

Antibody-Mediated Delivery of Anti-*KRAS*-siRNA *In Vivo* Overcomes Therapy Resistance in Colon Cancer

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Abstract

Purpose: *KRAS* mutations are frequent driver mutations in multiple cancers. *KRAS* mutations also induce anti-EGFR antibody resistance in adenocarcinoma such as colon cancer. The aim of this study was to overcome anti-EGFR antibody resistance by coupling the antibody to *KRAS*-specific siRNA.

Experimental Design: The anti-EGFR antibody was chemically coupled to siRNA. The resulting complex was tested for antibody binding efficiency, serum stability and ability to deliver siRNA to EGFR-expressing cells. Western blotting, viability, apoptosis, and colony formation assays were performed for efficacy evaluation *in vitro*. Furthermore, therapeutic activity of the antibody-*KRAS*-siRNA complexes was examined in *in vivo* xenograft mouse tumor models.

Results: Antibody-siRNA complexes were targeted and internalized via the EGFR receptor. Upon internalization, target gene

expression was strongly and specifically repressed, followed by a reduced proliferation and viability, and induced apoptosis of the cells *in vitro*. Clonogenic growth of mutant *KRAS*-bearing cells was suppressed by *KRAS*-siRNA-anti-EGFR antibody complexes. In xenograft mouse models, anti-EGFR antibody-*KRAS*-siRNA complexes significantly slowed tumor growth in anti-EGFR-resistant cells.

Conclusions: The coupling of siRNA against *KRAS* to anti-EGFR antibodies provides a novel therapy approach for *KRAS*-mutated EGFR-positive cancer cells *in vitro* and *in vivo*. These findings provide an innovative approach for cancer-specific siRNA application and for enhanced therapeutic potential of monoclonal antibody therapy and personalized treatment of cancer entities. *Clin Cancer Res*; 21(6): 1383-94. ©2015 AACR.

Introduction

Tumors are characterized by a complex molecular landscape, in which several genomic aberrations often coexist within the same sample (1). Targeting a single oncogenic pathway at a time may result in poor efficacy as the presence of other genomic lesions may compensate or bypass single inhibitors. Unfortunately, the

majority of these oncogenes are not easily druggable by current therapeutic approaches. *KRAS* ranks high among the frequently mutated oncogenes in human carcinomas (2, 3). Of relevance to this study, *KRAS* mutations (either primary or selected after prolonged administration of EGFR inhibitors) prevent the inhibitory effect of cetuximab or panitumumab in EGFR expressing colorectal cancer. One would expect that cotargeting *KRAS* and EGFR could overcome resistance and provide an effective therapeutic regimen for these cancers. The *KRAS* molecule is part of the RAS/RAF/MEK/ERK pathway, which links signal transduction from receptor tyrosine kinases to transcription factors by GDP and GTP binding and its intrinsic GTPase function (4). Mutations in the codons 12, 13, and 61 transform the GTPase activity of *KRAS* to a constitutively active conformation and thus induce a *KRAS* gain of function. Unfortunately, all attempts to design and test direct pharmacologic inhibitors of the *KRAS* oncogene have so far failed. Indeed, the GTP-binding pocket of *KRAS* is considered an undruggable target for a variety of reasons (4). The inability to target *KRAS* directly has prompted the search for inhibitors of effector kinases in its downstream pathway. These include MEK, PI3K, and AKT inhibitors. Most of these drugs are still in early clinical trials (5-7).

RNAi strategies can offer an alternative to directly target RAS. siRNA constitutes a class of molecules that allows effective and specific targeting the biosynthesis of oncogenic proteins. Indeed, siRNA has emerged as a major tool in molecular biology techniques and an important approach to identify suitable therapy targets in cancer (8). However, siRNA therapy approaches *in vivo*

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Translational Relevance

Anti-EGFR antibody therapy is successful in multiple cancers. Patients with colon cancer harboring *KRAS* mutations do not benefit from anti-EGFR antibody therapy. This article demonstrates that an anti-EGFR antibody–siRNA complex can partly overcome this resistance of adenocarcinoma cell lines, including colon cancer cells, *in vitro* and *in vivo*. Of note, the coupling of specific siRNAs to targeting monoclonal antibodies might enhance the efficacy of antibody therapies in cancer.

are scarce (9, 10). One of the problems that hinder siRNA development as therapeutic tool concerns their delivery, representing a challenge in clinical situations. Methods of siRNA delivery and stabilization include nanoparticles and cationic liposomes (11), cationic peptides such as protamine (12, 13) or poly-arginine (14).

Monoclonal antibodies might be suitable siRNA carriers, but so far have not been thoroughly tested in cancer therapy. Monoclonal antibody-based therapies have significantly improved therapy for multiple cancer types in the last 10 to 15 years (15–17). Prominent examples for antibodies targeting the EGFR family include trastuzumab and pertuzumab for breast cancer therapy, and cetuximab (monoclonal antibody against EGFR, here referred to also as α EGFR-mAB or anti-EGFR-mAB) or panitumumab for colorectal cancer. Cetuximab is an established and effective therapy in colon cancer (18). Its use in *KRAS* wild-type colon cancer improves response rates and survival as a monotherapy and particularly in combination with chemotherapy (4, 19). Unfortunately, colon cancer cells that harbor certain RAS mutations are highly resistant toward α EGFR-mAB even with a trend for inferior survival upon α EGFR-mAB treatment (20–22). Also in patients that develop anti-EGFR antibody resistance over time, the outgrowth of clones with *KRAS* mutations appears to be the predominant mode of resistance (20).

Because of the high relevance of RAS mutations for colon cancer therapy and the proven role of *KRAS* mutations for anti-EGFR antibody resistance, we designed an approach to inhibit *KRAS* with subsequent induction of sensitivity toward anti-EGFR antibody therapy. Using different model cell lines, we demonstrate an effective method to couple specific siRNAs onto therapeutic monoclonal antibodies. An esiRNA against *KRAS* that was coupled to α EGFR-mAB effectively overcame α EGFR-mAB resistance *in vitro* and *in vivo*. These findings suggest a novel approach to treat therapy resistance in cancer.

Materials and Methods

Coupling of anti-EGFR monoclonal antibody to protaminesulfate

Protamine sulfate (1.67 mmol/L) was amino-terminally coupled to the bifunctional cross-linker Sulfo-SMCC (Pierce No. 22622 in a 1:12 molar ratio in PBS buffer, pH8.5, left to react for 1 hour at room temperature; RT), then coupled to cysteine residues of anti-EGFR monoclonal antibody (mAB; 31 μ mol/L stock; cetuximab, Erbitux, Merck-Serono) in a 5:1 molar ratio at 4°C overnight. Nonreacted educts and protamine doublets were

separated from the high-molecular weight anti-EGFR mAB–protamine product by gel filtration chromatography in Zeba spin desalting columns (Pierce No. 89891). The anti-EGFR mAB–protamine adduct was stored at 4°C and was stable for several weeks.

siRNAs

For the estimation of siRNA coupling, stability, and internalization efficiency, anti-EGFR mAB–protamine was coupled to Allstars negative control siRNA–Alexa 488 ("scrambled," cat. no. 1027284; Qiagen). Treatment experiments were done using esiRNA duplexes against *KRAS* (*KRAS*-Mission esiRNA, EHU114431) and as a control anti-GFP esiRNA (EHU-GFP, both Sigma-Aldrich). Allstars negative control siRNA–Alexa 555 was used for *in vivo*-targeting visualization (cat. no. 1027286, Qiagen).

Coupling of siRNA to anti-EGFR mAB–protamine

siRNA duplexes were bound to anti-EGFR mAB–protamine in a 4- to 10-fold molar excess at 25°C for 3 hours. This complex was prepared freshly before use.

Estimation of siRNA load capacity and serum stability of the complex

Constant concentrations (2.5 μ mol/L) of control Allstars siRNA duplexes were preincubated with increasing amounts of anti-EGFR mAB–protamine up to a 40-fold molar excess for 1 hour at 4°C, subjected to agarose gel electrophoresis and stained by ethidium bromide. Anti-EGFR mAB–protamine complexed siRNA proved to be immobile in 2% agarose, whereas the unbound 25-bp siRNA duplex band traveled at expected size.

