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LABORATORY INVESTIGATION

Precision knockdown of EGFR gene expression using radio frequency electromagnetic energy

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Abstract Electromagnetic fields (EMF) in the radio frequency energy (RFE) range can affect cells at the molecular level. Here we report a technology that can record the specific RFE signal of a given molecule, in this case the siRNA of epidermal growth factor receptor (EGFR). We demonstrate that cells exposed to this EGFR siRNA RFE signal have a 30–70% reduction of EGFR mRNA expression and ~60% reduction in EGFR protein expression vs. control treated cells. Specificity for EGFR siRNA effect was confirmed via RNA microarray and antibody dot blot array. The EGFR siRNA RFE decreased cell viability, as measured by Calcein-AM measures, LDH release and Caspase 3 cleavage, and increased orthotopic xenograft survival. The outcomes of this study demonstrate that an RFE signal can induce a specific siRNA-like effect on cells. This technology opens vast possibilities of targeting a broader range of molecules with applications in medicine, agriculture and other areas.

Keywords Electromagnetic energy · Radio frequency · EGFR

Introduction

Radio frequency energy (RFE) exposure in the 3 kHz–3000 GHz range has a measurable effect on human cells [1, 2] and electromagnetic (EM) radiation in the RF range, can impact cellular function [3] in vitro and in vivo, without tissue heating. The magnetic field component of RF waves on living cells is likely a direct mechanism, as even weak magnetic fields affect cell function [4]. The hypothesis that molecular interaction has a stronger EM component than previously thought is supported by computational evidence [5].

Molecules in solution generate a weak magnetic field as they stretch, twist, tumble and vibrate in an aqueous medium [6, 7], and these magnetic fields are exceptionally weak, in the order of femto-Tesla in strength (data not shown). We hypothesize that the magnetic fields (as well as the electrostatic charge on the molecules) are critical for molecular recognition and non-covalent binding in many biological processes. Here, we demonstrate that specific electromagnetic modulations can be recorded and transmitted so that cells exposed to the specific electromagnetic modulation being transmitted behave as if the authentic molecule were present.

To record the specific RFE signal of various molecules, we developed a molecular magnetic field sensing system using superconducting quantum interference device (SQUID) technology, to record the dynamic magnetic fields of a pure population of molecules in solution. This chamber can attenuate external EM signal to as low as −80 dB. This device contains a specialized low temperature (liquid

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Helium) SQUID inside a shielded chamber housing a low temperature shielded Dewar connected to a data acquisition system (Fig. 1a, b). The low temperature SQUID coupled to the gradiometer detects small oscillations in the magnetic field over short time periods and converts these oscillations into current sequences. A full description of the recording system (designated as the Molecular Interrogation and Data System; MIDS) is given [8]. Previously we demonstrated [8] that the effects of paclitaxel could be recapitulated in an acellular system using the magnetic field recording of the active chemotherapy compound.

To demonstrate the specificity and cellular effects in vitro and in vivo, we used the human derived glioblastoma (GBM) cell line U-87 MG. We recorded the magnetic field signals of siRNA oligonucleotide molecule for the epidermal growth factor receptor (EGFR) gene, which plays a key role in cancer biology [9–13]. Here we show that we can specifically knock down EGFR gene expression, with resulting biological effects, in human U-87 MG and primary (GBM) cells by exposing these cells to physical-EGFR siRNA and RFE-siEGFR signal.

