Therapeutics, Targets, and Chemical Biology

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FGFR1 Induces Glioblastoma Radioresistance through the PLCγ/Hif1α Pathway

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Abstract

FGF2 signaling in glioblastoma induces resistance to radiotherapy, so targeting FGF2/FGFR pathways might offer a rational strategy for tumor radiosensitization. To investigate this possibility, we evaluated a specific role for FGFR1 in glioblastoma radioresistance as modeled by U87 and LN18 glioblastomas in mouse xenograft models. Silencing FGFR1 decreased radioresistance in a manner associated with radiation-induced centrosome overduplication and mitotic cell death. Inhibiting PLCγ (PLCG1), a downstream effector signaling molecule for FGFR1, was sufficient to produce similar effects, arguing that PLCγ is an essential mediator of FGFR1-induced radioresistance. FGFR1 silencing also reduced expression of HIF1α, which in addition to its roles in hypoxic responses exerts an independent effect on radioresistance. Finally, FGFR1 silencing delayed the growth of irradiated tumor xenografts, in a manner that was associated with reduced HIF1α levels but not blood vessel alterations. Taken together, our results offer a preclinical proof of concept that FGFR1 targeting can degrade radioresistance in glioblastoma, a widespread problem in this tumor, prompting clinical investigations of the use of FGFR1 inhibitors for radiosensitization. *Cancer Res; 76(10); 3036–44. ©2016 AACR.*

Introduction

Standard treatment of newly diagnosed glioblastoma associates, when feasible, surgery, radiotherapy, and concomitant daily temozolomide administration. However, despite this significant therapeutic advance, most patients with glioblastoma still die within 2 years because of a tumor resistance to radio-chemotherapy. This low tumor sensitivity to radiotherapy is because of the modulation of different biological signal transduction pathways in tumor cells but also to a cross-talk between the tumor cells and their microenvironment. Factors controlling the microenvironment have been described to decrease tumor cell sensitivity to radiotherapy by several teams (1–3), including ours, which focused on the role of basic fibroblast growth factor, FGF-2, in tumor radioresistance. Indeed, we previously showed that FGF-2, known to regulate microenvironment, induces a radioresistant phenotype through the small GTPase RhoB when overexpressed in tumor cells (4, 5). Furthermore, we showed that radioresistance of U87 glioblastoma xenografts is controlled by RhoB via the regulation of intrinsic radioresistance (6, 7) and factors controlling hypoxia and angiogenesis (8, 9) as integrins (10) or integrins downstream effectors (11). Moreover, we demonstrated that the coexpression of FGF-2 and αvβ3 integrin was associated with a poor response to radio-chemotherapy in tumors of patients with a non–small cell lung cancer (12). Finally, we very recently described the *in vitro* and *in vivo* radiosensitizer effect on U87 cells of the first in class compound that inhibits FGF-induced signaling linked to FGFR internalization at nanomolar concentrations, SSR128129E (13–15), enforcing the idea that targeting FGF2/FGFR pathways might be of interest in the aim to radiosensitize human glioblastoma.

The four types of FGF receptors (FGFR1, FGFR-2, FGFR-3, and FGFR-4) are composed of an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic domain containing the catalytic protein tyrosine kinase core. FGF-2 binds to FGFR1, 2, and 4 (for review, ref. 16). When recognizing their FGF ligands, FGFRs undergo dimerization. Consecutively, phosphorylation of specific intracellular receptor domains leads to the activation of cytoplasmatic effector molecules. Signalling mediated by fibroblast growth factors can cause mitogenesis, proliferation, differentiation, cellular migration, angiogenesis, and repair of tissue injury. FGFR1 is amplified in almost 10% of breast cancers (17) and in squamous cell lung cancer (18); translocation of FGFR1 gene with the transforming acidic coiled-coil (TACC) has been described in glioma (19). For our part, we recently demonstrated that the expressions in tumor cells of FGFR1 and αvβ3 integrin are independent bad prognostic factors of overall survival but also, for FGFR1 expression, of time to progression in patients treated with radio-chemotherapy for a glioblastoma (20). These data underline the central role of FGFR1 in carcinogenesis or response to therapy. Furthermore, we

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

C. Toulas and E. Cohen-Jonathan-Moyal contributed equally to the supervision of the study.

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previously demonstrated that a pan-FGFR inhibitor increases the radiosensitivity of U87 cells, which mainly express FGFR1 (13). These data led us to hypothesize that specifically inhibiting FGFR1 might increase the efficiency of radiotherapy in glioblastoma. In this paper, using *in vitro* and *in vivo* silencing strategies, we demonstrate that inhibiting FGFR1 radiosensitizes glioblastoma cells via PLCγ and HIF1α by controlling radiation-induced centrosome overduplication and radiation-induced mitotic cell death.

Materials and Methods

Cell culture

Human U87MG and LN18 glioblastoma cells were purchased from ATCC (LGC Standards) within the past 6 years, immediately frozen and passaged for fewer than 6 months after resuscitation. Cell line authentication was performed by ATCC using short tandem repeat DNA profiles. Cells were routinely maintained in DMEM-Glutamax (Invitrogen) supplemented with 10% fetal calf serum (FBS) at 37°C in 5% CO_2 humidified incubators.

