Amplification of the *EGFR* gene can be maintained and modulated by variation of EGF concentrations in *in vitro* models of glioblastoma multiforme

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Abstract

Glioblastoma multiforme (GBM) is the most common and lethal brain tumor in adults. It is known that amplification of the epidermal growth factor receptor gene (*EGFR*) occurs in approximately 40% of GBM, leading to enhanced activation of the EGFR signaling pathway and promoting tumor growth. Although GBM mutations are stably maintained in GBM *in vitro* models, rapid loss of *EGFR* gene amplification is a common observation during cell culture. To maintain *EGFR* amplification *in vitro*, heterotopic GBM xenografts with elevated *EGFR* copy number were cultured under varying serum conditions and EGF concentrations. *EGFR* copy numbers were assessed over several passages by quantitative PCR and chromogenic *in situ* hybridization. As expected, in control assays with 10% FCS, cells lost *EGFR* amplification with increasing passage numbers. However, cells cultured under serum free conditions stably maintained elevated copy numbers. Furthermore, EGFR protein expression positively correlated with genomic amplification levels. Although elevated *EGFR* copy numbers could be maintained over several passages *in vitro*, levels of *EGFR* amplification were variable and dependent on the EGF concentration in the medium. *In vitro* cultures of GBM cells with elevated *EGFR* copy number and corresponding EGFR protein expression should prove valuable preclinical tools to gain a better understanding of *EGFR* driven glioblastoma and assist in the development of new improved therapies.

Introduction

Glioblastoma multiforme (GBM) is the most common brain tumor in adults with a very dismal prognosis, despite a multimodal, intensive treatment regimen consisting of surgery, radio- and chemotherapy. Therapies are neither particularly effective nor durable with mean survival times of 12–15 months [1–3]. GBMs display a high intratumoral heterogeneity and are

characterized by invasive growth into surrounding tissue, making complete resection nearly impossible and favoring development of chemotherapy resistance [3,4].

The EGFR signaling pathway is a prominent regulator of proliferation, growth and survival of mammalian cells [5]. Upon binding of its extracellular ligand EGF or transforming growth factor α (TGFα), EGFR is activated, resulting in enhanced cell proliferation.

Alterations of the *EGFR* gene are considered as frequent driver mutations and are present in approximately 50% of GBM [6,7].

The most common *EGFR* aberration is genomic amplification (40%) [6], often due to extra-chromosomal material (double minutes) [8,9], leading to overexpression and enhanced signaling [6]. A multitude of signaling pathway cascades activated by EGFR are associated with GBM progression, e.g. activation of Cyclooxygenase-2 [10,11], K-RAS and AKT signaling [12], and mammalian target of rapamycin (mTOR) together with phosphatidyl-inositol-3-kinase (PI3K) pathways [13–16]. Hence, EGFR or components of its signaling pathway may be promising therapy targets, as shown for tyrosine kinase inhibitors (TKIs) in lung cancer or antibody therapies in colorectal cancer [17,18]. However, substances targeting EGFR or its downstream targets have yet to prove effective in clinical GBM trials [19–21]. Although genomic *EGFR* amplification can be well maintained in *in vivo* models of glioblastoma [22,23], research in a preclinical setting *in vitro* is hampered by the fact that common cell culture models of GBM lack *EGFR* gene amplification [24,25].

Our work shows that this major cell culture disadvantage may be circumvented by culturing the cells under serum free conditions with varying EGF concentrations, leading to the maintenance of *EGFR* gene copy numbers. Although *EGFR* amplification is lost according to EGF concentration, it can subsequently be restored by EGF depletion. This enables further studying of this important signaling pathway in cell lines with the same genetic background and either high or low *EGFR* amplification levels.

Material & methods

Cell culture

Cell lines were established from GBM tissue of three different heterotopic patient derived xenograft (PDX) tumors with varying *EGFR* copy numbers [22]. All PDX models were established by implanting GBM tissue received from the operation theater at the department of neurosurgery at the university medicine of Rostock subcutaneously into the flanks of immunodeficient NMRI Foxn[1] nu mice [22]. Specimen collection was conducted in accordance with the ethics guidelines for the use of human material, approved by the Ethics Committee of the University of Rostock (Reference number: A 2009/34) and with informed written consent from all patients prior to surgery. Prior to cell line establishment, the PDX underwent 2 (xHROG33 and xHROG59) or 5 (xHROG22) serial *in vivo* passages in NMRI Foxn[1] nu mice. Serial passaging *in vivo* was performed by excision of the subcutaneous xenograft tumors and implanting small pieces of tumor tissue (approximately 3 mm[3]) subcutaneously in the flanks of other NMRI Foxn[1] nu mice [22]. The obtained GBM PDX tissue was minced using sterile scalpels and passed through a 70 µm cell strainer to obtain single cell suspensions. Cells were divided equally among standard medium control (DMEM/Ham's F12, 2mM L-Glutamine, 10% FCS) and serum free media cultures (DMEM/Ham's F12, 2mM L-Glutamine, 1x B-27, 10ng/ml bFGF) with varying amounts of rhEGF (0 ng/ml, 0.5 ng/ml, 1 ng/ml, 1.5 ng/ml, 2 ng/ml, 2.5 ng/ml, 10 ng/ml and 30 ng/ml). Subsequently, all cultures were incubated at 37˚C, 5% CO_2 and 95% relative humidity. Multicellular spheroid cultures were passaged *in vitro* by passing the spheroids through a 70 µM cell strainer. When possible, cultures were sampled at every passage for further analysis.