For siRNA stability estimation, control Allstars siRNA coupled to anti-EGFR mAB–protamine was exposed to filtered HCT116 cell culture supernatant including FCS for indicated timespans, subjected to 0.4% agarose gel electrophoresis and stained by ethidium bromide. The anti-EGFR mAB–protamine–siRNA adduct was detectable as a barely mobile complex.

Cell culture

MDA-MB-435, HCT116, A549, SW620, and LoVo cells were maintained in DMEM supplemented with 10% FCS, 1% streptomycin and penicillin and 1% glutamine; HT29 was cultivated in IMDM and SW480, HCT15, and DLD1 cells in RPMI medium supplemented as above. MDA cells were *KRAS*-wild type and proved to be α EGFR-mAB sensitive (23). HCT116, A549, LoVo, and SW620 carry *KRAS* mutations in codon 12 or 13, respectively, leading to α EGFR-mAB resistance (24, 25). HT29 is BRAF mutated. Cell lines were obtained from ATCC (HT29, LoVo, A549, SW620, HCT-116, ATCC) or ECACC (DLD-1, SW480, HCT-15, ECACC). Identity was confirmed by short tandem repeat profiling before cells were taken for experiments.

Fluorescent microscopy

MDA, LoVo, and HCT116 cells were cultivated on chamber slides (Sigma C7057) and treated with anti-EGFR mAB–protamine or α EGFR-mAB alone incubated with Alexa Fluor 488-labeled Allstars control siRNA (Qiagen 1027284), at 1:10 molar ratio for 3 hours at 37°C and 5% CO₂, washed with PBS, methanol-fixed, stained with DAPI, mounted with Dako fluorescent mounting medium and photographed on a Zeiss Axioskop.

Flow cytometry

FITC-coupled anti-EGFR antibody (mouse monoclonal antibody no. 528) was purchased from Santa Cruz Biotechnology. For EGF receptor internalization studies cells were first treated with the α EGFR-mAB-based antibody constructs for 1 hour at RT and then stained for EGFR using the FITC-coupled anti-EGFR antibody.

Western blot analyses

Cells were treated with anti-EGFR mAB-protamine (50 nmol/L) coupled to the indicated siRNAs at 1:10 molar ratio once a day for 72 hours, and subjected to Western blot analysis using standard protocols with the following antibodies: anti-KRAS (ab55391, ABCAM), anti-ERK1/2 (4696), anti-phospho-ERK1/2 (4370), anti c-Myc (9402, all Cell Signaling Technology), anti EGFR (sc1005, Santa Cruz Biotechnology), anti-EZH2 (clone AC22, No. 3147 Cell Signaling Technologies), and anti β -Actin mAB (Clone AC-15, Sigma-Aldrich). Densitometric analysis of gel-electrophoretic bands was carried out using the NIH ImageJ package (<http://rsb.info.nih.gov/ij/>).

Proliferation assays

Bromodeoxyuridine (BrdUrd) incorporation analysis for cell-cycle analysis was carried out as described in Ji and colleagues (26).

Annexin V

Annexin V-PE/7AAD stainings (#556421, Becton-Dickinson) and 7-AAD staining (#559925 Becton-Dickinson) were carried out following the manufacturer's recommendations.

MTS assays

MTS viability assays (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega G5421) were carried out following the manufacturer's recommendations.

Clonogenic growth in soft agar

In brief, 5,000 trypsinized cells in 40 μ L full medium per sample were incubated with anti-EGFR mAB-protamine coupled to the indicated siRNAs at 50 nmol/L end concentration for one hour at RT, resuspended in 150 μ L of 0.5% soft agar in supplemented DMEM and cultivated for colony formation in 96-well format. A second treatment with 50 nm end concentrations was performed after 7 days of culture, fixed with 4% paraformaldehyde (PFA), counterstained with 0.1% crystal violet, photographed, and counted. All treatments were performed in groups of six wells each.

Mouse xenograft tumor model

Female CD1 nude mice (Charles River) were transplanted subcutaneously with 2×10^6 HCT116 cells or 1×10^7 HT29, DLD1, or SW480, respectively. Mice were randomized into groups of 6 and treated with cetuximab-protamine coupled to KRAS-specific esiRNA EHU114431, negative control esiRNA EHU-GFP (Sigma) or Alexa 555-labeled control siRNA (1027294324; Qiagen) or uncoupled α EGFR-mAB-protamine at 4 mg/kg twice a week intraperitoneally. For the bioavailability assessment, a single dose was used and tumors prepared 15 hours later. See Fig. 5 for details.

Quantitative real-time RT-PCR analysis

The method is described in the Supplementary Fig. S7.

Ki-67 staining

For Ki-67 staining, the tumors were isolated, washed in PBS, fixed in 4% paraformaldehyde (PFA) in PBS, embedded in paraffin, sectioned, blocked with 3% BSA, and stained with anti Ki-67 rabbit polyclonal antibody (clone MIB-1, DakoCytomation) according to standard methods. Counterstain was performed using hematoxylin.

Statistical analysis

All data are presented as mean \pm SD, if not indicated otherwise. The mean values of two groups were compared by the Student *t* test.

Results

Development of a receptor-specific siRNA carrier system

Cell type specific delivery of siRNA is a major problem for siRNA-based therapies. The coupling of specific siRNAs to therapeutic antibodies against cell surface receptors could be an effective tool for siRNA delivery. In addition, this approach could also enhance the antibody's efficacy and/or might overcome drug resistance. We tested several methods to couple antibodies with siRNA. The most effective method was chemical coupling using protamine as a siRNA complexing agent. Protamine is a positively charged molecule that is widely used as a heparin-antidote and has been extensively tested in humans in the form of protamine sulfate (28).

We coupled protamine to the monoclonal antibody against the EGF receptor (anti-EGFR mAB) using a sulfo-SMCC linker (Fig. 1A) in a 5:1 molar ratio, enabling the binding of multiple protamine molecules per molecule of mAB. siRNA binds to protamine by electrostatic interactions (13). The specific siRNA-binding capacity of protamine was analyzed by electrophoresis to determine bound versus free siRNA (14). On this low percentage gel, a degradation of siRNA would be seen as a decreased band intensity that represents the stable siRNA-protein conjugate. After ethidium bromide stain, no degradation of the high-molecular weight siRNA-protein complex was observed which indicated an increased stability (Fig. 1B).

Next, we tested protamine dependency of siRNA binding (Fig. 1C and D). A minimum of eight molecules of siRNA were able to bind to one molecule α -EGFR mAB-protamine, indicating a significant siRNA load for the antibody-protamine complex, while a higher molar excess of siRNA leads to an overflow of unbound siRNA.

Internalization of the antibody complex upon binding to the receptor (29) is required for intracellular siRNA activity. Therefore, we first incubated the anti-EGFR antibody either alone or as a complex with protamine and siRNA with HCT116 cells that express EGFR on their surface (Fig. 1E). FACS analysis using a FITC-labeled anti-EGFR antibody that binds to a different extracellular epitope of EGFR than cetuximab α EGFR-mAB revealed (Fig. 1E, second panel) that EGFR expression on the surface of HCT116 cells was no longer detectable when the cells were preincubated with α EGFR-mAB alone (Fig. 1E, third panel) or anti-EGFR mAB-protamine-siRNA complex (Fig. 1E, fourth panel). These analyses indicated that the α EGFR-mAB-siRNA complex and the chemical modification in combination with the highly anionic siRNA load did not interfere with EGF receptor binding and internalization.