Targets silencing

Cells were transfected with different small-interfering RNAs (siRNA): an aleatory sequence, SiScramble siRNA (5′-GAC-GUGGGACUGAAGGGGUdTdT-3′), and two siRNAs specific for FGFR1, si1b (5′-UCUGUGGUCAGGUUUGAAU-3′) and si1c (5′-UUUCUCUGUUGCGUCCGACUU-3′; Eurogentec). LN18 cells were transfected with 20 nmol/L of the different siRNAs using the Lipofectamine RNAiMax reagent according to the manufacturer conditions (Invitrogen). For generating clones constitutively silencing FGFR1, U87 cells have been transfected with a pool of 4 shRNAs directed against FGFR1 (5′-CGGGAAGCATAAGAATAT-CAT-3′; 5′-GGTGCTTCACTTAAGAAATGT-3′;5′-ACCACCTACT-TCTCCGTCAAT-3′;5′-GACCAAAGTGGCTGTAAGAT-3′ (SureSilencing shRNA Plasmids KH00372N; SABiosciences, Qiagen) or with an aleatory sequence, Scramble shRNA (ShScr) according to the manufacturer recommandation (SABiosciences, Qiagen). U87 Clones carrying shFGFR1 or shScr were selected and then maintained by continuously treating cells with G418 500 μg/μL. In the same way, U87 and LN18 cells were transfected with 20 nmol/L of the different siRNAs against PLCγ, siPLCγ 2(5′-CGG-AATCGTGAGGATCGTATA-3′) and siPLCγ 3 (5′-CACGCTCT-CTTTCTGGCGGAA-3′; Qiagen) using the Lipofectamine RNAi-Max reagent (Invitrogen).

Western blotting

Proteins were extracted with a buffer composed of 50 mmol/L Tris-HCl pH 7.5, 0.1% Triton X-100, EDTA 5 mmol/L, and a cocktail of proteases inhibitors. Proteins were separated on SDS-PAGE and then transferred onto a nitrocellulose membrane. Blots were probed with the following primary antibodies: anti-FGFR1 (D8E4; diluted 1:1,000; #9740, Cell Signaling, Ozyme), anti-HIF1α (diluted 1:1000; Cayman Chemical, Interchim), anti-PLCγ1 (diluted 1:1,000; #5690, Cell Signaling, Ozyme), anti-β-actin (1:20,000, #MAB1501, Millipore), anti-GAPDH (diluted 1:10,000; #sc-365062, Santa Cruz Biotechnology, CliniSciences). Detection was performed using peroxydase-conjugated secondary antibodies, Chemilluminescence Detection Kit (ECL, Pierce, ThermoFisher Scientific), and ChemiDoc imaging system (Biorad) and signals quantification using ImageLab software (Biorad).

Quantitative real-time PCR

To achieve quantitative gene expression assays for human FGFR1 gene, total RNA was isolated by using the RNeasy RNA Isolation Kit (Qiagen), FGFR1 mRNA expression was determined by real-time PCR, using Evagreen dye and ABI-stepone+ detection system (Applied Biosystems, Life Technologies). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization.

Radiation survival determination

Limited dilution cloning was used for the glioma cell line to measure the surviving fraction after 2 Gy irradiation as already described (10, 21).

Measurement of cell death

Mitotic cell death was determined by quantifying the percentage of giant multinucleated cells (11, 21). Five days after an 8 Gy irradiation, cells (2×10^4) were fixed in paraformaldehyde [3.7% (w/v) in PBS] for 15 min at room temperature. Coverslips were then rinsed three times with PBS and then incubated with 0.1 μg/mL DAPI (Life Technologies) at 37°C. Cells were viewed on a Zeiss microscope through an ×100 aperture immersion lens. For each sample, over 300 cells were examined in at least three independent experiments.

Evaluation of the centrosomes duplication

The number of centrosomes in U87 and LN18 cells was analyzed using immunofluorescence staining for γ-tubulin as already described (11, 22).

Tumor xenograft generation

Nude mice were housed aseptically in the Claudius Regaud Institute Animal Care-accredited facility and the Institution animal ethics committee approval was obtained for the use of the animal model and the study protocols. At 5 to 7 weeks of age, mice were inoculated by subcutaneously injection into the hind flank with 1×10^6 FGFR1-silenced clone 2, clone 7 cells, or ShScr cells suspended in serum free DMEM medium. Animals were assigned randomly to treatment groups when tumors attained a volume of 200 to 300 mm^3. Mice were locally irradiated at a dose of 5 Gy under anesthesia. Control animals were anesthetized and sham irradiated. Tumors were measured in three dimensions until they reached 3000 mm^3 when animals were sacrificed.

Immunohistochemistry experiments

For HIF1α staining and vessels detection, after a rinse, an endogenous peroxidase block was applied for 15 min. The sections were incubated for 90 min with a mouse anti-HIF1α (diluted 1:100; Cayman Chemical, Interchim) or a mouse monoclonal anti-collagen IV (diluted 1:100, Abcam).

To quantify HIF1α expression or vascularity, three phase-contrast images of each mice tumor stained with HIF1α antibody or collagen IV antibody were taken randomly (magnification, ×4). The rate of HIF1α expression or vascularity was then calculated as the average percentage of stained area using Image J software (Research Services Branch, National Institute of Health).

Statistical analysis

Student test was performed to compare the means of values from different experiments. Differences were considered statistically significant at $P < 0.05$.

