Inhibition of Receptor Signaling and of Glioblastoma-derived Tumor Growth by a Novel PDGFRβ Aptamer

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Platelet-derived growth factor receptor β (PDGFRβ) is a cell-surface tyrosine kinase receptor implicated in several cellular processes including proliferation, migration, and angiogenesis. It represents a compelling therapeutic target in many human tumors, including glioma. A number of tyrosine kinase inhibitors under development as antitumor agents have been found to inhibit PDGFRβ. However, they are not selective as they present multiple tyrosine kinase targets. Here, we report a novel PDGFRβ-specific antagonist represented by a nuclease-resistant RNA-aptamer, named Gint4.T. This aptamer is able to specifically bind to the human PDGFRβ ectodomain (Kd: 9.6 nmol/l) causing a strong inhibition of ligand-dependent receptor activation and of downstream signaling in cell lines and primary cultures of human glioblastoma cells. Moreover, Gint4.T aptamer drastically inhibits cell migration and proliferation, induces differentiation, and blocks tumor growth *in vivo*. In addition, Gint4.T aptamer prevents PDGFRβ heterodimerization with and resultant transactivation of epidermal growth factor receptor. As a result, the combination of Gint4.T and an epidermal growth factor receptor–targeted aptamer is better at slowing tumor growth than either single aptamer alone. These findings reveal Gint4.T as a PDGFRβ-drug candidate with translational potential.

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INTRODUCTION

The platelet-derived growth factors (PDGFs) signal through two structurally similar tyrosine kinase receptors (RTKs), PDGF receptors α and β (PDGFRα and PDGFRβ).[1–4] Pathogenic roles of altered PDGF/PDGFR signaling have been established for a number of human diseases including cancer. Preclinical studies have not only shown an important role for the overexpression, point mutations, deletions, and translocations of PDGFRβ in tumorigenesis and maintenance of the malignant phenotype,[2,5] but have also proven that the targeted inhibition of signaling cascades has significant anticancer effects.[6,7]

Our objective was to apply an aptamer-based approach to develop new PDGFRβ-targeting drugs for a specific and selective tumor therapy. Nucleic acid-based aptamers represent an emerging wave of targeted therapeutic molecules against RTKs.[8–14] They are short structured single-stranded RNA or DNA ligands that bind with high affinity to their target molecules and are now emerging as promising molecules to recognize specific cancer epitopes in clinical diagnosis and therapy.[15–17] Because of their high specificity and low toxicity, aptamers can successfully compete with the universally used antibodies for *in vivo*-targeted recognition as therapeutics or delivery agents for nanoparticles, small interfering RNAs, chemotherapeutic cargos, and molecular imaging probes.[18–20] Further, in contrast to monoclonal antibodies, aptamers are characterized by high stability and convenient synthesis and modification with minimal inter-batch variability. Different therapeutic aptamers are now being tested in clinical trials and one has been approved by the US FDA[15,16] thus supporting the potential effectiveness of aptamer-based approaches for therapeutic purposes.

So far, a number of tyrosine kinase inhibitors (such as Imatinib mesylate, Sunitinib malate and Sorafenib) that act on a wide spectrum of tyrosine protein kinases including PDGFRβ[21,22] are under development as antitumor agents. They might overcome molecular heterogeneity within or between cancer patients and therefore have a better chance of success; however, unnecessary targeting of multiple receptors could cause toxicity and limit drug effectiveness.[23]

Neutralizing antibodies for PDGF ligands and receptors have been used in experiments evaluating the importance of PDGF signaling in pathogenic processes but, to date, none of such antibodies has entered the clinic.[24–27] Furthermore, one aptamer against PDGF-B ligand has already entered clinical trials for the treatment of age-related macular degeneration.[28]

Aimed at generating antagonist PDGFRβ aptamers not only useful in their own right, but also as escorts for therapeutic or diagnostic reagents, we developed the first nuclease-resistant RNA-aptamer that binds to human PDGFRβ and internalizes into glioblastoma (GBM) target cells. In addition to exquisite cell specificity and antitumor effect in a xenograft model of GBM, this aptamer strongly cooperates with a previously described

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anti-epidermal growth factor receptor (EGFR) aptamer[9] to induce inhibition of tumor growth, providing the basis for further development of antitumor combination therapies.

Taken together, these results show that Gint4.T aptamer is a promising RNA-based molecule that can be developed as a more effective alternative to currently used PDGFRβ inhibitors.

RESULTS

The Gint4.T aptamer specifically interacts with the extracellular domain of the PDGFRβ

Gint4.T is a 33 mer-truncated version (**Figure 1a**) of the original 2′-fluoropyrimidine (2′F-Py) nuclease-resistant RNA-aptamer generated by a differential cell-SELEX approach on highly tumorigenic U87MG GBM cells.

As an attempt to identify the functional targets of Gint4.T, we first performed a phospho-receptor tyrosine kinase antibody array analysis that suggested that the target of Gint4.T could be PDGFRβ (**Supplementary Figure S1a**). Indeed, among the receptors whose serum-dependent phosphorylation was reduced following aptamer treatment, the greater inhibition was detected for PDGFRβ. To definitely establish the Gint4.T affinity and specificity for the target, we next performed a filter binding analysis with the soluble extracellular domains of human PDGFRα and PDGFRβ (here indicated as EC-PDGFRα and EC-PDGFRβ, respectively), used as targets. In the assay, an unrelated sequence was used as a negative control. As shown in **Figure 1b**, Gint4.T had a strong affinity for EC-PDGFRβ (Kd value of 9.6 nmol/l) whereas it did not bind to EC-PDGFRα (data not shown).

Further, we observed that the binding of Gint4.T to U87MG cells was strongly competed by the recombinant EC-PDGFRβ but not EC-PDGFRα (**Figure 1c**), thus supporting the ability of the aptamer to recognize target cells through the binding to the extracellular domain of PDGFRβ on the cell surface and proving that the aptamer is able to discriminate PDGFRβ from the structurally similar PDGFRα receptor. In agreement with these results, Gint4.T did not bind to PDGFRβ-negative non-small-cell lung carcinoma A549 cells and to U87MG target cells in which the expression of the endogenous PDGFRβ was abrogated by a specific short hairpin RNA (shRNA) (**Supplementary Figure S1b**).

To further characterize the binding identity of Gint4.T aptamer to PDGFRβ-expressing cells, 10 minutes-treatment of U87MG with FAM-labeled Gint4.T was combined with staining with a specific PDGFRβ antibody (**Figure 1d–g**). An extensive overlap of PDGFRβ antibody and FAM-Gint4.T fluorescent signals was observed in any field examined by confocal microscopy, thus indicating a clear co-localization of the aptamer and the antibody on the receptor expressed on cell surface. No FAM-Gint4.T binding to PDGFRβ-knockdown U87MG (**Figure 1h,i**) and PDGFRβ-negative A549 cells (**Figure 1j,k**) was observed.

Moreover, by cell-binding assays with radiolabeled aptamer we demonstrated that the Gint4.T aptamer is rapidly endocytosed into U87MG glioma cells, getting about 50% of cell internalization following 30 minutes-incubation and reached more than 70% following 2 hours of aptamer treatment (**Figure 1l**). Consistently, co-localization experiments of FAM-Gint4.T with the endocytosis markers, early endosome antigen 1 (EEA1) and lysosomal-associated membrane protein 1 (anti-LAMP1), confirmed the ability of the aptamer to rapidly enter into U87MG cells, showing the majority of internalized aptamer in compartments positive for EEA1 (early endosomes) and LAMP1 (late endosomes/lysosomes) following 30 minutes and 2 hours of incubation, respectively (**Figure 1m–t**).

Taken together, these results indicate that Gint4.T specifically recognizes PDGFRβ either if expressed on the cell surface in its physiological context or the purified soluble extracellular domain of the receptor. Furthermore, because of its ability to rapidly internalize into PDGFRβ-positive target cells, it is a highly promising candidate as cargo for tissue specific internalization.

Gint4.T inhibits the PDGFRβ-mediated signal pathways and migratory responses of GBM cells

As a next step, we asked whether, because of its binding to PDGFRβ, Gint4.T could interfere with ligand-dependent activation of the receptor and downstream signaling. As shown in **Figure 2a**, 200 nmol/l-Gint4.T treatment drastically reduced the tyrosine-phosphorylation of PDGFRβ following stimulation of T98G (left) and U87MG (right) cells with PDGF-BB, the primary activator of PDGFRβ, causing about 70% inhibition at 5 minutes of ligand treatment. No effect was observed in the presence of the unrelated sequence used as a negative control. Consistently, a substantial reduction of PDGF-BB-dependent phosphorylation of extracellular signal-regulated kinase 1 and 2 (Erk1/2) and PKB/Akt kinase was observed in the presence of Gint4.T treatment in both cell lines (**Figure 2b**). Furthermore, the neutralizing effect of the aptamer was also observed in primary cell cultures of malignant glioblastomas (**Figure 2c**).

Intracellular signaling initiated by PDGFRβ has been reported to be involved in the metastatic potential of cancer[5] and its inhibition *in vitro* results in impairment of cell migration,[29] thus we determined whether Gint4.T could affect migration of GBM cells.