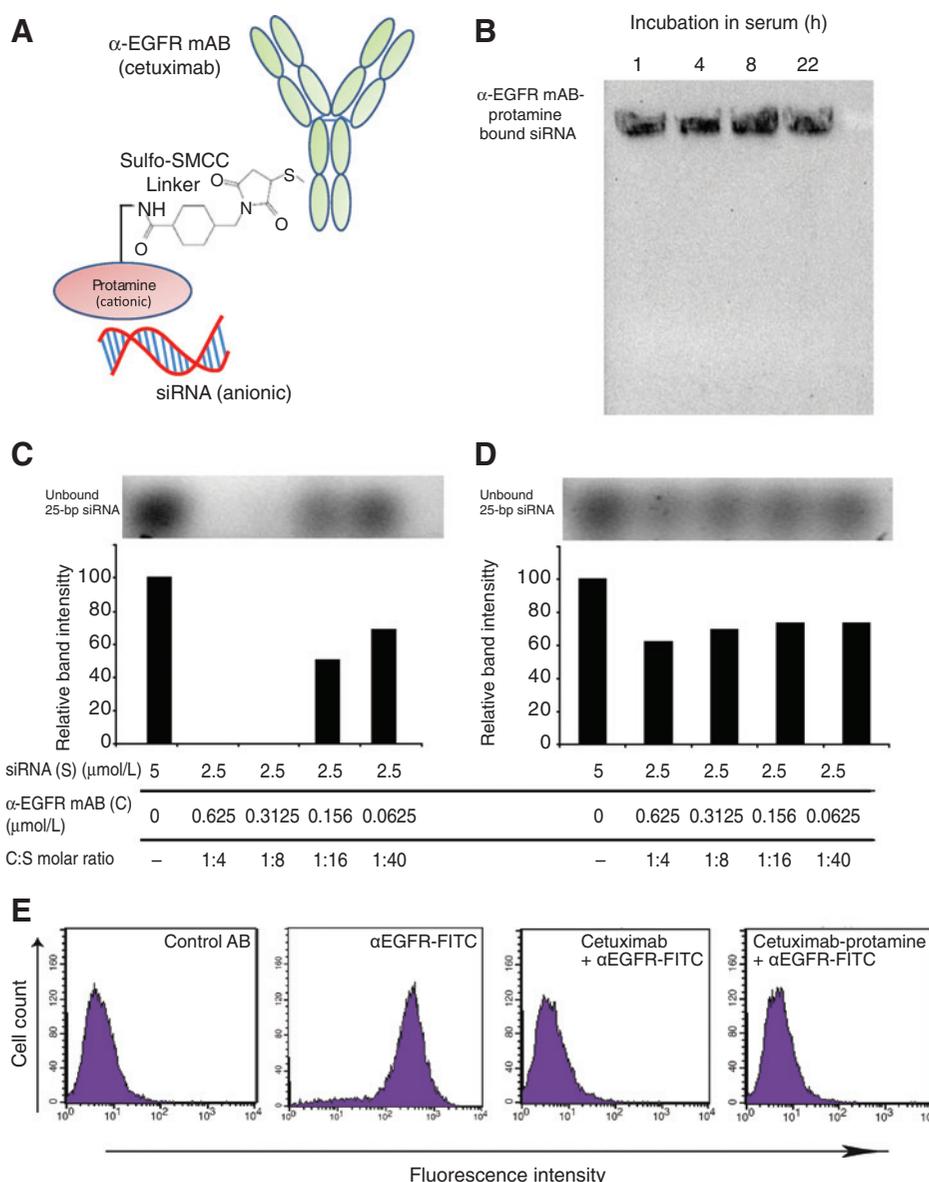


Figure 1.

Properties of the EGFR-targeting siRNA carrier system. A, graphical depiction of anti-EGFR mAb cetuximab-IgG coupling to protamine by bivalent cross-linker sulfo-SMCC. siRNAs bind to protamine via electrostatic interactions. B, an agarose gel (0.4%) electrophoretic analysis of anti-EGFR mAB-protamine-siRNA complex stability. Allstars negative control siRNA (Qiagen) coupled to anti-EGFR mAB-protamine was protected from enzymatic degradation during incubation in cell culture medium. The bands in the low concentrated agarose running higher than 25 kb represent the largely immobile antibody-siRNA conjugates and do not show band density decrease during incubation time. C and D, analysis of siRNA-binding capacity. Increasing amounts of anti-EGFR mAB-protamine (C) or the anti-EGFR mAB alone (D) were coupled to constant amounts of Allstars negative control siRNA. The nonbound 25-bp siRNA was visualized on an agarose gel. Densitometric analysis of the nonbound siRNA bands in the agarose gels revealed that anti-EGFR mAB-protamine binds up to 8 mol siRNA per mol of protein in contrast with uncoupled anti-EGFR mAB (C). Nonmodified anti-EGFR mAB does not bind siRNA (D). E, flow-cytometric analysis of EGFR receptor exposure on HCT116 cells before and after incubation with anti-EGFR mAB. EGFR surface expression was detected using a FITC-labeled anti-EGFR antibody that bound to a different extracellular epitope of EGFR than cetuximab. HCT116 cells showed high EGFR expression on their surface (" α EGFR-FITC," second panel, left) in comparison with a control antibody ("control AB," first panel). EGFR expression was no longer detectable due to internalization of the receptor after treatment with uncoupled cetuximab ("cetuximab + α EGFR-FITC," third panel) or cetuximab-protamine-siRNA control ("cetuximab-protamine + α EGFR-FITC," fourth panel) as shown by flow cytometry.

To further verify internalization, we coupled anti-EGFR mAB-protamine with scrambled control siRNA labeled with Alexa Fluor 488. Several carcinoma cell lines (HCT116, LoVo, and MDA cells) were incubated with the conjugate and showed

widespread and significant siRNA-antibody internalization as evident by the cytoplasmic localization of the green fluorescence. Detailed results are presented in Supplementary Fig. S1A.

Anti-EGFR mAB-directed RNAi reduces target gene expression in EGFR-expressing carcinoma cell lines

Because the anti-EGFR mAB–protamine–siRNA complexes were effectively taken up by cells, we next checked the intracellular functionality of RNAi. A potential problem with using siRNA as therapeutic agents is off target gene silencing effects. We used enzymatically produced (e)siRNA against *KRAS*, which consists of a complex pool of active inhibitory RNA fragments and possesses higher specificity and efficiency (30). *KRAS* in its mutated form in codons 12, 13, and 61 is a strong predictive biomarker for therapy resistance toward cetuximab/ α EGFR-mAB and panitumumab due to gain of function in its GTPase activity (4, 19). For suppression of *KRAS*, we made use of enzymatically digested (e) siRNAs, exhibiting maximal RNAi effects with minimal cross-silencing. As a control, esiRNA against xenoprotein GFP was used. The *KRAS* esiRNA bound to a concentration of 50 nmol/L anti-EGFR mAB–protamine (mAB-P) was sufficient to suppress *KRAS* protein expression in *KRAS*-mutant HCT116 and LoVo adenocarcinoma cell lines by more than 80% as analyzed by Western blotting (Fig. 2A). Control esiRNA did not affect *KRAS* expression level (Fig. 2A). The canonical RAS signaling pathway includes the RAF/MEK/ERK cascade involved in mitogenic signaling. Accordingly, ERK1/2 phosphorylation was markedly diminished in LoVo and HCT116 cells treated with α EGFR-mAB-*KRAS* esiRNA. A known target molecule of the ERK signaling is the c-Myc oncogene (31). Also, c-Myc protein expression was reduced in cells showing antibody-mediated *KRAS* knockdown and subsequent ERK inactivation (Fig. 2A).

As an independent experimental assay to verify the gene-specific effect, we used a specific siRNA against *EZH2*, a well-known oncogene in multiple solid tumors (32), which is highly expressed in adenocarcinoma cells. The exposure of α -EGFR mAB–P-*EZH2*-siRNA complex in HCT116 and A549 cells repressed *EZH2* expression in an antibody-specific fashion almost completely (Supplementary Fig. S2A). We next tested whether the α -EGFR mAB–siRNA complex inhibited tumor cells *in vitro*. As a functional assay, we determined anchorage-independent clonogenic growth in semi-solid medium as a readout for tumorigenic potential by self-renewal (33) of the respective cell line (Fig. 2B). As expected, α EGFR-mAB and α EGFR-mAB coupled to control esiRNA did not inhibit clonogenic growth in *KRAS*-mutant cells (Fig. 2B, left and middle). For example, HCT116 (Fig. 2B), LoVo (Fig. 2C), or A549 cells (Supplementary Fig. S4B). However, the α EGFR-mAB–protamine–*KRAS*-esiRNA complex inhibited clonogenic growth of *KRAS*-mutant (Fig. 2B–F), but not BRAF-mutant cell lines (Fig. 2G).