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Results

FGFR1 inhibition increases the glioblastoma cells sensitivity to ionizing radiation and radiation-induced mitotic cell death

To investigate whether the specific inhibition of FGFR1 may modify the cellular radiosensitivity, we selected radioresistant U87 [surviving fraction after a 2 Gy irradiation (SF2) value of 78%; ref. 13] and LN18 cell lines (SF2 value of 60%, data not shown), which mainly express FGFR1 (ref. 13 and Supplementary Fig. S1). As expected, FGFR1 was silenced in the two selected U87 clones [clones 2 and 7; FGFR1(−)U87 cells], which present a significant FGFR1 inhibition of 67.1% and 38.1%, respectively (Fig. 1A) and in LN18 cells [FGFR1(−)LN18 cells; $P < 0.001$; Fig. 1B). We then analyzed the impact of silencing FGFR1 on U87 and LN18 cell survival after irradiation by quantifying the SF2 value when FGFR1 has been silenced or not. FGFR1 inhibition made U87 and LN18 cells more sensitive to radiation as shown in Fig. 1C and D after a 2 Gy irradiation [SF2 value ratio vs. the control of 65.95% ($P < 0.01$) and 51.99% ($P < 0.001$) for FGFR1(−)U87 cells, and 66.9% for FGFR1(−)LN18 cells] or after higher irradiation doses (Supplementary Fig. S2A and S2B).

We then deciphered the biological pathways sustaining the radioprotective effect of FGFR1 in glioblastoma cells. Because we largely demonstrated that the radiation-induced cell death of solid tumor cells is mitotic cell death (6, 11, 21, 22), we first determined the number of giant multinucleated cells 5 days after 8 Gy irradiation when FGFR1 has been silenced or not. As shown in Fig. 2A, the percentage of radiation-induced giant multinucleated cells was significantly increased after irradiation when FGFR1 was inhibited compared to the control cells. Indeed, in irradiated U87 and LN18 control cells, the percentage of giant multinucleated cells was around 10%, but, when FGFR1 was downregulated, it increased to 35.9 % in U87 cells and to 57.5% in LN18 cells ($P < 0.01$). Because we previously demonstrated that the cell radioresistance controlled by post-mitotic cell death regulation is associated with the regulation of the radiation-induced centrosome overduplication (11, 22), we then quantified the number of cells containing more than two centrosomes, in irradiated cells (Fig. 2B). The percentage of cells presenting more than two centrosomes was significantly increased after irradiation in FGFR1 U87 and LN18 silenced cells compared to irradiated control cells (respectively by a 2.3- and 41-fold increase in U87 and LN18 cells; Fig. 2B). These results clearly demonstrate that silencing FGFR1 radiosensitizes glioblastoma by increasing radiation-induced mitotic cell death and radiation-induced centrosome overduplication.

FGFR1 radioprotective effect is mediated by PLCγ

One of the first events after FGFR binding to their ligands is the activation of phospholipase C gamma (PLCγ). PLCγ binds through its SH2 domain to a phosphotyrosine in the C-terminal tail of FGFRs and is phosphorylated at the tyrosine residues by the activated receptor tyrosine kinase. This tyrosine phosphorylation

