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Targeted siRNA Delivery and mRNA Knockdown Mediated by Bispecific Digoxigenin-binding Antibodies

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Bispecific antibodies (bsAbs) that bind to cell surface antigens and to digoxigenin (Dig) were used for targeted small interfering RNA (siRNA) delivery. They are derivatives of immunoglobulins G (IgGs) that bind tumor antigens, such as Her2, IGF1-R, CD22, and LeY, with stabilized Dig-binding variable domains fused to the C-terminal ends of the heavy chains. siRNA that was digoxigeninylated at its 3′end was bound in a 2:1 ratio to the bsAbs. These bsAb–siRNA complexes delivered siRNAs specifically to cells that express the corresponding antigen as demonstrated by flow cytometry and confocal microscopy. The complexes internalized into endosomes and Dig-siRNAs separated from bsAbs, but Dig-siRNA was not released into the cytoplasm; bsAb-targeting alone was thus not sufficient for effective mRNA knockdown. This limitation was overcome by formulating the Dig-siRNA into nanoparticles consisting of dynamic polyconjugates (DPCs) or into lipid-based nanoparticles (LNPs). The resulting complexes enabled bsAb-targeted siRNA-specific messenger RNA (mRNA) knockdown with IC_{50} siRNA values in the low nanomolar range for a variety of bsAbs, siRNAs, and target cells. Furthermore, pilot studies in mice bearing tumor xenografts indicated mRNA knockdown in endothelial cells following systemic co-administration of bsAbs and siRNA formulated in LNPs that were targeted to the tumor vasculature.

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Introduction

Bispecific antibodies (bsAbs) that recognize cell surface antigens and haptens can be used for targeted drug delivery. One recently developed targeting platform consists of immunoglobulin G (IgG)-derived bsAbs that bind to cell surface antigens on the one hand and to digoxigenin (Dig) coupled entities on the other hand. This delivery platform has impressively shown its potential for targeted delivery of small molecule drugs and fluorophores *in vitro* and *in vivo*.[1] A major advantage of IgG-derived bsAbs stems from the fact that its production is compatible with established upscale processes for recombinant IgGs. Furthermore, the modular approach of this technology yields the benefit that the targeting moiety and the payload can be produced separately, which circumvents potential incompatibilities with manufacturing processes and may allow individualized fine-tuning of the ratios between bsAb and payload. Despite recent advancements (including Alnylam's ALN-PCS02 and ALN-TTR01 clinical trials), the therapeutic success of small interfering RNA (siRNA) has often been hampered by safety issues regarding more often the delivery system for the siRNA rather than the siRNA technology. In search for a suitable delivery vehicle, a great variety of siRNA formulations with and without targeting moieties have been evaluated.[2–4] Appropriate delivery systems need to stabilize siRNA in systemic circulation, *i.e.*, prevent renal excretion and enable internalization by desired cell types or tissues. Moreover, once taken up by the cell, the siRNA has to be released intact into the cytosol in order to elicit its RNA interference (RNAi) effect.

For the pharmaceutical development of siRNA delivery systems it is mandatory that its components are not only well defined and robust, but can also be manufactured reproducibly at an industrial scale. Parameters that have to be addressed in this regard include (i) the linkage of the siRNA to antibody derivatives (defined positions and stoichiometry, stable in serum) without losing siRNA functionality or antibody-binding functionality, (ii) specificity of antibody-mediated siRNA delivery (in sufficient amounts with limited nonspecific delivery), and (iii) optional separation of siRNA or siRNA-containing vehicles from antibodies upon internalization to facilitate efficient transfer into the cytoplasm. Conceptually straightforward approaches aimed at targeted delivery of siRNAs are based on antibody derivatives as delivery vehicles,[5,6] and are frequently of limited therapeutic applicability. They are often difficult to produce and handle, and in many cases are instable or form rather undefined protein complexes.[6] Some of these show only poor siRNA transfer into the cytoplasm and hence require either high siRNA concentrations and/or systemic

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addition of nonspecific endosome-modulating agents such as chloroquine.[7]

The objective of the present work was to evaluate the usefulness of bsAbs in combination with dynamic polyconjugates (DPCs) or lipid-based nanoparticles (LNPs) for targeted delivery of siRNA. The most striking benefit of our approach is hereby the modular delivery concept. We demonstrate that bsAbs bind to cell surface antigens and transfer active siRNA into targeted cells *in vitro and in vivo*. Dig-binding bsAbs can be combined with defined and scalable drug delivery systems and therefore provide a robust platform for targeted delivery of siRNA.

Results

Cell surface targeting by Dig-binding bsAbs

The bsAbs that we applied for siRNA delivery in this study are derivatives of IgGs that bind to cell surface antigens and to Dig as described earlier.[1] The cell surface-binding functionalities of these molecules are positioned in the two Fab arms of the IgG moiety, with two disulfide-stabilized anti-Dig single-chain Fv modules recombinantly fused to the C-termini of the heavy chains (**Figure 1**). The VH and VL domains of the anti-Dig single-chain Fv[1] are held together by flexible linkers and by disulfide bonds between VH and VL to enhance stability (VHCys44 to VLCys100[8–10]). The cell surface targeting entities were derived from IgGs that bind the tumor associated antigens Her2, IGF1-receptor, CD22, VEGFR2, and the LeY carbohydrate antigen.[11–14] The bsAbs were produced in mammalian cells and purified with good yields from cell culture supernatants in the same manner as conventional IgGs (for details see ref. 1 and **Supplementary Data**).

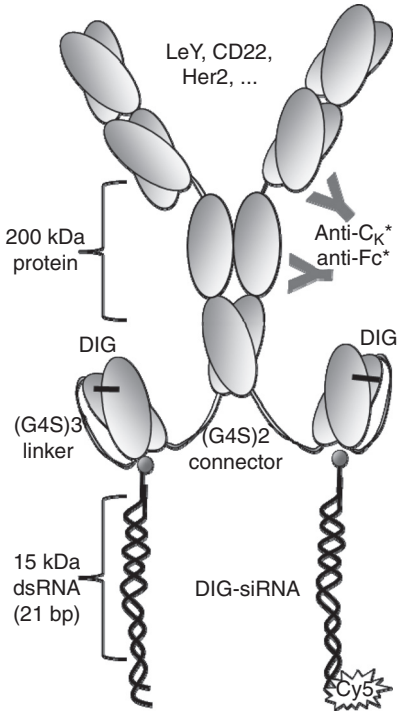

Figure 1 Bispecific antibodies (bsAbs) for small interfering RNA (siRNA) targeting. VH and VL domains of digoxigenin (Dig)-binding disulfide stabilized single-chain Fv's (scFv's) are connected to each other by flexible (Gly4Ser) modules. The same modules were also used to fuse Fv's to immunoglobulin G (IgG). The molecule can be detected by labeled antibodies that bind human Cκ or human IgG1 Fc (anti-Cκ* and anti-huFc*). Dig-coupled nucleic acids bind to bsAbs and form targeting complexes. siRNA has Dig covalently linked to the 3′ end of the sense strands. The sense strand may also be linked to fluorescent molecules for detection by fluorescence-activated cell sorting (FACS) or microscopy.

Dig-siRNA as a module for antibody binding

A prerequisite for our modular targeting approach is a linkage between the cargo, *i.e.*, the siRNA and Dig, without affecting either the siRNA functionality or the interaction between Dig and the Dig-binding moiety of the bsAb. We prepared Dig-siRNAs against the messenger RNAs (mRNAs) of the human activator of 90 kDa heat shock protein ATPase homolog 1 (AHA1) or the human kinesin-related motor protein (Eg5 also known as Kif11). We chose the 3′ end of siRNA for coupling to Dig because the 3′ end was previously found to be well suited for adding additional entities (such as cholesterol[15]) to siRNA. The potency of 3′-digoxigeninylated siRNA derivatives, *i.e.*, their ability to reduce target mRNA levels, was evaluated by branched DNA signal amplification assays following transfection in Hela cells.[16] These studies demonstrated that coupling of Dig to the 3′ end of the sense strand via C6-linkers generated Dig-siRNAs that retained full mRNA knockdown activity. Structures of Dig-siRNAs that were used in this study are shown in **Supplementary Figure S1**.