Isolation of genomic DNA and PCR analysis

Genomic DNA was isolated using a commercial Kit (Wizard® Genomic DNA Purification Kit, Promega, Mannheim, Germany) following the manufacturer's instructions. DNA concentration was determined with a spectrophotometer (NanoDrop 1000, Peqlab, Erlangen, Germany). *EGFR* copy numbers were determined by quantitative PCR using 30 ng DNA as template on a StepOne Plus Realtime PCR system (Applied Biosystems, Darmstadt, Germany) with SensiFastSYBR Hi-Rox-Kit (Bioline, Luckenwalde, Germany) (EGFR-for: 5'-TCCCATG ATGATCTGTCCCTCACA-3'; EGFR-rev: 5'-CAGGAAAATGCTGGCTGACCTAAG-3'). Commercially available normal Human Genomic DNA (Promega) served as calibrator and the repetitive element LINE1 as endogenous control (LINE1-for: 5'-TGCTTTGAATGCGTCCCA GAG-3'; LINE1-rev: 5'-AAAGCCGCTCAACTACATGG-3'). All reactions were performed in triplicate. The *EGFR* copy number was calculated with the ΔΔCt-algorithm.

Potential cross contamination of the samples with rodent genes was analyzed by PCR (94°C 3min, 35 cycles of 94°C 1min, 65°C 2min, 72°C 1min, final extension at 72°C 4min) using the DFS–Taq polymerase supplied by Bioron (Bioron GmbH, Ludwigshafen, Germany) following manufacturer's instructions with primers specific for murine *MLH1* (for: 5'-TGTCAATAGG CTGCCCTAGG-3', rev: 5'-TTTTCAGTGCAGCCTATGCTC-3') [26]. Human origin of the samples was verified by PCR with primers specific for human cytochrome B (for: 5'- TAGC AATAATCCCCATCCTCCATATTAT-3', rev: 5'- ACTTGTCCAATGATGGTAAAAGG-3' [27]) Results were analyzed by gel electrophoresis.

Paraffin embedding of GBM cells

Cells were harvested and washed with PBS twice. The cells were fixed immediately by resuspending the pellets in 4% buffered formalin (Formafix; Grimm, Torgelow, Germany). Cells were subsequently processed following established standard procedures [28] to form a conglomerate and then embedded in paraffin using the automated Excelsior AS system (Thermo Scientific, Dreieich, Germany) following standard procedures.

Tissue microarray

Hematoxylin and Eosin (H&E) stained sections of FFPE cell lines were reviewed by an experienced pathologist and regions suitable for analysis were chosen. Tissue microarrays (TMA) were created using a Manual Tissue Arrayer MTA-1 (Beecher Instruments, Sun Prairie, WI, USA) with 1mm diameter punches. From the donor blocks, 3 punches per sample were taken from the selected regions and transferred to an empty acceptor block. The block was heated to 50°C and the correct placement depth of the punches confirmed by microscopic examination. A section from the block was stained with H&E to confirm the presence of a sufficient amount of cells for further analysis.

Chromogenic *in situ* hybridization

In addition to quantitative PCR analysis, the *EGFR* amplification status was assessed by two-colored chromogenic *in situ* hybridization (2C CISH). 4 µM sections of FFPE tissue samples and TMAs were mounted to coated slides and *EGFR*-specific 2C CISH was performed using the ZytoDot 2C CISH implementation Kit with the ZytoDot 2C SPEC EGFR/CEN 7 Probe (Zytomed Systems, Berlin, Germany) according to manufacturer's protocols. Processed samples were analyzed by bright field microscopy. Red signals specifically represent the centromere of chromosome 7 for reference and ploidy, whereas green signals are specific for the

EGFR gene. A cell was considered to carry an *EGFR* amplification if the green / red ratio was >2 or the green signals occurred in clusters.

Immunohistochemistry

EGFR immunohistochemistry (IHC) was performed using a mouse derived anti-EGFR primary antibody (Zytomed Systems, clone 3G143, dilution 1:200). Slides were processed on an automatic IHC system, AutostainerLink48 (Dako, Hamburg, Germany), according to routine protocols.

Statistics

Statistical analysis was done using SigmaPlot 10.0 (Systat Software GmbH, Erkrath, Germany). A Kruskal-Wallis one way analysis of variance on ranks in combination with a Tukey test was used for statistical analysis of qPCR data.