As shown in **Figure 3a**, treating T98G and U87MG cells with Gint4.T aptamer strongly reduced cell migration, either stimulated by serum and by the PDGF-BB, as compared with the unrelated aptamer. In addition, monolayers of T98G and U87MG cells were scratched and images were taken at 0, 24, and 48 hours after wounding (**Figure 3b**). The wound closure was significantly delayed in the presence of Gint4.T treatment compared with controls, the effect of the aptamer being time dependent (see lower panels). Thus, in good agreement with previous reports, PDGFRβ inhibition by Gint4.T treatment results in cell migration impairment.

Gint4.T blocks GBM cell proliferation and induces cell differentiation

Based on the Gint4.T inhibitory potential on the activation of Erk1/2 and the PKB/Akt pathways, we determined whether the aptamer was also able to reduce cell viability and proliferation *in vitro*. As assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, treatment of T98G and U87MG with Gint4.T strongly inhibited cell viability in a dose-dependent (**Figure 4a,b**) and time-dependent (**Figure 4a,b**, inserts) manner. The effect was comparable or even stronger than in the presence of the anti-EGFR pro-apoptotic CL4 aptamer,[9] used as a positive control. Remarkably, no cytotoxicity was observed with the unrelated aptamer, even at high concentration.

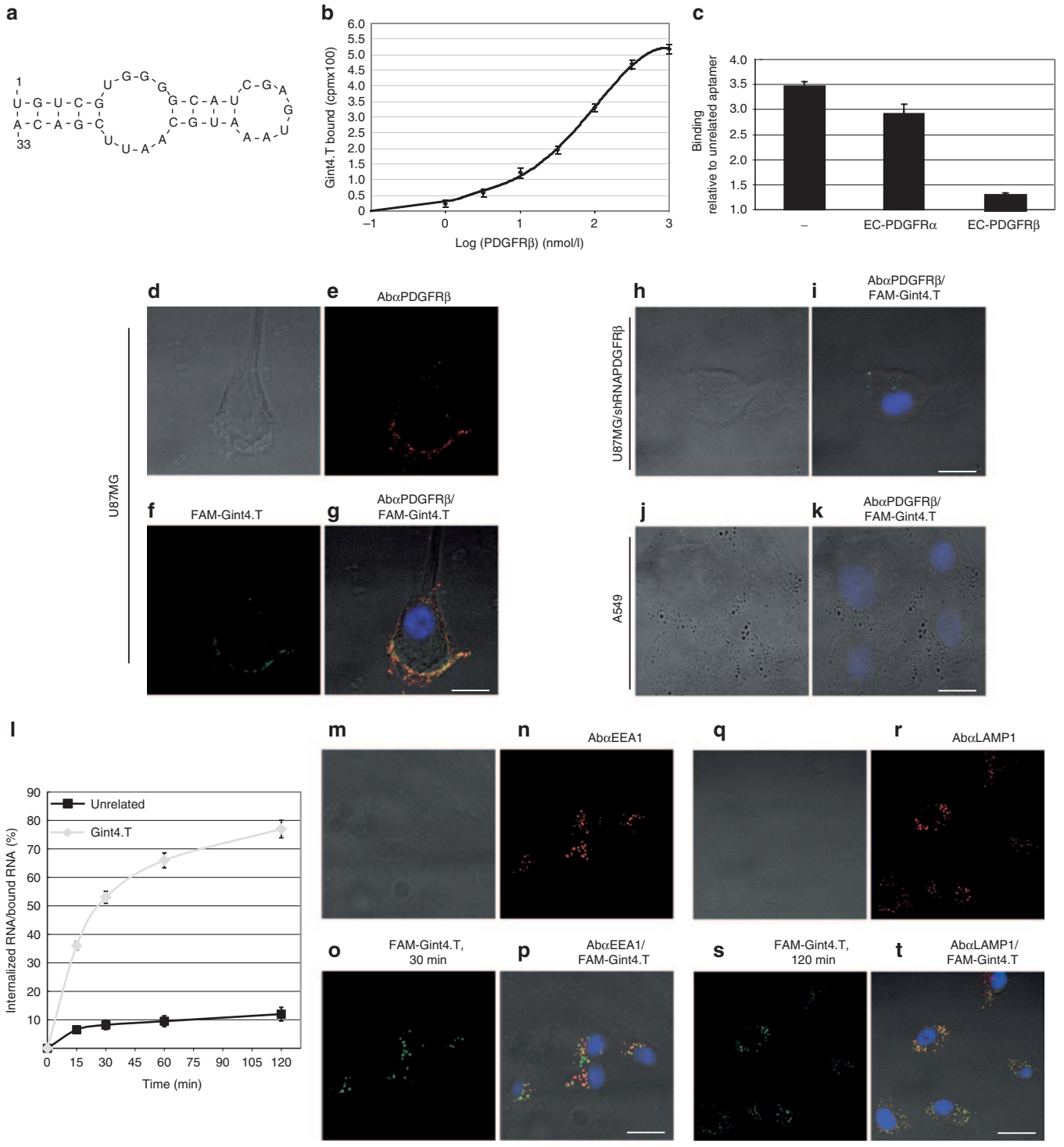

Figure 1 Gint4.T aptamer specifically binds to human PDGFRβ and rapidly internalizes into GBM cells. (**a**) Secondary structure of Gint4.T predicted by using DNAsis software. (**b**) Binding isotherm for Gint4.T: EC-PDGFRβ complex. (**c**) Binding of 100 nmol/l radiolabeled Gint4.T, prior incubated with 200 nmol/l EC-PDGFRα or EC-PDGFRβ for 15 minutes at 37 °C, to U87MG cells. In **b, c**, the results are expressed relative to the background binding detected with the unrelated aptamer, used as a negative control. (**d–k**) Following 10-minutes FAM-Gint4.T treatment, U87MG, U87MG/shRNAPDGFRβ (U87MG cells following 72 hour-transfection with a specific PDGFRβ short hairpin RNA) or A549 cells were stained with anti-PDGFRβ antibodies, visualized by confocal microscopy and photographed. (**l**) Internalization rate of radiolabeled Gint4.T and unrelated aptamer into U87MG cells. Results are expressed as percentage of internalized RNA relative to total bound aptamer. In **b, c, l** error bars depict mean ± SD ($n = 3$). (**m–t**) Following treatment with FAM-Gint4.T for the indicated times, U87MG cells were stained with anti-EEA1 (**m–p**) or LAMP1 (**q–t**) antibodies, visualized by confocal microscopy and photographed. (**d–k, m–t**) All digital images were captured at the same setting to allow direct comparison of staining patterns. Scale bars = 10 µm. The Manders' coefficients for the amount of co-localization were: M1, 0.975 and M2, 0.908 (**g**); M1, 0.620 and M2, 0.615 (**p**); M1, 0.980 and M2, 0.972 (**t**). EEA1, early endosome antigen 1; GBM, glioblastoma; LAMP1, lysosomal-associated membrane protein 1; PDGFRβ, platelet-derived growth factor receptor β.