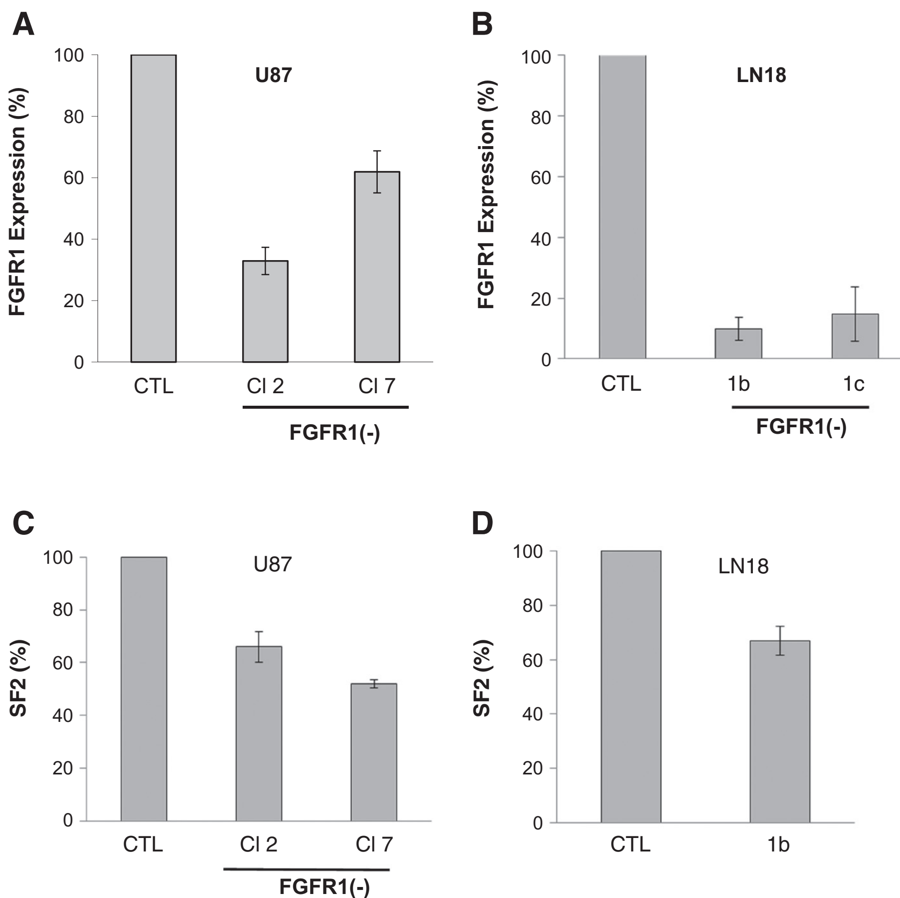

Figure 1.
Silencing FGFR1 in glioblastoma cells increases cell sensitivity to radiation *in vitro.* A and B, FGFR1 expression was determined by qPCR in U87 clone 2 (Cl2), clone 7 (Cl7), shScramble clone (CTL; A), and in FGFR1 silenced LN18 cells (si1b and si1c) and in siScramble (CTL) 48 hours after transfection (B). FGFR1 mRNA level was expressed as the ratio of FGFR1 expression in FGFR1-silenced cells versus control cells. C and D, FGFR1 silenced cells were plated in 96-well plates and irradiated 24 hours later as described in Materials and Methods. The SF2 value of U87-derived clones (C) and of FGFR1 inhibited LN18 cells (D) was calculated using limiting dilution test. Bars represent the mean ± SD of at least three different experiments.

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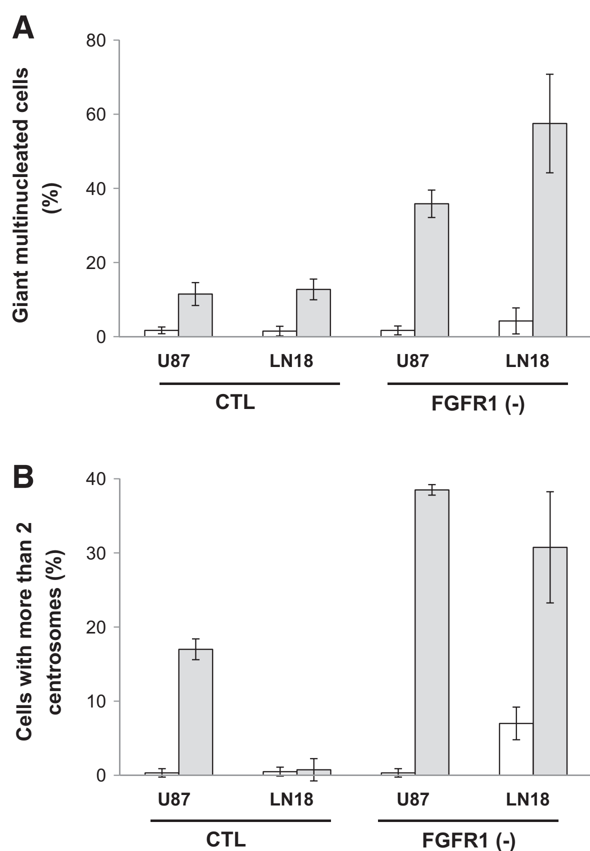

Figure 2.
Silencing FGFR1 increased radiation-induced mitotic cell death and centrosome overduplication. FGFR1(−) cells (U87Cl2 and si1bLN18 cells) were sham irradiated (white bars) or exposed to an 8 Gy irradiation (gray bars). A, mitotic cell death was quantified 5 days later after irradiation by counting the number of giant multinucleated cells (DAPI staining) among 100 cells fields. Bars represent the mean ± SD of at least three different experiments. B, to study centrosomal defects, the percentage of cells containing more than two centrosomes was determined 3 days later by staining cells with an anti-γ-tubulin antibody, as described in Materials and Methods. Bars represent the mean ± SD of at least three different experiments.

is necessary for the activation of PLCγ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to generate inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), conducing to the protein kinase C activation. To determine whether FGFR1 may exert its radioprotective effect through PLCγ, this enzyme was silenced by transfecting U87 and LN18 cell lines with two different siRNA directed against PLCγ. The expression of PLCγ determined by Western blot was respectively inhibited by 72% and by 45% in siRNAPLCγ2 U87 cells and in siRNAPLCγ3 U87 cells (Fig. 3A). Likewise in LN18 cells, siRNAPLCγ2 induced an 80% inhibition of PLCγ expression and the transfection with siRNAPLCγ3 decreased the PLCγ expression by 51% (Fig. 3A). As for FGFR1 silenced cells, compared to control cells, the SF2 value decreased by 28% and 33.9% in U87siPLCγ and LN18siPLCγ, respectively (Fig. 3B), demonstrating that PLCγ is an actor of FGFR1-dependent U87 and LN18 radioresistance. We then studied the effect of silencing PLCγ on radiation-induced mitotic cell death and centrosome overduplication. When PLCγ is downregulated, the percentage of giant multinucleated cells induced by radiation was increased by 12.5% ($P < 0.02$) and 47.75% ($P < 0.0002$) in U87 and LN18 cells, respectively (Fig. 3C) compared to control cells. Also, exposure of U87siPLCγ and LN18siPLCγ to radiation resulted in 8.03% ($P < 0.02$) and 38.25% ($P < 0.005$) increase of centrosome overduplication, respectively (Fig. 3D). These data strongly suggest that the FGFR1-dependent radioresistance pathway is mediated at least in part by PLCγ.