Stoichiometry of bsAbs and Dig–siRNA complexes

To determine the coupling stoichiometry between siRNA and bsAbs, a cyanine-5 (Cy5) fluorescently labeled Dig-siRNA (Dig-siRNA-Cy5, see **Supplementary Figure S1**) was added to Her2-Dig bsAbs in various molar ratios. Free and complexed siRNA was subsequently determined by size exclusion chromatography (**Figure 2**). At a ratio of two or less Dig-siRNAs per bsAb molecule all fluorescence was found in the high

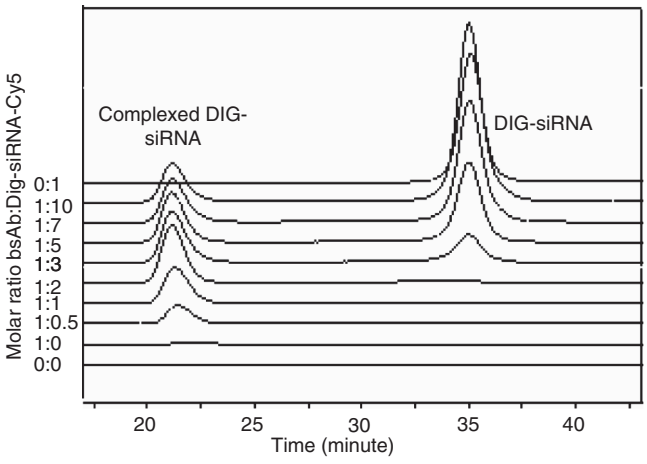

Figure 2 Stoichiometry of bsAb/siRNA charging. Dig-siRNA-Cy5 was titrated against constant amounts of Her2-Dig bsAb. After complex formation at room temperature (RT) for 1 hour, Dig-siRNA-Cy5 was detected by SEC with a fluorescence detector. Small interfering RNA (siRNA) that is captured by bsAb is detected in fractions that elute between 21 and 24 minutes; free siRNA elutes at ~35 minutes. Cy5, cyanine-5; Dig, digoxigenin.

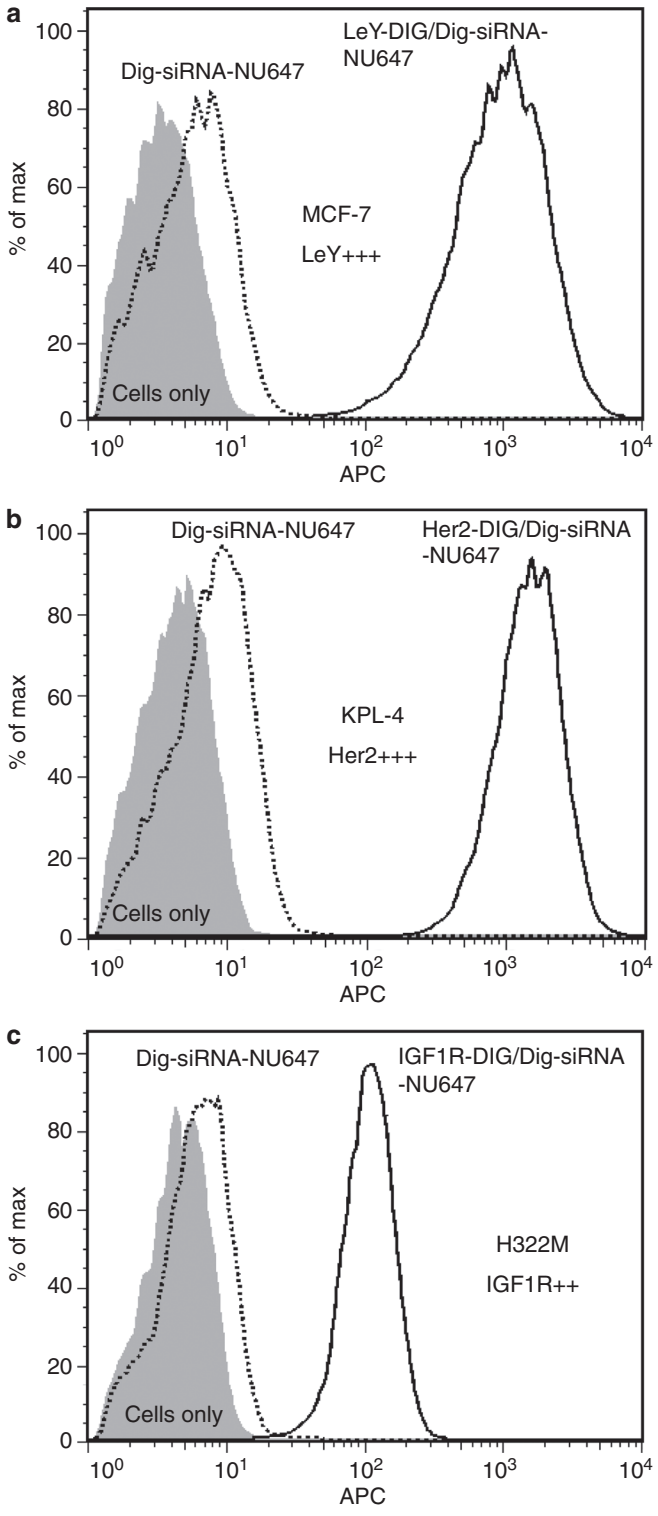

Figure 3 Specific delivery of Dig-siRNA. (**a**) MCF-7, (**b**) KPL-4, and (**c**) H322M were incubated in the absence (cells only) or presence of Dig-siRNA-NU647 (dotted line) or Dig-siRNA-NU647 complexed with Dig-bsAbs targeting LeY (LeY-Dig) on MCF7, Her2 (Her2-Dig) on KPL-4, and IGF1R (IGF1R-Dig) on H322M (solid line) and were analyzed by fluorescence-activated cell sorting (FACS) in the APC channel. bsAbs, bispecific antibodies. Dig, digoxigenin; siRNA, small interfering RNA.

molecular weight fraction representing antibody-complexed siRNA. Under these conditions, no free Dig-siRNA was detected. When Dig-siRNA-Cy5 was added at ratios >2 siRNAs per bsAb molecule, no further signal increase was observed for the fractions that contain the antibody complex. Instead, dose-dependent signal increases occurred only in fractions corresponding to the lower molecular weight fraction representing free Dig-siRNA-Cy5. These data demonstrate that bsAbs complex to siRNA with a defined stoichiometry of two siRNA molecules per one bsAb.

