CANCER

CRISPR-enhanced engineering of therapy-sensitive cancer cells for self-targeting of primary and metastatic tumors

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Tumor cells engineered to express therapeutic agents have shown promise to treat cancer. However, their potential to target cell surface receptors specific to the tumor site and their posttreatment fate have not been explored. We created therapeutic tumor cells expressing ligands specific to primary and recurrent tumor sites (receptor self-targeted tumor cells) and extensively characterized two different approaches using (i) therapy-resistant cancer cells, engineered with secretable death receptor–targeting ligands for "off-the-shelf" therapy in primary tumor settings, and (ii) therapy-sensitive cancer cells, which were CRISPR-engineered to knock out therapy-specific cell surface receptors before engineering with receptor self-targeted ligands and reapplied in autologous models of recurrent or metastatic disease. We show that both approaches allow high expression of targeted ligands that induce tumor cell killing and translate into marked survival benefits in mouse models of multiple cancer types. Safe elimination of therapeutic cancer cells after treatment was achieved by co-engineering with a prodrug-converting suicide system, which also allowed for real-time in vivo positron emission tomography imaging of therapeutic tumor cell fate. This study demonstrates self-tumor tropism of engineered cancer cells and their therapeutic potential when engineered with receptor self-targeted molecules, and it establishes a roadmap toward a safe clinical translation for different cancer types in primary, recurrent, and metastatic settings.

INTRODUCTION

Combined advances in the fields of biomedical research, drug development, medical imaging, and surgical techniques have translated into considerably improved outcomes of cancer patients over the last decades (*1*). The resulting impact of therapy improvement on even highly malignant tumors, which have previously been considered "untreatable," including lung cancer and melanoma, has recently led to excitement in the medical and scientific oncology fields. Nevertheless, numerous local and systemic cancer types, as well as many forms of metastatic disease, remain ultimately fatal, and treatment regimes in end-stage disease, especially in the recurrent setting, often lack evidence-based guidelines.

One of the major treatment hurdles of advanced-stage cancer is localized and distant tumor cell metastasis, resulting from vascular infiltration or penetration of anatomic boundaries (*2*, *3*). A growing body of evidence suggests that tumor progression at this stage may be enhanced by circulating cancer cells' ability of "self-seeding," a process involving cell dissemination into the vascular system away from a primary or metastatic tumor, followed by the cells rehoming to the site of origin (*4*). In recent years, several studies have tried to repurpose the tumor cells' self-homing properties for self-targeted delivery of anticancer agents to primary tumor sites. The most promising approaches included using tumor cells as a vehicle for delivery of oncolytic viruses (*5*, *6*), using engineered tumor cells expressing suicide genes to transfer death signals to neighboring tumor cells (bystander effect) (*7*, *8*), and targeting the tumor microenvironment by engineering cancer cells to express therapeutic agents that influence tumors' neovascular endothelium (*9*). Naturally, engineering tumor cells for self-targeted anticancer treatment is a double-edged sword: Premature cell death due to self-toxicity of introduced transgenes may limit their antitumor efficacy, whereas long-term survival of therapeutic cells may potentially cause secondary tumor formation, even if they are initially efficacious in treating the targeted self-tumor site. Consequently, to our best knowledge, none of the previous studies that involved rehoming of tumor cells followed a truly receptor-specific self-targeted approach, likely to avoid autocrine toxicity.

Much of the abovementioned progress recently seen in cancer therapies is ultimately a result of advances in the field of receptor-targeted therapies (*10*, *11*), where disease- or cancer-specific (over-) expression of cell surface receptors is targeted to modulate downstream pathways involved in functions such as proliferation/survival, cell cycle regulation, metabolism, angiogenesis, inflammation, or immune response. To improve therapy success, it has therefore become increasingly common to categorize patients according to their disease-specific (receptor) gene expression profiles. In cancer therapies, targeting proliferative or triggering proapoptotic receptor-mediated pathways has proven especially attractive (*12*–*14*). In previous investigations, the feasibility of combining receptor-targeted treatments with cell-based therapies and associated benefits have been shown in distinct tumor settings (*15*–*17*). Here, we screened a panel of different cancer types for their responses to surface receptor ligands/antagonists that target cell proliferation and death pathways and identified the secretory variant of TNF (tumor necrosis

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factor)–related apoptosis-inducing ligand [S-TRAIL (hereinafter ST)] as a promising antitumor agent because of its ability to strongly induce apoptosis in a wide variety of different cancer types, without causing considerable toxicity in normal cells (*18*–*20*).

Building on the above findings, we hypothesized that, by applying recent advances in bioengineering, we could simultaneously prevent receptor-mediated autocrine toxicity and combine the self-homing properties of cancer cells with the benefits of receptor-targeted treatment and inducible suicide system–related bystander effect/clearance. To test the clinical feasibility of this approach in first-line treatment of primary tumors, as well as in the therapy of recurrent and metastatic disease, we explored two different approaches: (i) an "off-the-shelf" allogeneic option for treatment of primary tumors with pre-engineered, therapy-resistant tumor cells, which, in a clinical setting, would be selected to match the patients' human leukocyte antigen (HLA) phenotype (Fig. 1A), and (ii) an "autologous" approach for treatment in the recurrent setting, which uses clustered regularly interspaced short palindromic repeats (CRISPR) technology to switch the treatment response phenotype of the therapeutic cells from therapy-sensitive to therapy-resistant before engineering with therapeutic molecules (Fig. 1B). We demonstrate that these approaches allow high expression of proapoptotic molecules without inflicting autocrine toxicity, which, in combination with self-homing and suicide system–mediated bystander effect/clearance, translates into marked survival benefits in mouse models of multiple cancer types.

RESULTS

Death receptor 4/5–targeted therapy demonstrates potent anticancer efficacy

To investigate the therapeutic efficacy of receptor-targeted molecules, we tested a panel of different cancer cell lines for their susceptibility to treatment with cell surface receptor ligands/antagonists targeting CD36 (thrombospondin-1), epidermal growth factor receptor (EGFR) (EGFR-blocking nanobody), heterodimeric interleukin-20 receptor (IL-20R) composed of subunits α and β (IL-24), and death receptors (DRs) (TRAIL) (Fig. 1C). A nodular (n) and an invasive (i) GBM cell line (Gli36Δ-EGFR and GBM8, respectively) were screened and found to be DR ligand (DR_L)–sensitive (s) (herein referred to as sGBMn and sGBMi). The following non-GBM cancer lines were also identified as DR_L-sensitive: PC3 (sPCm), Jurkat (sTCL), HCT116 (sCC), and MDA231-BrM2a (sBCm). In comparison to the other tested ligands, the DR_L TRAIL was the most effective agent, with potential to eliminate treated cell lines at 72 hours after treatment (Fig. 1C). In addition to the above DR_L-sensitive (s) cell lines, three DR_L-resistant (r) GBM cell lines were identified (GBM23, GBM64, and GBM46, herein referred to as rGBMi1, rGBMi2, and rGBMi3) (Fig. 1D). On the basis of these findings, DR4 and DR5 were chosen as the most suitable candidates to further investigate the therapeutic potential of receptor self-targeted therapies.

DR_L-resistant tumor cells can be engineered to express ST and have antitumor effects against DR_L-sensitive cells

To test whether DR_L-resistant glioblastoma cells (rGBM) can serve as a vehicle for DR_L delivery toward DR_L-sensitive tumors (sGBM) (Fig. 2A), we first analyzed DR expression of different rGBMs and sGBMi. Reverse transcription polymerase chain reaction (RT-PCR) analysis demonstrated that sGBMi expresses *DR4/5* (fig. S1A), which are necessary to activate TRAIL-induced apoptosis. Lack of *DR* expression was seen only in one of the three DR_L-resistant lines (fig. S1A), indicating that cells with preserved *DR* expression can nonetheless exhibit TRAIL resistance, likely due to activation of antiapoptosis mechanisms. Next, we tested the feasibility of expression and secretion of DR_L from invasive rGBMs (rGBMi) by engineering rGBMi1 and rGBMi2 with a secretable and potent variant of DR_L (ST) or green fluorescent protein (GFP) (control). Coculture of rGBMi1-ST and rGBMi2-ST with sGBMi engineered with lentivirus (LV) expressing firefly luciferase (Fluc)–mCherry (sGBMi-FmC) showed robust killing of sGBMi-FmC cells over time, mediated by caspase-induced apoptosis (Fig. 2, B to D). Furthermore, we transduced rGBMi1 and rGBMi2 with LV encoding a fusion variant of ST with the optical reporter Renilla luciferase [Rluc(o) (Rl)] (*21*). Both rGBMi1-Rl-ST and rGBMi2-Rl-ST demonstrated continued secretion of ST into culture medium without autocrine toxicity of the therapeutic cells, as demonstrated by a time-dependent increase of bioluminescent imaging (BLI) signal intensity (fig. S1B). Coculture assays of rGBMi1-Rl-ST or rGBMi2-Rl-ST with sGBMi-FmC further showed robust killing of sGBMi-FmC cells over time (fig. S1C). Together, these data show that engineered rGBMs can continuously secrete ST without inflicting autocrine toxicity and have the potential to serve as a delivery vehicle of ST toward DR_L-sensitive tumor cells. Because of the superior therapeutic efficacy observed for rGBMi2-ST compared to rGBMi1-ST, we selected rGBMi2-ST for further study.