FGFR1/PLCγ radioprotective effect is mediated by HIF1α

Because we previously shown that HIF1α controls glioblastoma radioresistance (11) and that the pan-FGFR blocker SSR128129E activates HIF1α degradation in U87 cells (13), we then investigated whether FGFR1 silencing may regulate HIF1α expression. As shown in Fig. 4A, silencing FGFR1 led to a significant diminution of HIF1α expression in the two cell lines [respectively 11.1-fold ($P < 0.001$) and 3.1-fold ($P < 0.05$) decrease in HIF1α expression in U87 and LN18 cells]. Because we previously demonstrated that PLCγ is an effector of FGFR1-mediated radioresistance, we then checked whether the FGFR1-controlled HIF1α expression could be also regulated via PLCγ. The expression of HIF1α was downregulated by PLCγ inhibition by 2.38-fold ($P < 0.02$) and 11.1-fold ($P < 0.001$) in U87 and LN18 cells, respectively (Fig. 4B). These results highly suggest that *in vitro* FGFR1-mediated radioresistance in glioblastoma cells is because of the regulation of radiation-induced centrosome overduplication and mitotic cell death through PLCγ and HIF1α.

FGFR1 inhibition in tumor xenografts increases the efficiency of radiotherapy

To explore the interest of specifically targeting FGFR1 to radiosensitize glioblastoma, we then performed an *in vivo* approach by generating FGFR1 U87 silenced xenografts with the two previously selected FGFR1-silenced U87 clones (clones 2 and 7) and with the control clone (shScr clone). Xenografts from these clones were then locally irradiated or sham irradiated with a single 5 Gy fraction when the tumor size reached 300 mm^3 (7). As shown in Fig. 5A, tumor growth was not reduced by FGFR1 silencing without irradiation. Although a single 5 Gy fraction irradiation has no effect on the tumor growth of control xenografts (shScr xenografts), tumor growth was delayed in irradiated mice when FGFR1 has been silenced in the two different clones 2 and 7.

Our *in vitro* results showed a HIF1 α decrease after FGFR1 silencing (Fig. 4A). Moreover, hypoxia and angiogenesis are known to regulate *in vivo* radiosensitivity. We thus investigated whether silencing FGFR1 *in vivo* might decrease HIF1α expression and modify angiogenesis. For this, we first studied HIF1α expression by immunohistochemistry in shScr or Clone 2 xenografts. Silencing FGFR1 in U87 cells reduced HIF1α expression in small tumors (100 mm^3; data not shown) but also in larger tumors reaching 3000 mm^3 (13-fold reduction, $P < 0.01$) as shown in Fig. 5B. We then checked whether this lower HIF1α expression might also be because of a regulation of vascularization when FGFR1 was inhibited. Angiogenesis was studied by labelling endothelial cells with collagen IV (23). As shown in Fig. 5C, silencing FGFR1 within the xenograft did not significantly modify either the density or the morphology of the vessels. These data confirmed that silencing FGFR1 downregulated HIF1α expression *in vivo*.

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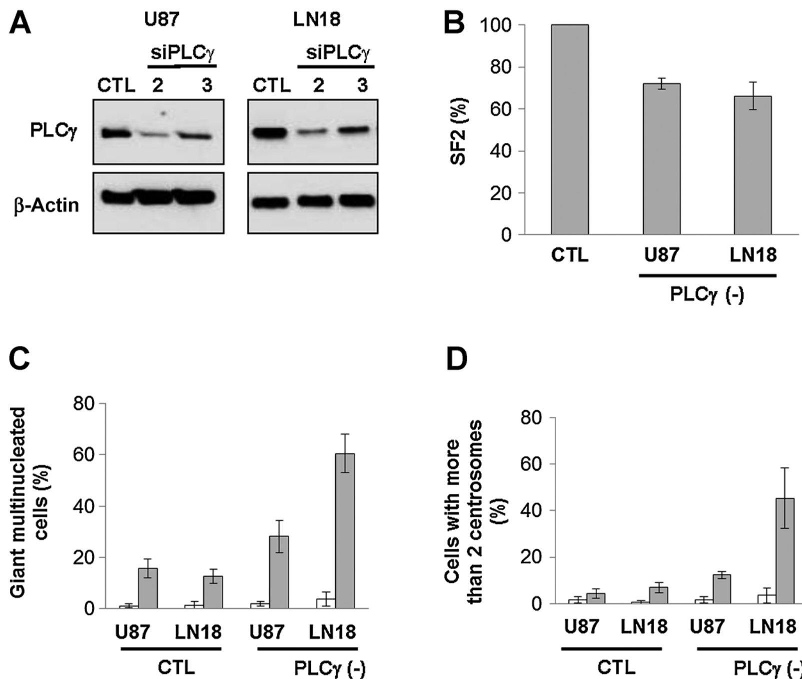

Figure 3.
FGFR1 regulates U87 radiosensitivity through PLCγ. A, U87 and LN18 cells were transfected with two different siRNA directed against PLCγ (siPLCγ2 and siPLCγ3) or with siScramble (CTL) as described in Materials and Methods and PLCγ expression was detected by Western blot 72 hours after transfection. B, U87 and LN18 cells transfected with siPLCγ2 (PLCγ−) or with siScramble (CTL) during 24 hours were plated in 96-well plates and irradiated as described in Materials and Methods. The SF2 values of U87- and LN18-transfected cells were calculated using limiting dilution test. Bars represent the mean ± SD of at least three different experiments. Twenty-four hours after the transfection of U87 and LN18 cells with siPLCγ2 [PLCγ(−)] or with siScramble (CTL), cells were plated on Lab-Tek II Chamber Slide System. Then cells were sham irradiated (white bars) or exposed to an 8 Gy irradiation (gray bars). C, mitotic cell death was quantified 5 days later by counting the number of giant multinucleated cells (DAPI staining) among 100 cells fields. Bars represent the mean ± SD of at least three different experiments. D, the percentage of cells containing more than two centrosomes was determined 3 days after irradiation by staining cells with an anti-γ-tubulin antibody, as described in Materials and Methods. Bars represent the mean ± SD of at least three different experiments.