Specific delivery of Dig-siRNA to target cells *in vitro*

To demonstrate the specificity of targeted siRNA delivery, we incubated MCF-7 breast cancer cells that express high levels of the carbohydrate antigen LeY[14] with bsAbs complexed with Nu647 fluorescently labeled siRNA. Subsequently, siRNA binding on these cells was analyzed by flow cytometry. Incubation of MCF-7 cells with Dig-siRNA-Nu647 alone yielded no significant Nu647 signal on the cell surface (**Figure 3a**). In contrast, when Dig-siRNA-Nu647 was preincubated with LeY-Dig bsAb, the majority of MCF-7 cells displayed fluorescence signals. Specific binding of Dig-siRNA complexed with Her-2-Dig bsAbs or with IGF-1R-Dig bsAb was also shown for Her-2-expressing KPL-4 breast cancer cells and for IGF-1R-expressing H322M lung cancer cells, respectively (**Figure 3b** and **c**). These observations were further confirmed by confocal microscopy studies using Cy5-siRNA combined with detection of bsAb with Alexa488-labeled secondary antibodies (see **Figure 1**). Her-2-Dig bsAbs efficiently delivered Dig-siRNA-Cy5 to the surface of KPL4 cells (**Figure 4**) whereas under the same conditions Dig-siRNA-Cy5 did not accumulate at Her-2-negative MDA-MB468 cells. Thus, bsAbs deliver the siRNA only to cells that express the cognate target antigen. This observation of antibody/antigen-specific delivery agrees with previous experiments in which bispecific Dig-binding antibodies were applied to deliver fluorescent payloads.[1]

Subcellular localization of antibody-targeted siRNAs

To analyze the antigen-specific uptake of Dig-siRNA-Cy5 in more detail, we incubated IGF-1R-positive H322M with IGF-1R-Dig bsAbs complexed with Dig-siRNA-Cy5 at a temperature of 37 °C for 10 minutes or 1 hour, respectively. Subsequent confocal microscopy visualized Dig-siRNA-Cy5 on the surface of IGF-1R expressing H322M cells following incubation with IGF-1R-Dig bsAb/Dig-siRNA complexes. Similarly, Her-2-Dig bsAb delivered siRNA to Her-2-positive KPL4 cells following 30 minutes of incubation (**Figure 4b**). Upon incubation for 1 hour at 37 °C, Dig-siRNA-Cy5 and IGFR-1-Dig bsAb were internalized and colocalized in vesicular compartments (**Supplementary Figure S2**). Of note, when Her2-positive KPL4 cells were incubated for 15 hours at 37 °C, separation of the Cy5 signals (from Dig-siRNA-Cy5) from the targeting Her2-Dig bsAb could be observed as indicated by a divergence of green-red colocalized fluorescence signal (**Figure 4b**). This may indicate that antibody and siRNA are routed into separate vesicular compartments. However, the Cy5-labeled siRNA molecules that appear to be detached from their delivery vehicle are still detectable only within vesicular compartments (**Figure 4b**). This may be explained either by their inability to

escape from vesicular compartments, and/or by their fluorescence signals being too faint to be detected if liberated into the cytosol. In agreement with this observation, siRNA-mediated mRNA knockdown could not be detected, even in cells in which siRNA accumulated at high levels (**Supplementary Figures S2** and **S3**). These results indicate that bsAb-siRNA complexes by themselves are insufficient to elicit RNAi.

mRNA knockdown by antibody-targeted DPCs *in vitro*

To promote access of the internalized siRNA payload to the RNAi machinery in the cytoplasm, we combined DPC technology with the bsAb-targeting platform. DPCs include an endosomolytic polymer that is shielded from nonspecific cell interactions by reversible covalent modification with polyethylene glycol (PEG). Both siRNA and targeting moieties are attached to the polymer by labile linkers.[17] In this study, the targeting moiety was incorporated by attaching Dig to the polymer. These digoxigeninylated DPCs were complexed with bsAbs at a 2:1 molar ratio to generate bsAb-targeted DPCs. The characterization of these DPCs by size exclusion chromatography in combination with multiple angle laser light scattering (SEC-MALLS) is described in detail in the **Supplementary Data** and shown in **Supplementary Figure S4**. Dig-polymer-siRNA DPCs without bsAb are a polydisperse solution with molecules of an estimated molecular weight between 300 and 720 kDa and a hydrodynamic radius from 7 to 10 nm (details in **Supplementary Figure S4a**). Addition of LeY-Dig bsAb to form LeY-Dig/Dig-polymer-siRNA DPCs increased the molecular weight range to 500–1,100 kDa and the hydrodynamic radius to 9–12.5 nm (**Figure S4b**). Thus, Dig-binding bsAbs can access and bind the Dig-moiety on the DPC module.

To demonstrate the potential for antibody-mediated specific targeting, DPCs were prepared with fluorescence-labeled polymer (Nu547) and bsAbs that recognize various cell surface

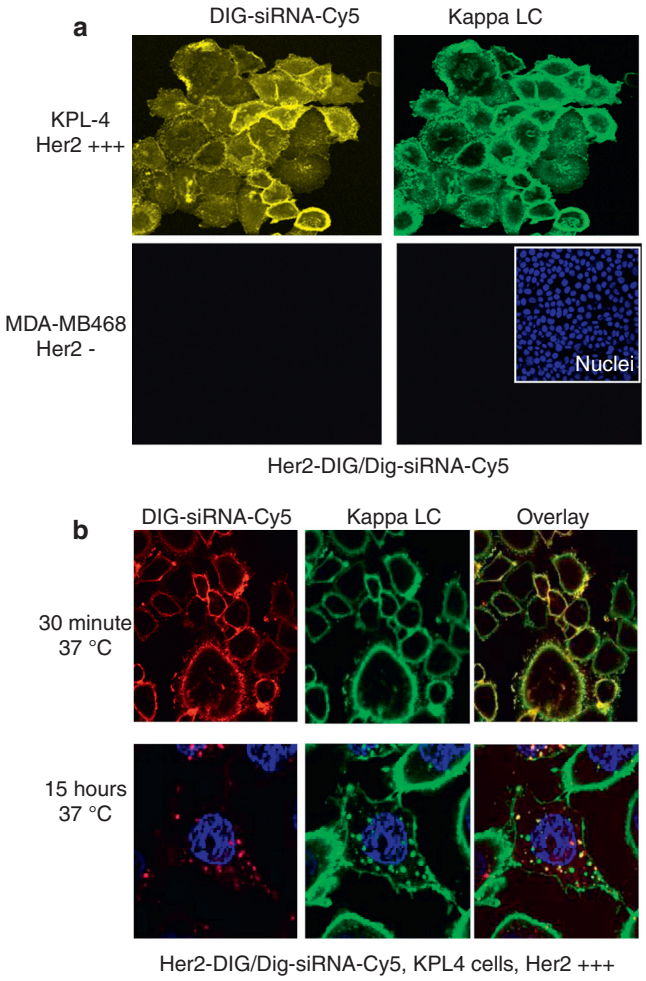

Figure 4 Targeting and internalization of Dig-siRNA. (**a**) KPL4 (Her2 positive) and MDA-MB468 (Her2 negative) incubated for 30 minutes at a temperature of 37 °C with Her2-Dig/Dig-siRNA-Cy5 at a concentration of 25 nmol/l. Inset shows nuclei to confirm presence of cells. The bsAb targets small interfering RNA (siRNA) only to Her2 expressing cells. Targeting was also demonstrated with IGFR1-R bispecific antibodies (bsAbs) on IGF1-R expressing H322M (Supplementary Figure S2). (**b**) KPL4 cells incubated for 30 minutes or 15 hours at a temperature of 37 °C with 25 nmol/l Her2-Dig/Dig-siRNA-Cy5. Note that the siRNA is internalized and the separation of the siRNA label and antibody signals after 15 hours at 37 °C. All cells were fixed and Alexa488-labeled antihuman-κ LC was used for visualization of the antibody. Cy5, cyanine-5; Dig, digoxigenin.

