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BRIEF COMMUNICATION

RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo

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RNA interference (RNAi) represents a powerful, naturally occurring biological strategy for inhibition of gene expression. It is mediated through small interfering RNAs (siRNAs), which trigger specific mRNA degradation. In mammalian systems, however, the application of siRNAs is severely limited by the instability and poor delivery of unmodified siRNA molecules into the cells in vivo. In this study, we show that the noncovalent complexation of synthetic siRNAs with low molecular weight polyethylenimine (PEI) efficiently stabilizes siRNAs and delivers siRNAs into cells where they display full bioactivity at completely nontoxic concentrations. More importantly, in a subcutaneous mouse tumor model, the systemic (intraperitoneal, i.p.) administration of complexed, but not of naked siRNAs, leads to the delivery of the intact siRNAs into the tumors. The i.p. injection of PEI-complexed, but not of naked siRNAs targeting the c-erbB2/neu (HER-2) receptor results in a marked reduction of tumor growth through siRNA-mediated HER-2 downregulation. Hence, we establish a novel and simple system for the systemic in vivo application of siRNAs through PEI complexation as a powerful tool for future therapeutic use.
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RNAi is induced by small 21–25 nt double-stranded small interfering RNAs (siRNAs), which become incorporated into the RNA-induced silencing complex (RISC) and serve as a guide for endonucleolytic cleavage of the complementary target mRNA ([1–3] and references therein). Since siRNAs play a pivotal role in this process, the critical factors that will determine the success of RNA interference (RNAi) approaches are the ability to deliver intact siRNAs efficiently into the appropriate cells. Numerous studies describe DNA vectors, which upon viral or nonviral transfection lead to intracellular expression of double-stranded RNA (eg,[4–10]). While this approach often results in robust downregulation of gene expression *in vitro*, it suffers *in vivo* from problems similar to those of gene therapy including nonspecific effects, low transfection efficiency, poor tissue penetration, and safety concerns. Alternatively, the transfection of chemically synthesized or *in vitro* transcribed siRNAs represents a direct method of inducing RNAi *in vitro*; however, problems *in vivo* include (i) the instability and rapid degradation of the siRNAs as well as (ii) their poor cellular uptake. It is clear that the efficient delivery of intact siRNAs will play a rate-limiting role in any mammalian gene therapy application and has so far widely limited *in vivo* applications of RNAi. Although limited data exist that the *in vivo* application of noncomplexed, 'crude' siRNA might work under certain circumstances, a system stabilizing siRNAs and enhancing their delivery into cells would clearly offer advantages.

Previously, it has been shown that polyethylenimine (PEI) is able to form noncovalent interpolyelectrolyte complexes with DNA[11] and RNA.[12] On this basis, PEIs with various molecular weights, degrees of branching, and other modifications have been used as transfection reagent in a variety of cell lines and live animals to establish its efficacy for DNA delivery (for a review, see Kichler[13] and Wagner *et al* [14] and references therein). This also includes antisense oligonucleotides and siRNA *in vitro*.[15,16]

To address the question of siRNA stabilization and protection against degradation through nucleases, we performed the PEI complexation of siRNA molecules with low molecular weight PEI. In the first set of experiments, we complexed ^{32}P-end-labeled siRNAs with a commercially available linear low molecular weight PEI (JetPEI), and the complexes were incubated at 37°C in the presence of fetal calf serum (FCS). At various time points, samples were subjected to gel electrophoresis and blotted. While in the case of noncomplexed siRNAs full degradation due to RNases in the FCS was observed already at the shortest time points assayed, PEI complexation led to almost complete protection of the siRNAs against degradation as indicated by the presence of radiolabeled bands representing the full-length siRNA molecule (Figure 1a).

To test for the cellular uptake and bioactivity of PEI-complexed siRNAs, we generated clonal, stably luciferase-expressing SKOV-3 ovarian carcinoma cell lines with

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various luciferase expression levels. We preferred this system over transient cotransfection of the pooled target gene+siRNA since the targeting of a stable, endogenously expressed gene represents a more relevant model for RNAi applications. For luciferase downregulation, we used commercially available synthetic siRNA duplexes with an optimized sequence and, as negative control, an unrelated siRNA. In the absence of serum in the culture medium, addition of the specific siRNA using the established transfection reagent TransIT-TKO under transfection conditions which were optimized for DNA plasmid transfections in our lab resulted in a robust, dose-dependent downregulation of luciferase activity (Figure 1b, upper left). The application of the same transfection conditions in the presence of serum, however, led to no reduction of luciferase activity indicating that here siRNA activity was lost most likely due to siRNA degradation (Figure 1b, upper right). In contrast, when siRNAs were complexed with PEI, RNAi-mediated decrease of luciferase activity in transiently (Figure 1b, lower left) or stably (Figure 1b, lower right) luciferase expressing SKOV-3 cells was again observed also in the presence of serum. The dose-dependent downregulation was detectable already at 24 h, reached its maximum after ~48 h and was stable for several days (Figure 1b, lower panel and data not shown). This demonstrates that upon PEI-complexation, siRNA is (i) protected against degradation also in the presence of nucleases, (ii) internalized by the cells, and (iii) released intracellulary displaying full bioactivity.