Prodrug-converting suicide system allows selective elimination of therapeutic rGBMs, and the bystander effect increases therapeutic efficacy of rGBM-ST

To ensure safety, a measure to efficiently eliminate therapeutic rGBMs had to be incorporated into our approach. Thus, we next engineered rGBMi2 to either express a prodrug-converting enzyme (HSV-TK) or coexpress ST, HSV-TK, and diagnostic GFl (rGBMi2-TK and rGBMi2-ST-TK-GFl, respectively) and tested the efficacy of HSV-TK/GCV–induced clearance of therapeutic rGBM in vitro. GCV treatment resulted in the dose- and time-dependent elimination of rGBMi2-TK cells in comparison to controls (Fig. 2E). In vivo noninvasive monitoring of intracranial rGBMi2-TK-GFl tumor growth in mice demonstrated that GCV treatment eliminated therapeutic rGBMi2-TK-GFl as compared to saline-treated control (Fig. 2F). In vivo GCV-induced cell clearance was further demonstrated in a clinically relevant 18F-FHBG PET imaging model using rGBMi2 co-engineered with ST and TK (rGBMi2-ST-TK) (Fig. 2G). After 10 days of daily GCV treatment, the tumor-specific PET signal was no longer visible intracranially, with only unspecific abdominal signal due to tracer clearance (*22*) remaining. Together, these results indicate that therapeutically engineered rGBMs can be selectively eliminated using HSV-TK/GCV in vitro and in vivo and additionally demonstrate that therapeutic cell fate can be clinically monitored using PET imaging.

To examine the bystander effect of HSV-TK/GCV, we tested the therapeutic efficiency of rGBMi2-ST-TK or rGBMi2-GFP via coculture with the DR_L-sensitive invasive GBM cell line sGBMi-FmC. A significant reduction in sGBMi-FmC viability was seen when cocultured with rGBMi2-ST-TK within 24 hours ($P < 0.05$, with and without GCV), and at 96 hours, sGBMi-FmC cells were completely eliminated when cocultures were treated with GCV and nearly completely eliminated under non–GCV-treated conditions (Fig. 2H).

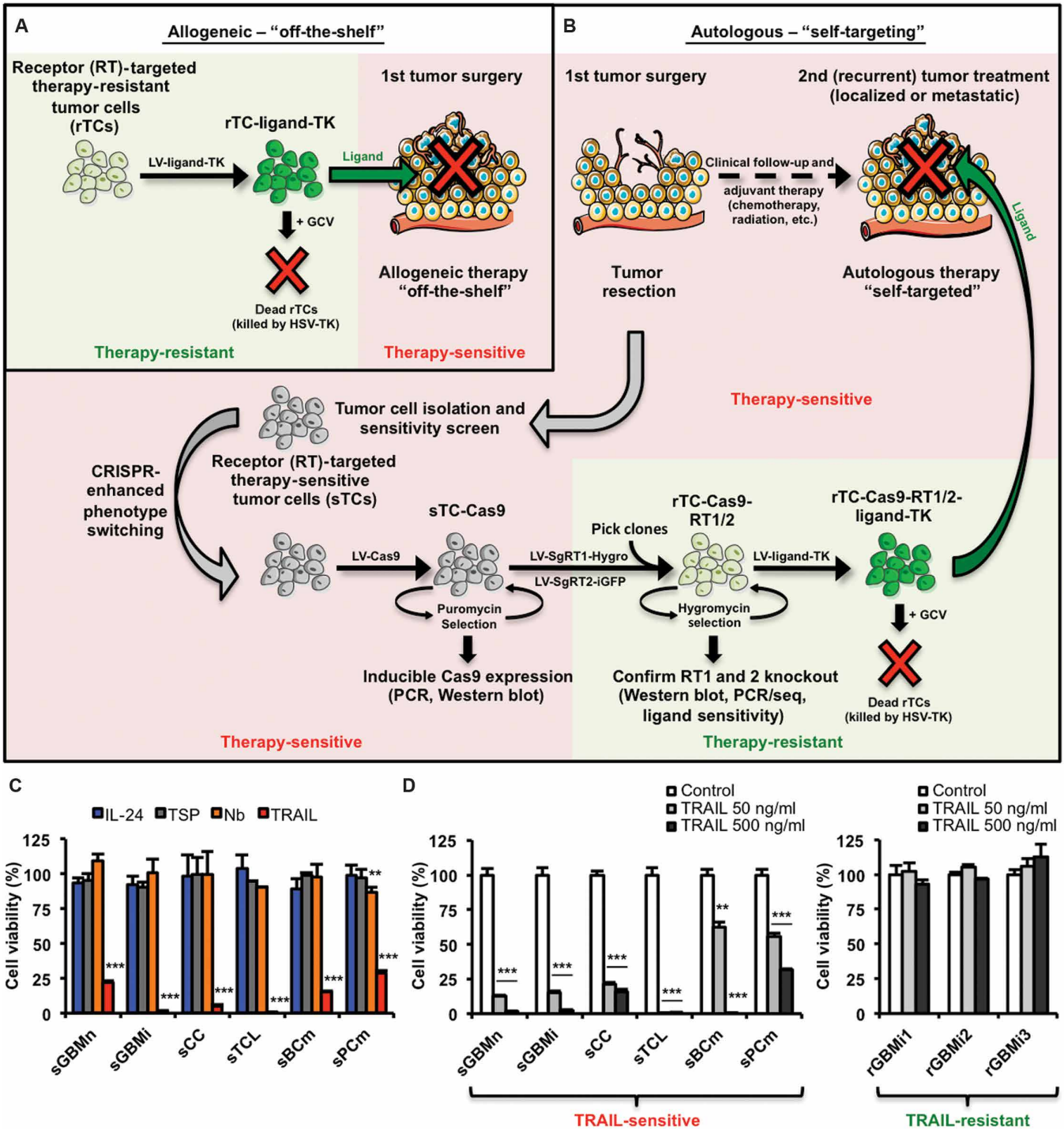

Fig. 1. Concept of study and identification of DR_L for cancer cell–based self-targeting therapies. (**A**) Allogeneic approach: Cancer cells resistant to receptor-targeted therapies can be used off-the-shelf for delivery of receptor ligands toward allogeneic cancers with ligand-sensitive phenotypes in the setting of primary treatment. Because they are co-engineered with a prodrug-activatable suicide system [herpes simplex virus thymidine kinase (HSV-TK)], therapeutic cancer cells can be eliminated after therapy using ganciclovir (GCV). (**B**) Autologous approach: Cancer cells harvested from patients during initial surgery and identified as sensitive to receptor-targeted therapies can be CRISPR-engineered to knock out the target receptors. Receptor knockout (KO) results in therapy resistance and allows engineering with receptor ligands and delivery toward autologous self-tumor sites in the setting of recurrence. (**C**) A panel of primary and metastatic cancer cell lines was treated with conditioned medium containing secretable variants of different receptor-targeted molecules, and viability was assessed 72 hours after treatment ($n = 3$ technical replicates). TSP, thrombospondin-1; Nb, nanobody. (**D**) Cancer cell lines were tested with varying concentrations of TRAIL to quantify TRAIL sensitivity ($n = 3$ technical replicates). TRAIL-sensitive (s) or -resistant (r), nodular (n) and invasive (i) glioblastoma (GBM), colon cancer (CC), T cell leukemia (TCL), metastatic breast cancer (BCm), and metastatic prostate cancer cells (PCm) were tested. All in vitro experiments were repeated at least twice. Means ± SD are shown. *P* values by unpaired *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$.