Our results clearly demonstrate that silencing FGFR1 in the glioblastoma cells decreases *in vitro* the cell radioresistance in part through PLCγ and HIF1α and *in vivo* increases tumor growth delay when associated to radiation.

Discussion

Our previous works have demonstrated the radioprotective effect of FGF-2 on tumor cells (5) through the regulation of mitotic cell death (4). We recently showed that inhibiting FGFRs in U87 cells with a first-in class FGFR blocker SSR128129E, radiosensitized *in vitro* and *in vivo* U87 cells and decreased HIF-1α expression (13). We investigate here the specific role of FGFR1 in the modulation of glioblastoma radioresistance. We first demonstrate that specifically inhibiting FGFR1 by silencing strategies increases glioblastoma cells radiosensitivity *in vitro* and when associated with radiotherapy induces a significant tumor growth delay *in vivo*. This data clearly identify FGFR1 as a key factor of glioblastoma response to radiotherapy. It is well known that FGFRs, and more particularly FGFR1, participate to carcinogenesis by genetic alterations as mutations, amplification or translocation to generate fusion proteins in breast, bladder or non–small cell lung cancer (NSCLC) carcinomas. In pilocytic astrocytoma, the most common childhood brain tumor, mutation of two hotspot residues in the kinase domain of FGFR1 have been reported (24). In glioblastoma, mutations in the kinase domain have been less frequently reported ((25) but around 3% of glioblastoma harbour transforming in-frame fusion of the tyrosine kinase coding domains of FGFR genes (FGFR1 or FGFR3) with the TACC (19). FGFR1 has already been involved in resistance to other therapy as hormone therapy or targeted drugs in different cancer models. Indeed, it has been shown that FGFR1 amplification drives endocrine therapy resistance in amplified breast cancer cell lines *in vitro* and, according to our results, FGFR1 silencing reverses this resistance to hydroxytamoxifen (26). More recently, an FGFR1–FGF2 autocrine pathway has been identified as a novel pathway of acquired resistance to EGFR-specific TKIs in NSCLC cell lines bearing activating mutations in EGFR (27). FGFR1 has also been involved in resistance to nontargeted chemotherapies as cytosine arabinoside in leukemia (28) or cisplatin in ovarian cancer and, in this case, the resistance of the SKOV3 cell line to this drug is also reversed by silencing this receptor (29). Other growth factor receptors as EGFR (3), HGF/Met (30), or IGF1 (31) have already been involved in tumor cell radioresistance and inhibitors of these pathways have been evaluated through clinical trials in association with radiotherapy (for review, ref. 32). However, up to now, the specific involvement of FGFR1 in the *in vitro* and *in vivo* resistance of glioblastoma cells to radiotherapy, which is one of the main treatments for this pathology, has never been reported. Moreover, our present work strongly suggests a regulation of hypoxia through HIF1α. A link between FGFR1 and hypoxia regulation has been once reported in prostate cancer cells by demonstrating that a conditional activation of FGFR1 increases HIF1α expression (33). The regulation of HIF1α by other growth factor receptors known to regulate radioresistance, as EGFR, has been described in head and neck squamous cell carcinoma cells (34), suggesting that the modulation HIF1α expression by growth factors receptors may be an important pathway of radioresistance. Moreover, we previously reported, using a pan FGFR blocker, that HIF1α expression was regulated by FGFR pathways through GSK-3β in U87 cells (13). We confirm here *in vivo* that silencing FGFR1 in tumor cells significantly decreases HIF1α expression even in large tumors, strongly suggesting that silencing FGFR1 in tumor cells decrease tumor hypoxia *in vivo*. By its dual effect on tumor cell intracellular radiosensitivity and hypoxia regulation, FGFR1 is certainly a central actor of glioblastoma radioresistance.

