantly associated with high FGFR4 expression (Fig. 2B and Additional File 5: Table S3). To dissect the underlying cell biological mechanisms in an isogenic background, we overexpressed wild-type GFP-tagged FGFR4-388Gly (FGFR4-388Gly-GFP; Fig. 2C) in the endogenously FGFR4low GBM patient-derived cell models BTL1529 and BTL53, and the stable cell line U251-MG. In parallel, GBM models were transduced with a GFP-tagged, dominant-negative FGFR4 version (loss-of-function point mutation K504M; FGFR4-KD). Stable mRNA and protein expression as well as FGFR4 functional activity and intracellular localization were confirmed in all GBM sublines (Additional File 1: Figure S5A-D). The effect of empty- and GFP-vector transduction on cell proliferation was negligible (Additional File 1: Figure S6A). Overexpression of wild-type FGFR4-388Gly resulted in significantly increased clonogenicity of all GBM models (Fig. 2D) and promoted proliferation capacity of the low-passage primo-cell cultures (Fig. 2E, left; Additional File 1: Figure S6B) but not the high-passage GBM cell line (Fig. 2E, right). In contrast, the migratory potential of all FGFR4-388Gly-expressing cells was distinctly enhanced in Boyden chamber (Fig. 2F) and wound-healing assays (Additional File 1: Figure S6C). Stimulation with FGF19 significantly promoted wound closure selectively in FGFR4-388Gly-expressing models BTL1528 and BTL1529 cells, respectively, with the FGFR4-specific activating ligand FGF19.
Fig. 1 (See legend on previous page.)
Fig. 2 FGFR4 overexpression promotes GBM aggressiveness. A Volcano blot showing differentially expressed genes (DEGs) of TCGA-GBM RNA sequencing data in FGFR4 high (red) versus FGFR4 low (blue) GBM. Top 15 genes are annotated and FGF19 is highlighted. Adjusted p-value < 0.05. B Gene sets significantly enriched in the FGFR4 high GBM subgroup are indicated. GSEA of DEGs (in A) were performed. Selected gene ontologies are plotted. C Scheme of the FGFR4-388Gly-GFP protein. D+E Clonogenicity D and proliferation capacity E of the FGFR4-388Gly-overexpressing and GFP-transduced, endogenously FGFR4 low GBM models are shown (means ± SEM from three independent experiments). F Filter-migration capacities of FGFR4-388Gly-overexpressing normalized to GFP-transduced endogenously FGFR4 low GBM models are shown (mean ± SEM from three experiments). Representative photographs are shown. G Wound-healing capacity of FGFR4-388Gly-overexpressing and GFP-transduced U251-MG cells in response to FGF19 stimulation (50 ng/ml) is shown at the indicated time points. Results were normalized to the respective FGF19-unstimulated conditions (means ± SD from three experiments). Red asterisks: Significance FGF19-stimulated versus -unstimulated. Statistical analyses: 2-way ANOVA/Bonferroni correction in D,E,G; Student’s t-tests in (F). *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant.
Together, this suggests that FGFR4 exhibits a pronounced growth- and migration-promoting function. This effect is more comprehensively reflected in primary patient-derived cell explants as compared to high-passage GBM cell lines. 

**Inactivation of FGFR4 attenuates GBM cell aggressiveness**

The consequences of FGFR4 inactivation in GBM were assessed by two approaches (Fig. 3A). First, the above described *FGFR4-KD-GFP(KS04M)* vector was transduced generating stable FGFR4-inactivated GBM models (compare Additional File 1: Figure S5). Second, for transient FGFR4 blockade, an adenoviral construct encoding a truncated FGFR4 gene variant (*tFGFR4*) with the intracellular kinase domain exchanged by CFP was applied (Fig. 3A, Additional File 1: Figure S7A). *tFGFR4* transduction into all tested glioma cells led to significantly impaired clone formation (Fig. 3B) and proliferation potential (Fig. 3D) of GBM cell models. Concerning stemness, *tFGFR4* significantly reduced sphere formation capacity in GSC models (compare Fig. 3C). In parallel, the spontaneous cell death rate in several GBM models increased upon *tFGFR4* infection (Additional File 1: Figure S7A-B and Fig. 3E). Accordingly, introduction of *FGFR4-KD(KS04M)* impaired GBM clonogenicity and cell proliferation, especially in the endogenously *FGFR4high* glioma models (Fig. 3F; Additional File 1: Figure S7C, respectively). This effect was less pronounced as compared to *tFGFR4* infection (compare Fig. 3B), possibly due to cellular compensatory mechanisms upon FGFR4 inactivation in the stable *FGFR4-KD(KS04M)*-overexpressing cell models. Accordingly, genes encoding for FGFR2, FGFR3 and the universal FGFR-activating ligand *FGF*1 were upregulated in response to *FGFR4-KD(KS04M)* expression (Additional File 6: Table S4).