As expected, α EGFR-mAB–protamine alone reduced clonogenic growth in *KRAS* wild-type MDA cells (Supplementary Fig. S4A) independent of the attached siRNA and targeting with *KRAS*-siRNA did not enhance therapy response toward anti-EGFR antibody in *KRAS* wild-type cells.

Overcoming cetuximab/ α EGFR-mAB resistance in *KRAS*-mutated colorectal cell lines *in vitro*

In a MTS viability-assay, we exposed HCT116, LoVo, DLD1, SW480, and HT29 cell lines to increasing concentrations of α EGFR-mAB–control esiRNA or α EGFR-mAB-*KRAS* esiRNA ranging from 0 to 500 nmol/L (Fig. 3A). All three cell lines proved to be resistant to α EGFR-mAB–control esiRNA, probably caused by mutation-dependent enhanced and constitutive *KRAS*/BRAF signaling, but the *KRAS*-mutant cell lines showed a significant

sensitivity to α EGFR-mAB coupled to *KRAS* esiRNA (Fig. 3A and B), whereas the BRAF-mutant HT29 cell line remained resistant to α EGFR-mAB despite *KRAS* esiRNA treatment (Fig. 3A–C, right hand). Of note, the EGFR-negative cell line SW620 showed neither reaction upon α EGFR-mAB–control nor α EGFR-mAB–esi*KRAS* treatment, indicating that the efficacy entirely depended on the expression of EGFR (Supplementary Fig. S3).

Treatment response in LoVo and HCT116 cell did not differ in the *in vitro* applications although LoVo expressed more EGFR than HCT116 (Supplementary Fig. S2B). This finding suggested that α EGFR-mAB-*KRAS* esiRNA therapy is active in cancer cells with varying levels of EGFR expression, although a threshold expression of EGFR is necessary. On the other hand, the cell lines used here varied in their microsatellite stability index, the majority of the responsive cell lines were stated microsatellite instable, whereas SW480 was MSS without change of responsiveness to the α EGFR-mAB-*KRAS* esiRNA therapy.

HCT116, LoVo, DLD1, SW480, and HT29 cell lines were analyzed for their cell-cycle status using BrDUrd incorporation (Fig. 3B). BrDUrd incorporation was strongly reduced for both cell lines after α EGFR-mAB-*KRAS*-esiRNA exposure (Fig. 3B, first to fourth panel) compared with the control samples. The BRAF-mutant HT29 cells, as expected, did not respond to this treatment (Fig. 3B, right hand).

Last, the same *KRAS*-mutated cell lines exhibited signs of apoptosis in an Annexin V staining assay when treated with mAB-*KRAS*-esiRNA, but not control samples (Fig. 3C, first to fourth panel). The BRAF-mutated HT29 cells did not undergo apoptosis following treatment with any of the complexes (Fig. 3C, right hand).

Efficacy of *KRAS*-siRNA–anti-EGFR complexes in mouse tumor xenografts

Next, we analyzed the effect of α EGFR-mAB–esiRNA complexes *in vivo*. First, we checked for the bioavailability of the antibody–siRNA conjugate *in vivo* by coupling fluorescence-labeled Alexa 568 siRNA to α EGFR-mAB–protamine in concentrations of 4 mg/kg mouse body weight intraperitoneally or subcutaneously close to a HCT116 tumor. Cryosections from tumors treated with α EGFR-mAB–Alexa 568–control siRNA exhibited accumulations of red fluorescent cellular signals in the rim region of the tumor (Fig. 4A and B), whereas naked Alexa 568–control siRNA administered showed no significant enrichment of fluorescence signals (Fig. 4D and E).

To test the therapeutic effect of the antibody–esiRNA treatment, α EGFR-mAB–resistant SW480, DLD1, HCT116, or HT29 carcinoma cells were subcutaneously injected and mice with acceptable tumor growth were randomized into groups. Mice were treated twice weekly by i.p. injections with anti-EGFR mAB–protamine (mAB-P) alone or with mAB-P–esiRNA complexes and tumor growth was measured (Fig. 5A). Cetuximab-resistant tumors continued to grow despite anti-EGFR mAB–protamine therapy (Fig. 5B–D, blue rhombs) and also with control esiRNA coupled to anti-EGFR mAB–protamine (Fig. 5B–D, red squares). In contrast, anti-EGFR mAB–protamine–*KRAS*-esiRNA complexes significantly inhibited tumor growth (Fig. 5B–D, green triangles), including complete loss of tumors in one out of six cases of the mAB–protamine–*KRAS*-esiRNA group in DLD1 and SW480. At the defined end of the therapy, tumor volumes were reduced to about 25% in SW480 and DLD1 compared with the volumes of the fast growing control groups and to less than half