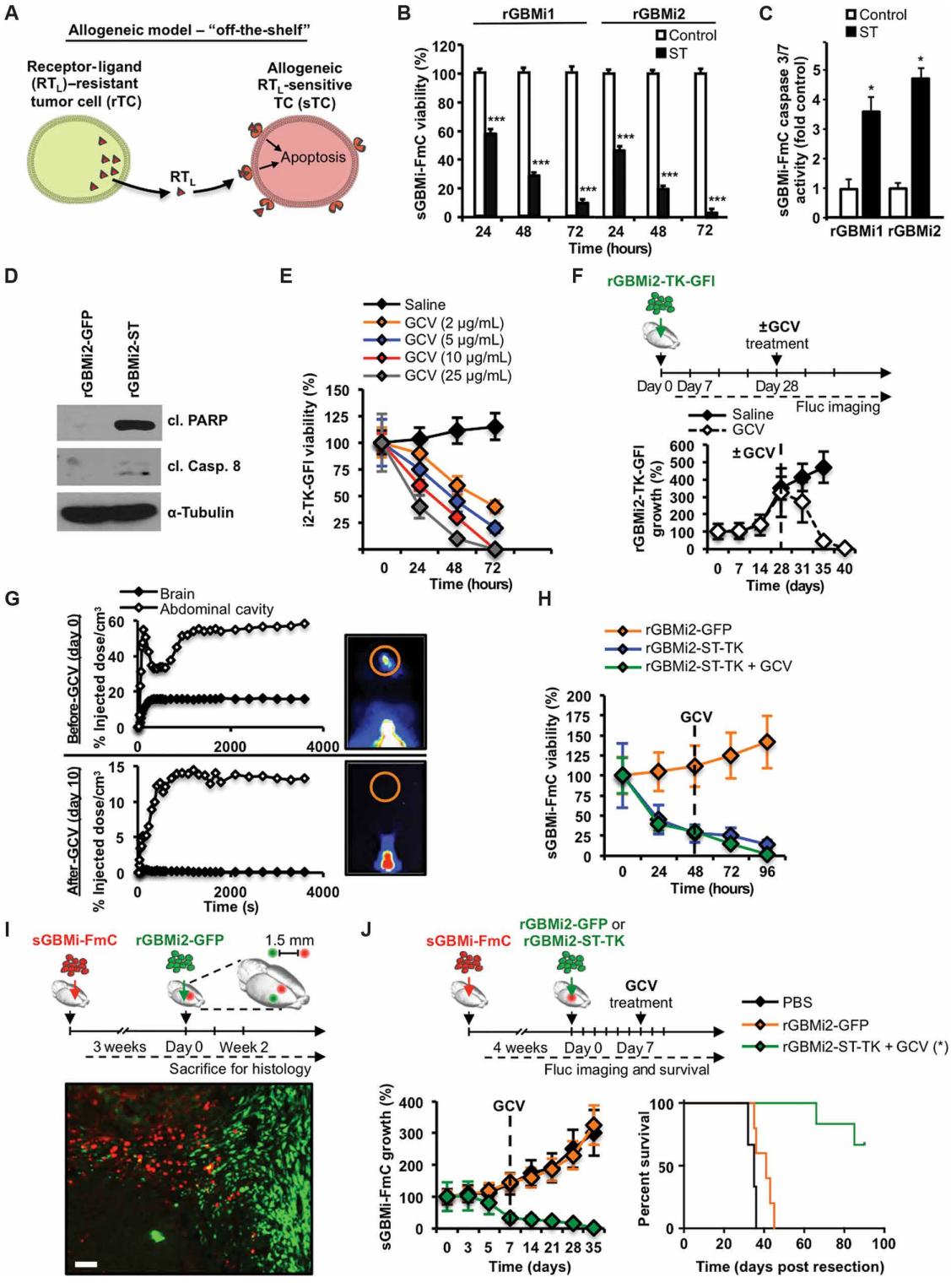

Fig. 2. Off-the-shelf therapy using DR_L-resistant self-targeting tumor cells. (**A**) Concept of off-the-shelf approach: Receptor ligand (RT_L)–resistant tumor cells can be engineered to secrete RT_L, which can induce cell death in RT_L-sensitive allogeneic cancer cells. (**B**) Cell viability of DR_L sGBMi-FmC cells during time course coculture with DR_L-resistant cancer cell lines (rGBMi1 or rGBMi2) expressing secretable DR_L ST ($n = 3$ technical replicates). (**C**) Caspase 3/7 activity in sGBMi-FmC 8 hours after start of coculture with ST-expressing rGBMs ($n = 2$ technical replicates). (**D**) Western blot analysis of poly(adenosine 5′-diphosphate–ribose) polymerase (PARP), caspase 8 cleavage, and α-tubulin in DR_L sGBMi-FmC 8 hours after the start of coculture with either GFP or ST-expressing rGBM. (**E**) In vitro cell viability ($n = 3$ technical replicates) and (**F**) in vivo growth of DR_L-resistant cancer cells (rGBMi2) co-engineered with prodrug-converting enzyme HSV-TK and GFP-Fluc (GFI) in the absence or presence of GCV over time ($n = 5$ per group). (**G**) In vivo positron emission tomography (PET)–based monitoring of rGBMi2 co-engineered with ST and HSV-TK (rGBMi2-ST-TK) with and without GCV treatment ($n = 3$ mice per group). (**H**) Evaluation of bystander effect of rGBMi2-ST-TK cells on cocultured sGBMi-FmC cells over time ($n = 3$ technical replicates). (**I**) sGBMi2-GFP cells (5×10^5) were implanted at a distance of 1.5 mm from established sGBMi-FmC tumors. Representative fluorescence photomicrograph shows cell populations 2 weeks after injection of sGBMi2-GFP ($n = 2$ mice). Scale bar, 200 μm. (**J**) Top: Experimental outline for testing efficacy of rGBMi2-ST-TK in mice bearing intracranial sGBMi-FmC tumors. Bottom: Estimate of relative tumor volume in treatment groups based on Fluc signal of sGBMi-FmC–bearing mice (left) and the respective Kaplan-Meier survival curves (right); $n = 3$ for phosphate-buffered saline (PBS), $n = 5$ for rGBMi2-GFP, and $n = 6$ for rGBMi2-ST-TK + GCV. All in vitro experiments were repeated at least twice. Means ± SD are shown for in vitro experiments and means ± SEM are shown for in vivo experiments. *P* values by unpaired *t* test (B and C) or Mantel-Cox (log-rank) test (**J**), $^*P < 0.05$, $^{***}P < 0.001$.

These results demonstrate that the introduction of HSV-TK may not only serve to eliminate therapeutic cells but also further increase the therapeutic efficacy of ST-mediated apoptosis via additional bystander effect. However, likely because of the highly TRAIL-sensitive phenotype of sGBMi-FmC, the observed difference between GCV-treated and non–GCV-treated conditions was not statistically significant ($P > 0.05$ at the 96-hour time point).

Therapeutic rGBMs show efficacy against DR_L sGBMs

To investigate the in vivo growth pattern of therapeutic rGBMs and their potential for targeting of allogeneic DR_L sGBMs, mice bearing sGBMi-FmC tumors were implanted with rGBMi2-GFP at a distance of 1.5 mm from the sGBMi-FmC tumor site. Fluorescence imaging of mouse brain sections showed extensive tracking of invading sGBMi-FmC cells by rGBMi2-GFP, suggesting rGBMi2-GFP migration toward preimplanted sGBMi-FmC in vivo (Fig. 2I). Next, mice with established sGBMi-FmC tumors were injected intratumorally with either rGBMi2-GFP (control) or therapeutic rGBMi2-ST-TK cells. BLI revealed a marked reduction of sGBMi-FmC tumor sizes in the rGBMi2-ST-TK group, indicating a robust induction of cell death in sGBMi-FmC cells (Fig. 2J). After GCV treatment, the sGBMi-FmC tumors regressed further, and the therapeutic response translated into significant survival benefits for the mice ($P < 0.05$; Fig. 2J). Together, these data indicate that therapeutically armed DR_L rGBM cells can efficiently deliver cell surface receptor-specific antitumor ligands toward established sGBM and mediate efficacy in vivo.

Cas9-mediated DR-KO in tumor cells confers resistance to DR-targeted therapy

To translate the above findings into a clinical scenario that would potentially allow the use of patients' own (autologous) tumor cells for therapeutic self-targeting in the event of tumor recurrence (Figs. 1B and 3A), we first engineered DR_L-sensitive tumor lines of various cancer types with CRISPR-associated protein 9 (Cas9) RNA-guided DNA endonuclease. Inducible Cas9 expression was confirmed by Western blotting (Fig. 3B) or RT-PCR (fig. S2A). Cas9 tumor lines were then engineered with single guide RNA (SgRNA) expression vectors targeting *DR4*, *DR5*, or both receptors to create DR-KO cell lines. Target efficacy of SgRNAs was semiquantitatively evaluated by Western blotting for DR4 and DR5 in mixed populations (fig. S2B), followed by clonal selection and screening of individual clones for DR-KO status with Western blotting (showing DR4, DR5, and DR4/5 KO of sGBMn; Fig. 3C). In addition, flow cytometry analysis confirmed marked reduction in surface expression of DR4, DR5, or DR4/5 (Fig. 3D), and genomic DNA sequencing identified indel mutations at SgRNA-targeted exonic gene segments of DR4, DR5, and DR4/5 double-KO clones (Fig. 3E). Together, these results demonstrate successful CRISPR engineering of DR_L sGBM cells to knock out DR4, DR5, or both DR4 and DR5.

Next, we tested whether DR-KO correlates with a DR_L-resistant phenotype in vitro. Cell viability assay of DR4/5 intact sGBMn cells (GBMn-control and GBMn-Cas9), as well as their DR4, DR5, and DR4/5 DR-KO clones, identified DR5 as the more dominant receptor in TRAIL-induced apoptosis (Fig. 3F). However, complete resistance to DR_L-mediated apoptosis was only observed in the third double-KO clone, GBMn-DR4/5-3 (Fig. 3F). Cleaved caspase 8 and cleaved PARP analysis of ST-treated sGBMn cells further confirmed reduced activation of TRAIL-mediated apoptosis in sGBMn-DR single-KO clones (sGBM-DR4-2 and sGBM-DR5-1) compared to DR-intact sGBMn cells and the fully resistant phenotype in the rGBMn-DR4/5 double-KO clone rGBMn-DR4/5-3 (herein referred to as $rGBMn^{DR4/5}$; Fig. 3G).

After verifying our DR-KO strategy, we next aimed to extend the panel of DR4/5 double-KO cell lines with the other cancer cell lines previously identified as TRAIL-sensitive (Fig. 1D). Using the same approach, we achieved DR4/5 double KO of ST sPCm, sCC, and sBCm cell lines. In addition, DR4/5 double KO of sTCL and the invasive GBM cell line sGBMi was established by engineering with DR4/5 KO constructs as described above, followed by in vitro selection with ST exposure. Western blotting was used to confirm establishment of double-KO lines (Fig. 3H). Sequencing of genomic DNA from wild-type and DR4/5 double-KO sBCm clone DR4/5-2 (herein referred to as $rBCm^{DR4/5}$) further confirmed indel mutations of targeted exonic gene sequences (fig. S2C). In conclusion, our DR-KO strategy can be applied to a variety of cancer types.

DR-KO tumor cells co-engineered with DR_L and a suicide system exhibit self-targeting efficacy in autologous recurrent tumor models in vitro

To mimic the clinical scenario of using autologous cells, established after primary tumor surgery, for self-targeted ligand delivery (outlined in Fig. 1B), we next aimed to establish mouse models of recurrent tumors as a platform to test the self-targeting efficacy of autologous CRISPR-engineered therapeutic cells. Furthermore, with this approach, we wanted to gain insight into whether adjuvant therapy, as commonly initiated after first-time tumor surgery, alters the TRAIL sensitivity phenotype of tumors (Fig. 4A). sGBMn and sGBMi (Fig. 1C) were transduced with LV to express FmC (fig. S3A) and implanted intracranially into severe combined immunodeficient (SCID) mice. Tumor-bearing mice were treated with temozolomide (TMZ), and therapy response was observed, as indicated by a reduction of the tumor cells' BLI signal (Fig. 4B). After tumor recurrence, mice were sacrificed, tumor cells were harvested using fluorescence microscopy as guidance, and recurrent GBM cells (sGBMnRec and sGBMiRec) were established in culture. Cell viability assays of these recurrent GBM lines revealed increased resistance to TMZ, whereas sensitivity to DR-targeting ST was unchanged or increased compared to the primary GBM cells (Fig. 4C). These findings suggest that adjuvant in vivo treatment with TMZ induces a TMZ-resistant phenotype in recurrent tumors but does not negatively influence response to DR-targeted therapy.