**FGFR4 inactivation attenuates GBM cell migration and endothelial barrier disintegration**

Overexpression of *FGFR4-KD(KS04M)* significantly reduced migration capacity in all tested primo-GBM models, but not in the stable cell line U251-MG (Fig. 4A). Furthermore, the wound-healing capacity was significantly impaired upon *FGFR4* inactivation as representative shown for BTL1528 (Fig. 4B). For in vitro investigation of GBM cell invasiveness, spheres of BTL1376 *FGFR4-KD(KS04M)* or GFP control cells were transferred onto a monolayer of blood endothelial cells. The endothelial cell layer invasion capacity of *FGFR4high* GBM cells was drastically impaired upon *FGFR4* inactivation (Fig. 4C). To dissect the underlying mechanisms, we performed GSEA of the *FGFR4-KD(KS04M)* subclones of both endogenously *FGFR4high* GBM models. The *KEGG Focal Adhesion* pathway appeared amongst the highest enriched ontologies in BTL1528 as well as in BTL1376 (Fig. 4D and Additional File 7: Table S5, Additional File 1: Figure S8A and Additional File 8: Table S6, respectively). This translated well into regulated focal adhesion kinase (FAK) expression and phosphorylation levels, as well as talin protein expression in response to *FGFR4* manipulation (Fig. 4E, Additional File 1: Figure S8B), resulting in significantly diminished adhesion potential to cell culture polystyrene in several GBM (Fig. 4F; Additional File 1: Figure S8C+D, left) and GSC (Additional File 1: Figure S8E) models upon *FGFR4* blockade. Corroboratively, the re-differentiation capacity was impaired by *FGFR4-KD(KS04M)*, especially in endogenously *FGFR4high* GBM models (Fig. 4G). Regardless of endogenous *FGFR4* expression levels, *tFGFR4* transduction significantly reduced the re-differentiation capacity of all tested GBM (Additional File 1: Figure S8F) and GSC (Additional File 1: Figure S8G) cultures. Furthermore, pharmacological FGFR4 inhibition by either BLU554 or ponatinib distinctly reduced expression of the stemness marker nestin (Additional File 1: Figure S8H), correlating with significantly impaired all-trans retinoic acid (ATRA)-induced differentiation of NCH644 GSC (Additional File 1: Figure S8I).
Fig. 3 (See legend on previous page.)
revealed alterations in REACTOME pathways including extracellular matrix organization, NCAM signaling for neurite outgrowth, and collagen formation following FGFR4 inactivation. By applying identical GSEA settings to the more comprehensive TCGA-GBM RNA sequencing dataset, all three above listed pathways were ranked as the most significantly enriched REACTOME processes in the FGFR4-high versus FGFR4-low subgroup (padj=0.002, NES=1.79; padj=0.0004, NES=1.92; padj=0.0004, NES=1.92, respectively; Additional File 5: Table S3). Enrichment network analyses of BTL1528 subclones identified these gene sets clustering together in one functional module (Additional File 1: Figure S10B, violet module). Accordingly, collagen coating distinctly promoted FGFR4-dependent GBM cell adhesion (Fig. 5D compare Fig. 4F, middle panels of Additional File 1: Figure S8C + D). In addition to decreased expression of collagen network genes upon FGFR4 inactivation (compare Fig. 5C and Additional File 1: Figure S9C), we also found loss of integrin-cell surface interactions-related genes in both GBM models (BTL1528: Fig. 5E and BTL1376: Additional File 1: Figure S9D). This translated well into consistently downregulated integrin-mediated cell adhesion via various integrin isoforms and heterodimers in FGFR4-KD(K504M)-overexpressing FGFR4-high BTL1528 cells (Fig. 5F). Accordingly, expression of several integrin members including integrin αV was changed following FGFR4 manipulation (Additional File 1: Figure S9E + F). A functional impact of the diminished integrin αV protein expression was also suggested by reduced adherence capacity towards fibronectin (Additional File 1: Figure S8C + D right, S9G). Strikingly, FGFR4-KD(K504M) expression led to distinct hypersensitivity towards the Arg-Gly-Asp (RGD) motif-specific integrin inhibitor cilengitide in both GBM models (Fig. 5G, Additional File 1: Fig. S9H). Also the FAK inhibitor defactinib, targeting focal adhesion-related integrin downstream signaling, was hyperactive in FGFR4-KD(K504M) subclones (Additional File 1: Figure S9I + J), suggesting cooperation between FGFR4 and integrin-mediated signals promoting GBM cell survival. This hypothesis was supported by synergistic actions of pharmacological FGFR4 inhibition with both the multi-FGFR inhibitor ponatinib or the FGFR4-specific tyrosine kinase inhibitor (TKI) BLU554 with cilengitide in the endogenously FGFR4-high model BTL1528 (Fig. 5H).