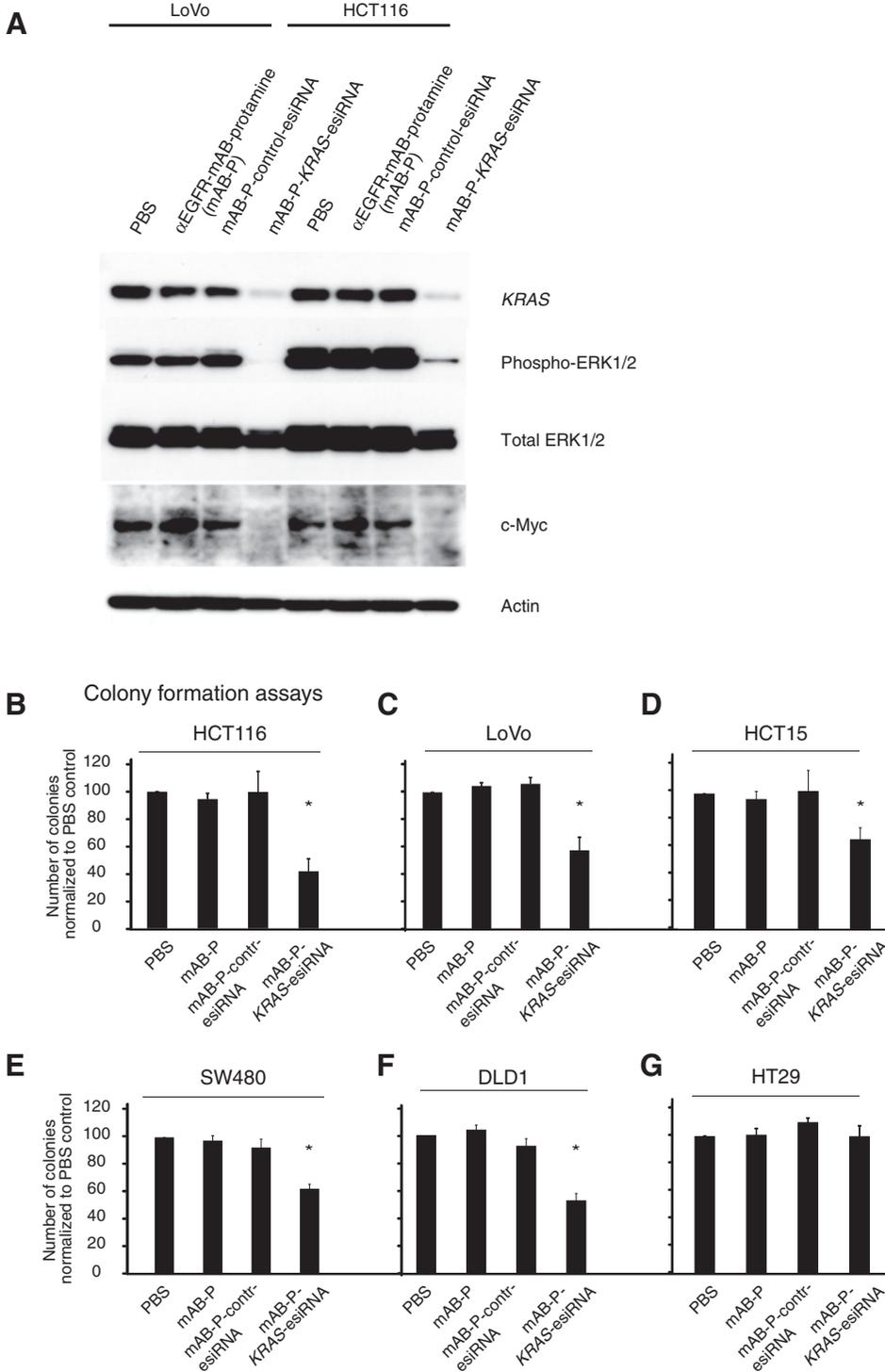


Figure 2. Anti-EGFR antibody-directed RNAi suppresses target gene expression and clonogenic growth in EGFR-expressing carcinoma cell lines. A, HCT116 and LoVo cells were exposed to 50 nmol/L of anti-EGFR mAB-protamine ("mAB-P") coupled to GFP control esiRNA ("mAB-P-control esiRNA") or to *KRAS* esiRNA ("mAB-P-*KRAS* esiRNA") for 72 hours at 37°C. Western blot analysis was performed for *KRAS*, phospho-ERK1/2, total ERK1/2, cMyc, and actin as loading control. Expression of *KRAS* and c-Myc and phosphorylation of ERK1/2 were suppressed upon mAB-P-*KRAS* esiRNA treatment. Shown here is one representative of three independent experiments. B-G, for colony formation assays, cells from different colon carcinoma lines were treated with anti-EGFR mAB-protamine (mAB-P) esiRNA at 50 nmol/L final concentration, resuspended in 96-well plates in soft agar and analyzed for clonogenic growth. After 7 days, a second treatment was performed. Colonies were fixed, stained with crystal violet, photographed, and counted after 3 weeks. Significance: *, $P < 0.005$, of mean values anti-EGFR mAB-protamine-*KRAS*-esiRNA group versus anti-EGFR mAB-protamine-control group, respectively. Graphs depict mean of three independent experiments \pm SD normalized to PBS mean.

the size of the anti-EGFR-P mAB only treated HCT116 tumors. BRAF-mutated HT29 tumors did not respond to anti-EGFR mAB-protamine-*KRAS*-esiRNA complexes (Fig. 5E). The therapy success was also mirrored by the significantly lower average tumor weights depicted in Fig. 5F-H in *KRAS*-mAB-treated SW480, DLD1, and HCT116. Again, average tumor weight was unchanged between the groups of treated HT29 tumors (Fig. 5I). In contrast,

there was no significant variation of total mouse weight development during the treatment period in the three groups, indicating lack of toxicity (Supplementary Fig. S5). Nonetheless, the anti-EGFR antibody specifically reacts with human EGFR and EGFR-mediated human side effects cannot be evaluated in this model.

Further experiments were performed to analyze pharmacodynamic activity *in vivo*. We performed real-time RT-PCR analysis on

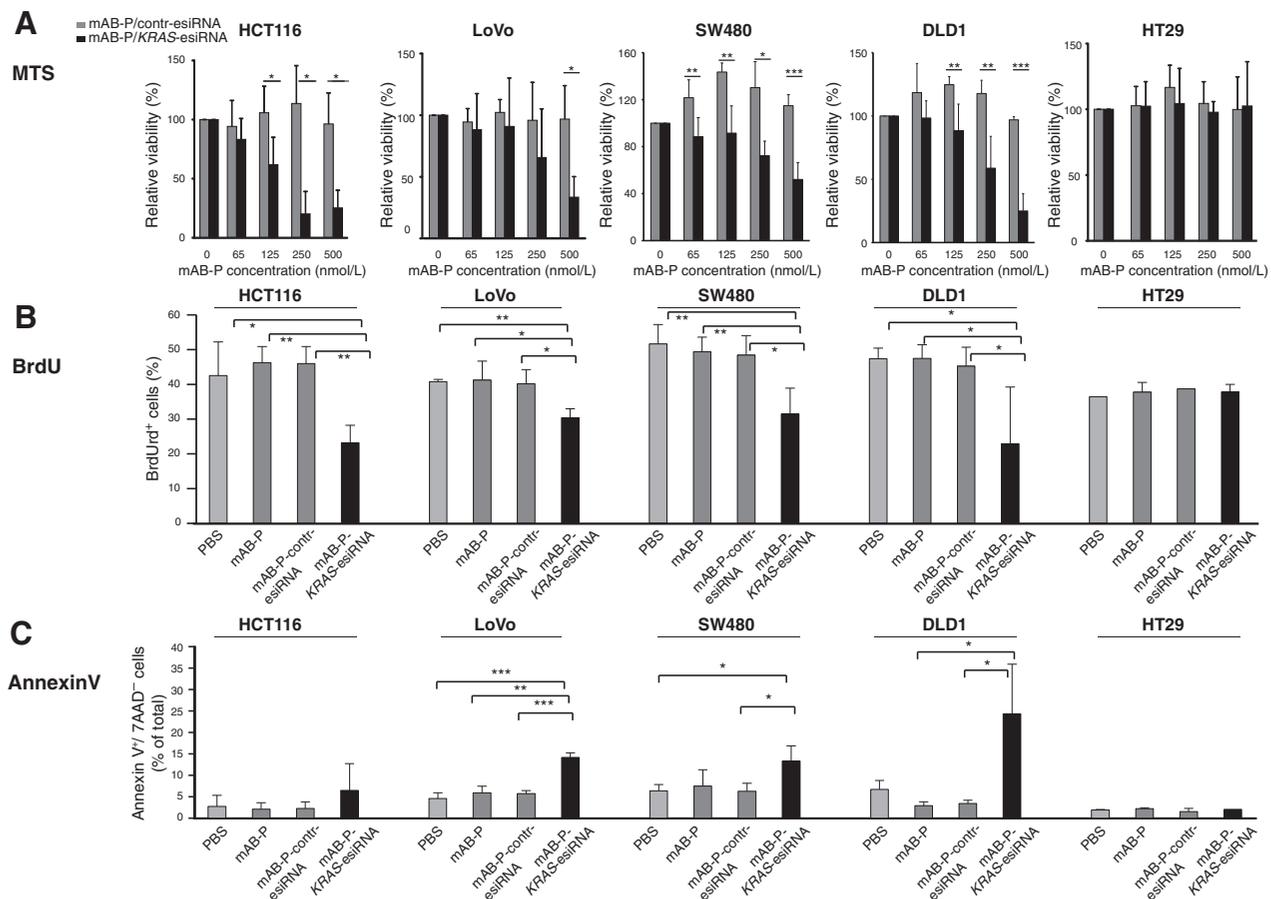


Figure 3.

Overcoming anti-EGFR antibody resistance in *KRAS*-mutated adenocarcinoma cell lines *in vitro*. We exposed cells to α EGFR-mAB in a complex with either control or anti-*KRAS* siRNA and analyzed the cells for proliferation and apoptosis using MTS assays (A) and the incorporation of BrdUrd (B). We used eight different adenocarcinoma cell lines expressing EGFR, four of them exhibiting the G13D *KRAS* mutation (HCT116, HCT15, DLD1, and LoVo), one cell line containing the G12V *KRAS* mutation (SW480), another the G12S mutation (A549), and finally a *KRAS* wt cell line with a BRAF V600E mutation (HT29). An overview of the characteristics of cell lines used in this study is given in Supplementary Fig. S2B and S2C. Cells were tested for their EGFR expression status which differed widely among different cell lines, including the G12V *KRAS*-mutated SW620 colon carcinoma cells, which do not express EGFR. A, MTS viability assay: cells were exposed to increasing concentrations of anti-EGFR mAB-control esiRNA and anti-EGFR mAB-*KRAS* esiRNA. HCT116 and LoVo, SW480 and DLD1 cells (first to fourth panel) showed significantly decreased viability by increased mAB-*KRAS* esiRNA, but not mAB-control esiRNA. BRAF-mutant HT29 cells (right) did not differ in viability upon treatment. Significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Graphs depict mean of three independent experiments \pm SD. B, proliferation/BrdUrd incorporation assay: cells were treated with 50 nmol/L of mAB-*KRAS*-esiRNA, labeled with BrdUrd and analyzed by FACS. HCT116 and LoVo SW480 and DLD1 showed significantly (*, $P < 0.01$) reduced proliferation when treated with mAB-*KRAS* esiRNA, but not mAB-control-esiRNA (first to fourth panel). BRAF-mutant HT29 cells did not show a decreased proliferation upon treatment (right). Graphs depict mean of three independent experiments \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. C, apoptosis induction: cells were exposed to 50 nmol/L mAB-esiRNA and stained with anti-annexin V antibody and 7AAD for FACS analysis. Annexin V-positive/7AAD-negative cells represent the apoptotic cell population and were determined by FACS. HCT116 and LoVo SW480 and DLD1 cells treated with mAB-*KRAS* esiRNA showed increase of cells in early apoptosis (first to fourth panel, * = $P < 0.002$ for LoVo cells). BRAF-mutant HT29 cells treated with the same combination did not show an increase in apoptosis (right). Graphs depict mean of three independent experiments \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