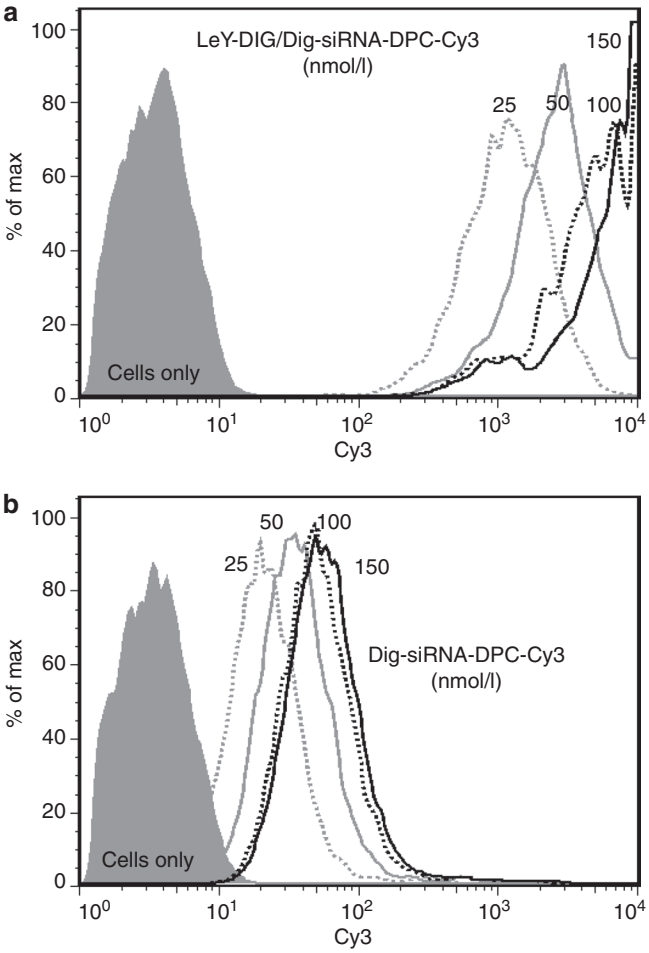

Figure 5 Specific delivery of Dig-siRNA-DPCs. MCF7 cells were incubated with (**a**) the complex of LeY-Dig bsAb and Dig-siRNA-DPCs-Cy3 or with (**b**) Dig-siRNA-DPCs-Cy3 alone and analyzed by fluorescence-activated cell sorting (FACS) in the Cy3 channel. Dig-siRNA-DPCs-Cy3 was added in increasing concentrations (25, 50, 100, and 150 nmol/l). Dig, digoxigenin; DPCs, dynamic polyconjugates; siRNA, small interfering RNA.

antigens. Incubation of MCF-7 cells, which express the LeY antigen, with fluorescent LeY-Dig/Dig-polymer-siRNA DPC demonstrated a strong target-specific and dose-dependent binding (**Figure 5a**). In contrast, exposure of MCF-7 cells to Dig-polymer-siRNA DPCs alone (no LeY-Dig bsAb) resulted in pronounced reduction of fluorescent signals (**Figure 5b**). We also determined whether bsAb-DPCs maintained the ability to internalize into the cells similar to complexes of bsAb and Dig-siRNA DPCs (**Figure 6**). MCF-7 cells were incubated with LeY-Dig/Dig-polymer-siRNA (Nu547-labeled siRNA) and assessed by confocal microscopy. Following incubation for 30 minutes, MCF-7 cells exhibit clear binding of LeY-Dig/Dig-DPCs to the cell-surface membrane (**Figure 6b**). Conversely, incubation of MCF-7 cells with Dig-polymer-siRNA DPC not complexed with LeY bsAb (**Figure 6a**), or complexed with CD33 bsAb that does not bind to MCF-7 cells, showed an absence of DPC binding (**Figure 6c**). As expected, the Dig moiety is an essential component of the DPC module to affect

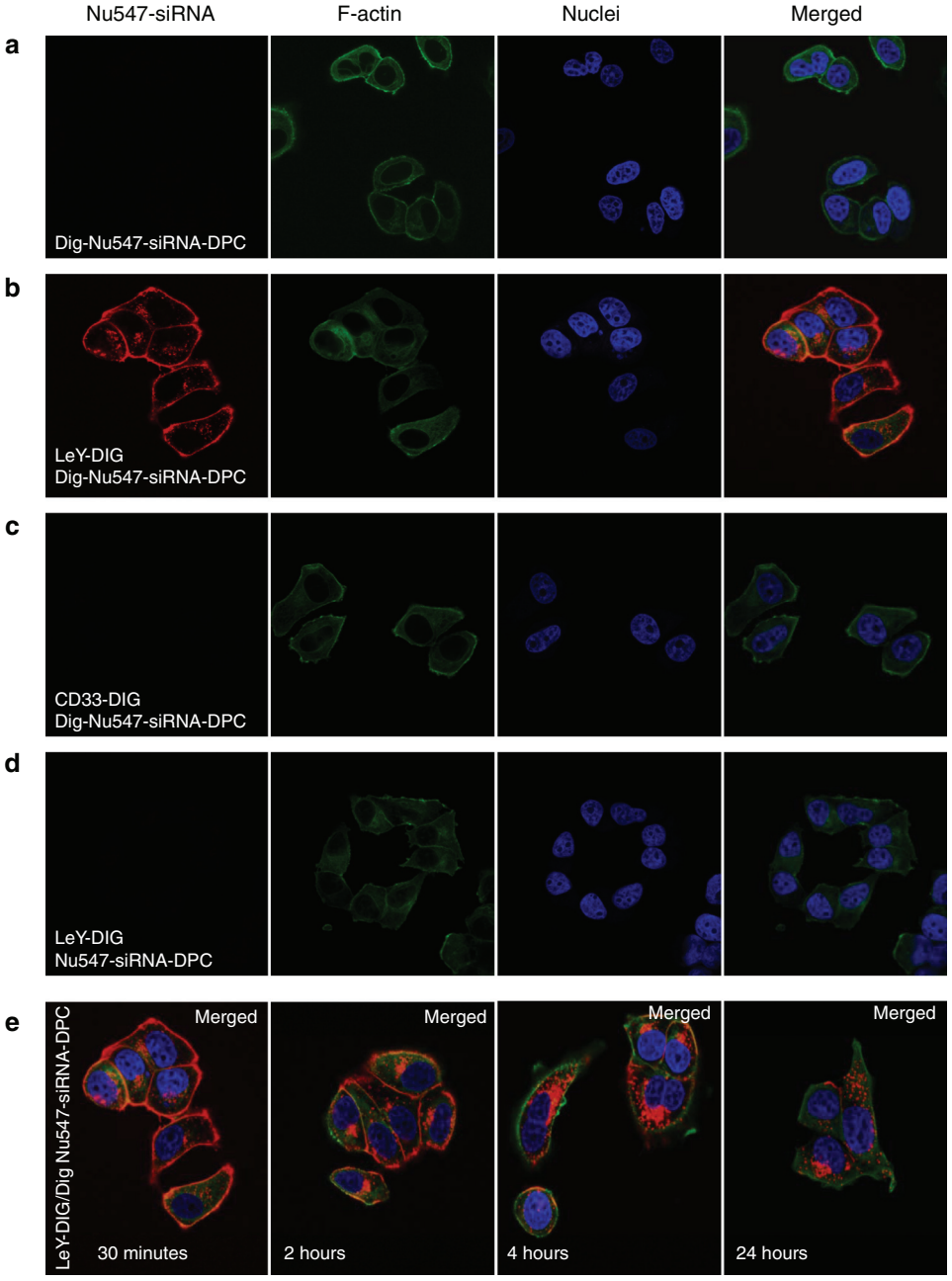

Figure 6 Targeting and internalization of LeY-Dig/Dig-siRNA-DPC complexes. MCF-7 cells were incubated with dynamic polyconjugates (DPCs) for 30 minutes (Nu547-siRNA = 285 nmol/l), washed with phosphate-buffered saline (PBS) after DPC exposure, fixed, and stained for F-actin (Alexa 488-phalloidin) and nuclei (ToPro-3). Images were acquired with a laser scanning confocal microscope. (**a**) Dig-Nu547-siRNA-DPC without bsAb; (**b**) LeY-Dig/Dig-Nu547-siRNA-DPC; (**c**) CD33-Dig/Dig-Nu547-siRNA-Dig; (**d**) LeY-Dig/Nu547-siRNA-DPC (no Dig on DPC). (**e**) MCF-7 cells were incubated with LeY-Dig/Dig-Nu547-siRNA-DPC for 30 minutes (Nu547-siRNA = 285 nmol/l), washed with PBS after DPC exposure, and further incubated for to the indicated time periods. Dig, digoxigenin; siRNA, small interfering RNA.

complex formation between bsAb and DPC, and is required for binding to MCF-7 cells (**Figure 6d**).