FGFR4 promotes GBM tumorigenicity
One endogenously FGFR4-high (U251-MG) and one FGFR4-high (BTL1528) GBM cell model as well as their FGFR4-altered sublines were next tested for xenograft formation in SCID mice. While both U251-MG sublines were tumorigenic in all animals tested (Fig. 6C), upregulation of wild-type FGFR4-388Gly in the endogenously FGFR4-low U251-MG cells resulted in significantly enhanced tumor volumes (Fig. 6A) and shorter overall survival (Fig. 6B) as compared to GFP-control tumor-bearing mice. FGFR4 inactivation in the FGFR4-high BTL1528 model resulted in significantly reduced tumor volumes (Fig. 6D) and longer overall survival times of the animals (Fig. 6E). In contrast to the notoriously tumorigenic GFP variants, FGFR4-KD(K504M) transplants engrafted only in 2/7 mice in BTL1528 (Fig. 6F). Transgene positivity of the xenograft tumors was validated by GFP fluorescence on cryo-sections (Fig. 6C+F, right). The profound impact of FGFR4 inactivation on tumor take might again be related to the above-described impact of FGFR4 on FAK expression, which was confirmed in vivo by immunoblots of xenograft protein extracts (Fig. 6G). The in vivo effects of FGFR4 on BTL1528 aggressiveness were even more distinct at the orthotopic implantation site. Intracranial injection
Fig. 4 (See legend on previous page.)
of BTL1528 GFP cells led to tumor bulk formation in 100% of cases (n=5) (Fig. 6H + l), while none of the BTL1528 FGFR4-KD(K504M)-implanted mice (n=5) developed detectable tumors (Fig. 6H). In addition to mouse xenotransplantation models, the impact of FGFR4 on GBM aggressiveness was tested in the zebrafish larvae (Danio rerio) model system. Again, FGFR4 high GFP cell fluorescence widely persisted at the injection site, while BTL1528 FGFR4-KD(K504M) tumors significantly regressed after 2 days (Fig. 6 left). Additionally, significantly less cells migrated away from the primary tumor site in case of the FGFR4-blocked BTL1528 model, as compared to its GFP subline (Fig. 6 right and photomicrographs), confirming a key role of FGFR4 on the invasive potential of this FGFR4 high GBM model.