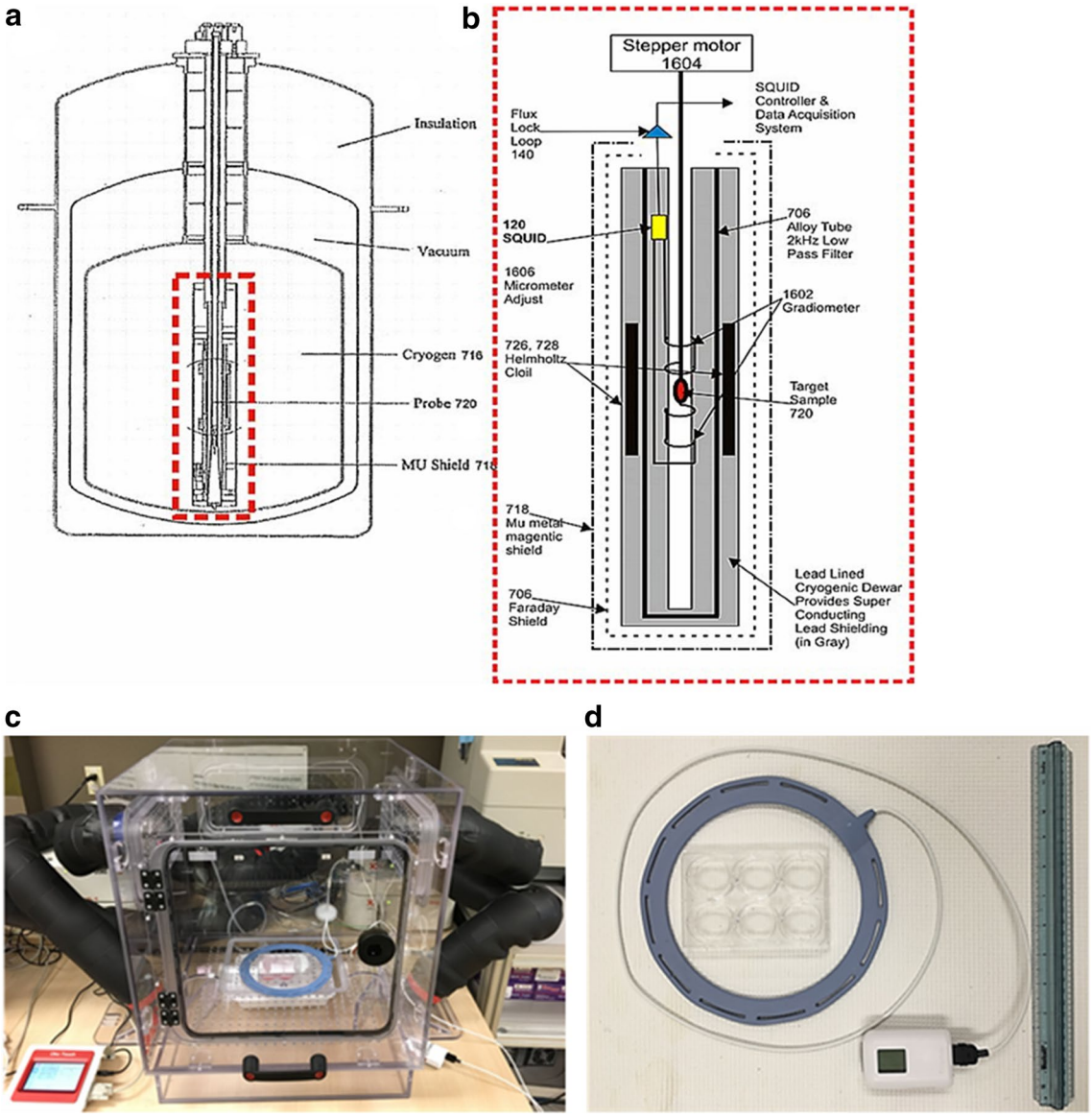


Fig. 1 Schema for Molecular Interrogation and Data System (MIDS) recording system, RFE exposure incubator and the transmission system with coil. **a** Construction diagram for the MIDS EMF attenuation chamber and cryogenic Dewar; **b** diagram of the SQUID/gradiometer sample analyzer set-up inside the cryogenic Dewar. **c** Acrylic Incubator set-up in the laboratory, with the transmitter and coil in place. **d** The transmitter and the coil (antenna) used to transmit the EGFR siRNA RFE or the scrambled siRNA RFE signals. Ruler on the right is a standard 12 inch ruler

Materials and methods

The electrostatic surface potential of molecules can be indirectly measured and recorded as an oscillating magnetic field using a 'Super Conducting Quantum Interference Device' (SQUID)-based magnetometer. Transducing these highly precise ulRFE profiles (cognates) into biological systems have been show to produce precise biological responses [14]. It is theorized that transduction of these cognates induces selective electron and charge transfer, or charge redistribution in a defined bioactive target, thus altering cell dynamics, which can produce therapeutic effects [15, 16].