tumors after α EGFR-mAB-*KRAS* or α EGFR-mAB-control esiRNA treatment. In HCT116 tumors treated with α EGFR-mAB-*KRAS* esiRNA, *KRAS* mRNA levels were consistently suppressed compared with α EGFR-mAB-control treatment (Supplementary Fig. S7).

Following up *in vitro* results, we also analyzed Ki-67 as proliferation marker in tumors from mice treated *in vivo*. In the *KRAS*-mAB tumors, Ki-67 staining was significantly reduced (Fig. 6C: SW480, and 6F: DLD1; Supplementary Fig. S6, bottom left and right hand: HCT116), while Ki-67 positive cells were not reduced in treated HT29 tumors (Fig. 6I, compared with 6G and H). In addition, control esiRNA did not show a significant effect on Ki-67 expression (Supplementary Fig. S6, top).

Interestingly, HCT116 cells contain a PIK3CA activating mutation H1047R besides the *KRAS* G13D activating mutation (34, 35). We performed colony assays (details presented in Supplementary Fig. S8) with PIK3CA and *KRAS* esiRNA coupled to α EGFR mAB. Of note, HCT116 cells treated with α EGFRmAB carrying combined *KRAS* and PIK3CA esiRNAs showed a cooperative effect further reducing colony growth. This clearly indicates that complete molecular characterization of the signaling pathways (see also Supplementary Fig. S8B) driving malignant behavior of the respective cell type would enable us to tailor the siRNA delivery system described to optimal activity.

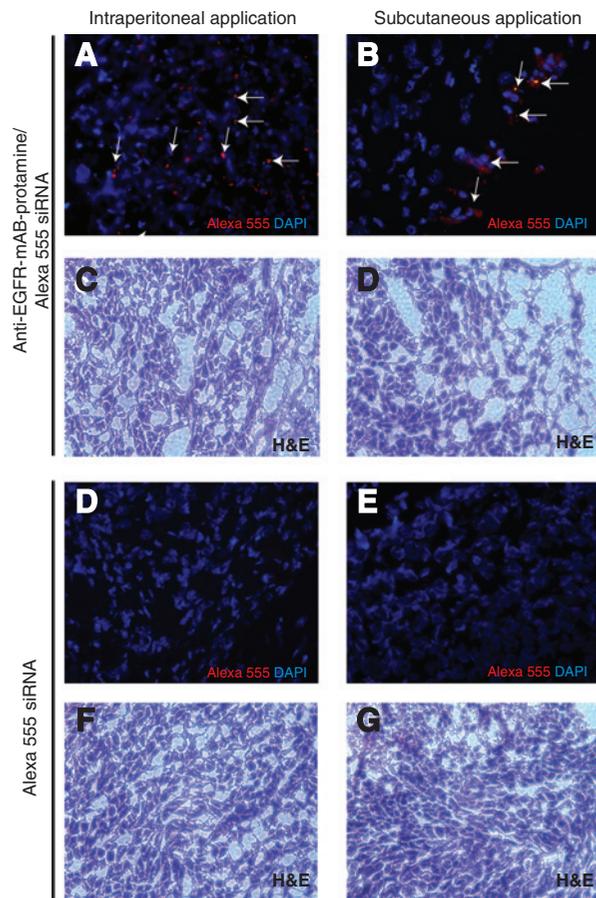


Figure 4. Antibody-dependent siRNA targeting *in vivo*. A total of 2×10^6 cetuximab-resistant HCT116 colon adenocarcinoma cells were subcutaneously implanted in CD1 nude mice. After tumor size reached an average of 200 mm^3 , mice were injected with 4 mg/kg anti-EGFR mAb-Alexa 555-labeled Allstars negative control siRNA or with the uncoupled negative control siRNA. Tumors were resected 15 hours after injection. In mice injected intraperitoneally (A) or subcutaneously (B) with mAb-555-siRNA, tumors showed Alexa 555 fluorescent signals in the tumor rim. C and D depict parallel sections of A and B stained hematoxylin and eosin (H&E). In mice injected with uncoupled siRNA-Alexa 555 (D and E), no fluorescent signals were detectable. F and G again show parallel sections of D and E stained H&E.

Discussion

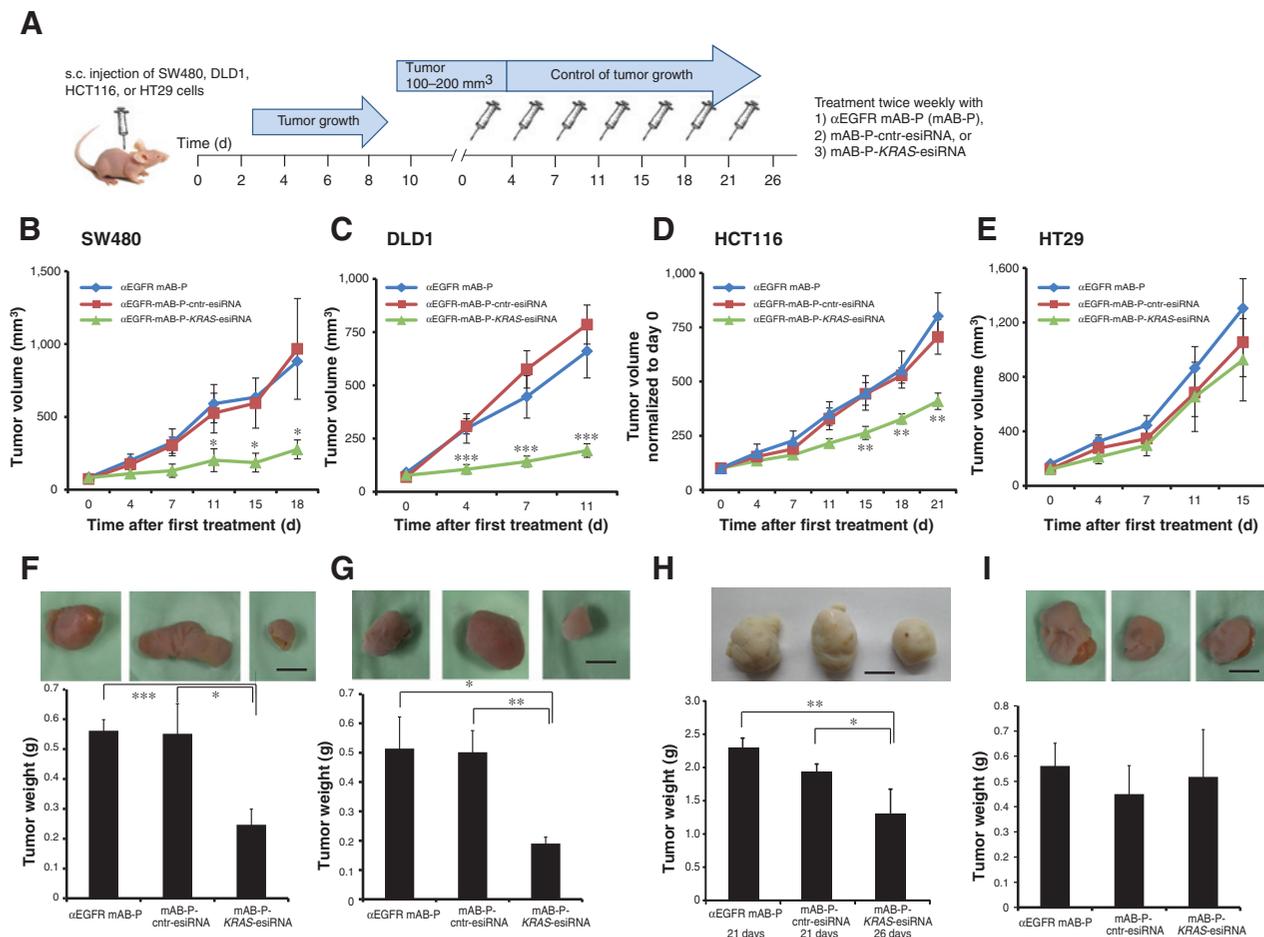
The specific targeting of oncogenic drivers and resistance mechanisms for conventional therapies offers substantial hope for improved therapies for cancer. However, the therapeutic targeting of many driver mutations in cancer has so far remained elusive. *KRAS* ranks high among the most frequently mutated genes in carcinomas and as a mediator of therapy resistance against EGFR antibodies such as cetuximab (4, 36). Unfortunately, despite the long available knowledge about mutant *KRAS*, no successful therapeutic approaches have entered clinical practice. One targetable molecule MEK might help to block signaling downstream of mutated RAS and several inhibitors of MEK are currently in clinical trials (6, 7, 37). Only recently, a new MEK inhibitory substance was reported to rescue cetuximab resistance in *KRAS*-mutated colorectal cancer cell lines (38). RAS mutations are not only drivers in tumorigenesis, but also mediators of