To determine the fate of bsAb complexes after binding to the cell surface, fluorescently labeled siRNA was visualized by confocal microscopy at different time points after exposure to cells at 37 °C. MCF-7 cells incubated with LeY-Dig/Dig-polymer-siRNA DPC (30 minutes incubation followed by washing out unbound bsAb-DPC and continued incubation) initially exhibited intense DPC staining/binding to the cell surface. In addition, some intracellular punctuate staining was observed, suggesting that some DPCs had already internalized into vesicular (presumably endosomal) compartments. Continued incubation resulted in a gradual fading of cell surface staining and an increased intracellular accumulation of DPCs in vesicular compartments. At 24 hours after incubation the fluorescence intensity at the cell surface was reduced to a minimum (**Figure 6e**).

The specificity of bsAb-targeting in combination with the DPC delivery system to mediate RNAi was also tested for Aha1-mRNA knockdown: MCF-7 cells incubated with LeY-Dig/Dig-polymer-siRNA DPCs exhibited almost 80% knockdown of Aha1-mRNA levels (**Figure 7a**). Incubation with Dig-siRNA DPCs alone or with Dig-siRNA DPCs complexed with control CD33-Dig bsAb or with LeY-Dig bsAb and Dig-control (CD45) siRNA did not result in Aha1-mRNA knockdown. Hep3b cells express the LeY antigen at the cell surface at significantly (~60-fold) lower degree as compared with MCF-7 cells. When Hep3b cells were evaluated for Aha1-mRNA knockdown using the same DPCs as used for the MCF-7 cells, LeY-Dig/Dig-Aha1-siRNA-DPCs caused <20% specific knockdown (**Figure 7b**). Overall, the combination of bsAb with DPCs resulted in a potent mRNA knockdown and the effect was highly specific for the targeted antigen as well as for the mRNA-specific siRNA.

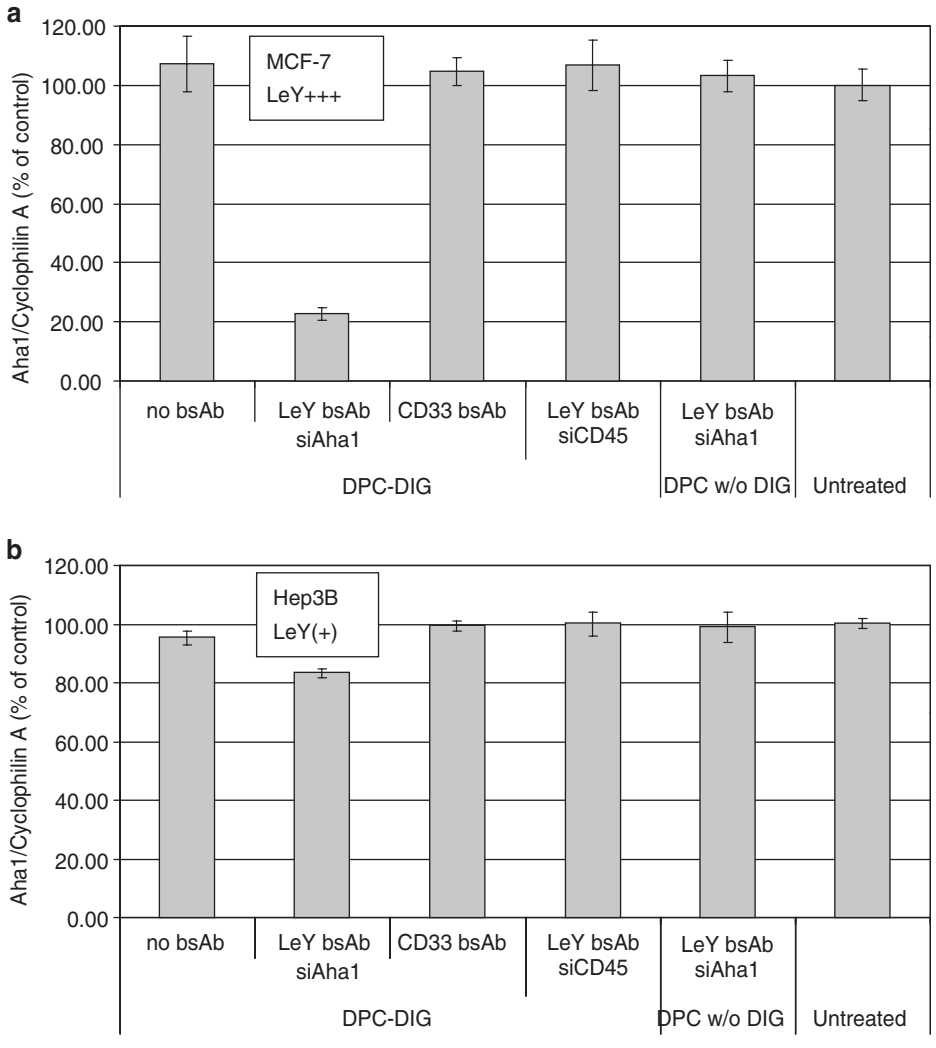

Figure 7 Specific messenger RNA (mRNA) knockdown by LeY-Dig/Dig-siRNA-DPC complexes. Cells were incubated with dynamic polyconjugate (DPC) formulations for 30 minutes. Short incubation periods with 1.14 µmol/l small interfering RNA (siRNA) was applied to avoid DPC disassembly and subsequent nonspecific transfection during the exposure period. After 30 minutes of incubation with DPCs, cells were washed with phosphate-buffered saline (PBS), and further grown for 24 hours. The ratio of mRNA levels of ATPase homolog 1 (AHA1) to Cyclophilin A was assessed by quantitative RT-PCR assay. (**a**) MCF-7 cells; (**b**) Hep3B cells. Dig, digoxigenin.

mRNA knockdown by antibody-targeted LNPs *in vitro*

A second delivery system that enables cytoplasmic entry of siRNAs into a variety of cell types are LNPs. The LNPs used in this study contain PEG-lipids in which lipophilic acyl chains anchor the hydrophilic PEG molecules in the particle. This mediates stability and structural particle integrity. The acyl chains of the PEG-lipids can be of various lengths, which influences the residence time of the molecules in the particle following i.v. injection. The LNPs used herein contain PEG-lipids with either a relatively long C18 anchor that consist of 18 methanediyl groups and is considered nonexchangeable, or with a shorter C16 anchor that consist of 16 methanediyl groups and is considered exchangeable. The composition and optimization of Dig-LNPs for bsAb targeting is described in detail in **Supplementary Data** and **Supplementary Figure S5**. To investigate the targeting ability of bsAb in combination with LNPs, we attached a Dig molecule to the PEG-moiety of PEG-lipids (**Supplementary Figure S5**). Thereby, Dig becomes exposed and is amenable for bsAb binding. To ensure a stable association of Dig and the LNP, and subsequently of the LNP and the bsAb, Dig was attached to PEG-lipids containing the C18 lipid anchors that are stably connected to LNPs. The remaining PEG-lipids of Dig-LNPs contained either C16 or C18 lipid anchors at various ratios (for details see **Supplementary Data**). The final Dig-LNP formulations, which also enclosed either Aha1 or luciferase siRNA, contained a total of 1.4 mol% PEG-lipids, including those that were 1, 0.4, 0.04, or 0 mol% Dig-modified. The remaining Dig-free PEG-lipids contained C16 lipid anchors to enable effective LNP deshielding and high siRNA transfer/transfection potency.[18–20]