To implement these measurements, a specialized, low temperature (liquid Helium) 'SQUID' based magnetometer was constructed inside a shielded chamber, connected to a data acquisition system. The SQUID magnetometer detects small oscillations in the magnetic field over short time periods and converts these oscillations into current sequences that are recorded using a Keithley KPCI-3108 data acquisition board operating in differential mode. Signals were recorded for 60-s intervals, digitized using proprietary software and stored as a WAV file (Microsoft format). Signals were selected from sets of 1 min recordings done over a period of a few weeks using a frequency analysis package in FlexPro8 (DEWETRON GmbH, Parkring 4, A-8074 Graz-Grambach, Germany). The digitized data from FlexPro8 plots of the signals were converted from the time domain into the frequency domain using a Fast Fourier Transform algorithm. An anti EGFR siRNA signal was selected due to its high signal-to-noise ratio, low interference and low entropy.

The flux densities applied to the cells are in the range of 10–50 milli-Gauss. The frequencies applied to the cells are in the range of DC through 44.1 kHz. Energy levels applied to the transmission coil is ~25 mW, with temperature changes of ±0.2 °C during temperature sampling runs [8]. The energy levels are constant and applied 24 h a day, 7 days a week. Heating due to RF is not a significant source of cell effects.

The establishing intracerebral xenograft has been described previously [17]. Microarray profiling was performed using SA Biosciences platforms and analyzed by vendor online software. Real-Time PCR was assessed using Taqman mRNA probes and reagents (Applied Biosystems). All animal work was done at UCSF in accordance with approval from UCSF institutional animal care guidance. A statistical analysis was conducted suing Graphpad Prism software. Full Methods are available in the online version of the paper.

Results

Recording RFE signal

The use of the RFE transmitter (Voyager) and the EGFR siRNA RFE required that cells were exposed to the signal in a non-metallic (non-ferrous) incubator, to eliminate the potential for magnetic field distortion. A custom built acrylic incubator was designed and built for the in vitro work (Fig. 1c). The coil (a simple solenoid in a rubber mold) and Voyager system were used to expose U-87 MG cells to the specific RFE of the siRNA signal we had acquired from the EGFR siRNA (Fig. 1d). As negative