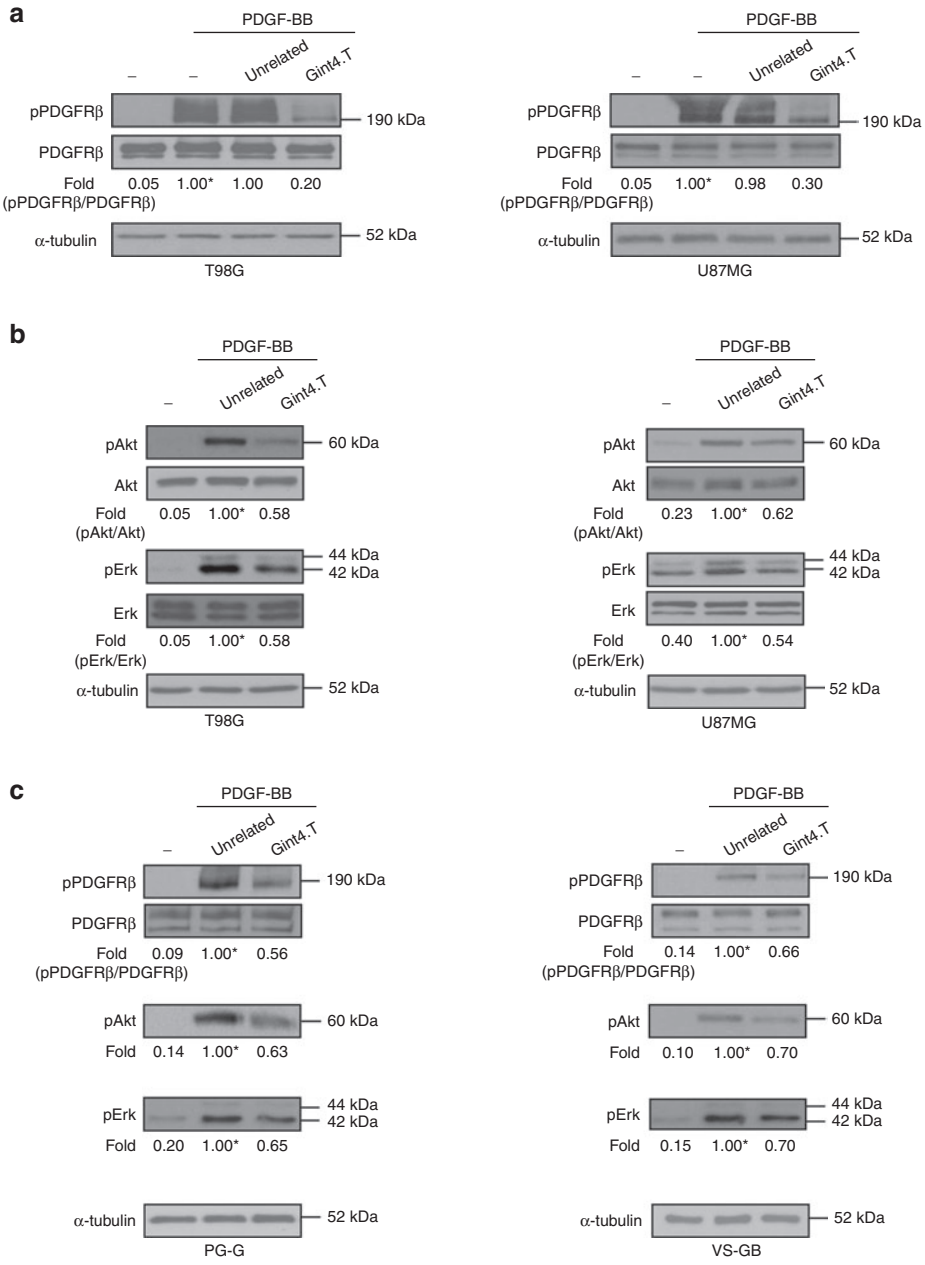

Figure 2 Gint4.T inhibits PDGF-BB-dependent PDGFRβ activation. (**a–c**) Serum-starved T98G, U87MG, or primary glioma cells from two patients (PG-G and VS-GB) were either left untreated or stimulated with PDGF-BB in the presence of Gint4.T or the unrelated aptamer (used as a negative control), as indicated. Cell lysates were immunoblotted with anti-pPDGFRβ, anti-PDGFRβ, anti-pERK, anti-pAkt. Filters were stripped and reprobed with anti-Erk and anti-Akt antibodies, as indicated. Values below the blots indicate signal levels relative to PDGF-BB stimulated cells in the absence (**a**) or in the presence (**b, c**) of unrelated aptamer, arbitrarily set to 1 (labeled with asterisk). (**a–c**) Equal loading was confirmed by immunoblot with anti-α-tubulin antibody. Molecular weights of indicated proteins are reported. PDGFRβ, platelet-derived growth factor receptor β.

To establish whether Gint4.T inhibits cell proliferation, we performed flow cytometry of cells stained with anti-5-bromodeoxyuridine and propidium iodide. Interestingly, 72-hours treatment of T98G (**Figure 4c**) and U87MG (**Figure 4d**) with Gint4.T (left) depleted cells at G2/M phases, with a consistent increase of the cell population in S-phase compared with the unrelated aptamer-treated cells (right). Analyzing the anti-5-bromodeoxyuridine incorporation profile (see inserts), we observed that the Gint4.T treatment did not prevent T98G and U87MG cells from entering the S-phase, even if cells displayed an evident defect in intra-S, but caused a failure of the cells to complete S and move toward G2 phase.

Accordingly, growth curve experiments confirmed that the aptamer exerted a severe inhibitory effect on T98G and U87MG cell proliferation (almost 80% inhibition at day 6) with respect to cells mock-treated or treated with the unrelated sequence, that proliferated at comparable rates (**Figure 5a,b**). Furthermore, to determine whether GBM cells would resume proliferation upon removal of Gint4.T, T98G cells were left in culture over a 11-day time period in the presence of Gint4.T (**Figure 5a**, solid line) or at

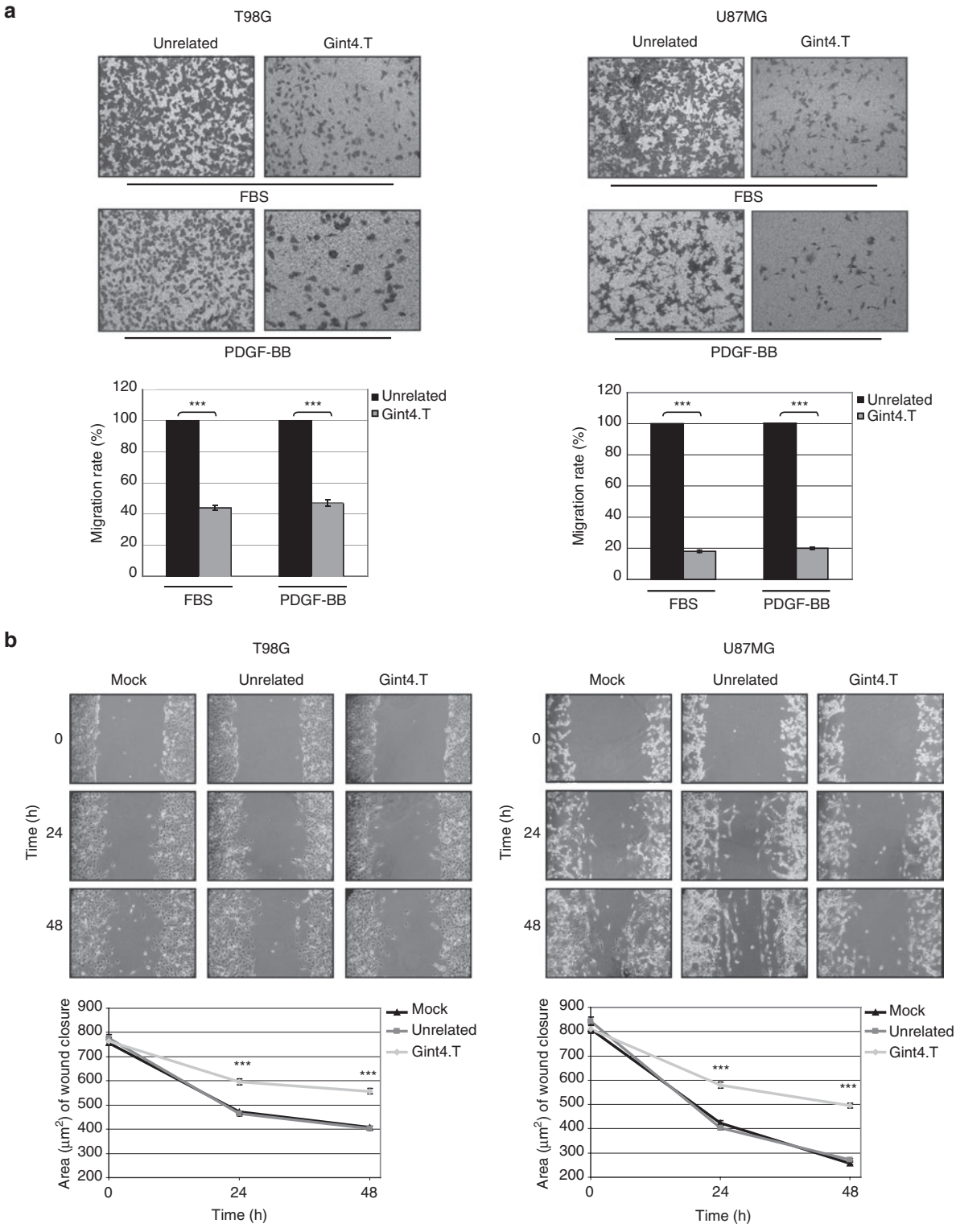

Figure 3 Gint4.T inhibits GBM cell migration. (**a**) Motility of T98G and U87MG cells was analyzed by Transwell Migration Assay in the presence of Gint4.T or the unrelated aptamer, used as a negative control, for 24 hours toward 10% FBS or PDGF-BB (50 ng/ml) as inducers of migration. The migrated cells were stained with crystal violet and photographed. Representative photographs of at least three different experiments were shown. The results are expressed as percent of migrated cells in the presence of Gint4.T with respect to cells treated with the unrelated aptamer. (**b**) Confluent monolayers of T98G and U87MG cells were subjected to scratch assays and mock-treated or treated with Gint4.T or the unrelated aptamer for 24 and 48 hours. Phase-contrast microscopy images were taken at the indicated time and the extent of wound closure was calculated (magnification 4×). (**a, b**) ***$P < 0.0001$ relative to unrelated ($n = 3$). Error bars depict means ± SD. FBS, fetal bovine serum; PDGF, platelet-derived growth factor.