To evaluate the potency of siRNA-containing Dig-LNPs, *in vitro* knockdown experiments were performed. MCF-7 cells were incubated with various concentrations of LNPs, and reduction of Aha1-mRNA was measured by branched DNA amplification assay.[14] The results of these experiments (**Supplementary Figure S6a**) revealed that transfection functionality was retained for Dig-LNPs with Dig content of 0.04 Dig-PEG (>90% knockdown with IC50 of 1.7 nmol/l, respectively), similar to LNPs without Dig (>90% knockdown with IC50 of 1.6 nmol/l). In contrast, LNP formulations containing 0.4 or 1 mol% Dig-PEG exhibited a reduction of the siRNA transfection potency. This loss of potency was not attributable to the attachment of Dig, but rather due to increased amounts of nonexchangeable PEG-lipid since a corresponding reduction in potency could be observed when the same amount of exchangeable C16 anchored PEG was replaced with nonexchangeable C18 (without Dig, **Supplementary Data** and **Supplementary Figure S5b**).

To assess whether Dig molecules at the end of PEG-lipids in functional LNPs are accessible to bsAb, the average size of Dig-LNPs was determined by dynamic light scattering (DLS) in the presence and absence of bsAbs. In the absence of bsAbs, Dig-LNPs were on average 132 nm in size, similar to LNPs not containing Dig-lipid. This indicated that Dig has no or only a minor influence on particle size and shape. In the presence of bsAbs, the average size of Dig-LNPs increased to 158 nm. The size of LNPs not containing Dig-lipid did not increase in the presence of Dig-binding bsAbs, indicating that the interaction between bsAbs and LNPs is dependent on the presence of Dig. The polydispersity indices (Pdi) of these particles were determined as a measure for the size heterogeneity of LNPs in a mixture. The Pdi's were <0.1 in all samples that we analyzed (**Supplementary Figure S5b** and **c**). This indicates that the applied LNPs and antibody complexes are quite homogeneous. To further evaluate the potential of Dig-LNPs and antibody-complexed Dig-LNPs to aggregate, LNPs were incubated with LeY-DIG bsAbs at room temperature for 3 hours. Determination of size and Pdi (via DLS) of the particles after 0.5, 1, 2, and 3 hours of incubation revealed that the Pdi was <0.1 in all samples, demonstrating that LNPs have a homogenous size distribution and do not change within 3 hours.

To evaluate whether bsAb–LNP complexes cause a specific mRNA knockdown, as a measure of targeted delivery, we incubated LeY-positive and CD22-negative MCF-7 cells with Dig-LNPs alone, or with Dig-LNPs that were preincubated with either LeY-Dig or CD22-Dig bsAb (**Figure 8a**). LeY-Dig, but not CD22-Dig bsAb, caused an efficacious and specific mRNA knockdown in combination with Dig-LNPs. Formulations containing either 0.4 or 0.04 mol% Dig-lipids caused a significantly improved and target-specific knockdown when complexed with LeY-Dig bsAb as compared with the same LNPs complexed with CD22-Dig bsAb or LNPs alone. The activity of LNPs without Dig-lipid was not affected by the presence of bsAb, indicating that antibody binding and receptor targeting are dependent on the Dig–antibody interaction. LeY-targeted LNPs revealed in dose–response experiments that LNPs containing 0.04 or 0.4 mol% Dig-lipids exhibited IC_{50}-values eightfold or tenfold lower as compared with untargeted LNPs (**Supplementary Figure 6b**). To investigate whether the observed targeting effects could be generalized, similar experiments were repeated using an IGF1R-Dig bsAb in combination with MCF-7 cells, as well as CD33-Dig bsAb in combination with MOLM-13, Kasumi-1, or MV4-11 cells. In all cases, the bsAbs that recognized antigens present on target cell surfaces increased the knockdown efficiency of Dig-LNPs (**Figure 8b**).

Targeting of Dig-LNPs to the tumor vasculature *in vivo*

Mice bearing MCF-7 tumor xenografts were injected at two consecutive days with VEGFR2-Dig/ Dig-LNPs (which target both human and mouse VEGFR receptor 2). The bsAb-complexed LNPs contained either CD31-siRNA or luciferase-siRNA. For each *in vivo* administration, 0.4 mg/kg antibody and 4 mg/kg Dig-LNPs were injected intravenously (see **Supplementary Data** for further experimental details). Both LNP preparations had the same composition as those described earlier for the *in vitro* analyses, *i.e.*, 0.04 mol% PEG –Dig with the C18 anchor. MCF-7 xenografts express CD31 mRNA in endothelial cells of the tumor vasculature. This murine mRNA is not expressed in the human tumor cells.[21] CD31 mRNA quantification thus permits a selective determination of siRNA-mediated knockdown in the epithelial cells of the tumor vasculature. **Figure 9** summarizes the results of CD31 mRNA quantification in the xenografts: Dig-LNPs containing CD31-siRNA (without antibody) mediated ~40% knockdown of CD31 mRNA 48 hours after start of treatment (24 hours after the second siRNA application), whereas LNPs containing luciferase-siRNA had a much smaller effect of <20% on CD31 mRNA levels (**Figure 9**). The observation of siCD31-dependent knockdown indicates that Dig-LNPs have access to the tumor vasculature and deliver