therapy resistance to anti-EGFR antibodies. The frequently encountered RAS mutations in colon carcinoma preclude treatment with anti-EGFR antibodies such as cetuximab and panitumumab for many patients.

In the current study, we demonstrate that a *KRAS*-specific siRNA can be delivered into tumor cells via cetuximab-protamine and can effectively inhibit tumor growth by downregulation of *KRAS* and subsequent deactivation of ERK and the MAPK pathway in *KRAS*-mutated but not BRAF-mutated cell lines. This targeting is dependent of the antibody action of finding, binding, and internalizing EGFR on the tumor cell and as a second step, inhibiting MAPK signaling by *KRAS* RNAi. One of the possible consequences of ERK signaling activity depicts the regulation of stability of the oncogene *c-Myc* by its phosphorylation (31, 39). Here, *c-Myc* expression was diminished upon *KRAS* knockdown along with the phosphorylation of ERK. Overexpression of Myc proteins in cultured cells and in transgenic animals blocks differentiation and induces neoplastic transformation (40). Accordingly, it is possible that reduced *c-Myc* activity contributes to the reduction of colony formation, viability, and tumor growth effect of the α EGFR-mAb cetuximab-esi*KRAS* conjugate therapy. Of note, we provide evidence that a threshold expression of EGFR is required to enable cetuximab-*KRAS* siRNA response, these data are in line with current clinical practice: anti-EGFR antibody therapy is indicated for EGFR-expressing colorectal cancers.

Cetuximab monotherapy delays tumor growth in patients with colorectal carcinoma with less than 10% of patients achieving a partial response according to RECIST criteria (41). Also, only approximately 10% of xenografts transplanted from patient-derived, metastatic colorectal cancer samples showed tumor reduction upon cetuximab treatment (42). As a clinical consequence, cetuximab in colorectal cancer is usually used as combination therapy.

Here, we transplanted CD1 nude mice subcutaneously with four colorectal cancer cell lines harboring different cetuximab-resistance connected mutations, for example, *KRAS* G12V in the SW480 cell line, or the G13D in HCT116 and DLD1, as well as the BRAF V600E-mutant HT29 cell line. Upon α EGFR-mAb cetuximab-*KRAS*-esiRNA treatment, the xenografted *KRAS*-mutated cell lines reacted with diminished proliferation resulting in considerable reduced growth of treated tumors compared with control groups, including tumor extinction in one out of six tumors in SW480 as well as DLD1 in the α EGFR-mAb cetuximab-*KRAS* siRNA group. The cell line HCT116 represents one of the most cetuximab-resistant colorectal cancer cell lines harboring additional aberrations that potentially confer therapy resistance such as mutated PIK3CA, low PTEN expression, and mutated β -catenin (24, 35, 43). Therefore, we did not anticipate the conjugate monotherapy against *KRAS* to induce major remissions *in vivo*. Instead, the HCT116 xenograft tumors showed a significant delay of tumor growth to about half the size of the controls. Keeping in mind that signaling downstream from EGFR can be mediated by the MAPK as well as PI3K pathway, targeting a crucial checkpoint of PI3K by the cetuximab-siRNA conjugate might be a logical consequence. The conjugation of a PIK3CA esiRNA to cetuximab and consequent treatment of HCT116 cells in colony assays reduced the colony formation in a comparable manner like *KRAS* esiRNA, whereas the combination of both *KRAS* and PIK3CA esiRNAs acted cooperatively and reduced colony numbers by 80%, indicating that both aberrant pathways are involved in and are necessary for cetuximab resistance in an cooperative fashion.

**Figure 5.**

Overcoming anti-EGFR antibody resistance in *KRAS*-mutated adenocarcinoma tumors *in vivo* in CD1 nude mice. A, outline of the anti-EGFR antibody-siRNA treatment regimen. B-E, a total of 2×10^6 to 1×10^7 colon adenocarcinoma cells were subcutaneously implanted in CD1 nude mice. After tumors reached an acceptable size, three groups of mice ($n = 6$ each) were treated with anti-EGFR mAB-protamine (α EGFR mAB-P), anti-EGFR mAB-protamine-*KRAS*-esiRNA (α EGFR mAB-P-*KRAS*-esiRNA), or anti-EGFR mAB-P-control-esiRNA (α EGFR mAB-P-cntr-esiRNA) at 4 mg/kg twice a week by intraperitoneal injection. For a single dose, 750 pmol of anti-EGFR mAB-protamine was coupled to 3.5 nmol of the respective esiRNA. Tumor growth was followed with standard caliper measurements in a blinded fashion twice a week. Tumor volumes were calculated by the formula length \times width² \times 0.52 (27). At the end of the experiment, animals were euthanized by cervical dislocation in deep CO₂ anesthesia, primary tumors were surgically removed, and tumor weight was determined. Values represent absolute tumor volumes if not stated otherwise. *KRAS*-mutated colorectal cancer xenograft-tumor-bearing mice treated with anti-EGFR mAB-P-*KRAS* esiRNA complexes showed a markedly delayed growth compared with anti-EGFR mAB-P and anti-EGFR mAB-P-control esiRNA. Tumor growth was followed by caliper measurements. E, in BRAF-mutated HT29 colorectal cancer tumors α EGFR mAB-P-*KRAS*-esiRNA did not show significant influence on tumor development. Error bars in Fig. 6B-E represent SEM. *P* values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. F-I, at the end of the experiment, tumors were prepared and photographed and weighted. Photographs show representative examples. Average weight of anti-EGFR mAB-protamine-*KRAS* esiRNA treated *KRAS*-mutated tumors was significantly lower than that of the control groups, including complete loss of tumors in 1 out of 6 cases in SW480 as well as DLD1; $n = 5/6$ (mAB-P-*KRAS* esiRNA group). BRAF-mutated tumors did not show any influence upon treatment. Note that tumor weight includes a connected section of mouse dermis along with tumor for histologic evaluation leading to elevated weights, especially in small tumors; $n = 6$ (other groups; contr. = GFP control). *P* values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Bar, 1 cm.

A potential problem with using siRNA as therapeutic agents is off target gene silencing effects. We minimized cross-silencing effects by using enzymatically produced esiRNA rather than chemically synthesized siRNA. It was shown that presenting a multitude of enzymatically produced specific silencing RNA fragments have higher specificity and efficiency (30). Of note, the α EGFR-mAB cetuximab-*KRAS*-esiRNA treatment was able to overcome therapy resistance induced by *KRAS* mutations. Resistance based on absence of EGFR expression or on BRAF mutations was not affected by cetuximab-*KRAS*-esiRNA. These findings provide further evidence for the dual specificity of the observed therapeutic effects.