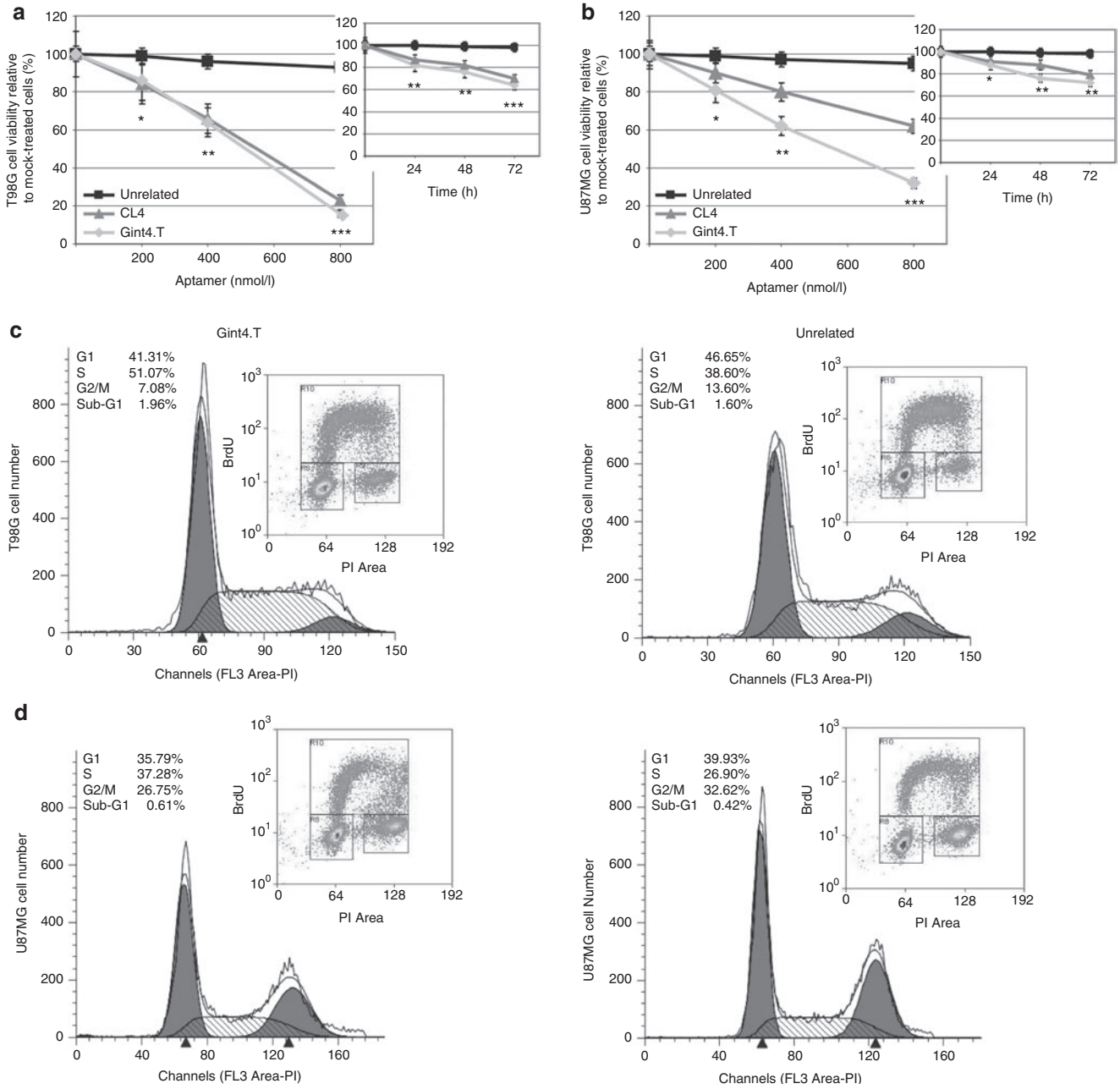

Figure 4 Gint4.T inhibits GBM cell survival and proliferation. T98G (**a**) and U87MG (**b**) cells were mock-treated or treated for 24 hours with increasing amounts of Gint4.T, CL4 or the unrelated aptamer as a negative control, or with 200 nmol/l final concentration of each aptamer for the indicated incubation times (inserts). Cell viability was analyzed and expressed as percent of viable treated cells with respect to mock-treated cells. (**a, b**) *P* values for Gint4.T and CL4 relative to unrelated are: ***$P < 0.0001$; **$P < 0.005$; *$P < 0.05$ ($n = 6$). Error bars depict means ± SD. T98G (**c**) and U87MG (**d**) cells were treated for 72 hours with Gint4.T or the unrelated aptamer, as indicated. Cell-cycle profile were determined by BrdU incorporation and PI staining. Percentages of cells in each cycle phase are indicated. BrdU, anti-5-bromodeoxyuridine; PI, propidium iodide.

day 4 medium containing Gint4.T was replaced with aptamer-free medium and incubation was further prolonged for 7 days (**Figure 5a**, dashed line). As shown, a slight increase of cell growth was observed indicating that Gint4.T was able to induce an almost full inhibition of glioma cells proliferation over the entire period of observation.

Consistently with the inhibitory effects of Gint4.T on cell viability and proliferation, treating U87MG and T98G cells with the aptamer caused a time-dependent reduction of [^{3}H]-thymidine incorporation (**Supplementary Figure S2**).

The block of T98G and U87MG cells proliferation was accompanied by a dramatic morphological change of the cells that became spindle shaped with long processes; the effect was evident starting from 2 days of Gint4.T treatment and more pronounced after 6 days (**Figure 5c**). Since these morphological alterations are also suggestive of cellular differentiation we monitored the expression of glial fibrillary acidic protein (GFAP), a determining factor for astrocytic cell shape.[30] Our results showed that the expression of GFAP was remarkably increased in Gint4.T-treated cells, compared with untreated

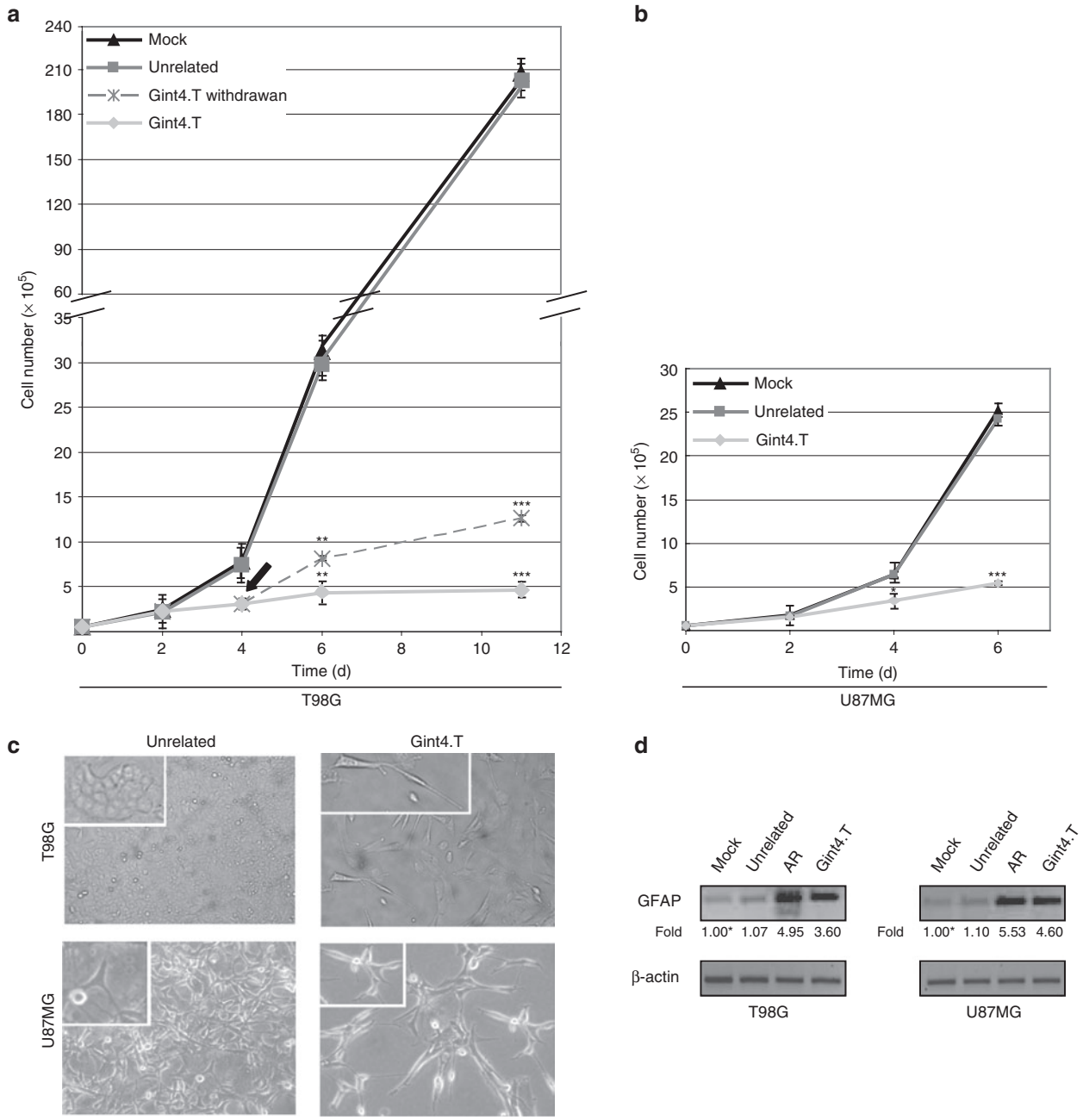

Figure 5 Gint4.T induces GBM cell differentiation. T98G (**a**) and U87MG (**b**) cells were either mock-treated or treated with Gint4.T or the unrelated aptamer, used as a negative control, by renewing the aptamer treatment each 24 hours and the cell number was counted at the indicated time points. In (**a**), at day 4 of Gint4.T treatment, the aptamer was removed from the culture medium (the arrow) and incubation prolonged (dashed line). (**a,b**) Growth curves represent the average of three independent experiments. ***$P < 0.0001$; **$P < 0.005$; *$P < 0.05$ relative to unrelated ($n = 6$). Error bars represent mean ± SD. (**c**) T98G and U87MG cells were treated for 6 days with Gint4.T or the unrelated aptamer and photographed by phase-contrast microscopy (magnification 4×). (**d**) Cells were treated as in (**c**) or with 1 µmol/l ATRA and GFAP mRNA levels were analyzed by RT-PCR. ATRA, all-trans retinoic acid; GFAP, glial fibrillary acidic protein.