In light of the highly TRAIL-resistant phenotype observed after DR-KO, we next wanted to test whether the established DR4/5 double-KO lines could serve as a cellular vehicle for continuous delivery of ST toward autologous self-cells and ultimately be eliminated. To test this hypothesis, we transduced $rGBMn^{DR4/5}$ and $rGBMi^{DR4/5}$ with LVs to express ST or co-express ST and HSV-TK, followed by autologous coculture with their respective TMZ-resistant recurrent cell lines (sGBMnRec-FmC and sGBMiRec-FmC; Fig. 4D). DR4/5 double-KO cell lines engineered to express ST showed robust TRAIL expression and secretion as compared to GFP-transduced counterparts (fig. S3, B and C). TRAIL secretion from CRISPR-engineered therapeutic cancer cell lines was further quantified via enzyme-linked immunosorbent assay (ELISA) time course (fig. S4). Autologous coculture of $rGBMn^{DR4/5}$-ST and $rGBMi^{DR4/5}$-ST-TK with their respective recurrent lines further revealed high potential for self-targeting,

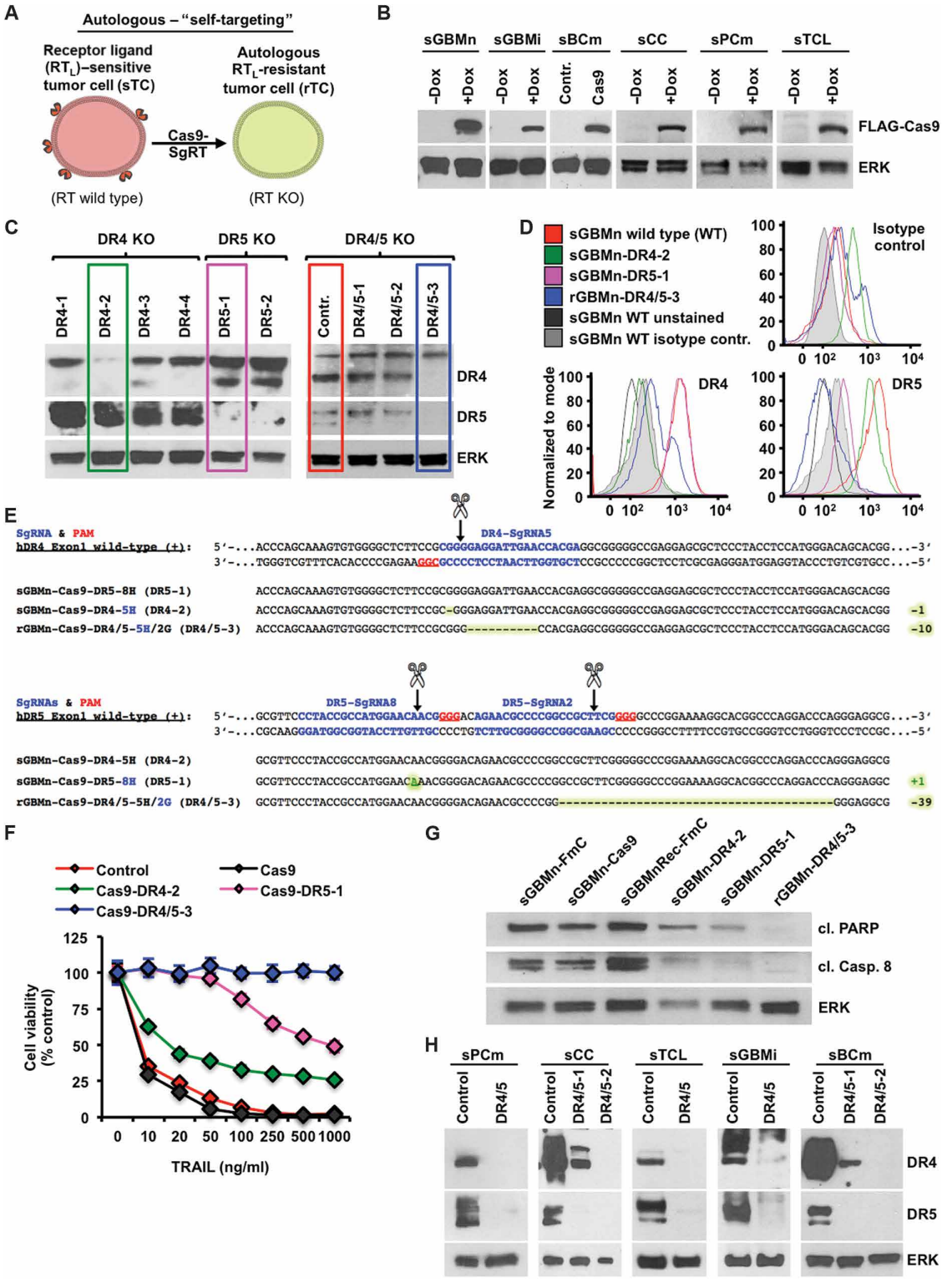

Fig. 3. CRISPR-mediated DR-KO and influence on sensitivity to DR_L TRAIL. (**A**) Concept of autologous approach: CRISPR-mediated receptor KO changes the phenotype of cancer cells from RT_L-sensitive to RT_L-resistant before engineering with secretable RT_L. (**B**) Western blot analysis of DR_L-sensitive tumor lines transduced with doxycycline-inducible FLAG-Cas9 or constitutively expressed FLAG-Cas9 (sBCm) constructs, blotted for FLAG and total extracellular signal–regulated kinase (ERK). (**C**) Single-clone Western blot analysis of CRISPR-targeted DR4, DR5, and total ERK expression in sGBMn. (**D**) Flow cytometry analysis of DR4 and DR5 surface expression in wild-type cells and KO clones identified in (C). (**E**) Single-clone sequencing of genomic DNA from wild-type and KO sGBMn clones identified in (C). (**F**) Titration of DR-KO clones with DR_L TRAIL (n = 3 technical replicates). (**G**) Western blot analysis of PARP and caspase 8 cleavage and total ERK in DR wild type (sGBMn-FmC, sGBMn-Cas9, and sGBMnRec-FmC) and DR single- or double-KO clones identified in (C) 8 hours after treatment with DR_L TRAIL. (**H**) Western blot analysis of DR4, DR5, and total ERK expression in other DR-KO CRISPR-engineered tumor cell lines.

resulting in marked reduction of the targeted recurrent GBM cells in a dose-dependent manner as compared to coculture with GFP controls (Fig. 4D).

Treatment of both nodular and invasive $rGBMn^{DR4/5}$-ST-TK and $rGBMi^{DR4/5}$-ST-TK with GCV resulted in dose-dependent cell elimination as opposed to non-TK control lines (Fig. 4E). To further test whether HSV-TK bystander effect might also provide efficacy against TRAIL-resistant autologous self-cells (which may be needed in clinical settings of tumor recurrence with change in TRAIL sensitivity phenotype), $rGBMn^{DR4/5}$ was engineered to express FmC and cocultured with $rGBMn^{DR4/5}$-ST-TK cells, followed by treatment with and without GCV (fig. S5). Addition of GCV resulted