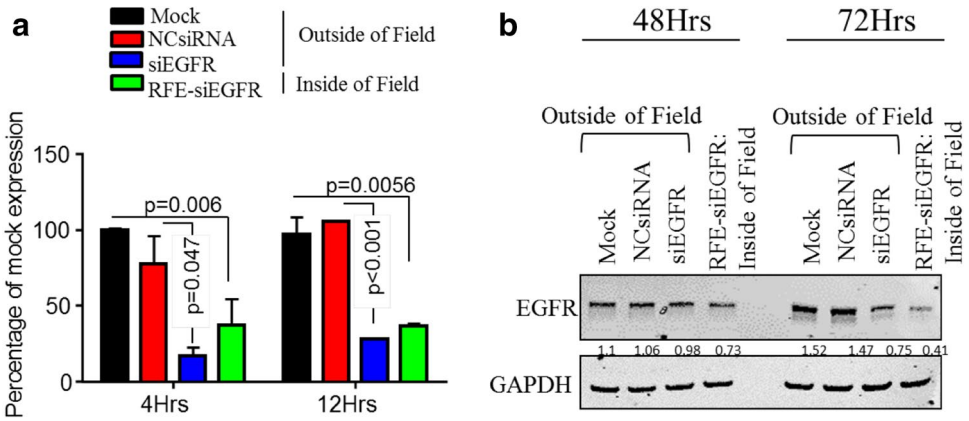


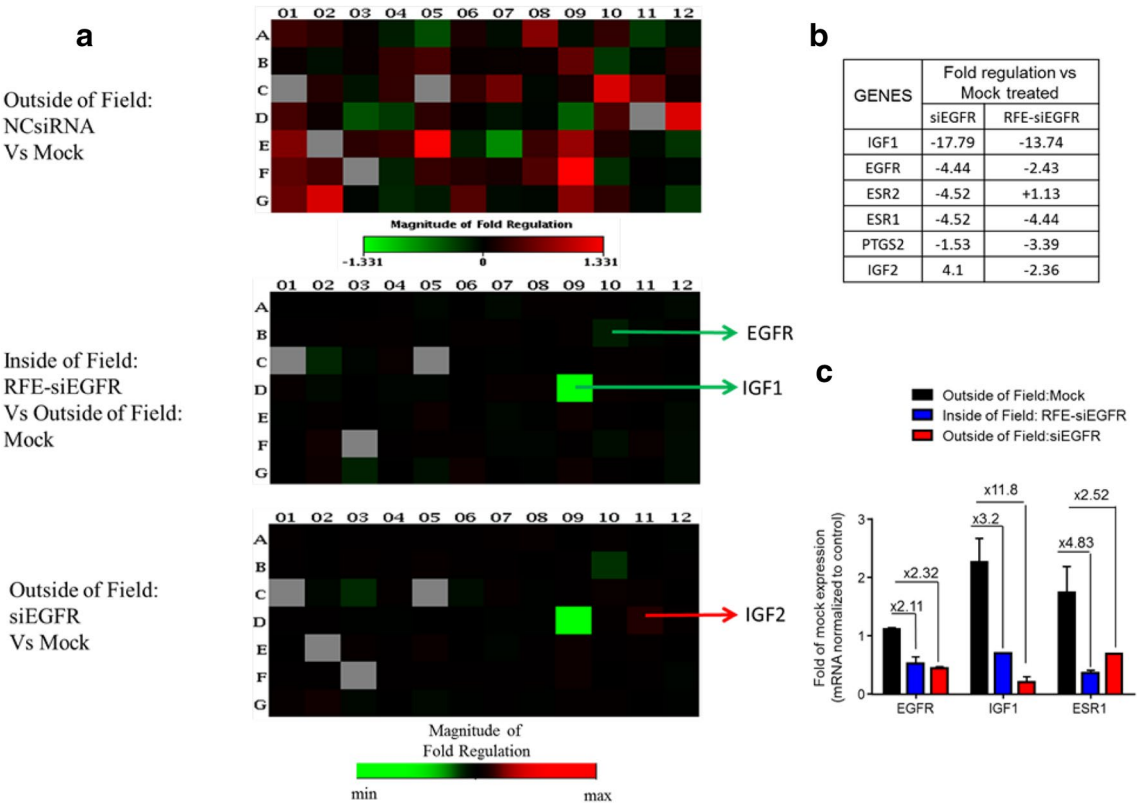
Fig. 2 Radiofrequency energy (RFE) inhibits EGFR expression in glioma cells. **a** RT-PCR analysis of EGFR transcripts 4 and 12 h after exposure with Mock, non-coding siRNA (NCsiRNA), siEGFR (EGFR specific siRNA) both are "Outside of Field" and RFE encodes siRNA against EGFR ("Inside of Field"). Experiment performed three times, data presented as percentage of mock expression ±SD. **b** Protein assessment of EGFR expression detected at 48 and 72 h post treatment by western blotting. Experiment performed three times and data from single experiment following by densitometry analysis is presented

controls, we transfected U-87 MG cells with either no (Mock-"Outside of Field") or scrambled noncoding siRNA (NC-siRNA). In addition, a signal for scrambled siRNA was used as another negative control (RFE-siScrambled). As a positive control we transfected cells with the physical siRNA sequence to EGFR (siEGFR) that was used to record the EGFR siRNA RFE. Control and experimental cells were obtained from an identical source (ATCC, Manassas, VA), and experiments were performed concurrently in the same non-metallic incubator under identical conditions, except that the control cells were grown in the upper chamber of the incubator ("Outside of Field", Supplementary Fig. 1) outside the electromagnetic field of the RFE treated cells.

RFE gene knockdown

We tested the knockdown of EGFR with siEGFR and RFE-siEGFR. After 4 h of exposure we observed significant (72 and 63%) reduction of EGFR mRNA expression in U-87 MG cells treated with siEGFR ($p = 0.047$ vs. NCsiRNA, T test) and RFE-siEGFR ($p = 0.006$ vs. mock, T test). At 12 h, the siEGFR and RFE-siEGFR treated cells demonstrated similar levels of EGFR RNA inhibition, consistent with that observed at 4Hrs after exposure (~70% inhibition, Fig. 2a). At the protein level, the loss of EGFR mRNA expression after 4 and 12 h exposure with siEGFR and RFE-siEGFR translated into inhibition of protein expression detected in U-87 MG cells. At 48 and 72 h, EGFR inhibition by RFE reduced the level of EGFR protein by 27 and 73% respectively (Fig. 2b). These data indicate that RFE can inhibit gene expression at the transcriptional and protein levels, similar to what is observed with physical siRNA.