T98G and U87MG cells, with the highest expression observed at 6-day treatment (Figure 5d). Expression levels were similar to those observed in cells treated with 1 µmol/l all-trans retinoic acid that has been reported to induce differentiation with upregulation of GFAP in both U87MG and T98G cells.[31] Taken together, the results indicate that Gint4.T inhibits growth by inducing S-phase cell-cycle arrest and differentiation in GBM cells.

Gint4.T prevents PDGFRβ-mediated EGFR transactivation in GBM cells

It has been reported that PDGFR stimulation transactivates EGFR in rat aortic vascular smooth muscle[32] and in medulloblastoma[29] and that receptor heterodimerization is an essential mechanism for PDGF-induced EGFR transactivation. Thus, we asked whether interfering with PDGFRβ expression and function by a specific shRNA (Figure 6a) or Gint4.T aptamer (Figure 6b), respectively,

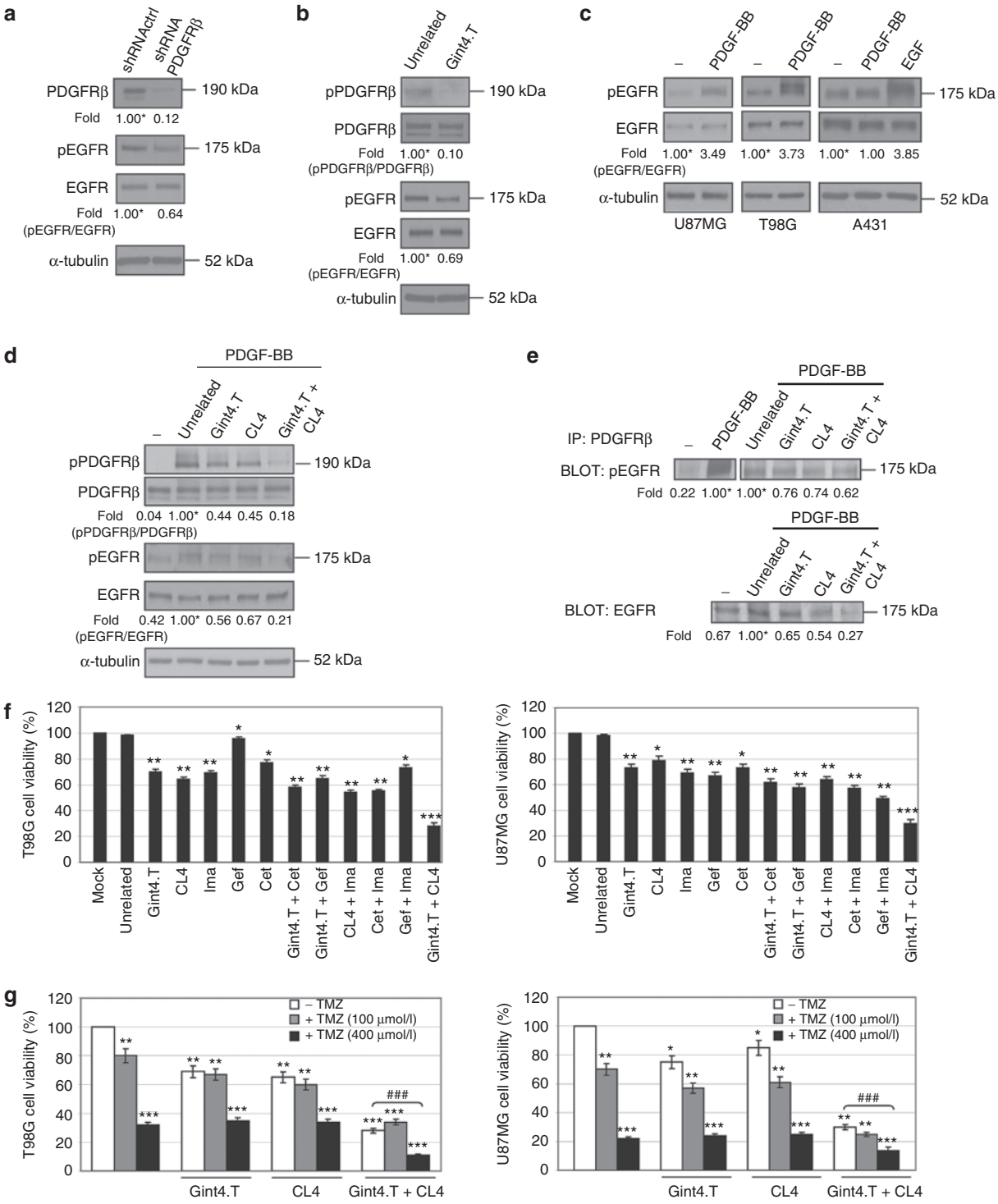

Figure 6 Gint4.T prevents PDGFRβ-mediated EGFR transactivation. (**a, b**) Lysates from T98G cells, following transfection with shRNAPDGFRβ or shRNActrl (**a**) or treated for 6-hours with Gint4.T or the unrelated aptamer as a negative control (**b**), were immunoblotted with anti-pPDGFRβ, anti-PDGFRβ, anti-pEGFR, anti-EGFR antibodies, as indicated. (**c**) Serum-starved U87MG, T98G, and A431 cells were either left untreated or stimulated with PDGF-BB or EGF and cell lysates were immunoblotted with anti-pEGFR, anti-EGFR antibodies, as indicated. (**d, e**) Serum-starved T98G cells were either left untreated or stimulated with PDGF-BB in the absence or in the presence of 200 nmol/l Gint4.T, 200 nmol/l CL4, 200 nmol/l Gint4.T plus 200 nmol/l CL4, or 400 nmol/l unrelated aptamer, as indicated. Cell lysates were either immunoblotted with anti-pPDGFRβ, anti-PDGFRβ, anti-pEGFR, anti-EGFR antibodies (**d**) or immunoprecipitated with anti-PDGFRβ antibody and immunoblotted with anti-pEGFR and anti-EGFR antibodies (**e**). (**a–e**) Values below the blot indicate signal levels relative to each control, arbitrarily set to 1 (labeled with asterisk). (**a–d**) Equal loading was confirmed by immunoblot with anti-α-tubulin antibody. Molecular weights of indicated proteins are reported. (**f, g**) T98G and U87MG cells were mock-treated or treated for 72 hours with 200 nmol/l Gint4.T, 200 nmol/l CL4, and (**f**) 10 μmol/l Imatinib (indicated as Ima), 5 μmol/l Gefitinib (indicated as Gef), 1 μmol/l Cetuximab (indicated as Cet), or (**g**) 100 μmol/l and 400 μmol/l TMZ, as single agents or in combination, as indicated and cell viability was analyzed. (**f**) As a negative control, cells were treated with the unrelated aptamer at a concentration of 400 nmol/l. ***$P < 0.0001$; **$P < 0.005$; *$P < 0.05$ relative to mock-treated ($n = 6$). ###$P < 0.0001$. Error bars depict means ± SD. EGFR, epidermal growth factor receptor; PDGFRβ, platelet-derived growth factor receptor β; TMZ, temozolomide.

could inhibit basal EGFR phosphorylation in T98G cells that express high levels of both EGFR and PDGFRβ. As shown, we observed about 30% of inhibition of EGFR phosphorylation with both the approaches thus indicating that PDGFRβ can transactivate EGFR under basal unstimulated cell condition.

Further, we determined whether PDGF-BB stimulation could enhance the EGFR transactivation observed in unstimulated cells and, if that was the case, whether inhibiting PDGFRβ and EGFR with Gint4.T and CL4, respectively, could affect this event. As shown, PDGF-BB stimulation significantly activated EGFR in U87MG and T98G cells but not in A431 cells, which overexpress EGFR but lack PDGFRβ, indicating that PDGF stimulates EGFR in PDGFRβ-dependent manner (**Figure 6c**). Remarkably, PDGF-BB dependent transactivation of EGFR in T98G cells was decreased by Gint4.T as well as CL4 treatment (**Figure 6d**) thus likely occurs via receptor heterodimerization. Further, by combining Gint4.T and CL4 treatment, a more pronounced reduction of PDGFRβ and EGFR phosphorylation (about 80%) was observed (**Figure 6d**). In addition, we found that the amount of pEGFR/EGFR coimmunoprecipitated with PDGFRβ increases following PDGF-BB stimulation of the cells and that Gint4.T as well as CL4 inhibited PDGF-induced PDGFRβ/EGFR heterodimers and EGFR transactivation (**Figure 6e**). This suggested that the binding of each aptamer to the extracellular domain of its related receptor interfered with the heterodimers formation, again the inhibition results stronger by using the two aptamers in combination (**Figure 6e**). In agreement with these observations from T98G cells also in U87MG cells, both aptamers were able to block PDGF-induced EGFR transactivation (**Supplementary Figure S3**).