Discussion

Members of the FGF/FGFR signaling network were suggested to regulate several hallmarks of GBM aggressiveness, such as invasion, self-renewal, tumor growth, and therapy resistance [4, 23]. However, a potential role of FGFR4 has not been comprehensively addressed yet. Based on extensive in silico and wet lab analyses of GBM tissues, tumor explants, and GSC models, we identified FGFR4 mRNA and protein overexpression in a distinct GBM subgroup, which was traced back to the original patient tumor samples by IHC. Based on functional in vitro investigations in stable cell lines and patient-derived explant models, we identified FGFR4 as central mediator of GBM cell aggressiveness, as previously published for melanoma [21]. FGFR4 blockade significantly diminished GBM invasiveness in zebrafish and site-specifically impacted on mouse xenograft formation. In particular, tumorigenicity was completely abolished at the original site in case of the FGFR4-blocked BTL1528 model, as compared to its FGFR4 high GFP transduced BTL1528 subclones (right). Exemplary photomicrographs are shown. Outspread cells after 60 min were counted microscopically and normalized enrichment score, padj = adjusted p-value. D Adhesion capacities of BTL1528 and respective GFP cells towards collagen as percentage of cell-coated well surfaces (means ± SEM of three experiments (left)). Exemplary photomicrographs are shown. Outspread cells after 60 min were counted microscopically and normalized to GFP control cells (right). E Integrin-mediated cell adhesion arrays of FGFR4-KD(K504M)- and GFP-transduced BTL1528 subclones are shown (means of two experiments). G+H Viability of BTL1528 FGFR4-KD(K504M) and GFP-expressing GBM cells in response to single-agent cilengitide (G), combined-agent cilengitide + ponatinib (pon.) (H left) and cilengitide + BLU554 (H middle) was evaluated by MTT assays. For each panel, one representative out of three experiments is shown as mean ± SD from triplicates. Combination indices (CI) are given for selected drug concentrations combining cilengitide with ponatinib (blue) or BLU554 (black) (H right). CI values < 0.9 were considered synergistic [22]. Statistical analyses: 2-way ANOVA/Bonferroni correction (D left, F–H), Student’s t-tests (D right). *p < 0.05, **p < 0.01, ***p < 0.001, n.s. = not significant, n.d. = not detected.
Fig. 5 (See legend on previous page.)
the relationship between FGFR4 overexpression and mesenchymal differentiation does not seem straightforward and needs further in-depth investigations.

The FGF19/FGFR4 axis has been connected to dismal patient prognosis and disease progression in different tumor entities including HCC and breast cancer [5, 12, 23, 30, 31]. Additionally, FGFR4 was identified as key factor inducing proliferation, metastatic disease, and cell dedifferentiation in aggressive luminal A-like breast cancer [31]. Accordingly, we detected shorter overall survival of the FGFR4high patient subgroups from several datasets, and FGFR4-KD(K504M) tumor-bearing mice survived significantly longer as compared to endogenously FGFR4high control xenografts. Regarding GBM, we found significantly elevated expression of FGFR4 and associated activating ligand genes in the majority of radio-/chemotherapy-refractory recurrent tumors, based on both in silico and surgical specimen analyses. Consistently, FGFR4 appeared among the highest upregulated genes in the FGFR4high subgroup. Consequently, these findings further support a role of FGFR4 in GBM recurrence in a highly aggressive GBM subset based on an autocrine loop connecting FGFR4 and FGFR4, as previously reported in breast cancer [30].

By introducing activating and dominant-negative FGFR4 constructs into an isogenic GBM background, a broad impact of FGFR4 on the malignant phenotype of GBM cells in vitro was elucidated. While transient expression of the kinase domain-truncated FGFR4 version efficiently induced cell death, transduction with the point-mutated, kinase-dead FGFR4 variant allowed stable clone selection. This suggests that FGFR4KD(K504M)-transduced cells partially bypass the FGFR4 blockade, probably via a so-called receptor tyrosine kinase (RTK)-switch. Indeed, we found other FGFR family members distinctly upregulated upon FGFR4-KD(K504M)-mediated blockade. In addition, overexpression of FGFR4-KD(K504M), despite lacking downstream signaling activation, might still deliver kinase-independent, tumor-promoting signals to GBM cells. Interactions of FGFR4 extracellular and transmembrane domains with alternative binding partners such as N-Cadherin or NCAM have been proposed [12]. Furthermore, FGFR4 was reported to form complexes with N-Cadherin and MT1-MMP in the cell membrane, altering cell adhesion properties and facilitating protease-dependent collagen invasion [12].