Results

GBM PDX derived cell lines

In order to identify cell culture conditions, which would allow sustained *EGFR* amplification of GBM cells *in vitro*, cell lines were established from 3 different GBM PDX with varying *EGFR* copy numbers as determined by qPCR and 2C CISH (Fig 1). Prior to cell line establishment, HROG33 and HROG59 PDX underwent 2 serial passages *in vivo*, HROG22 PDX underwent 5 serial passages in NMRI Foxn[1] nu mice.

HROG33 and HROG59 PDX showed high genomic *EGFR* amplification levels (Fig 1C and 1E), whereas *EGFR* amplification was absent from HROG22 PDX (Fig 1A). The HROG22 PDX also showed a weaker staining in immunohistochemistry analysis of EGFR protein expression, compared to the two *EGFR* amplified PDX, HROG33 and HROG59 (Fig 1B, 1D and 1E). Nevertheless, HROG22 was included in the study to determine if *in vitro* EGF exposure could select for HROG22 cells carrying *EGFR* amplifications. Cell line establishment of xHROG22 and xHROG33 was successful for all conditions tested (serum free with 0, 0.5, 1, 1.5, 2, 2.5, 10, 30 ng/ml EGF and 10% FCS controls). In the case of xHROG59, cell line establishment failed at 2 ng/ml EGF and the 10% FCS control, but was otherwise successful. Contamination of the cell lines with murine DNA was excluded by PCR specific for murine *MLH1* and their human origin was verified by PCR specific for human cytochrome B (S1 Fig, [26,27]). All cell lines established under serum free conditions grew as non-adherent multicellular spheroids regardless of EGF concentration, whereas the 10% FCS controls grew as adherent monolayers. Establishment of spheroid cultures with 10% FCS supplemented medium using anti-adhesive tissue culture dishes failed in all three cases. Although initial spheroid formation was observed, the cells failed to reform spheroids after splitting. Cells from all successful conditions were further analyzed over several passages. In the case of multicellular spheroid cultures, a passage was defined as the time required for single cells growing in suspension to form spheroids. Spheroid formation was considered as complete when they appeared solid in form with no lose single cells attached to the sphere and reached a diameter of approximately 100μM.

EGFR amplification is maintained in serum-free *in vitro* models of GBM and correlates with EGFR protein expression

Amplification of the *EGFR* gene was analyzed by qPCR over several *in vitro* passages for all culture conditions tested. As expected, *EGFR* amplification was lost in the 10% FCS control of
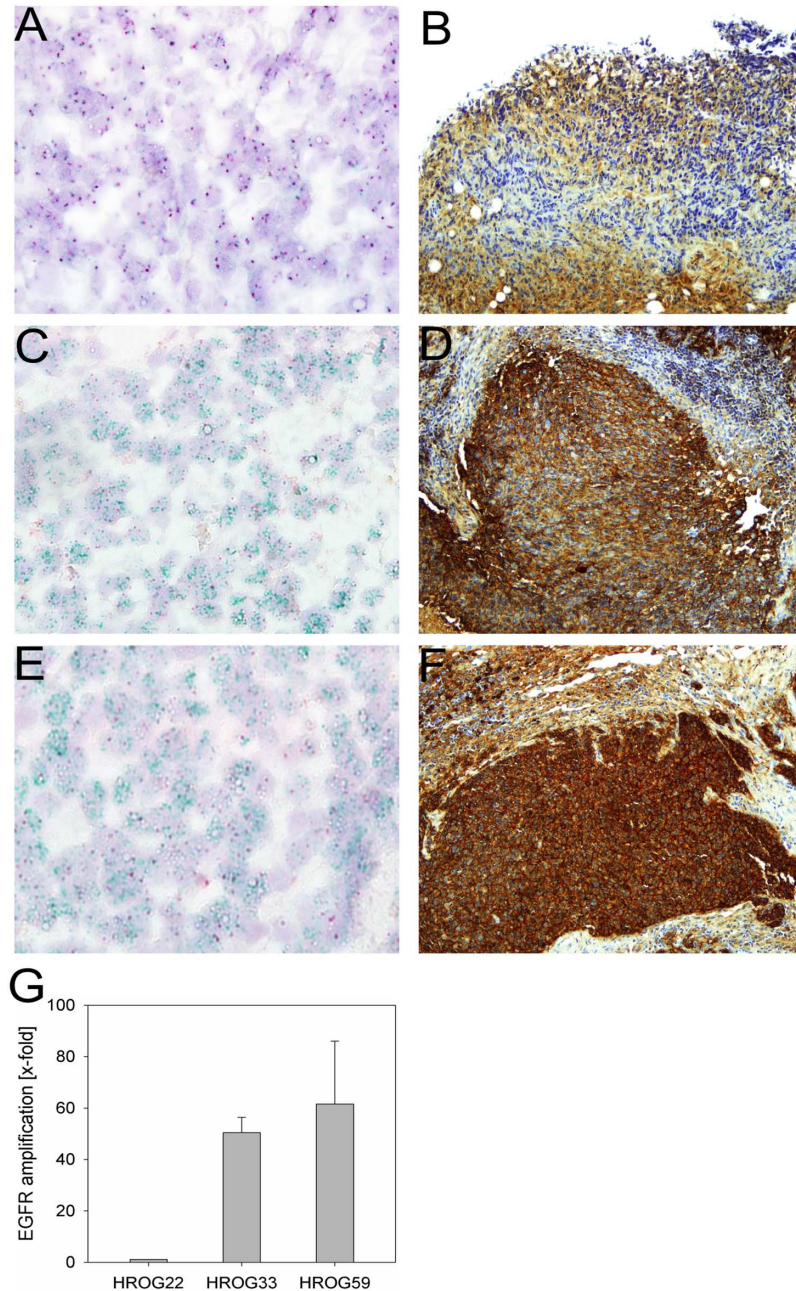
Fig 1. Analysis of *EGFR* amplification by 2C CISH and qPCR and corresponding EGFR protein expression by immunohistochemistry in GBM PDX tissue of HROG22, HROG33 and HROG59. A) 2C CISH of HROG22 (400x magnification), B) EGFR IHC of HROG22 (200x magnification), C) 2C CISH of HROG33 (400x magnification), D) EGFR IHC of HROG33 (200x magnification), E) 2C CISH of HROG59 (400x magnification), F) EGFR IHC of HROG59 (200x magnification); red signals: centromere of chr. 7; green signals: *EGFR* in the 2C CISH analyses; G) qPCR analysis of GBM PDX tissue, error bars represent the standard deviation of triplicate analyses.