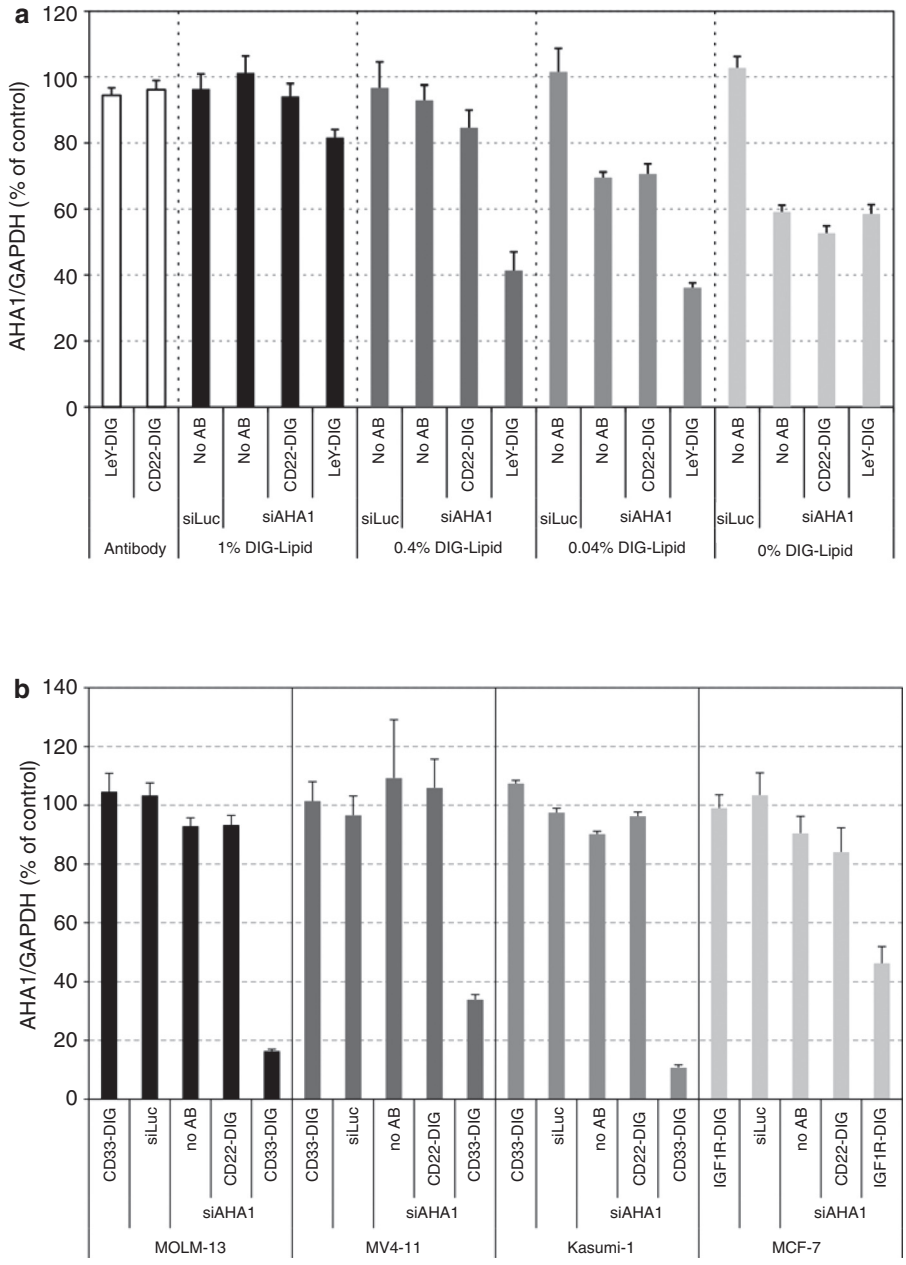

Figure 8 Specific messenger RNA (mRNA) knockdown by bsAb/Dig-siRNA-LNP complexes. The mRNA levels of ATPase homolog 1 (AHA1) normalized against GAPDH were assessed by bDNA assays. (**a**) MCF-7 (LeY positive, CD22 negative) were incubated with various LNP formulations. (**b**) MOLM-13, MV4-11, and Kasumi-1 cells, which express CD33 (but low/no CD22), were incubated with LNP formulations directed against CD33 or CD22. MCF-7 cells, which express intermediate levels of IGF1R, show also small interfering RNA (siRNA) targeting when exposed to IGF1R bsAb complexes. Dig, digoxigenin.

siRNAs into endothelial cells. Complexation of Dig-LNPs with VEGFR2-Dig bsAbs improved the degree of CD31 mRNA knockdown in the tumor vasculature: VEGFR2-targeted LNPs reduced the levels of CD31 mRNA to ~30% remaining mRNA, compared with levels of ~80% in the control siRNA-treated group and ~60% levels in the untargeted siCD31-LNP group. CD31 mRNA levels were reduced to a much lesser degree in animals that received targeted complexes containing luciferase (control) siRNA. Our results therefore indicate that our bsAb-targeting approach works *in vivo* and that the antibody-enhanced increase of CD31 mRNA knockdown *in vivo* is specifically caused by the targeted siRNA.

Discussion

Antibody-mediated siRNA targeting approaches are still hampered by practical hurdles. Delivery systems such as protamine fusion particles or nanoparticle conjugates are often difficult to be produced in the proper scale or quality, are unstable, or have undesired biophysical properties such

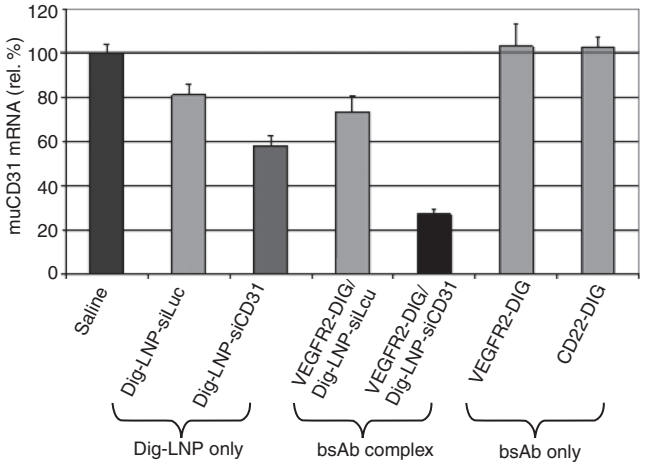

Figure 9 Targeted messenger RNA (mRNA) knockdown *in vivo*. MCF-7 tumor xenografts were exposed to bsAb–LNP complexes directed at VEGFR2 in the tumor vasculature. The level of CD31 mRNA (normalized to GAPDH), which is expressed in the tumor vasculature, was determined by bDNA assays. Data are presented as mean and SD of the $N = 7$ groups.

as a high tendency to multimerize or aggregate.[6] To date, most targeted delivery vehicles are thus not suitable for pharmaceutical drug development. Hapten-binding bsAbs, however, use well-defined and stable components that can be produced with good yields with technologies that are fully compatible with existing scale-up processes for antibody production.[1] These bsAbs behave like normal IgGs and bind to Dig-conjugated payloads such as siRNA or nanoparticles with a distinct stoichiometry. Dig is an established hapten for coupling to nucleic acids and subsequently for bsAb binding.[1] To achieve efficient antibody targeting, the cognate antigen has to be expressed at sufficiently high levels on the cell surface to enable effective accumulation of bsAb-siRNA and subsequent internalization into the cell. Our results indicate, however, that just this process is, by itself, not sufficient to cause effective mRNA knockdown. The likely reason for that is that the vast majority of internalized Dig-siRNA remains in vesicles, most likely endosomes or lysosomes, and is not released to the cytoplasm. This shortcoming was solved by encapsulating the siRNA into delivery systems (DPCs or LNPs) to which a Dig-moiety was conjugated. Since bsAbs, siRNA, and the delivery systems are generated by separate methods, manufacturing processes can be optimized for these individually. Thus, technologies that are specially designed for each component can be applied.

The DPC technology involves a positively charged scaffold that incorporates the siRNA payload and is shielded by PEG. The PEG shield is stable at basic or at neutral pH, thereby minimizing nonspecific membrane interactions. At acidic pH (*e.g.*, within endosomes), the PEG shield detaches and reveals the positive-charged DPC components that cause endosomal release. The combination of the bsAb-targeting platform with DPCs facilitated effective antibody-mediated specific siRNA targeting and target mRNA knockdown. The second siRNA delivery option that we investigated applied LNPs composed of a mixture of lipids and cholesterol including defined amounts of PEG-lipids and Dig-PEG-lipids. One major factor that determines endosome release potency of LNPs is the presence of exchangeable PEG-lipids. To provide a stable anchor for our bsAbs, we had to replace a fraction of exchangeable PEG-lipids within the LNPs with nonexchangeable Dig-PEG-lipid. A small amount of 0.04 mol% Dig-PEG-lipid did not affect the transfection potency but was sufficient for effective cell-type-specific targeting by bsAbs. It provided a preferential accumulation in target cells at high enough levels to enable uptake and target mRNA knockdown. Increasing the amount of Dig-PEG-lipid to 0.4 mol% generated LNPs with reduced nonspecific transfection potency, which, however, were still potent enough to elicit mRNA knockdown when combined with bsAb targeting. The IC50 values that we achieved with LNPs as siRNA delivery vehicles were in the low nanomolar range. Since IC_{50} values of delivery systems for siRNA are frequently obtained under serum-free conditions, we want to further stress the point that all our experiments and potencies were obtained under "normal" cell culture conditions, *i.e.*, in the presence of serum and without additives such as chloroquine.