Consistently, in vitro and in vivo (zebrafish larvae) data demonstrated a supportive role for FGFR4 in GBM cell migration, invasion, and adhesion, which was corroborated by gene expression analyses of our FGFR4-engineered glioma cell models and the TCGA-GBM data cohort. The most significantly altered gene ontology in relation to FGFR4 functionality were consistently associated with cell adhesion and integrin-related extra-cellular matrix (ECM) interaction mechanisms. This is well in agreement with the massive inhibitory effect of FGFR4-KD(K504M) expression on wound-healing capacity, reflecting in many aspects the invasive tumor leading edge. Integrins constitute a heterodimeric transmembrane glycoprotein receptor family for ECM components frequently associated with tumor progression. Multiple studies suggested complex formation between different RTKs and integrins, leading to therapy resistance and tumor progression [32, 33]. Integrin αV is part of the so called RGD-binding integrin subgroup, a target motif in ECM components like fibronectin and vitronectin [33], and is particularly important in glioma pathogenesis [34]. We found that FGFR4 blockade consistently attenuated integrin αV expression as well as dimerization with various β-isomers. Functionally, integrin-mediated cell adhesion towards several coatings was distinctly impaired upon FGFR4 inactivation. In addition, clear-cut hypersensitivity of FGFR4-KD(K504M) subclones towards two integrin axis-targeting compounds, the integrin antagonist cilenitide and the FAK inhibitor defactinib, was detected. Focal adhesion assembly involving integrin dimers is closely regulated by FAK and talin [33],
Fig. 6 (See legend on previous page.)
which were reduced in FGFR4-KD(K504M)-transduced GBM cells and in associated xenografts. Pharmacological FGFR inhibition by either the multi-TKI ponatinib or the FGFR4-specific drug BLU554 sensitized FGFR4-high glioma cells towards integrin-targeting. Hence, combined FGFR4 and integrin-/FAK inhibition might be a highly active therapy strategy for the here described aggressive GBM subpopulation. The feasibility of this approach needs to be confirmed in further (pre-)clinical studies.

Conclusion
Although widely absent in non-malignant brain, here we revealed a pro-tumorigenic function of FGFR4 in GBM. FGFR4 overexpression was identified in a subgroup of GBM patients, predicting shorter survival times. In parallel, the specific receptor-activating ligand FGF19 was co-regulated in this patient cohort, suggesting an oncogenic feedback loop mediated by the FGF19-FGFR4 axis. Expression levels of FGFR4 and its specific ligands were significantly enhanced in recurrent diseases. Screening a broad collection of GBM cell models corroborated a distinct FGFR4-high subgroup in vitro. By genetic overexpression and knock-down experiments we proved that FGFR4 regulates several classical hallmarks of GBM, including clonogenicity, proliferation, differentiation, migration, and eventually invasiveness by closely interconnecting with the integrin signaling network. FGFR4 blockade significantly reduced tumor growth and progression in subcutaneous murine and in zebrafish xenografts, respectively, and completely diminished tumor formation in mouse brains. Consequently, our data suggest combination approaches targeting both FGFR4 and integrin signaling as novel therapeutic concept in FGFR4-high GBM patients.

Supplementary Information
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Author contributions
Conceptualization: WB, LG, DL; Methodology: LG, CNJ, SvS, BE, CP, JG, VW, DK, CS, AW, SG, MD, JS, SK, TM; Validation: LG, CNJ, SvS, MS, PV, LM, MLF, ALae, ALan; Formal Analysis: LG, DL, CNJ, SvS, SK, TM; Investigation: LG, CNJ, SvS, BE, CP, PV, MS, LM, MLF, JG, VW, DK, ALae, CS, AW, SG, MD, JS; Resources: LG, WB, DL, JG, SSK, TM, SK, MD, JS, FE, DJD, BM, BGK, MG, BK; Data Curation: LG, DL, WB, CNJ, SvS, SK, TM; Writing-Original Draft: LG, WB, DL (with feedback from all authors); Writing-Review and Editing: LG, WB, DL, JG, CP, CNJ, LM, MG, SSK; Visualization: LG, SK, CNJ, TM, AW, CS, MD, SG; Supervision: WB, DL; Funding Acquisition: LG, WB, DL, LM, MD.

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Availability of data and material
The REMBRANDT and TCGA-GBM mRNA expression data sets were downloaded from public sources [Gene expression omnibus: GSE108474 and GDC data portal, respectively]. mRNA expression data sets from patient-derived GBM cell models will be available from the corresponding author on reasonable request. GSEA data are provided in the supplementary tables.

Declarations
Ethics approval and consent to participate
We kindly thank the patients and their families for generously donating tumor tissue for research purposes. All procedures involving human material were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the ethics committee of Upper Austria, Linz (E-39–15) and ethics committee of Medical University of Vienna [EK Nr:1616/2020]. All patients signed an informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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