To confirm that the RFE-mediated inhibition of EGFR was specific, we used an 80 gene PCR-based array to determine if RFE-siEGFR treatment of U-87 MG cells caused the same specific RNA knockdown pattern as that caused by the authentic EGFR siRNA treatment. U-87 MG cells were exposed to mock, NCsiRNA, siEGFR (all "Outside of Field") and RFE-siEGFR ("Inside of Field") for 12 h,



GENES	Fold regulation vs Mock treated	
	siEGFR	RFE-siEGFR
IGF1	-17.79	-13.74
EGFR	-4.44	-2.43
ESR2	-4.52	+1.13
ESR1	-4.52	-4.44
PTGS2	-1.53	-3.39
IGF2	4.1	-2.36

Fig. 3 RFE-siEGFR inhibits EGFR related gene expression in the U87 cells. **a** Quantitative comparison of transcript levels in Mock, NCsiRNA, siEGFR (both are "Outside of Field"), RFE-siEGFR ("Inside of Field")-treated cells at 12 h using the RT2-Profiler Cancer Target PCR Array (SA-Biosciences-Qiagen). Changes in transcript level are expressed as fold change relative to "Outside of Field": Mock treated cells and presented as *red* or *green* channels in the heat diagram; **b** summary of gene expression in siEGFR and RFE-siEGFR treated cells with respect of gene expression in the Mock-treated cells; **c** quantitative PCR analyses of Mock, NCsiRNA, siEGFR (both are "Outside of Field"), RFE-siEGFR ("Inside of Field") treated cells. Data suggest inhibition of EGFR, ESR1 and IGF1 mRNAs in the presence of siEGFR ("Outside of Field"), RFE-siEGFR ("Inside of Field") signals. Fold reduction is presented at the plot

and the expression levels of 80 cancer genes were measured by qRT-PCR (Fig. 3a). We observed no significant difference in gene expression between mock and NCsiRNA treated cells. EGFR siRNA treatment inhibited expression of EGFR (4.4 fold), IGF1 (17.8 fold) and ESR1 (4.52 fold vs. NCsiRNA expression) genes, that have been described earlier with respect of EGFR signaling [18–21]. Similarly, the RFE-siEGFR treatment inhibited EGFR (2.4 fold), IGF1 (13.7 fold) and ESR1 (4.4 fold, Fig. 3b). Real-time PCR analysis confirmed differences in gene expressions of EGFR, ESR1 and IGF2 between siEGFR and RFE-siEGFR treated samples (Fig. 3c). These data show a correlation between specific gene knockdown patterns in an 80 gene array in the cells treated with siEGFR and RFE-siEGFR, and indicate a specific EGFR RNA knockdown effect in the U-87 MG cells with RFE-siEGFR.

Next, we sought to confirm that the knockdown of EGFR by RFE-siEGFR was specific at the protein level. Since EGFR transactivates PLCγ1, a cellular kinase of the Ras/MAPK pathway [22, 23], we investigated the effect of siEGFR RNA and RFE-siEGFR on the expression of EGFR-related phosphorylated and unphosphorylated proteins in the U87 cells by antibody array blot-based chemiluminescence. As shown in Fig. 4a, siEGFR RNA and RFE-siEGFR treated cells both exhibit significant knockdown of protein expression of unphosphorylated EGFR (98 and 71.7% vs. NCsiRNA or Mock expression), MEK2 (50.6 and 30.8% vs. NCsiRNA or Mock expression) and PLCγ1 (52 and 37.4% vs. NCsiRNA or Mock expression). Additionally, the Mock or NCsiRNA-treated cells exhibited a high level of basal phosphorylated PLCγ1 or ERK1/2. However, in siEGFR-exposed cells this basal level was reduced by 47 and 75% vs. expression in NCsiRNA treated cells, respectively (Fig. 4b), suggesting that lack of PLCγ1 and ERK1/2 phosphorylation changes in the RFE-siEGFR-treated cells can be attributed to the specificity of EGFR inhibition mediated by specific RFE ablation.