Pilot tumor xenograft experiments gave evidence for targeted RNAi *in vivo* by our bsAb/Dig-LNP delivery platform. Tumor vasculature targeting was demonstrated by combining VEGFR2-binding bsAb and Dig-LNPs. Specific mRNA knockdown efficacy of Dig-siRNA-LNPs became increased compared with all control samples when the cell-targeting antibody was attached (**Figure 9**). Independent of which control group was used as reference, LNPs containing siCD31 caused much greater CD31 mRNA knockdown than LNPs with control siRNA (demonstrating siRNA specificity). A clear enhancement of knockdown can also be demonstrated with the targeting antibody (demonstrating antibody-mediated targeting specificity). Some minor reduction of target mRNA was also observed in control groups that received untargeted LNPs, or targeted LNPs containing control siRNA. However, in these groups, no antibody-targeted enhancement of activity was observed. Thus, any nonspecific effect is clearly nontargeted. It may be associated with induction of the interferon response or inflammatory cytokines via immune receptors (for antibodies) or toll-like receptors (for liposomes or siRNA).[24] Compared with that "background," however, LNPs containing siCD31 caused much greater CD31 mRNA knockdown (demonstrating siRNA specificity), and a clear enhancement of knockdown with the targeting antibody (demonstrating antibody-mediated targeting specificity). Furthermore, *in vitro* analyses of interferon response gene expression (shown in **Supplementary Data**, **Supplementary Figure S7**, and **Supplementary Table S1**) showed lack of induction of interferon response in cells that were exposed to complexes that contained the different applied antibodies and different siRNAs (while poly I:C lead to significant interferon response under the same assay conditions). This and the observation of specific antibody-targeted mRNA knockdown make it unlikely that an unspecific response (interferon response) is a major driver of the (target-specific) knockdown that we observed.

The target cells that we chose to address in our first *in vivo* evaluations were not the tumor cells, but they are

endothelial cells of the tumor vasculature. Size is one factor that influences (and limits) tissue penetration and distribution. In contrast to the tumor cells, endothelial cells of the tumor vasculature are directly accessible to intravenously applied large entities such as bsAb-complexed siRNA delivery vehicles that have a size of 158 nm. Cell surface antigens of the tumor vasculature may therefore be suitable targets for large siRNA delivery vehicles.

Taken together, our data suggest that Dig-binding bsAbs provide a robust and modular platform for targeted delivery of siRNA-containing nanoparticles.

Materials and methods

bsAbs: plasmids, expression, and purification. Methods for manipulation of DNA are described by Sambrook and Russel[22] and molecular biological reagents were used according to the manufacturer's instructions. Gene segments encoding bsAbs components were generated by automated gene synthesis (Geneart AG, Regensburg, Germany), subcloned into expression vectors and expressed and purified as described earlier.[1] Further details are listed in **Supplementary Data**.

Fluorescence-activated cell sorting. Cells were seeded in a 96-well rounded bottom plate to a final density of 3×10^5 and incubated with or without Dig-siRNA-NU647 or Dig-siRNA-NU647 complexed with the indicated Dig-bsAbs for 1 hour on ice. Then, cells were washed with phosphate-buffered saline and analyzed in the APC channel with a fluorescence-activated cell sorting canto II (BD Biosciences, Franklin Lakes, NJ). Cells incubated with Dig–DPC complexes were treated equally, but analyzed in the Cy3 channel.

Confocal microscopy. For confocal microscopy, cells were grown on glass coverslips to a density of ~50–70%, treated with 25 nmol/l Her2-Dig complexed with Dig-siRNA-Cy5 for the indicated time at 37 °C, and thereafter fixed with paraformaldehyde. Fixed cells were washed, treated with the blocking reagent GSDB and incubated with a rabbit antihuman κ-light chains antibody (DAKO, Glostrup, Denmark) at a concentration of 6.5 µg/ml for 1.5 hours in a humidity chamber. After another wash, the cells were incubated with an Alexa488-labeled goat anti-rabbit antibody (Molecular Probes; Life Technologies, Paisley, UK) in a concentration of 28.6 µg/ml for 1.5 hours in a humidity chamber. The cells were then washed, DNA stained with DAPI (Roche, Mannheim, Germany) at a concentration of 10 µg/ml for 2–3 minutes, washed again and covered with mounting medium. For confocal microscopy (Leica SP20 confocal microscope; Leica Microsystems, Wetzlar, Germany) of LeY-Dig/Dig-siRNA-DPC complexes, MCF-7 cells grown on glass coverslips were treated with the complexes (285 nmol/l Dig-Nu547-siRNA) for 30 minutes, washed with phosphate-buffered saline, and further grown to the indicated time points. At harvest, cells were washed and fixed as described above and stained for F-actin (Alexa 488-phalloidin) and nuclei.

SEC-MALLS and DLS analyses. Dig-DPC-siRNA alone or complexed with the LeY-Dig bsAb were analyzed by SEC-MALLS and quasi elastic light scattering. For SEC, a HPLC pump, degasser, and autosampler from the Dionex Ultimate 3000-Series was applied. One hundred and ninty microliters of 5 mg/ml solution of Dig-DPC-siRNA or 190 µl of a 3.3 mg/ml solution of the LeY-Dig/Dig-DPC-siRNA complex was applied to a Superose 6 10/300 GL SEC column (GE Healthcare, Uppsala, Sweden), with phosphate-buffered saline as eluent and a flow rate of 0.25 ml/minute. The samples were detected by a differential refractive index detector (Optilab rEx), a three-angle laser light scattering detector (miniDAWN Treos, GaAs laser 658 nm, 50 mW, K5 cell), and a DLS detector (WyattQELS; Wyatt Technology, Santa Barbara, CA). The calculation of the molecular weights using Zimm plots and hydrodynamic radii was done by ASTRA for the Windows Software, Version 5.3.4.13. Dig-LNP-siRNA alone or complexed with the bsAb in phosphate-buffered saline were analyzed by DLS to determine their hydrodynamic radii and Pdi. To determine the potential of LNPs to aggregate, LNPs were incubated with <LeY-DIG> bsAbs at room temperature (~25 °C) for up to 3 hours. After 0.5, 1, 2, and 3 hours of incubation, size and polydispersity of the particles were determined by DLS.

bDNA assays. bDNA (branched DNA) assays detect the amount of specific mRNAs in cells.[23] Cells were seeded into 96-well plates to attach overnight and transfected on the next day with siRNA or treated with the agents whose effect on the mRNA-level should be analyzed. Twenty four hours later, the QuantiGene kit (Affymetrix, Santa Clara, CA) protocol was applied according to the manufacturer's instructions. In brief, cell lysates were transferred to a capture plate in the presence of a gene-specific probe set, incubated at 53 °C overnight, washed, and subsequently incubated at 53 °C with an amplifier and an alkaline phosphatase-linked label probe (with washes between incubations). After the final wash, the luminescent alkaline phosphatase substrate dioxitane was added and incubated for 30 minutes at 53 °C. Luminescence was detected using an InfiniteF200 luminescence reader (Tecan, Maennedorf, Switzerland).

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Supplementary Material

Figure S1. Composition of digoxigenated siRNA derivatives.
Figure S2. Targeting and internalization of Dig-siRNA.
Figure S3. bsAb-mediated targeting of Dig-siRNA does not cause mRNA knockdown.
Figure S4. SEC-MALLS of Dig-siRNA-DPC and LeY-Dig/Dig-siRNA-DPC complexes.
Figure S5. Composition of Dig-LNPs and LNP complexes.
Figure S6. Optimization and targeting specificity of Dig-LNP complexes.
Figure S7. Induction of interferon response genes in MCF7 cells.
Table S1. Induction of interferon response genes in MCF7 cells.
Supplementary Data

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)