As a next step, we compared their inhibition on cell viability to that of three commercially available inhibitors that are currently in clinical use as anticancer therapeutics for EGFR, Gefitinib (tyrosine kinase inhibitor), and Cetuximab (monoclonal antibody), and for PDGFRβ, Imatinib (tyrosine kinase inhibitor). In dose- and time-dependent experiments, T98G and U87MG cells resulted highly resistant at both the EGFR inhibitors (see previous report[33] and **Supplementary Figure S4**). Regarding Imatinib, an appreciable reduction of cell viability was observed only starting from a concentration of 10 μmol/l (see previous report[34] and **Supplementary Figure S4**). Further, single drugs and pairwise combinations were analyzed in T98G and U87MG for cell viability (**Figure 6f**). No additive or synergic effect was observed with each drug combinations except that for the treatment with CL4 plus Gint4.T that appeared to be the best combination for inhibiting cell viability with additive interaction, reaching about 70% inhibition when compared with mock-treated cells or cells treated with the unrelated aptamer (**Figure 6f**, **Supplementary Figure S5**).

Ultimately, as shown in **Figure 6g**, the massive decreasing of T98G and U87MG cell viability level after the combined treatment with Gint4.T and CL4 at a total concentration of 400 nmol/l is comparable with that obtained with a concentration of temozolomide (TMZ), the chemotherapeutic agent used to treat glioblastomas, even much higher than that used in clinic.[35,36] Further, almost 80% inhibition of cell viability was observed in both cell lines by combined treatment of the two aptamers with TMZ.

Altogether, these results establish that the use of the two aptamers in combination causes a drastic reduction of PDGF-BB-dependent activation of the receptors that results in the inhibition of cell viability even stronger than that caused by high concentration of approved PDGFR and EGFR inhibitors and of TMZ.

Gint4.T inhibits tumor growth and enhances antitumor activity of the CL4 anti-EGFR aptamer

To assess tumor targeting by Gint4.T, mice bearing palpable (~60 mm^3) xenograft tumors from GBM U87MG-luc cells ($PDGFR\beta^+$) and breast MCF7-luc cells ($PDGFR\beta^-$) on left and right flanks, respectively, were treated with a single intravenous injection of 1,600 pmol (1.04 mg aptamer/kg mean body-weight) of Alexa-labeled Gint4.T or Alexa-labeled unrelated aptamer. The affinity of Gint4.T for PDGFRβ was unchanged by the labeling procedure (data not shown). The aptamer amount in the tumors was thus monitored at different times by evaluating the intensity of fluorescent signal per bioluminescence as measure of tumor mass. As shown in **Figure 7a**, the signal of Gint4.T, normalized to that of the unrelated aptamer, consistently increased from 60 to 120 minutes and remained high up to 24 hours in U87MG target tumors but not in MCF7 nontarget tumors, thus indicating that Gint4.T still preserves its binding specificity *in vivo*. Further, 15 days after aptamer injection the bioluminescence increased approximately four times in unrelated aptamer control tumors while remained unchanged in Gint4.T-treated tumors (**Figure 7b**) thus indicating that a single aptamer treatment is sufficient to cause a significant tumor growth inhibition at least in mice bearing small tumors. Therefore, to test the efficacy of Gint4.T and CL4 in combination in U87MG-derived mouse xenografts, treatments were initiated at 24 days after cell inoculation, when tumor mean volume was ~150 mm^3, and tumor growth was monitored by bioluminescence imaging (**Figure 7c**) and calipers measuring (**Figure 7c**, insert) for further 10 days. CL4 and Gint4.T were administered intravenously individually or in combination at day 0, 3, 5, and 7. As shown, xenografts of CL4-treated and Gint4.T-treated mice grew at a significantly slower rate than xenografts of unrelated aptamer and vehicle control-treated mice, Gint4.T revealing more effective in inhibiting tumor growth than CL4. Further, reproducing the cell culture findings, the combined treatment of the two aptamers inhibited tumor growth (**Figure 7c**) and decreased the extent of EGFR and PDGFRβ tyrosine phosphorylation (**Figure 7d**) more efficiently than the treatment with each single agent.

The antitumor activity of Gint4.T was also confirmed by immunohistochemical staining for Ki-67 that revealed a strong reduction of the number of proliferating Ki-67-positive cells in tumors from Gint4.T-treated mice compared with tumors from mice vehicle-treated (**Figure 7e**). This inhibition of GBM-derived tumor growth was further enhanced when Gint4.T was used in combination with the CL4 aptamer (**Figure 7e**). Notably, the inhibiting effect of Gint4.T and CL4, both if administrated alone or in combination, culminated in a strong induction of caspase-3 cleaved fragments, a hallmark for induction of apoptosis[37] (**Figure 7f**).

At last, in order to exclude nonspecific immune activation in response to aptamer treatments, we observed that the expression levels of interferon-inducible IFIT1 (P56) and OAS1 genes were not increased in liver and spleen of treated animals (**Figure 7g**).