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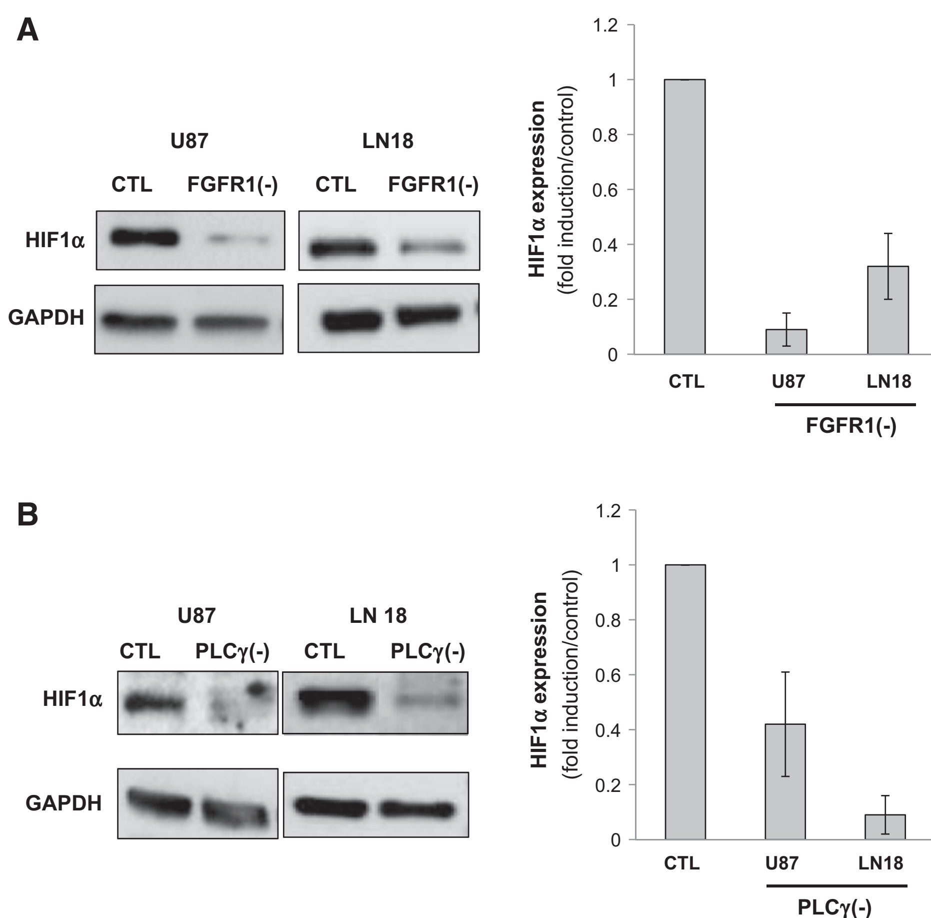

Figure 4.
Silencing FGFR1 or PLCγ decreases HIF1α expression. A, Western blot analysis determined HIF1α expression in previously described cells. Quantification was performed as described in Materials and Methods. Controls represent U87 shScramble and LN18 siScramble, FGFR1(−)U87–designed U87Cl2 and FGFR1(−)LN18, si1bLN18 cells. Bars represent the mean of fold induction versus the control ± SD of at least three different experiments. B, Western blot analysis was performed to follow HIF1α expression in U87 and LN18 cells silenced for PLCγ (siPLCγ2) and in control cells (siScr). Quantification was performed as described in Materials and Methods. Bars represent the mean of fold induction versus the control ± SD of at least three different experiments.

The FGFR1-dependent radioresistance pathway is mediated at least in part through one of the first downstream FGFR effector, PLCγ. Indeed, silencing this enzyme led to the reduction of survival after irradiation of glioblastoma cells while increasing radiation-induced centrosome duplication and mitotic cell death. PLCγ has been involved in resistance of tumor cells to chemotherapy. In fact, depletion of PLCγ expression or inhibition of its activity significantly increases cisplatin-induced apoptosis in gastric cancer cells (35). However, little is known concerning the involvement of PLCγ in the radioresistance mechanisms. PKC, known to be regulated by PLCγ, has been reported since 1994 as a regulator of cell response to ionizing radiation (2). PLCγ by itself has been shown to be a transducer of the *in vitro* radiosensitizer effect of Imatinib in pancreatic cancer cells (36) or of the MET inhibitor SU11274 in NIH3T3 cells (37) but the direct effect of specifically inhibiting PLCγ on survival after irradiation has never been reported. Our present work also demonstrates a regulation of HIF1α by this enzyme and FGFR1. Up to date, only a regulation loop of hypoxia mediated by PLCγ has been reported. Indeed, under intermittent hypoxia conditions, PLCγ activation is required for HIF1α accumulation (38). For our part, we clearly establish here that silencing this enzyme in glioblastoma cells leads to a decrease of HIF1α expression that we have shown to be induced by irradiation and that in turn regulates glioblastoma cells radioresistance (11).

We then deciphered the biological pathways controlled by FGFR1 involved in the cell radiosensitivity regulation and demonstrated that silencing FGFR1 or PLCγ increased the radiation-induced centrosome overduplication and the radiation-induced mitotic cell death. Aberrant centrosome number is a common feature of tumor cells. Extracentrosomes induced by DNA damage as ionizing radiation occur from an uncoupling between cell cycle checkpoints and centrosome duplication whereas centrosome duplication continues during a radiation-induced cell cycle arrest at G_2–M (39, 40). CDK2/cyclin-A/E activity is required for the production of excess centrosomes after irradiation (41). Regulation of centrosome duplication cycle by growth factors has been reported but in absence of exposure to radiation: endothelial cells exposed to elevated VEGF or FGF-2 present subnumerary centrosomes, cyclin E/Cdk2 hyperactivity, and NPM hyperphosphorylation (42). For our part, we previously demonstrated the role of NPM in radioresistance of HeLa cells (43), which is dependent of FGF-2 (5), a ligand of FGFR1. Our present results clearly establish for the first time that a growth factor receptor, FGFR1, is an upstream regulator of radiation-induced centrosome overduplication.