The problem to successfully deliver siRNA into cancer cells has remained a major obstacle for the further development of siRNA-based therapies (44). There are several strategies to deliver siRNA nonspecifically. For example, we have previously shown that injection of naked plasmids that encode shRNAs can inhibit metastatic development in mouse models (45, 46). Because of the short half-life of siRNA after injection into the blood stream (44), siRNAs are often directly injected into different organs or tumors (10). This approach is unlikely to yield major systemic responses and is therefore also unlikely to succeed for metastatic cancer. On the other hand, use of nanotechnology and/or the

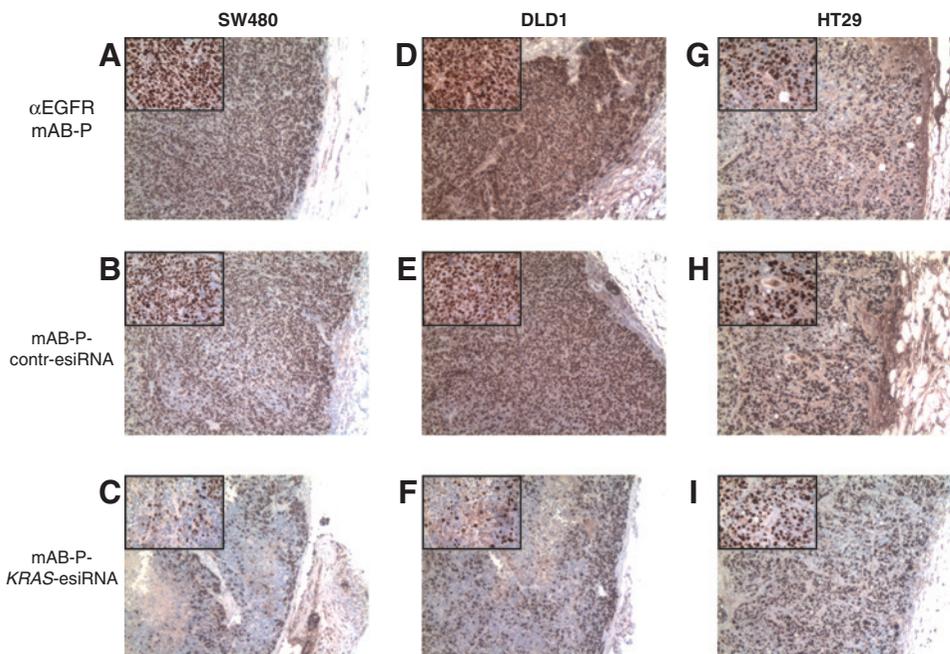


Figure 6. mAB-P-*KRAS* esiRNA treatment induced proliferation control in colorectal cancer xenograft tumors. In histologic sections of SW480, DLD1, and HT29 xenograft tumors from the experiment presented in Fig. 5, we observed a markedly reduced expression of the general proliferation antigen Ki-67 judged by brown DAB IHC in low-power ($\times 100$) and high-power magnifications ($\times 400$, inset) seen in bottom row C and F, whereas this effect was not detectable in tumors from mice treated with mAB-control-esiRNA or antibody alone (top and middle row; A/D and B/E). However, in BRAF-mutated xenografts, mAB-P-*KRAS* esiRNA treatment did not have influence on Ki-67 expression pattern (G, H, and I). Nuclear counterstain was performed using hematoxylin solution.

complexing of the siRNA with lipophilic agents are strategies that are followed by several academic groups and by biopharmaceutical companies (11). A first in-man study followed this strategy, combining siRNAs against VEGF and KSP in lipid nanoparticles was effective in patients with hepatocarcinoma (9). A recent publication underlines the importance to target *KRAS* with siRNA: Here, the authors chose to apply *KRAS* siRNA with the help of miniature polymer capsules locally implanted to xenografted pancreatic tumors (47). All of these therapeutic approaches, especially the local administrations of siRNA would benefit from the possibility of specific targeting to tumor cells to receive systemic effects (48).

The most remarkable progress in cancer therapy in the last 15 years is based on the use of monoclonal antibodies. Monoclonal antibodies have entered clinical practice for multiple entities, including hematologic and solid cancers. In many instances, the antibody is not coupled to any drug and the mechanisms of action might depend on direct cytotoxicity or antibody-dependent cellular cytotoxicity (49). In recent years, several drug conjugates have been introduced into clinical practice. First, anti-CD33 antibody coupled to ozogamicin has activity in acute myelogenous leukemia (50). The coupling of trastuzumab to the cytotoxic drug emtansine (TDM1) has shown significant clinical activity even in trastuzumab-resistant breast cancers (51). Drug conjugates, for example, anti-CD30 antibodies (brentuximab) or anti-CD20 (britumomab-tixetan) have been approved by the FDA and the EMA for routine clinical use (52). Thus, the coupling of antibodies allows cell-specific delivery of drugs. So far, the drug conjugates are mainly of cytotoxic or radioactive nature with still significant side effects and probably similar resistance mechanisms as for other cytotoxic drugs. The coupling of siRNAs to monoclonal antibodies might thus improve therapeutic efficacy and broaden the therapeutic range. Systemic delivery of antibody-siRNA complexes is feasible as has been demonstrated for an anti-CD7-siRNA construct that was used to treat HIV infection in humanized mice (14). This construct was based on a scFV single

chain antibody with a c-terminal histidine that was coupled to a small cationic peptide via a disulfide bond. Ultimately, the positively charged peptide bound the siRNA for delivery by electrostatic charge. Here, we went on to develop a much simpler strategy. Protamine, a positively charged peptide, was covalently linked to the monoclonal antibody. Protamine effectively bound siRNA with a significant excess of siRNA for each molecule of antibody (12, 13).

Protamine is an endogenous protein most highly expressed in sperm. It has long been used to delay the activity of insulin (28). Also, protamine is commonly used to inhibit heparin activity, for example, after overdosing. Thus, there is ample experience with the administration of protamine to patients. Given that protamine is simply used as a linker at low concentration in the antibody-siRNA conjugate, it is likely that the complexes can be used with minimal side effects due to protamine. This approach might allow early proof-of-principle studies in patients with *KRAS*-mutant cancer. Pharmacokinetic estimates roughly comparing cetuximab doses clinically used with our *in vivo* protocol hint at the possibility to reach therapeutic activity with our siRNA delivery system with antibody doses at least a log-step lower than the cetuximab dose. However, detailed pharmacokinetic studies have to be performed in the frame of clinical phase I studies to define best clinical protocols.

Taken together, chemically coupled antibody-(e)siRNA complexes, designed similar to previously reported immune constructs transporting DNAs (53), are a new and potentially powerful approach for targeted anticancer therapy following a simple building-block strategy. A coupling of anti-*KRAS* siRNA to cetuximab might overcome the *KRAS*-mutation mediated primary or secondary resistance toward anti-EGFR antibodies in clinical use. Most important, the combination of multiple siRNAs conjugated to cetuximab targeting independent oncogenes in combination underlines the flexibility and the high potential of this approach. Furthermore, by targeting overexpressed EGFR and mutated *KRAS* and potentially other

molecules within one molecule, this new treatment represents a dual specificity approach.

This therapeutic principle might also be applicable to other cancer-related receptor-targeting monoclonal antibodies and, of course a multitude of siRNA targets. With the high loading capacity of the protamine for siRNA, one even can envisage loading of siRNA molecules targeting different oncogenic molecules tailored to the individual tumor specifications within one antibody construct. In fact, this principle can contribute to a personalized anticancer therapeutic approach where tumors can be treated with specific siRNAs against several driver mutations and genes at the same time. Thereby, antibody-siRNA conjugates can provide a valuable alternative especially in rare cancer entities when the cost-effective design of conventional inhibitory drugs is difficult.

Disclosure of Potential Conflicts of Interest

C. Müller-Tidow reports receiving speakers bureau honoraria from Merck. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: S. Bäumer, N. Bäumer, S. Schelhaas, E. Wardelmann, W.E. Berdel, C. Müller-Tidow

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