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xHROG33 after few passages (Fig 2D, S2 Fig). However, under serum free conditions, *EGFR* amplification was maintained in xHROG33 and xHROG59 over at least 10 *in vitro* passages, although at varying degrees in dependence on the EGF concentration (Fig 2B and 2C, S2 Fig). Of note, GBM cells cultured with high amounts of EGF (30 ng/ml) showed the same rapid loss
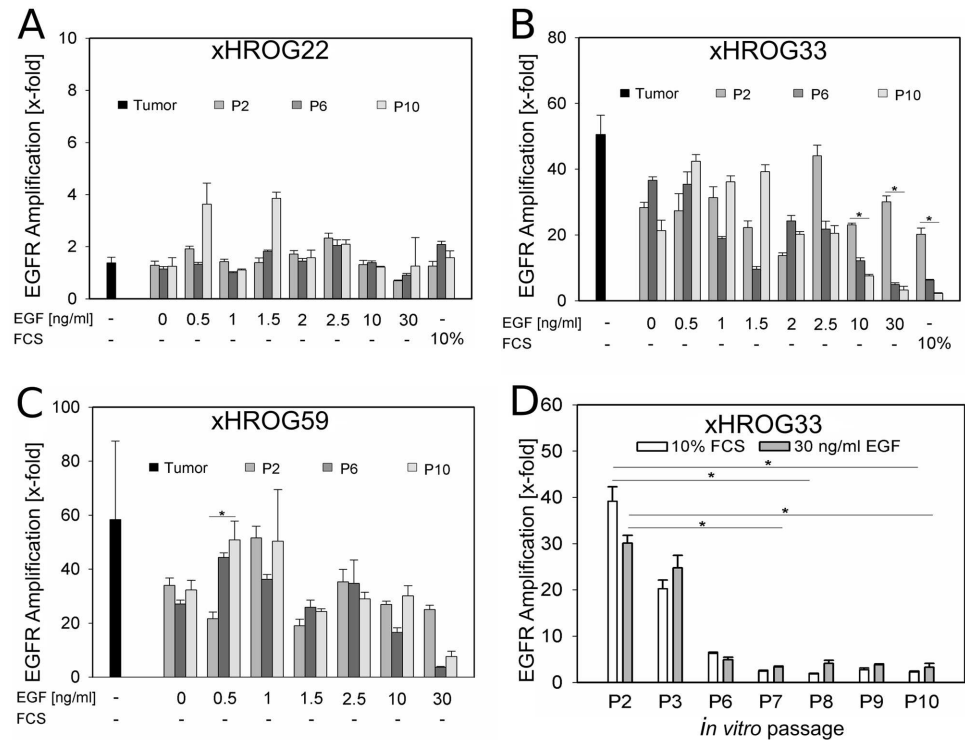
Fig 2. *EGFR* amplification is maintained over several *in vitro* passages, but lost in the 10% FCS control and with high EGF concentrations. qPCR analysis of A) xHROG22, B) xHROG33, C) x HROG59 cell lines grown under all culture conditions at passage 2 (grey bars), passage 6 (dark grey bars) and passage 10 (light grey bars). Black bars represent the respective PDX prior to cell culture establishment. D) qPCR analysis of the 10% FCS control (white bars) and the 30 ng/ml EGF cell line (grey bars) of xHROG33 over several *in vitro* passages. Error bars represent the standard deviation of triplicate analyses. * p<0.05, Tukey test.