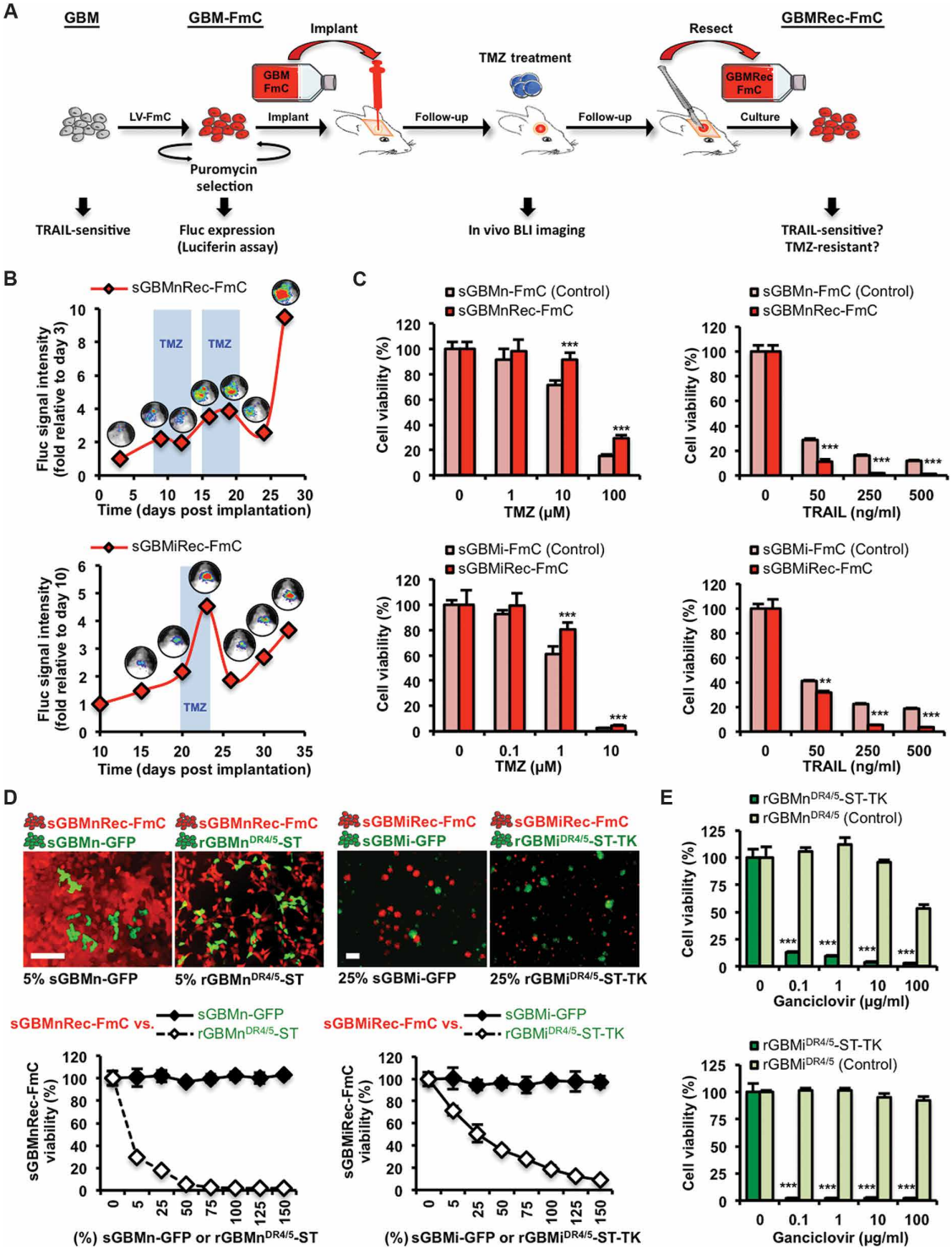

Fig. 4. In vitro autologous self-targeting efficacy of DR-KO tumor cells co-engineered with a secretable DR_L and a suicide system. (**A**) Strategy for the establishment of autologous recurrent glioblastoma models using in vivo TMZ treatment. (**B**) Effect of in vivo TMZ treatment on intracranial growth of nodular (sGBMnRec-FmC) and invasive (sGBMiRec-FmC) tumors as monitored by BLI ($n = 1$ each; see also tables S1 and S2). (**C**) Recurrent tumor lines established in (B) and their respective primary lines were titrated with TMZ (left) and DR_L TRAIL (right) to identify differences in their sensitivity to TMZ and TRAIL treatment ($n = 3$ technical replicates each). (**D**) Representative photomicrographs (top) and assessment of viability (bottom) of DR_L sGBMnRec-FmC or sGBMiRec-FmC cocultured with increasing percentages (0 to 150%, as indicated on the *x* axis) of their respective autologous TRAIL-secreting cell lines or autologous GFP-transduced controls ($n = 3$ technical replicates). Scale bars, 200 μm. (**E**) GCV titration of DR4/5 KO cancer lines engineered with or without prodrug-converting suicide system HSV-TK in vitro ($n = 3$ technical replicates). All in vitro experiments were repeated at least twice. Means ± SD are shown. *P* values by unpaired *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$.

in not only cell death in the rGBMn$^{DR4/5}$-ST-TK therapeutic line but also elimination of TRAIL-resistant DR4/5 KO self-cells (rGBMn$^{DR4/5}$-FmC) via the bystander effect (fig. S5B). Thus, should tumors acquire a DR_L-resistant phenotype during adjuvant clinical therapy and tumor recurrence, the TK-induced bystander effect of therapeutic self-cells might still provide robust treatment efficacy (fig. S5, A and B).

CRISPR-engineered tumor cells expressing cytotoxic molecules show autologous self-targeting efficacy in primary and metastatic tumors

To translate our in vitro findings of CRISPR-enhanced autologous therapies into in vivo settings, we established three mouse models, each closely mirroring a different clinical scenario of cancer treatment that might particularly benefit from tumor self-targeting: (i) implantation of autologous self-targeting cells into resection cavities of mice undergoing surgery for recurrent nodular tumors derived from the TMZ-resistant sGBMnRec-FmC, (ii) direct stereotactic intratumoral implantation of autologous self-targeting cells into mice bearing recurrent invasive tumors derived from the TMZ-resistant sGBMiRec-FmC tumor line, and (iii) injection of autologous self-targeting cells into the carotid artery of mice bearing established intracranial metastatic sBCm-FmC breast cancer deposits. The three outlined scenarios feature the potential of self-targeting for (i) local treatment of surgically controllable (primarily nodular) tumor recurrence, (ii) local treatment of recurrent (primarily invasive) cancers for which surgical debulking is not indicated, and (iii) systemic treatment for disseminated/metastatic disease.

(1) Nodular recurrent GBM resection model. On the basis of our previous work with orthotopic GBM resection mouse models, local retention of therapeutic cells can be achieved by encapsulation of therapeutic cells into biodegradable synthetic extracellular matrix (sECM) before implantation into the resection cavity (*16*). First, efficacy of sECM-encapsulated rGBMn$^{DR4/5}$-ST-TK cells against sGBMnRec-FmC self-cells was confirmed via in vitro coculture, indicating efficient release of ST out of the sECM (Fig. 5A). Furthermore, rGBMn$^{DR4/5}$-ST-TK was co-engineered to express GFl followed by sECM encapsulation and intracranial implantation into SCID mice to test for in vivo growth and efficiency of GCV-induced (HSV-TK–mediated) cell clearance. Implanted sECM-encapsulated rGBMn$^{DR4/5}$-ST-TK-GFl localized to the implantation site and demonstrated in vivo growth, and GCV treatment resulted in successful cell clearance, as indicated by a drop of BLI signal back to baseline by day 9 after initiation of GCV therapy (Fig. 5B). Together, these data show that ST can be released from sECM-encapsulated CRISPR-engineered therapeutic tumor cells and that these cells retain their potential for in vivo growth but can be eliminated with GCV. On the basis of these observations, we next tested the in vivo antitumor efficacy of sECM-encapsulated rGBMn$^{DR4/5}$-ST-TK in a clinically relevant resection model of mice bearing recurrent GBM. Mice implanted with the recurrent TMZ-treated autologous GBM cell line sGBMnRec-FmC (Fig. 4, A to C) underwent either no treatment (control) or fluorescence microscopy–guided subtotal tumor resection with or without simultaneous implantation of sECM-encapsulated rGBMn$^{DR4/5}$-ST-TK into the resection cavity followed by treatment with or without GCV (Fig. 5C). As expected, tumor volume was significantly reduced immediately after GBM resection, as indicated by a drop of the BLI signal after surgery ($P < 0.01$; Fig. 5C). Mice undergoing tumor resection and rGBMn$^{DR4/5}$-ST-TK implantation demonstrated a marked survival benefit compared to nonresected control mice and mice with resection alone. Survival was further extended in mice receiving GCV treatment (Fig. 5C).

(2) Invasive recurrent GBM model. To investigate antitumor efficacy in the clinical setting of nonresectable recurrent tumors, we chose the highly invasive recurrent sGBMiRec-FmC model (Fig. 4, A to C) and used direct stereotactic implantation of rGBMi$^{DR4/5}$ (control) or therapeutic rGBMi$^{DR4/5}$-ST-TK into the tumor site (Fig. 5D). A marked reduction of tumor burden was observed in rGBMi$^{DR4/5}$-ST-TK–treated mice in comparison to control mice (Fig. 5D). In comparison to controls, therapy with rGBMi$^{DR4/5}$-ST-TK and GCV resulted in significant improvements of survival ($P < 0.01$; Fig. 5D). Although initially effective in reducing sGBMiRec-FmC tumor growth, treatment with rGBMi$^{DR4/5}$-ST-TK alone (without GCV) did not significantly improve mouse survival. This is likely a consequence of therapeutic tumor cell growth and underlines the importance of GCV treatment when using therapeutic tumor cells.

(3) Metastatic breast-to-brain cancer model. To extend the applicability of the above-outlined autologous self-targeting approaches to other tumor models, we further investigated their efficacy in a metastatic cancer model using the DR_L-sensitive breast-to-brain metastatic cell line sBCm (Fig. 1, C and D), which was previously established by Bos *et al.* (*23*) from the cell line MDA-MB-231. DR4/5 double-KO clone rBCm$^{DR4/5}$ (sBCm-DR4/5-2; Fig. 3H) was engineered to express ST and TK; TRAIL expression and secretion were confirmed by Western blotting and ELISA analysis of conditioned medium over time (figs. S3, B and C, and S4). In vivo, mice bearing sBCm tumors engineered to express Rl-mCherry (sBCm-RmC; fig. S3A) were treated by injecting autologous rBCm$^{DR4/5}$-ST-TK cells into the ICA as previously described (*24*). In comparison to control mice, mice treated with rBCm$^{DR4/5}$-ST-TK and GCV showed marked reduction in tumor growth and prolonged survival (Fig. 5E).

Analysis of brain sections from mice bearing sGBMn-FmC and sGBMi-FmC using H&E staining and fluorescence microscopy confirmed close proximity of therapeutic cells and targeted tumor deposits [Fig. 5, C (bottom) and D (bottom)]. To explore the time-dependent migration potential of CRISPR-engineered cells, mice bearing established sGBMiRec-FmC tumors were implanted with rGBMi$^{DR4/5}$-GFP cells at a distance of 1.5 mm from the established tumor site. Our data indicate a directed migration of rGBMi$^{DR4/5}$-GFP cells starting at 1 week after implantation, with a steady increase of migrating cells over the follow-up period of 1 month, and cells covering a distance of more than 2 mm in this time period (Fig. 6). Together, our in vivo data indicate that CRISPR-modified cancer cells engineered to secrete receptor-targeted therapeutic molecules can specifically target and kill autologous self-cells in mouse models of recurrent and metastatic cancers and that treatment increases the survival of mice.