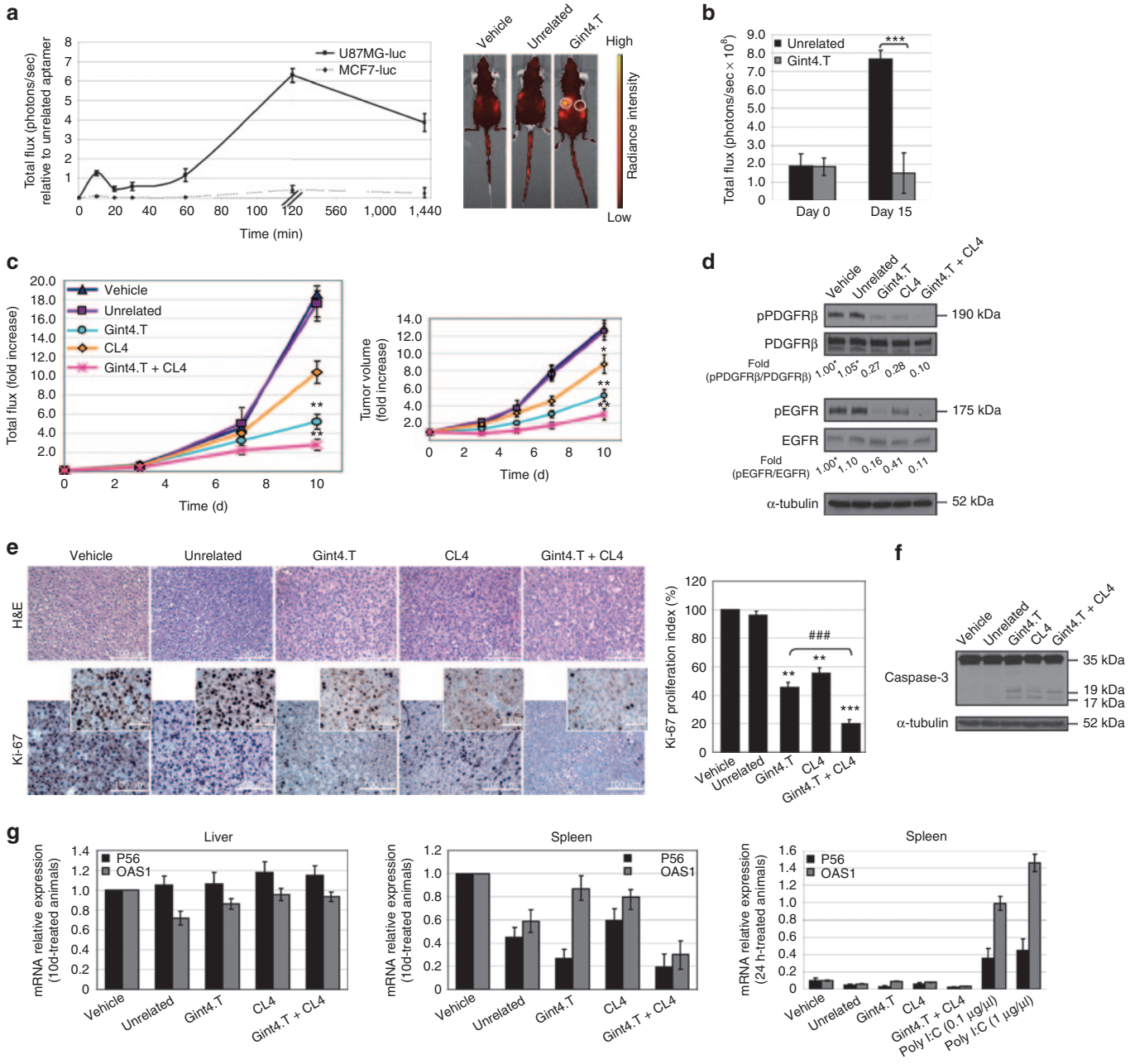

Figure 7 Gint4.T cell specificity *in vivo* and inhibition of tumor growth. (**a, b**) Mice bearing MCF7-luc (right-flank) and U87MG-luc (left-flank) xenografts (tumor mean volume: 60 mm^3) were injected intravenously either with Alexa-labeled Gint4.T or the unrelated aptamer, used as a negative control. (**a**) Aptamer amount was monitored by evaluating the intensity of fluorescent signal normalized for the bioluminescence and measured at the indicated times. Example shows fluorescence signal in one representative animal from each treatment group at 120 minutes after injection. The circles indicate where the tumors are located. (**b**) Tumor growth inhibition was measured as bioluminescence intensity (photons/second). ***$P < 0.0001$ ($n = 5$). (**c**) Mice bearing U87MG-luc xenografts (tumor mean volume, 150 mm^3) were injected intravenously with Gint4.T, CL4, Gint4.T plus CL4, unrelated aptamer or PBS (vehicle) at day 0, 3, 5, and 7. Tumor volumes were measured by bioluminescence and calipers (insert) and experimental raw data (expressed as fold increase) were interpolated with no curve fitting or regression analysis. **$P < 0.005$; *$P < 0.05$ relative to vehicle ($n = 5$). In (**b, c**) day 0 marks the start of treatments. (**d**) Immunoblot with anti-pPDGFRβ, anti-PDGFRβ, anti-pEGFR, anti-EGFR, and anti-α-tubulin antibodies of pooled lysates from recovered tumors. Values are expressed as relative to vehicle, arbitrarily set to 1 (labeled with asterisk). (**e**) Representative sections of tumors from each groups were stained with H&E and Ki-67 antibody, and Ki-67 proliferation index was calculated. ***$P < 0.0001$; **$P < 0.005$ relative to vehicle ($n = 5$). ###$P < 0.0001$. Scale bars are indicated. (**f**) Immunoblot with anti-caspase-3 and anti-α-tubulin antibodies of pooled lysates from recovered tumors. (**g**) P56 and OAS1 mRNAs expression relative to control arbitrarily set to 1. PBS and Poly (I:C)-treated mice were used as a negative and positive control, respectively. In (**a**–**c, e, g**) error bars depict means ± SD. EGFR, epidermal growth factor receptor; PDGFRβ, platelet-derived growth factor receptor β.

DISCUSSION

The crucial roles that PDGFRβ plays in tumorigenesis and tumor progression,[2,5] together with the absence of specific anti-PDGFRβ strategies in clinic, prompted us to develop more effective cancer drugs that specifically target PDGFRβ.

In this study, we prove that a 33 mer nuclease-stabilized RNA aptamer, named Gint4.T, acts as a neutralizing ligand for human PDGFRβ by inhibiting the receptor activity and downstream signaling in GBM cells and *in vivo*.

We show that Gint4.T dramatically inhibits *in vitro* GBM cell migration and blocks cell proliferation. The Gint4.T-dependent inhibition of cell proliferation is accompanied by a profound U87MG and T98G morphological transformation indicative of cell differentiation, which is further supported by the upregulation of glial differentiation marker GFAP. As recently emerged, targeting PDGFRβ (by siRNA and small inhibitors) in self-renewing tumorigenic glioma stem cells, attenuates glioma stem cell self-renewal and tumor growth and induces cell differentiation.[38,39] We are currently investigating whether Gint4.T could as well induce glioma stem cells differentiation and reduce the ability of these cells to propagate tumors *in vivo*.

Accordingly with the remarkable specificity of aptamers that can distinguish targets on the basis of subtle structural differences, as assessed by different biochemical approaches, Gint4.T binds to PDGFRβ at high affinity and discriminate between the β and the α receptors. It has been shown that human GBM have a different cellular distribution of PDGFRα and PDGFRβ and that the two receptors can stimulate distinct signals once activated by PDGFs.[38,40] Thus, Gint4.T may help to understand the role of individual PDGF receptors on glioma cellular biology and signaling. From a therapeutic standpoint, because no specific antagonists exist for discrimination of the two receptor subtypes, Gint4.T shows a great potential with respect to conventional pharmacological approaches for GBM treatment.

Interestingly, the co-activation of c-Met and PDGFR in GBM has been suggested to be one of the mechanisms that potentially leads to GBM resistance to anti-EGFR therapy and limits the efficacy of therapies targeting single receptors.[41,42] Here we first demonstrate that PDGFRβ induces transactivation of EGFR in GBM cells under basal condition that is further increased following PDGF-BB stimulation of the cells. Remarkably, we found that the treatment of the cells with the anti-PDGFRβ Gint4.T aptamer inhibits PDGF-BB-induced EGFR transactivation. This is in good agreement with previous reports showing that Imatinib blocks migration and invasion of medulloblastoma cells by concurrently inhibiting activation of PDGFRβ and transactivation of EGFR.[29] Further, the combination of Gint4.T and the anti-EGFR CL4 aptamer inhibits the PDGF-BB-induced EGFR transactivation at a higher extent compared with single aptamer treatment. Accordingly, the use of the two aptamers in combination causes a drastic inhibition of cell viability even stronger than that caused by high concentration of approved PDGFRβ and EGFR inhibitors (imatinib, gefitinib, and cetuximab) and of TMZ, the benchmark agent for the management of GBM. Further, almost 80% inhibition of cell viability was observed by combined treatment of GBM cell lines with the two aptamers and TMZ.

Importantly, the potent inhibitory effect of Gint4.T extended to a xenograft model of GBM. Indeed Gint4.T, administrated with a single intravenous injection in mice bearing small tumors (60 mm^3), induces a remarkable tumor growth inhibition, thus in good agreement with the effective block of cell proliferation observed *in vitro*. Synergistic tumor growth inhibition is obtained by the combination treatment of Gint4.T and CL4 aptamers in mice bearing large tumors (150 mm^3), again, confirming the relevance of the cell culture data to *in vivo* models. Notably, no non-specific immunostimulatory effects were observed with CL4 and Gint4.T aptamers, as expected by the use of chemically-modified nucleotides.[43,44]

Furthermore, we show that when systemically administrated Gint4.T is able to discriminate between target tumors (U87MG, expressing the related receptor) and nontarget tumors which do not express PDGFRβ (MCF7), thus providing exquisite aptamer cell specificity not only in cell culture but also *in vivo*.

Moreover, the potential impact of Gint4.T as therapeutic, is strongly highlighted by the finding that the aptamer not only binds to the PDGFRβ at high affinity and inhibits its activity, but also rapidly and specifically internalizes within the target cells. Thus, based on the recent development of aptamer-siRNA/miRNA bioconjugates,[19,20] Gint4.T appears as a prime candidate tool for delivering cell-selective gene knockdown, the major challenge for translating RNAi into therapy.

Whether a Gint4.T fraction sufficient for therapeutic benefit could penetrate on its own the blood–brain barrier after systemic injection,[45] remains to be determined. Alternatively, Gint4.T could breach the blood–brain barrier once conjugated to nanoparticles. Indeed, paclitaxel loaded nanoparticles cross the blood–brain barrier and reduce GBM growth in animal models,[46–48] suggesting the potential of increasing the affinity and specificity of these molecules through conjugation to Gint4.T aptamer.

Collectively, our study represents an initial development of novel aptamer-based therapies that in combinations with conventional therapeutics will allow to face with human glioblastomas.

MATERIALS AND METHODS

Cell lines and transfection. Growth conditions for human GBM U87MG and T98G, epidermoid carcinoma A431, non–small-cell lung carcinoma A549 (American Type Culture Collection, Manassas, VA) were previously reported.[9] U87MG-luc2 (herein indicated as U87MG-luc) and human breast MCF7-luc-F5 (herein indicated as MCF7-luc) (Caliper Life Sciences, Hopkinton, MA) were grown following the provider indications.

Primary cell cultures from GBM specimens were derived and grown as described previously.[11]

For PDGFRβ gene silencing, U87MG and T98G cells (3.5×10^5 cells per 6-cm plate) were transfected with shRNA PDGFRβ or shRNActrl (2 μg; Open Biosystems, Huntsville, AL) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM I reduced serum medium (Invitrogen). After 5-hours incubation, complete culture medium was added to the cells and incubation was prolonged up to 72 hours.

Cell-internalization SELEX and aptamers. Following 14 rounds of selection performed onto U87MG cells as previously described,[11] the enriched pool was incubated onto U87MG for 30 minutes (first internalization round) and 15 minutes (second internalization round) at 37 °C and unbound aptamers were removed by five washes with Dulbecco's modified Eagle medium (DMEM) serum free. To remove surface-bound aptamers,

target cells were treated with 0.5 μg/μl proteinase K (Roche Diagnostics, Indianapolis, IN) for 30 minutes, washed with DMEM serum free and internalized RNA aptamers were then recovered by RNA extraction and RT-PCR as described.[11,12]

Gint4.T, 5′ FAM-labeled Gint4.T, CL4[9] and the unrelated 2′F-Py RNAs were purchased from ChemGenes corporation (Wilmington, MA).

Gint4.T aptamer: 5′UGUCGUGGGGCAUCGAGUAAAUGCAAU UCGACA3′.

The scrambled sequence of CL4 aptamer[9] has been used as a negative control; herein indicated as unrelated:

5′UUCGUACCGGGUAGGUUGGCUUGCACAUAGAACGUGU CA3′.

For *in vivo* experiments, aptamers have been internal-labeled with Alexa Fluor 647 fluorescent probe following the provider indications (Invitrogen).

Before each treatment, the aptamers were subjected to a short denaturation-renaturation step (85 °C for 5 minutes, snap-cooled on ice for 2 minutes, and allowed to warm up to 37 °C).