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of *EGFR* amplification as the 10% FCS control (Fig 2D). In case of xHROG22, no marked differences in *EGFR* amplification were observed under all conditions applied with copy numbers below four (Fig 2A, S2 Fig).

To further confirm the qPCR data, cells at passage 10 were fixed in formalin and embedded in paraffin for *EGFR* specific CISH analyses (Fig 3). The 2C CISH analyses showed a high number of *EGFR* gene copies in xHROG33 cell lines cultured serum free with low amounts of EGF, whereas culture at 10 ng/ml EGF was accompanied by lowered amplification levels. Parity of chromosome 7 specific and *EGFR* specific signals indicating the loss of *EGFR* amplification was observed in xHROG33 cells cultured with 30 ng/ml EGF in the medium and the 10% FCS control (Fig 3). Similar results were obtained in xHROG59 (Fig 3). As expected, there was no increase of *EGFR* signal in xHROG22 (Fig 3). Thus, the 2C CISH results are well in line with the results obtained by qPCR analyses of *EGFR* amplification.

In order to determine if *EGFR* amplification levels also result in increased EGFR protein expression, TMA sections of embedded GBM cells were subjected to EGFR IHC staining (Fig 4). In all cases, the obtained results accorded with the 2C CISH and qPCR data (Figs 2 and 3).

Loss of *EGFR* amplification can be restored by EGF withdrawal

Genomic amplification of *EGFR* appears to be dependent on the EGF concentration present in the medium (Figs 2 and 3, S2 Fig). We observed a decrease in *EGFR* amplification with 10 ng/
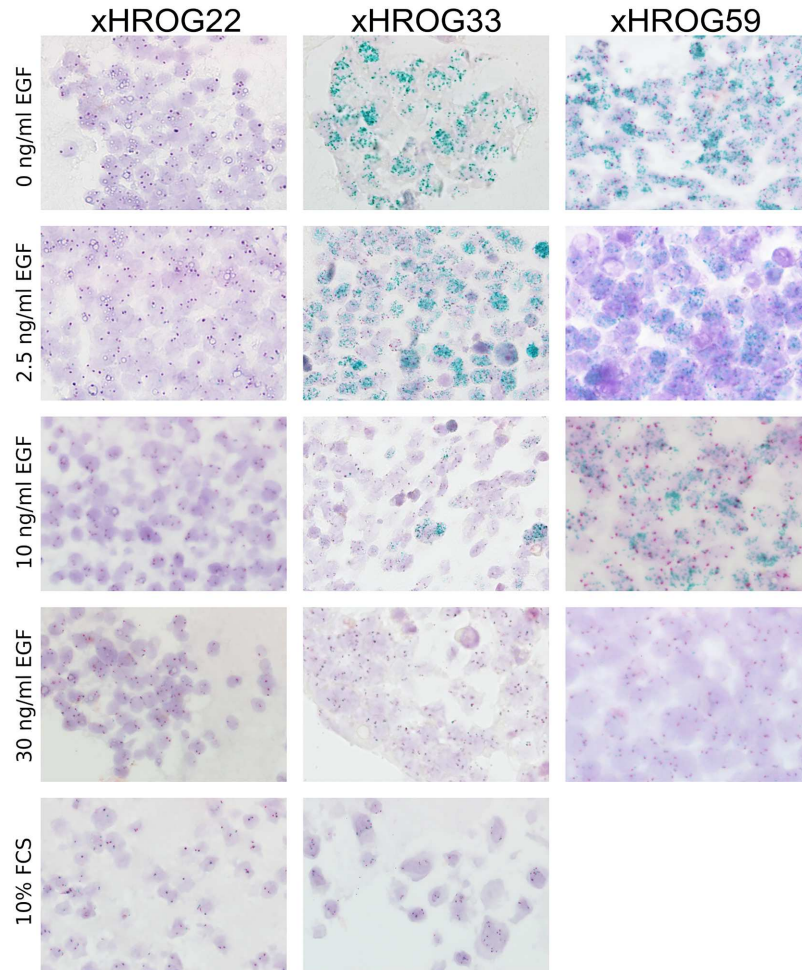
Fig 3. 2C CISH analysis confirms qPCR data of *EGFR* amplification. 2C CISH analyses of xHROG22, xHROG33 and xHROG59 cell lines established under different conditions as indicated at passage 10 of cell culture; 400x magnification.

ml EGF in xHROG33 and xHROG59 and, additionally, for xHROG33 an almost complete loss of *EGFR* amplification with 30 ng/ml EGF approaching the level of the 10% FCS control. In both cases, after culture with 30 ng/ml EGF for 10 passages, EGF was withdrawn and *EGFR* amplification was analyzed by qPCR, 2C CISH and EGFR IHC staining after 5 *in vitro* passages post EGF depletion. *EGFR* amplification and protein expression were gradually restored after EGF withdrawal (Fig 5). In the case of the 10% FCS control of xHROG33, the cells failed to adapt to the change from 10% FCS to serum free without EGF followed by cell death.