DISCUSSION

This study demonstrates the therapeutic potential of using engineered tumor cells and their self-homing properties for developing receptor-targeted therapeutics for various cancers. We show the feasibility and clinical translatability of this approach by using (i) off-the-shelf tumor cells resistant to DR_L, which could be used for targeting of allogeneic patient tumors in clinical scenarios of primary tumor treatments, and (ii) inherently DR_L-sensitive tumor cells, which, after CRISPR-mediated DR-KO, can be used in autologous

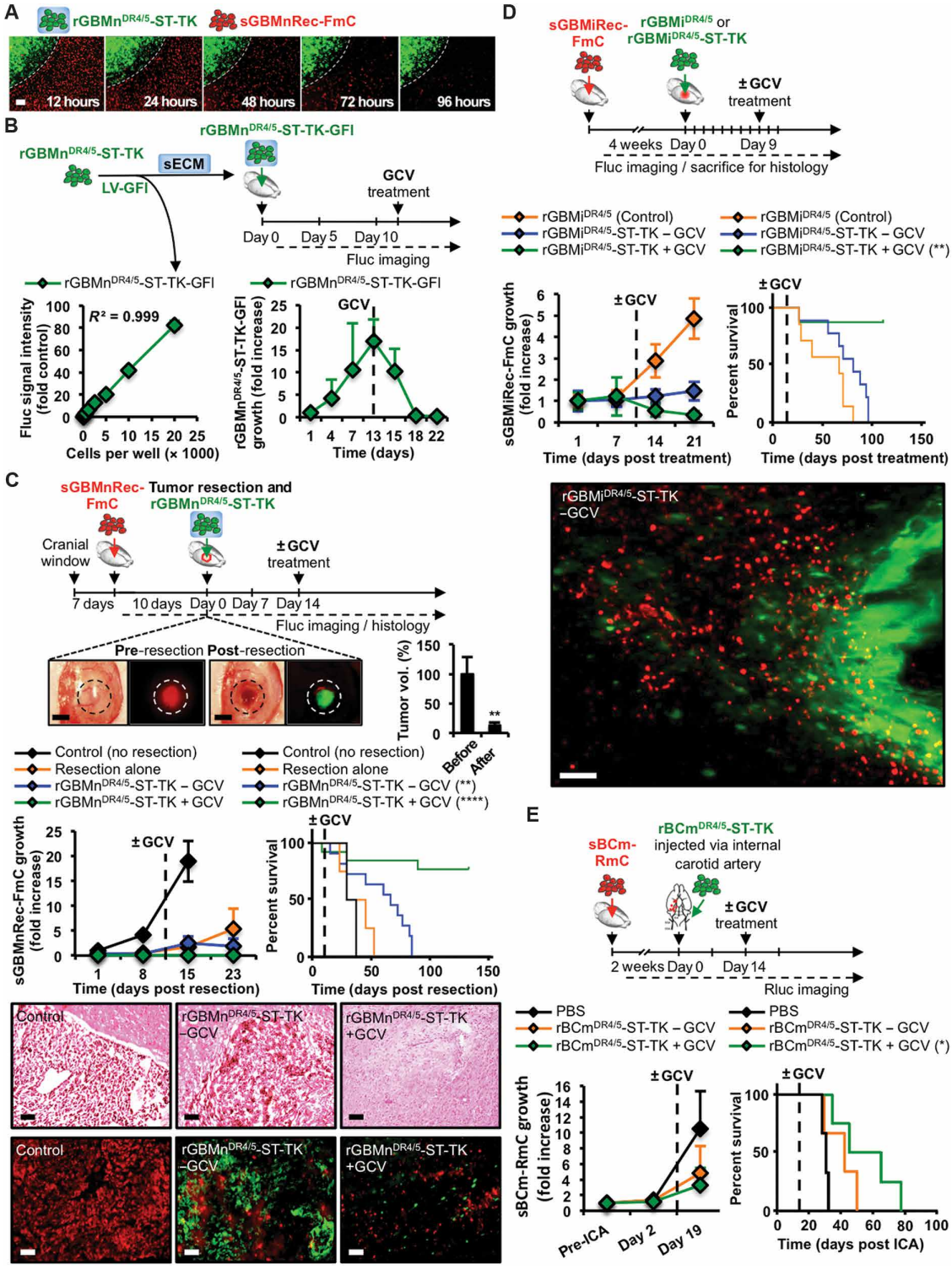

Fig. 5. In vivo autologous self-targeting efficacy of DR-KO tumor cells co-engineered with a secretable DR$_L$ and a suicide system. (**A**) Photomicrograph time course of sECM-encapsulated ST-secreting rGBMn$^{DR4/5}$-ST-TK cocultured with their autologous DR wild-type parental cells (sGBMnRec-FmC). Scale bar, 200 μm. (**B**) rGBMn$^{DR4/5}$-ST-TK was engineered with GFI. The graph on the left shows in vitro correlation of Fluc signal with cell number. rGBMn$^{DR4/5}$-ST-TK-GFI was encapsulated in sECM, followed by intracranial implantation into SCID mice. The graph on the right shows Fluc signal of rGBMn$^{DR4/5}$-ST-TK-GFI before and after GCV treatment (n = 2 mice). (**C**) Top: Experimental outline for testing efficacy of sECM-encapsulated rGBMn$^{DR4/5}$-ST-TK in mice with resected sGBMnRec-FmC tumors. Photomicrographs show light and fluorescence photos of intact and resected intracranial tumors after implantation of therapeutic cells. Scale bars, 1 mm. Black/white dashed circles indicate tumor area. The bar graph on the right shows mean tumor volume estimated on the basis of Fluc signal before and after resection (n = 28). Middle: Estimate of relative tumor volume after resection in treatment groups based on Fluc signal intensity of sGBMnRec-FmC-bearing mice (left). Kaplan-Meier survival curves are shown on the right (control, n = 4; resection alone, n = 4; rGBMn$^{DR4/5}$-ST-TK-GCV, n = 11; rGBMn$^{DR4/5}$-ST-TK + GCV, n = 13). Bottom: Representative hematoxylin and eosin (H&E)–stained sections and immunofluorescence photomicrographs of nonresected control versus resected sGBMnRec-FmC tumors treated with therapeutic rGBMn$^{DR4/5}$-ST-TK with or without GCV. Scale bars, 200 μm. (**D**) Top: Experimental outline for testing the efficacy of rGBMi$^{DR4/5}$ (control) or rGBMi$^{DR4/5}$-ST-TK in mice bearing intracranial sGBMiRec-FmC tumors. Middle: Estimate of relative tumor volume in treatment groups based on Fluc signal of sGBMiRec-FmC–bearing mice (left) and respective Kaplan-Meier survival curves (right) (rGBMi$^{DR4/5}$, n = 7; rGBMi$^{DR4/5}$-ST-TK – GCV, n = 9, rGBMi$^{DR4/5}$-ST-TK + GCV, n = 8). Bottom: Immunofluorescence photomicrograph of sGBMiRec-FmC–bearing mice injected with rGBMi$^{DR4/5}$-ST-TK (no GCV treatment). Scale bar, 200 μm. (**E**) Top: Experimental outline for testing efficacy of rBCm$^{DR4/5}$-ST-TK injected via the internal carotid artery (ICA) in mice bearing intracranial sBCm-RmC tumors. Bottom: Estimate of relative tumor volume increase based on RI signal intensity of sBCm-RmC–bearing mice (left) and respective Kaplan-Meier survival curves (right) (PBS, n = 3; rBCm$^{DR4/5}$-ST-TK – GCV, n = 3; rBCm$^{DR4/5}$-ST-TK + CGV, n = 4). Means ± SEM are shown. P values by unpaired t test (C, top) or Mantel-Cox (log-rank) test (survival curves), *P < 0.05, **P < 0.01, ****P < 0.0001.

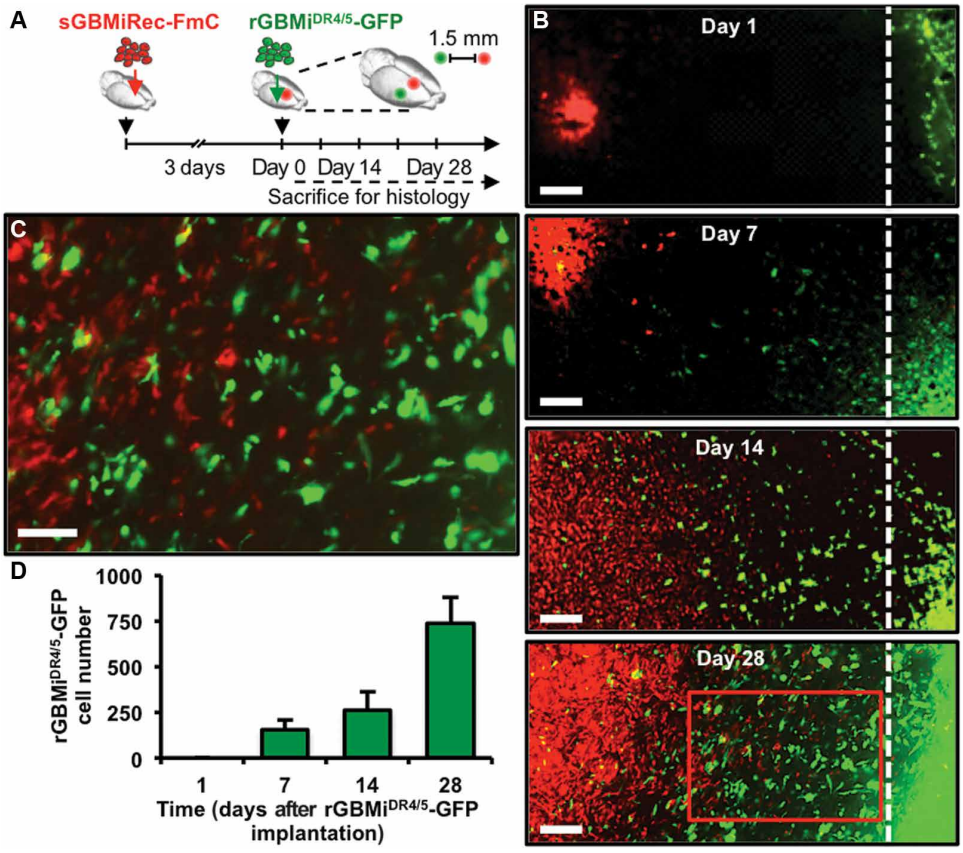

Fig. 6. Migratory potential of CRISPR-engineered therapeutic tumor cells toward recurrent self-tumor sites. (**A**) Experimental outline: 5×10^5 sGBMiRec-FmC cells were implanted into the right hemisphere of SCID mice, followed by injection of 5×10^5 rGBMi$^{DR4/5}$-GFP cells at a distance of 1.5 mm laterally 3 days later. Mice were sacrificed at days 1, 7, 14, and 28 after rGBMi$^{DR4/5}$-GFP implantation ($n = 2$ for each time point) to assess migration of CRISPR-engineered rGBMI$^{DR4/5}$-GFP cells toward the sGBMiRec-FmC self-tumor site. (**B**) Representative fluorescence photomicrographs showing the location of sGBMiRec-FmC (red) and rGBMi$^{DR4/5}$-GFP (green) tumor cell populations at the time points outlined above. The dashed line was placed adjacent to the rGBMi$^{DR4/5}$-GFP implantation site to facilitate quantification of migration toward the established sGBMiRec-FmC tumor site. The red box marked in the photomicrograph for day 28 is magnified in (C). Scale bars, 200 μm. (**C**) Magnified fluorescence microphotograph from day 28. Scale bar, 100 μm. (**D**) Quantification of rGBMi$^{DR4/5}$-GFP migration toward the sGBMiRec-FmC tumor site at different time points based on rGBMi$^{DR4/5}$-GFP cell count from the left part of (B), excluding the nonmigratory established tumor site shown to the right of the dashed line. Means ± SD are shown. Two biological replicates per time point.

settings of recurrent or metastatic disease. Moreover, this study highlights the advantages of combining tumor cell–based receptor targeting with prodrug-activatable suicide systems and, using optical and PET imaging, demonstrates the feasibility, therapeutic efficacy, and safety of this approach in clinically relevant mouse models of primary, recurrent, and metastatic disease.