Our results lead us to postulate that specifically targeting FGFR1 opens a new field of therapeutic application to radiosensitize human glioblastoma. Preclinical or clinical trials evaluating the antitumor effect of FGFR inhibitors are under current, most of them concerning tyrosine kinase inhibitors targeting FGFR as single agents or in combination with other drugs (for review, ref. 44) or for some of them with radiotherapy (45). For our part, we recently reported preclinical evidence that targeting

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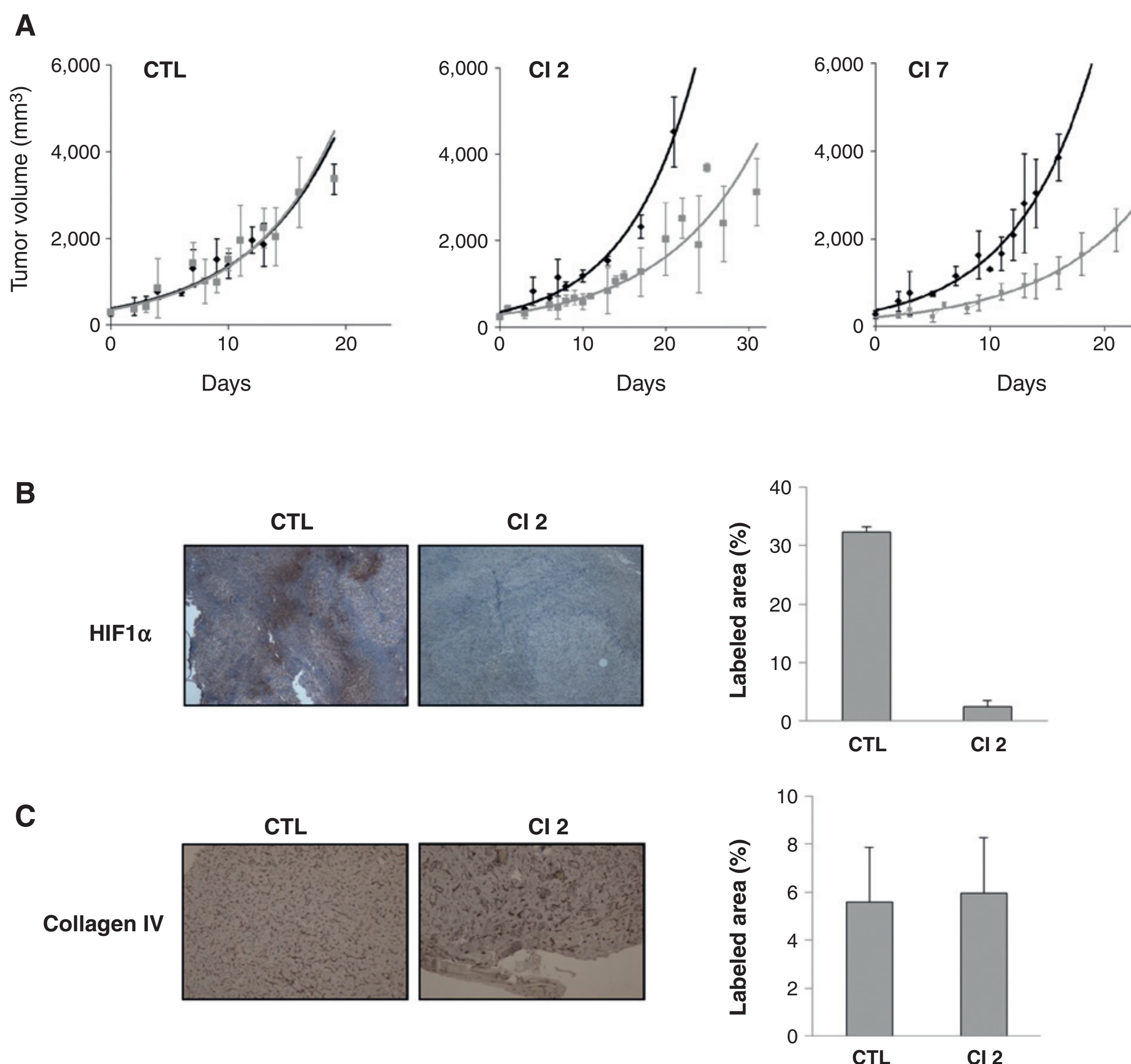

Figure 5.
Silencing FGFR1 in U87 xenografts increases the survival of mice after irradiation and decreased HIF1α expression. A, xenografts were generated from U87 shFGFR1 clone2 (Cl2) and clone 7 (Cl7), shScr (CTL) and sham irradiated (black curves), or irradiated (gray curves) as described in Materials and Methods. Tumors were measured as described in Materials and Methods. Dots and bars represent the mean ± SD of tumor size at least eight different mice. B and C, xenografts were generated from U87 shFGFR1 clone2 (Cl2) and shScr (control) as described in Materials and Methods. Immunohistochemistry was performed as described in Materials and Methods on 3,000 mm^3 tumors. B, HIF1α staining was performed (×4) and quantified between xenografts generated from shFGFR1 clone2 (Cl2) and shScr (control) as described in Material and Methods. Bars represent the mean ± SD of at least three different experiments. C, tumor vascularization was studied and quantified by collagen IV staining (23) between xenografts generated from shFGFR1 clone2 (Cl2) and shScr (control) as described in Materials and Methods. Bars represent the mean ± SD of at least three different experiments.

FGFR1 in association with radiotherapy could overcome the radioresistance of glioblastoma cells, sustaining the hypothesis that FGFR inhibitors might be not used as single agents but in combination with radiotherapy (13) in high-grade glioma treatment. Preclinical and then clinical trials should be designed to test the combination of drug specifically blocking FGFR1 with radiotherapy in the treatment of *de novo* glioblastoma and define the biomarkers allowing a correct selection of patients who could benefit from the combined treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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