Discussion

Glioblastoma multiforme remains a lethal brain tumor despite intensified treatment strategies, highlighting the need for more effective therapies [1,2]. Nearly half of GBM cases display a genomic amplification of *EGFR* and subsequent hyperactivation of the PI3K/AKT/mTOR signaling pathways [5,13,14]. However, preclinical *in vitro* studies are complicated by the rapid loss of genomic *EGFR* amplification under standard cell culture conditions [24,25]. GBM *in vitro* models with stable *EGFR* amplification would represent an experimental system more
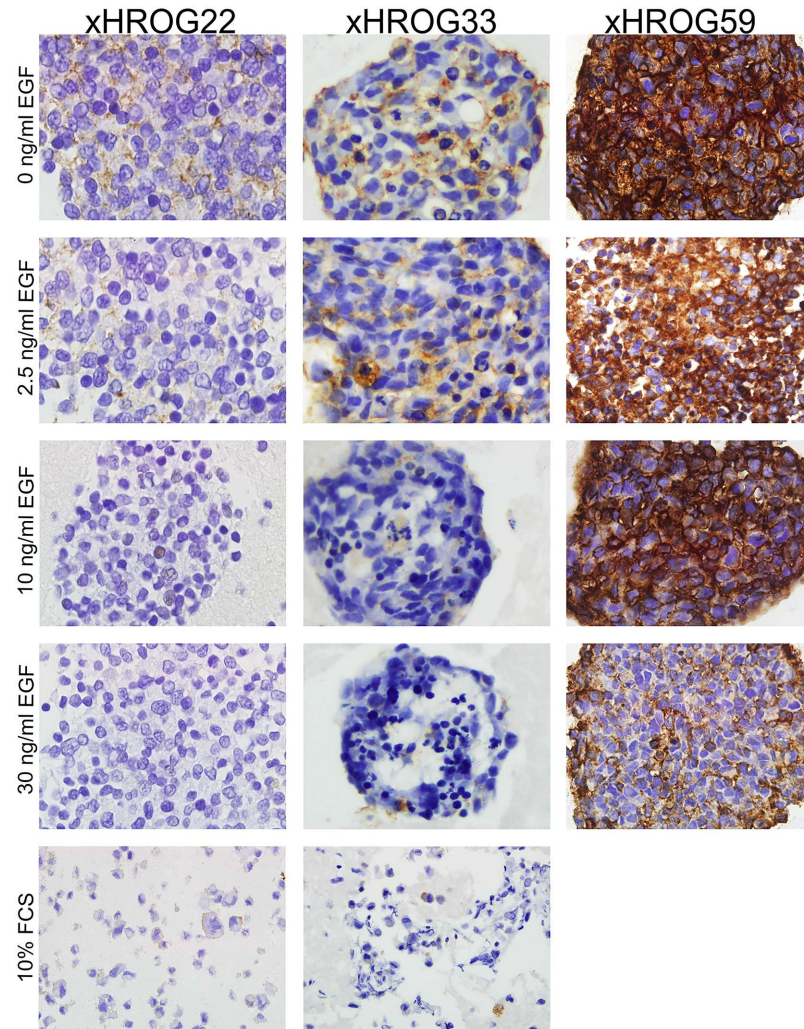
Fig 4. EGFR protein expression is concordant with genomic *EGFR* amplification. EGFR immunohistochemistry staining of paraffin embedded xHROG22, xHROG33 and xHROG59 cells grown under different conditions as indicated at passage 10 of cell culture; 200x magnification.

faithfully representing the original tumor. The *in vitro* models in this study were established from GBM PDX instead of directly patient derived GBM tissue. We confirmed the human origin of all PDX models as well as their resemblance to the primary GBM in a previous study [22]. All PDX models were histologically analyzed by a neuropathologist and found to fulfill requirements for a GBM diagnosis [22]. Additionally, the human origin of the established cell lines was verified (S1 Fig), suggesting that the findings of this study would likely be translatable to directly patient derived *in vitro* models.

Although the exact doubling times of the established cell lines were not determined, we did not observe distinct differences in cell proliferation between cell lines cultivated with low EGF and high EGF concentrations. However, other studies observed higher proliferation rates with primary GBM cell cultures established under high EGF concentrations compared to cell lines of the same origin cultured without EGF supplementation [29].