Despite great leaps in the treatment of malignant neoplasms over the past decades, cancer remains the second most common cause of death in the western world, slightly surpassed only by heart disease, and currently accounts for nearly one of every four deaths in the United States (*25*, *26*). Consequently, new therapeutic approaches are desperately needed, especially in cases of recurrent and metastatic disease, where standard therapy has failed and evidence-based options for salvage treatments are limited or lacking. Preclinical data increasingly indicate that cell-based therapies enabling local delivery of therapeutic agents might provide a valuable option for these cases, and multiple studies are currently ongoing with the goal of translating these approaches into clinical settings (*27*–*30*). Associated advantages are manifold and include achievement of continuously high local concentrations of secreted agents with reduction of systemic toxicity, delivery of molecules with short half-lives that are inefficacious when used systemically, exposure or aid in detection of tumor-specific antigens, and consecutive activation of the immune system, as well as the application of the pathotropic abilities of therapeutic cells to track tumor microdeposits.

Traditionally, the main focus of research in the field of cell-based therapies has been on stem cells (SCs). In addition to the possibility of modifying these cells to express various proapoptotic and antiproliferative molecules, SCs' inherent pathotropic properties and their intrinsic antitumor effects have rendered them promising tools in the treatment of multiple cancers (*29*, *31*). In the setting of GBM in particular, SC transplantation may enable local delivery of molecules that cannot cross the blood-brain barrier when administered systemically. However, despite the apparent advantages of using SCs for certain cancer types, several inherent and external roadblocks are still restricting widespread clinical translation of SC-based therapies: (i) Adult SCs are often slow-growing in vitro and, unless artificially immortalized, (ii) have a limited passage number, which makes engineering them with therapeutic molecules difficult and reduces treatment efficacy because of short in vivo survival (*32*); (iii) donor SCs, prepared from a healthy individual or from a pool of healthy donors, may not (or only partially) match the recipient's HLA status, possibly causing adverse immune responses and/or toxicity, as well as premature SC clearance by the recipient's immune system (*33*); and (iv) autologous SC transplantation would be ideal but is time-consuming and, therefore, currently not practical in first-line treatment or for patients with end-stage cancer because SCs have to be harvested, reengineered with therapeutic molecules, and expanded before reapplication can be considered (*34*). Moreover, SC harvesting from patients necessitates further interventional procedures, therefore adding to the overall risk of clinical complications, especially for late-stage and immunocompromised patients after chemotherapy.

Numerous studies have investigated the mechanisms that influence tumor progression and eventually contribute to metastasis (*2*, *3*). One concept suggests that, during tumor evolution, cancer cells gain the ability of "self-seeding," a process involving cell dissemination into the vascular system away from the primary or metastatic tumor, followed by the cells rehoming to the site of origin (*4*, *35*, *36*). The exact factors involved in this mechanism are still poorly understood,

but studies suggest that, besides leaky primary tumor vasculature with impaired barrier function, cytokine-receptor interactions between the primary tumor and circulating cancer cells may play a major role (*36*–*38*). On the basis of these findings, several studies have investigated approaches to repurpose the self-homing properties of tumor cells for self-targeted delivery of anticancer agents to primary tumor sites (*5*–*9*). Exploring the clinical scenario associated with this approach, cancer cells harvested at the time of tumor surgery would be introduced to culture conditions and engineered with anticancer agents, followed by local or systemic reapplication of autologous therapeutic cells upon tumor recurrence. Possible advantages, in comparison to the abovementioned SC-based therapies, are enhanced homing of therapeutic cancer cells toward the primary tumor site, ease of engineering with therapeutic molecules due to robust growth in vitro, prolonged therapeutic cell survival resulting in enhanced therapeutic efficacy in vivo, and their ready availability for autologous therapy because tumor biopsy is part of standard management for the vast majority of cancer patients. In comparison to allogeneic approaches, treatment with autologous cells additionally does not increase the risk of adverse immune response and/or toxicity, as well as premature therapeutic cell clearance by the recipient's immune system.

Previous studies exploring self-targeted cancer therapies, to our best knowledge, have focused on three different approaches: (i) the use of cancer cells as a vehicle for delivery of oncolytic viruses (*5*, *6*), (ii) the use of bystander effect by engineering tumor cells with suicide genes (*7*, *8*), and (iii) engineering tumor cells to secrete TNF-α, a cytokine known for its ability to induce hemorrhagic necrosis in solid tumors due to its damaging effect on tumors' neovascular endothelium (*9*, *39*). Although approaches (i) and (ii) incorporate therapeutic tumor cell elimination, a safety feature indispensable for possible future clinical translation, anticancer efficacy of these approaches might be limited because of spatiotemporal limitations if therapeutic cells are eliminated or die before reaching the primary or metastatic tumor sites. In addition, bystander effect due to suicide gene expression is only a one-time effect, which subsides once therapeutic cells are eliminated. TNF-α–secreting tumor cells (*3*) have shown promising preclinical results (*9*). However, TNF-α is a rather unspecific anticancer agent because its primary target receptor, TNF receptor 1, is ubiquitously expressed and mediates a large variety of effects (*40*), including inflammation and tissue degeneration, which may be associated with unwanted side effects (*41*).

Our study explored combining the advantages of suicide system–induced therapeutic cancer cell elimination with the continuous expression of a receptor-targeted anticancer agent. The DR-targeted apoptosis-inducing ligand, TRAIL, was specifically chosen for its superior anticancer efficacy when tested against other receptor-targeted molecules in a panel of tumor cell lines including solid and nonsolid, as well as primary, recurrent, and metastatic lines. Besides TRAIL's receptor-targeted properties and its ability to strongly induce apoptosis in a wide range of human cancer cell lines, it has the advantage of not inducing cytotoxicity in normal cells and can be engineered in a secretable form (ST) (*42*–*44*). However, although these properties seem ideal for self-targeted therapy, TRAIL-sensitive cancer cells cannot readily be engineered with ST because of autocrine toxicity. We therefore explored different self-targeting approaches to avoid TRAIL-induced self-toxicity, each aimed at specific, clinically relevant cancer treatment scenarios. In models of primary cancer treatment, we show that TRAIL-resistant cancer cells can be readily engineered with ST and used off-the-shelf for targeting TRAIL-sensitive GBM. However, ideally, one would like to use autologous cells for self-targeting, thereby avoiding possible immune-mediated premature elimination of therapeutic cells and adverse effects. Our studies indicate that such an autologous approach can be realized by using CRISPR technology to knock out TRAIL receptors DR4 and DR5, thereby reversing the TRAIL-sensitivity phenotype and allowing engineering of previously TRAIL-sensitive cancer cells with ST.

Considering the time needed for engineering of autologous cell lines, treatment with KO lines should be aimed at therapy of recurrent or systemic/metastatic disease. Consequently, we used autologous KO lines either in mouse models of tumor recurrence or in a metastatic breast-to-brain model using the cell line sBCm, which was established through several rounds of brain selection of breast carcinoma cells (*23*). Besides reflecting different cancer models (primary, recurrent, and metastatic), cell lines used in this study were further selected on the basis of their in vivo growth characteristics. sBCm was specifically chosen to investigate self-targeting in metastatic settings based on a previous report (*36*), which demonstrated sBCm's highly efficient self-targeting phenotype in mouse models of primary breast cancer and lung metastasis (using lung metastatic derivative MDA231-LM2). The TRAIL-sensitive recurrent cell lines sGBMiRec-FmC (invasive growth) and sGBMnRec-FmC (nodular growth), on the other hand, reflect the different in vivo phenotypes of recurrent GBM to mimic the clinical scenarios where a second (recurrent) tumor resection is possible (nodular), versus the case of a nonresectable recurrent GBM (invasive model). On the basis of these in vivo growth characteristics, we further adapted techniques for in vivo therapy. In the nodular recurrent model (sGBMnRec-FmC), we aimed to retain therapeutic cancer cells within close proximity to remaining tumor tissue by encapsulating therapeutic cells into synthetic biodegradable ECM before implantation into the resection cavity. In models of highly invasive recurrent GBM, however, resection is not an option, which is why direct implantation of non-encapsulated therapeutic cancer cells was used instead. Moreover, to promote homing to invasive tumor deposits, therapeutic cells were not ECM-encapsulated whenever direct implantation into invasive tumors was used. Our migration studies indicated that non-encapsulated CRISPR-engineered cells retain their ability for targeted delivery of therapeutics toward self-tumor sites. Although the exact mechanisms underlying the self-targeting of tumor cells remain to be further elucidated, studies have suggested multiple theories to explain this phenomenon. In addition to common cytokine-receptor interactions that mediate directed cell migration, the establishment of a favorable tumor microenvironment, which supports survival of migrating tumor cells, may play a crucial role in this process (*36*, *45*, *46*). In addition, it has been demonstrated that IL-6 and IL-8 might serve as tumor-derived attractants, and fascin actin-bundling protein 1 and matrix metalloproteinase 1 might additionally be involved in mediating migration (*23*, *36*, *47*–*50*).