Inhibition of EGFR expression by RFE in U87 cells inhibits cell viability in vitro and in vivo

As shown in Fig. 5a, both siEGFR RNA and RFE-siEGFR inhibited cell viability (as determined by Calcein-AM assay) of U-87 MG by 63.1 and 45.4%, respectively. To investigate the mechanism of cell toxicity of the two treatments, we performed a cell integrity assay. As shown in Fig. 5b, cellular toxicity was significantly increased in the U-87 MG siEGFR RNA and RFE-siEGFR treated cells, with 72 h exposure, by up to 45%. Of note, further western blotting analyses of siEGFR and RFE-siEGFR treated cells

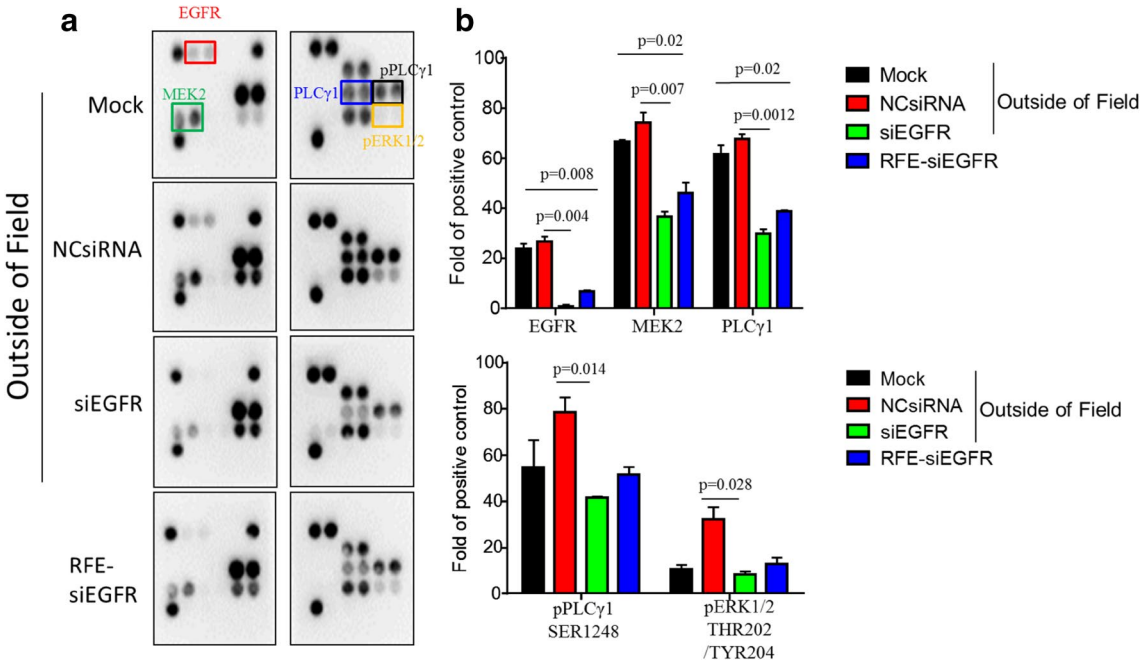


Fig. 4 RFE-siEGFR affects EGFR signaling. **a** Simultaneous detection of the phosphorylated/unphosphorylated forms 26 receptor tyrosine kinases (RTKs) related to EGFR pathway in the U87 cells treated with Mock, NCsiRNA, siEGFR ("Outside of Field") or RFE-siEGFR ("Inside of Field") for 72 h; **b** densitometry analyses of protein expression detected in (**a**) and normalized in agreement with vendor recommendations. The densitometric ratios of the EGFR, PLCγ1, MEK2, phosphor-PLCγ1 and phospho-ERK1/2 spots of siEGFR and RFE-siEGFR vs. positive control. Experiment was performed twice in duplicates and average of two independent sets was plotted as bar diagram. In comparison with the Mock treated cells, siEGFR and RFE-siEGFR exhibits reduced level of PLCγ1, EGFR and MEK2 proteins