For cell incubation longer than 24 hours, the aptamer treatment was renewed each day and the RNA concentration was determined to ensure the continuous presence of at least 200 nmol/l concentration, taking into account the 6 hours-half-life of the aptamer in 10% serum.

Binding assays. Aptamer binding to cells was performed as described.[9] Filter binding analysis with EC-PDGFRα and EC-PDGFRβ (R&D Systems, Minneapolis, MN) was performed by incubating 1 nmol/l of radiolabeled aptamers with 1, 3.2, 10, 32, 100, 320, and 1,000 nmol/l of EC-PDGFRβ or EC-PDGFRα as described.[9]

To check the endocytosis rate, 100 nmol/l radiolabeled Gint4.T or the unrelated aptamer was incubated onto U87MG cells for increasing incubation times (from 15 minutes up to 2 hours) and at desired times, cells have been treated with 0.5 μg/μl proteinase K (Roche Diagnostics) at 37 °C. Following 30-minutes treatment, the amount of RNA internalized has been recovered and counted.

Immunoprecipitation, Immunoblot, and Immunofluorescence analyses. Cell extracts, immunoprecipitation, and immunoblot were performed as described.[49] The primary antibodies used were: anti-phospho-PDGFRβ (Tyr771, indicated as pPDGFRβ), anti-PDGFRβ, anti-phospho-EGFR (Tyr1068, indicated as pEGFR), anti-EGFR, anti-phospho-44/42 MAPK (D13.14.4E, indicated as p-Erk), anti-phospho-Akt (Ser473, indicated as pAkt), anti-Akt, anti-caspase 3 (Cell Signaling Technology Inc., Danvers, MA); anti-Erk1 (C-16; Santa Cruz Biotechnology, Santa Cruz, CA); anti-α-tubulin (DM 1A; Sigma, St. Louis, MO). RTK antibody arrays (R&D Systems) were performed as recommended. Densitometric analyses were performed on at least two different expositions to assure the linearity of each acquisition using ImageJ (v1.46r). Blots shown are representative of at least four independent experiments.

To assess the effect of the aptamers on ligand-dependent PDGFRβ and EGFR activation, cells (1.5×10^5 cells per 3.5-cm plate) were serum-starved overnight, pretreated with 200 nmol/l aptamer for 3 hours and then stimulated for 5 minutes with 50 ng/ml PDGF-BB or EGF (R&D Systems) in the presence of 200 nmol/l aptamer.

For immunofluorescence, cells grown on glass coverslips were treated at different incubation times with 2.5 μmol/l FAM-Gint4.T, washed five times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. The coverslips were washed three-times in PBS and then blocked in PBS, 1% bovine serum albumin for 30 minutes. Cells were incubated with anti-PDGFRβ (R&D Systems), anti-EEA1 and anti-LAMP1 (Abcam, Cambridge, MA) diluted in PBS, 1% bovine serum albumin for 1 hour at 37 °C. Coverslips were washed three-times with PBS and treated with Alexa Fluor 568 Goat Anti-Rabbit IgG (H+L) (Invitrogen) for 30 minutes at 37 °C. Coverslips were washed, mounted with Gold antifade reagent with DAPI (Invitrogen) and the cells were visualized by confocal microscopy. For co-localization experiments of Gint4.T with EEA1 and LAMP1, cells have been permeabilized with PBS, 0.5% Triton X-100 for 15 minutes at room temperature before blocking. The immunofluorescence images have been analyzed for quantization of colocalized spots using the ImageJ plugin Coloc2 and Manders' coefficients (M1 and M2)[50] have been calculated.

Cell viability and proliferation. Cell viability was assessed as reported.[10] For combined treatment with Gint4.T, we used Gefitinib (LC Laboratories, Woburn, MA), Cetuximab (provided by Prof. G. Tortora), Imatinib mesylate (Santa Cruz Biotechnology), TMZ (Sigma).

For cell proliferation assay, T98G and U87MG cells (2×10^4 cells/well in 24-well plates) were mock-treated or treated for 24 and 48 hours with Gint4.T or the unrelated aptamer as previously described.[9] To assess cell-cycle analysis, anti-5-bromodeoxyuridine (BD Biosciences, Heidelberg, Germany) incorporation was performed according to the manufacturer's protocol and then analyzed by fluorescence activated cell sorting (FACS).

Cell migration. Transwell migration assay on T98G and U87MG cells were performed as described[10] by using PDGF-BB (50 ng/ml) or 10% fetal bovine serum as inducers of migration. For wound healing assay, T98G and U87MG cells were plated in six-well plates and grown to confluence. After serum starvation overnight in the absence or in the presence of 200 nmol/l Gint4.T or the unrelated aptamer, cells were scraped to induce a wound. Culture medium with 0.5% fetal bovine serum with/without treatment with aptamers was added and the wounds were observed using phase-contrast microscopy. The extent of wound closure was quantitated by measuring the wound areas obtained from 10 independent fields using ImageJ (v1.46r).

Reverse transcription-PCR analysis. RNA was extracted by TRiZol (Invitrogen) and 1 μg total RNA was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and the resulting cDNA fragments were used as PCR templates.

Primers used were: GFAP, Fwd 5′GAGTCCCTGGAGAGGCAGAT3′, Rev 5′CCTGGTACTCCTGCAAGTGG3′; β-actin, Fwd 5′CAAGAGAT GGCCACGGCTGCT3′, Rev 5′TCCTTCTGCATCCTGTCGGCA3′. Amplifications were performed by using the following conditions: GFAP, 30 cycles: 1 minute at 95 °C, 1 minute at 61 °C, and 1 minute at 72 °C; β-actin, 15 cycles: 30 seconds at 95 °C, 30 seconds at 57 °C, and 1 minute at 72 °C. Densitometric analyses was performed by using ImageJ (v1.46r).

Animal model studies. Athymic CD-1 nude mice (*nu/nu*) were housed in a highly controlled microbiological environment, thus to guarantee specific pathogen free conditions.

For the *in vivo* evaluation of Gint4.T tumor-binding specificity, mice were injected subcutaneously with 5×10^6 U87MG-luc and 8×10^6 MCF7-luc cells on the left and right sides of the animal, respectively. When tumor mean volume reached 60 mm^3, mice (five for group) were treated by caudal vein injection with 1,600 pmol in 100 μl (1.04 mg aptamer/kg mean body-weight) Alexa Fluor 647-labeled Gint4.T or unrelated aptamer. For imaging analysis, the CALIPER IVIS Spectrum has been used, and the images were processed by using Caliper living image software 4.1.

To follow tumor growth inhibition, mice were injected subcutaneously with 5×10^6 U87MG-luc cells. When tumor mean volume reached 150 mm^3, mice (five for group) were treated by caudal vein injection with 1,600 pmol of Gint4.T, CL4, Gint4.T plus CL4, and unrelated aptamer. Saline (PBS) treated animals were used as control. Tumor growth was measured with calipers or bioluminescence. Animals were sacrificed following 10 days. All tumors were recovered, processed for protein and pooled for immunoblot.

To determine immune response, livers, and spleens of treated animals at 24 hours and 10 days following aptamer treatment were excised, lysed for RNA extraction and pooled. As a positive control, spleens from mice (five for group) treated for 24 hours with Poly (I:C; 10 μg and 100 μg in 100 μl, Sigma) were processed for total RNA. P56

and OAS1 mRNAs were analyzed by RT-qPCR as reported.[44] GAPDH expression was used for normalization of the qPCR data. Primers used were: P56, Fwd 5′TCAAGTATGGCAAGGCTGTG3′, Rev 5′GAGGC TCTGCTTCTGCATCT3′; OAS1, Fwd 5′ACCGTCTTGGAACTGGTC AC3′, Rev 5′ATGTTCCTTGTTGGGTCAGC3′; GAPDH, Fwd 5′AAC TTTGGCATTGTGGAAGG3′,Rev5′ACACATTGGGGGTAGGAACA3′.

Histology and immuno-histochemistry. Histological examinations for hematoxylin and eosin (H&E, BDH Laboratory Supplies) and Ki-67 was performed as described.[10] Ki-67 proliferation index was calculated as the percentage of Ki-67 positive cells/total cell count for 10 randomly selected 20× microscopic fields.

Statistics. Statistical values were defined using Graphpad Prism 6. A *P* value of 0.05 or less was considered significant.

Ethics Statement. All the animal procedures were approved by the Ethical Committee for the Animal Use (CESA) of the Istituto di Ricerche Genetiche Gaetano Salvatore (IRGS) and where communicated to the national authorities accordingly with national and European rules. Primary tumor cultures were derived from surgical biopsies. The study protocol was approved by the local Ethics Committee of the University of Cologne. Written informed consent was acquired prior to surgery from every patient for further studies on primary glioma cultures.

SUPPLEMENTARY MATERIAL

Figure S1. Gint4.T specifically interacts with PDGFRβ.
Figure S2. Gint4.T reduces [3H]-thymidine incorporation.
Figure S3. Gint4.T cooperates with CL4 in preventing EGFR transactivation in U87MG cells.
Figure S4. Effect of Gefitinib, Cetuximab and Imatinib on cell viability.
Figure S5. Combined effect of Gint4.T and CL4 on cell viability.

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