In metastatic disease, resection is also often not possible because of metastatic tumor location and/or the large number of metastatic deposits. To mirror this scenario, breast-to-brain metastatic cell line sBCm was implanted intracranially into mice, followed by application of therapeutic cells via ICA injection, thereby reflecting systemic treatment of metastatic disease. Using the above-outlined models, our studies demonstrated that CRISPR-modified therapeutic cancer cells can directly kill self-cells via TRAIL-induced apoptosis in vitro and in vivo and that, in combination with their self-homing properties,

these effects increase the survival of mice bearing autologous recurrent or metastatic tumor deposits. Although it is known that sBCm exhibits a highly efficient self-targeting phenotype (*36*), future studies will have to explore whether systemic treatment scenarios are also feasible for metastatic models of other cancer types.

One of the main concerns for treatments using therapeutic cancer cells is their tumorigenic potential. Here, we incorporated a prodrug-activatable suicide system to address this concern. Our in vivo data demonstrate that therapeutic cancer cells expressing HSV-TK can be safely eliminated, and we did not observe recurrences of therapeutic cells after in vivo GCV treatment. This is in line with clinical studies, which demonstrated a robust safety profile of HSV-TK systems when used on proliferating cells in patients (*51*, *52*). The importance of GCV-induced therapeutic cancer cell elimination is highlighted by our in vivo survival studies, which showed that self-targeted therapy without GCV treatment did not provide overall survival benefit in nonresected tumor models. This is likely the case because therapeutic tumor cells retain their potential for in vivo growth and, even if very efficacious in treating the self-tumor site (as shown by the drop in BLI signal), will therefore eventually outgrow the targeted tumor cells and result in premature animal death if no GCV treatment is administered. Therefore, if these are considered for clinical translation, therapeutic cancer cells should be confirmed to have stable HSV-TK expression, and GCV will need to be administered to all patients. Moreover, adding a second suicide system (*53*–*55*) should be considered, and larger-scale preclinical studies focused on analyzing the safety profile of this approach should be performed. In case of clinical translation, an advantage of the HSV-TK system is that it can be used to noninvasively monitor the fate of therapeutic cells via PET imaging in combination with radioactive substrates, such as the 18F-FHBG used in this study (*56*, *57*).

Besides ensuring safety, our in vitro data demonstrate that HSV-TK–induced cell elimination is associated with a bystander effect, which may contribute to the overall treatment efficacy in cases of secondary TRAIL resistance. In clinical settings, HSV-TK–mediated tumor cell elimination might further boost therapeutic efficacy via exposure of tumor antigens followed by tumor-specific immunoactivation, which may be especially helpful in cases of tumors with heterogeneous DR_L sensitivity (*58*–*60*).

Here, engineering and generation of therapeutic cell lines required transduction with multiple vectors and clonal selection, which was time-consuming and labor-intensive. Further studies are necessary to make this process more efficient and increase clinical feasibility. Lessons learned from the recent introduction of chimeric antigen receptor (CAR) T cells into clinical practice and current efforts to increase CARs' efficacy via multistep engineering may provide valuable expertise during this process. Another challenge during clinical translation may be immune reactions to engineering vectors or the expressed transgenes, especially Cas9 (*61*, *62*). Although implementation of an inducible Cas9 system, as used in this study, will limit Cas9 expression after KOs are achieved, nonconstitutive expression strategies would be preferred.

Moreover, although DR_L-based therapies have demonstrated great efficacy in many preclinical studies, efforts for their clinical translation have so far been disappointing. We believe that the two main reasons for the failure of previous clinical studies using TRAIL therapy are likely (i) inefficient patient stratification as a result of missing screening of patients for their tumors' TRAIL sensitivity phenotype and (ii) reduced efficacy of TRAIL treatment due to spatiotemporal (short half-life, rapid clearance) and dose toxicity issues when TRAIL was administered systemically (*63*–*65*). This study demonstrates that (ii) might be solved by local delivery of TRAIL via transplantation of therapeutic cells directly into the vicinity of the targeted tumor site. Because of the continued secretion of TRAIL at the tumor site (in contrast to intermittent systemic treatment), this approach not only overcomes toxicity problems reported for systemic treatment but also addresses issues of inadequate local TRAIL concentration. Here, we additionally addressed the issue of inefficient patient selection (i) by screening tumor cell lines for TRAIL sensitivity. This approach reflects a clinical scenario where patients' tumor cells are screened for TRAIL sensitivity before treatment initiation. To translate this screening process into future patient therapy, one could try to isolate patients' circulating cancer cells upon admission and screen them for DR expression when an allogeneic off-the-shelf approach is favored (*66*). Another option is to isolate and culture patients' own tumor cells after the first surgery and test them for TRAIL sensitivity in vitro before CRISPR modification and engineering with ST. Each patient's own therapeutic cells could then be readministered once the patient is readmitted for recurrent surgery (autologous approach), assuming that the recurrent tumor will retain its TRAIL-sensitive phenotype (as our data suggest).

Recent studies on the TRAIL-inducing small-molecule ONC201/TIC10 have shown promising results in a variety of cancers (*67*–*69*). ONC201 induces apoptosis in a p53-independent manner via selective antagonism of D2-like dopamine receptors (DRD2) and ultimately results in TRAIL induction. ONC201 is orally active and crosses the blood-brain barrier, thus making it a promising agent for future therapy of GBMs (*68*). However, because ONC201 (unlike TRAIL) does not directly act on DRs, its clinical efficacy not only may depend on DRD2 and DR expression in tumor cells but also further relies on preservation of (downstream) pathways for Akt, ERK, and TRAIL. This might potentially reduce the number of targetable tumors and may further increase the potential for resistance. Therefore, local cell-based secretion of TRAIL as suggested in this study may provide higher efficacy.

In conclusion, our studies reveal the fate, therapeutic efficacy, and safety of engineered receptor-targeted human tumor cells in xenograft mouse models of primary, recurrent, and metastatic cancer. This study supports clinical development of cancer therapy that uses genetically engineered allogeneic or autologous tumor cells in cancer patients. We envision that, after the removal of the main tumor mass, patients' own cancer cells will be ex vivo engineered with receptor-targeted antitumor agent(s), as well as an inducible suicide system before they are readministered via different routes, depending on the type and clinical stage of cancer. These cells would result in killing of residual, invasive, and metastatic tumor deposits with the ultimate goal of improving outcomes.

MATERIALS AND METHODS

Study design

This study was designed to evaluate the fate, therapeutic efficacy, and safety of receptor-targeted ligand-secreting human tumor cells. This objective was addressed by (i) determining suitable receptor-targeted ligands, (ii) evaluating the self-targeting efficacy of inherently ligand-resistant tumor cells for therapeutic use against inherently sensitive cancer cell lines of the same cancer type, (iii) assessing the feasibility of CRISPR-mediated KO of ligand receptors to switch

cancer cells' ligand-sensitivity phenotype from sensitive to resistant before engineering with previously self-toxic ligand, and (iv) assessing the in vitro and in vivo self-targeting efficacy of these ligand-secreting therapeutic tumor cells.

In animal studies, mice were randomized to groups according to tumor volume at the start of treatment. The number of mice per group varied between experiments and is specified in the figure legends. The primary end point was survival. All in vivo procedures were approved by the Subcommittee on Research Animal Care at Brigham and Women's Hospital (BWH) and Massachusetts General Hospital (MGH). All in vitro and in vivo results are representative of two to five independent experiments. The investigators were not blinded during the study.

Statistical analysis

Data were expressed as means ± SD for in vitro studies and means ± SEM for in vivo studies and analyzed by Student's *t* test when comparing two groups. Survival times of mouse groups were analyzed and compared using log-rank test. GraphPad Prism 5 Software was used for all statistical analysis and also to generate Kaplan-Meier survival plots. Differences were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. DR expression, engineering of DR_L-resistant cancer cells with RI-ST, and in vitro coculture efficacy.
Fig. S2. Screening and identification of CRISPR-induced DR-KO.
Fig. S3. Engineering of cell lines for in vivo BLI and ST expression from DR4/5 KO cell lines.
Fig. S4. ELISA quantification of secreted TRAIL from CRISPR-engineered therapeutic cancer cell lines.
Fig. S5. Concept of autologous cancer cell–based self-targeting strategies and possible role of GCV-activated HSV-TK suicide system in case of DR_L nonresponsive tumor recurrence.
Table S1. Establishment of sGBMnRec-FmC recurrent cell line via in vivo TMZ treatment of sGBMn-FmC (provided as an Excel file).
Table S2. Establishment of sGBMiRec-FmC recurrent cell line via in vivo TMZ treatment of sGBMi-FmC (provided as an Excel file).
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Abstract

One-sentence summary: CRISPR-engineered receptor-specific self-targeted tumor cells demonstrate anti-tumor efficacy in vitro and in vivo.

Editor's Summary:
Cellular double agents

Tumor cells exhibit a "self-homing" behavior, whereby cells released into the circulation can home back to the main tumor site. To take advantage of this behavior and use the cells as vehicles to deliver therapies to the main tumor site, Reinshagen *et al*. engineered self-targeting tumor cells. These cells were designed to secrete death receptor–targeting ligands to which they were resistant to kill the main tumor but not destroy themselves. Conversely, they could be eliminated on demand using a drug-triggered cellular suicide system to prevent them from repopulating the tumor site. The authors then tested the efficacy and safety of this method in mouse models of primary, recurrent, and metastatic tumors.