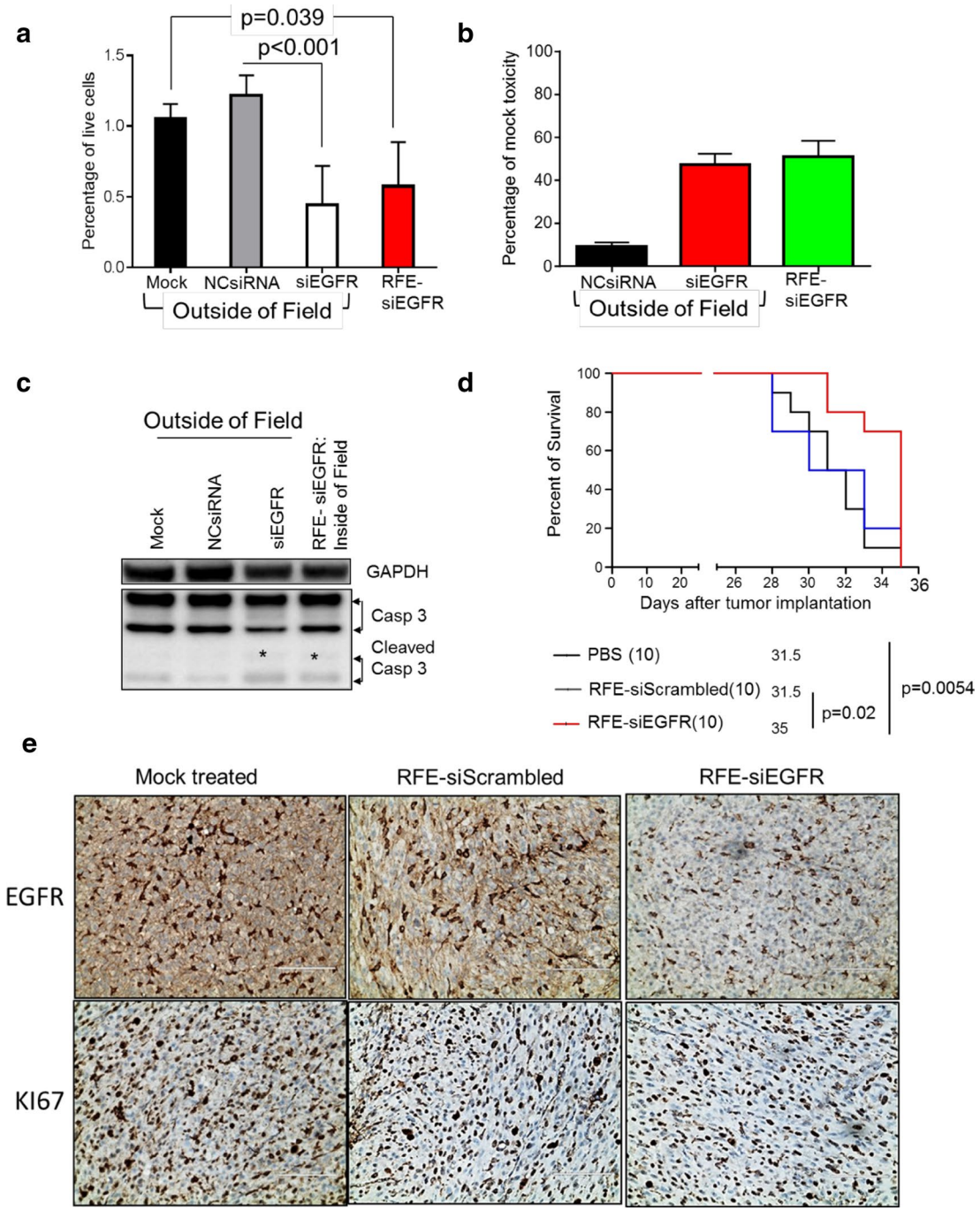


Fig. 5 RFE-siEGFR induces cell toxicity in U87 cells. **a** Calcein AM cell viability of U87 cells treated with RFE-siEGFR. Cell viability was assessed in U87 glioma cells treated with Mock, NCsiRNA, siEGFR, RFE-siEGFR for 72 h. The data represent means ± SD derived from two independent experiments (n = 9 biological replicates in each group). *P < 0.05, compared with NCsiRNA or Mock treated cells (two-tailed *t* test); **b** membrane integrity is compromised upon exposure to RFE-siEGFR. Cellular toxicity was determined using Lactate dehydrohenase release (LDH) 72 h after exposure cells with Mock, NCsiRNA, siEGFR or RFE-siEGFR. Data represent percentage of positive control ± SD derived from two independent experiments (n = 6 biological replicates in each group); **c** effect of RFE-siEGFR on U87 cells apoptosis as determined by western blot analysis of caspase 3 cleavage. Both full length and cleaved forms (p17/p15) of caspase 3 are shown. 10 µg of cell lysates obtained from U87 cells treated with Mock, NCsiRNA, siEGFR or RFE-siEGFR were loaded per gel and then probed with antibodies against caspase 3. Data from 1 experiment is shown. *Asterisks* indicates accumulation of proapoptotic caspase 3 cleaved form in the siEGFR or RFE-siEGFR treated cells; **d** Kaplan–Meier survival curve of mice with U87 intracranial xenografts treated with no-RFE, RFE-NCsiRNA or RFE-siEGFR. 300,000 U87 cells were implanted into the right caudate-putamen of athymic mice and 11 days later the mice were exposed to various treatments; **e** the human KI67 and EGFR stained images of representative mouse brains that were collected at the time of death. Representative images were acquired at 40× magnification

revealed an activation of apoptosis detected using cleaved caspase 3 (Fig. 5c). Additionally, since EGFR is involved in cell proliferation, we applied RFE-siEGFR signal to primary human GBM expressing various level of EGFR (Supplementary Fig. 3a), growing in vitro in stem cell conditions. As shown in the western blotting data presented in Supplementary Fig. 3b, RFE-siEGFR decreases EGFR expression by 20–40%. Moreover, inhibition of EGFR by RFE-siEGFR ("Inside of Field") in GBM2 and GBM3 led to reduction of total neurospheres by 15 and 45% vs. Mock: "Outside of Field" (Supplementary Fig. 3c and d).