We observed spontaneous spheroid formation in all cell lines under all applied serum free conditions independent on the EGF concentration in the media. Since the culture method
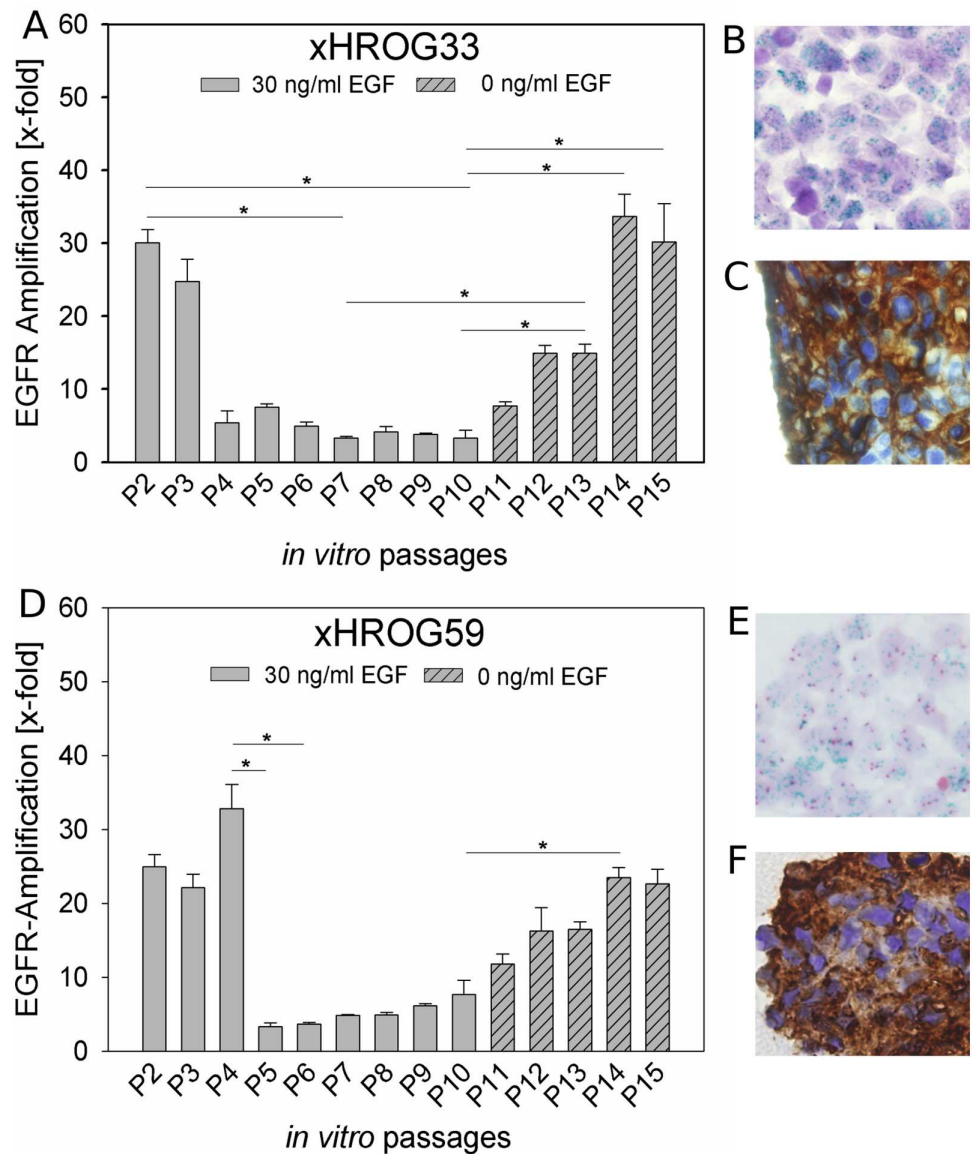
Fig 5. EGF withdrawal restores *EGFR* amplification. A and D) qPCR analysis of xHROG33 (A) and xHROG59 (D) cultured with 30 ng/ml EGF until passage 10 (grey bars) and without EGF for 5 additional passages (dashed bars), respectively. Error bars represent the standard deviation of triplicate analyses; B and E) 2C CISH analysis of paraffin embedded samples of xHROG33 (B) and xHROG59 (E) after 5 passages post EGF withdrawal (P15), C and F) EGFR immunohistochemistry staining of paraffin embedded samples of xHROG33 (C) and xHROG59 (F) after 5 passages post EGF withdrawal (P15). 400x magnification. * p<0.05, Tukey test.

applied in this study (serum free media supplemented with B27 and FGF) is often used to enrich cancer stem-like cells (CSCs) of GBM we hypothesized that the spontaneous spheroid formation we observed under those conditions may be attributable to CSCs [30,31].

In the case of the *EGFR* unamplified xHROG22, obtaining *EGFR* amplified cells under the applied *in vitro* conditions proved unsuccessful. It is likely that an initial event to establish extrachromosomal *EGFR* amplification, like unscheduled DNA synthesis and replication or inverted duplications, occurs during tumorigenesis [32]. In case of HROG22 such an event

apparently did not occur, suggesting that this tumor is not dependent on *EGFR* amplification but rather on other mechanisms and pathways. It was not possible to induce *EGFR* amplification *in vitro* under the conditions applied in this study, suggesting that the xHROG22 cell line is similarly not dependent on *EGFR* amplification.