To determine if we could inhibit U-87 MG tumor growth in a mouse model of intracranial tumor growth, we treated mice with intracranial xenograft implants of U-87 MG with either no treatment, RFE signal to scrambled siRNA or RFE-siEGFR. Inhibition of EGFR expression via RFE signal prolonged mouse survival by 20% ($p < 0.05$, Fig. 5d). Histological assessment of the brain tumor sections with U-87 MG xenografts revealed a decrease of EGFR and KI67 expression (Fig. 5e) in the presence of RFE-siEGFR. The observed decrease was graded in a blinded fashion by a veterinarian pathologist. Overall, these data suggest that exposure of cells to the RFE-siEGFR signal decreases proliferation of primary and established GBM cells, which reflects the biological significance of EGFR signaling for glioma pathobiology.

Conclusions

In this report, we present evidence of a novel technological innovation. We have developed the ability to measure and record the specific electromagnetic radiofrequency emission of a molecule in solution using SQUID technology. Furthermore, using this approach, the recorded signal of a given molecule can be transmitted back through a solenoid in such a way that living cells within the electromagnetic field will be exposed to the specific RFE signal of the recorded molecule, and will react to this signal as if the authentic molecule were present. Specifically we have shown that we can record the electromagnetic signature of the EGFR siRNA molecule, and transmit this information to human cancer cells with resulting knockdown of EGFR mRNA, protein and cell viability. These findings are the first, to our knowledge, that demonstrate specific molecular gene knockdown using RFE energy.

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Author contributions MB and JB developed Voyager technology and designed devices; IU, HF, JGY and PH designed experimental set up, performed cell culture experiments, and analyzed the data; IU, CC, XF, and HF wrote and corrected paper. MP, TN and TO designed, conducted, data analyzed in vivo experiments.

Compliance with ethical standards

Conflict of interests J.B. and M.B. are employed full time at Nativis, Inc. I.U. and X.F. are partially employed by Nativis, Inc. CC serves as a consultant for Nativis, Inc.

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