Two cell lines analyzed in this study (xHROG33 and xHROG59) stably maintained *EGFR* amplification under low EGF conditions ranging from 0 to 2.5 ng/ml over at least 10 *in vitro* passages in long-term cell culture (12 to 15 months). *EGFR* amplification levels decreased rapidly with higher EGF concentrations in both cases.

These results are well in line with previous studies, demonstrating maintained *EGFR* amplification under serum free conditions without EGF supplementation as well as decreased *EGFR* amplification with high EGF concentrations in primary GBM cell lines [23,29,33]. Hence, *EGFR* amplification levels appear to be directly influenced by the EGF concentration *in vitro*.

However, Schulte *et al.* reported an almost complete loss of *EGFR* amplification in primary GBM cell lines cultivated with 10 ng/ml EGF [29]. In the case of xHROG33 a decrease in *EGFR* amplification was observed in qPCR results, but 2C CISH analysis confirmed the presence of *EGFR* amplified cells. However, in the case of xHROG59 cells established under serum free conditions with 10 ng/ml EGF, we did not observe a major decrease of *EGFR* amplification in qPCR, 2C CISH or EGFR IHC analysis.

In case of xHROG33 we observed a rapid decrease of *EGFR* amplification in the 10% FCS control similar to the serum free culture with 30 ng/ml EGF. Although the morphology of the cells is different–spheroids in the serum free culture and adherent monolayer with 10% FCS–we deemed it unlikely that the morphology plays a significant role for decreased *EGFR* amplification of the 10% FCS cell line of xHROG33. Would the loss of *EGFR* amplification be attributable to cell attachment, we would have expected no similarly rapid decrease of *EGFR* amplification in the spheroid culture of xHROG33. Although not further investigated, we hypothesize that the variety of growth factors present in FCS has a similar effect on the *EGFR* amplification of the cells as the supplementation of high EGF amounts.

Our qPCR and 2C CISH data are concordant with EGFR protein expression as determined by IHC staining of EGFR, indicating that the genomic amplification is functional.

Furthermore, restoration of genomic *EGFR* amplification after high *in vitro* EGF-induced loss was possible by complete EGF withdrawal in both cases analyzed (xHROG33 and xHROG59). Previous studies demonstrated restoration of *EGFR* amplification in primary GBM cell lines by reducing EGF concentrations from 20 ng/ml to 5 ng/ml EGF [33]. However, other studies failed to restore *EGFR* amplification after withdrawal of EGF in primary GBM cell lines with high EGF-induced *EGFR* amplification loss [29,31]. Interestingly, similar restoration effects have been described in a model of EGFR inhibitor resistance [34]. Nathanson *et al* found extrachromosomal *EGFR* present in untreated GBM cells, which was lost under erlotinib treatment, but restored subsequently to drug removal [34]. Although in our study the withdrawal of EGF and not discontinued treatment with an EGFR inhibitor lead to restoration of *EGFR* amplification *in vitro*, the underlying mechanism might be similar. A marker chromosome including *EGFR* positive homogeneous staining regions (HSR) might serve as *EGFR* reservoir, enabling tumor cells to regain extrachromosomal *EGFR* amplification in response to microenvironmental stimuli like discontinued EGFR inhibitor exposure or, possibly, EGF withdrawal [34–36].

However, this was not further investigated in our study and thus remains speculative.

In sum, the GBM *in vitro* models described here stably maintain *EGFR* amplification under low EGF conditions. Furthermore exposure of cell lines that were initially derived from the same PDX tissue showed decreased *EGFR* amplification when high amounts of EGF were

supplemented. Thus, these *in vitro* models are a useful tool for in depth analysis of EGFR expression level dependent effects in an isogenic background.

Supporting information

S1 Fig. PCR analysis of murine *MLH1* and human specific *cytochrome B*. A) Analysis of gDNA pools of xHROG22 (xH.22), xHROG33 (xH.33) and xHROG59 (xH.59) at passage 2 (P2) and passage 10 (P10). Ctr M: DNA derived from mouse tail tissue as positive control for murine *MLH1* (~350bp), Ctr H: DNA derived from a GBM cell line established from patient derived GBM tissue B) Verification of human origin of the cell lines by human specific *cytochrome B* PCR (~130bp product).
(TIF)

S2 Fig. qPCR analysis of *EGFR* amplification of all successfully established cell lines. Tukey test, * p<0.05.
(TIF)

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Supervision: Doreen William, Michael Linnebacher, Carl Friedrich Classen, Andreas Erbersdobler, Björn Schneider.

Validation: Doreen William, Björn Schneider.

Writing – original draft: Doreen William, Björn Schneider.

Writing – review & editing: Doreen William, Poroshista Mokri, Nora Lamp, Michael Linnebacher, Carl Friedrich Classen, Andreas Erbersdobler, Björn Schneider.

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