To test our system *in vivo*, we chose a target gene with proven relevance in tumor therapy in man. The HER-2 (neu/c-*erb*B-2) proto-oncogene belongs to the epidermal growth factor (EGF) receptor family with heterodimeric, HER-2 containing receptor combinations showing super-

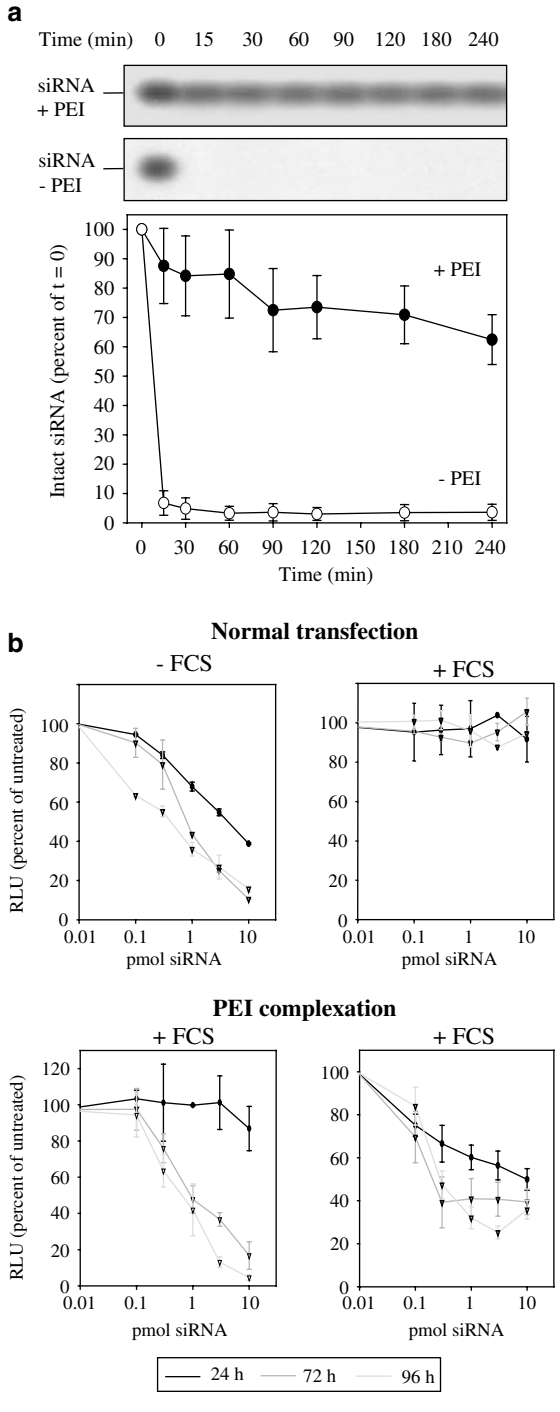

Figure 1 *Stabilization and activity of siRNAs upon PEI complexation. ^{32}P-end-labeling of siRNAs was performed using T4 polynucleotide kinase according to standard protocols with 8 μg siRNA and 50 μCi γ-[^{32}P]ATP. For PEI complexation, 100 pmol specific and/or nonspecific siRNAs (Dharmacon, Lafayette, CO, USA) were dissolved in 200 μl 150 mM NaCl, pH 7.4, and incubated for 10 min. 1.25 μl 4xJetPEI (Qbiogene, Wiesbaden, Germany) was dissolved in 200 μl 150 mM NaCl, pH 7.4, and after 10 min pipetted to the siRNA solution resulting in N/P ratio = 10. After vortexing, the mixture was incubated for 1 h at room temperature prior to use. (a) Determination of siRNA stability in vitro. ^{32}P-labeled siRNAs, complexed (closed circles) or not complexed (open circles) with PEI, were subjected to treatment with 1% fetal calf serum at 37°C. At the time points indicated, 1% sodium dodecyl sulfate was added and mixtures were heat-denatured for 5 min at 100°C. The samples were analysed by agarose gel electrophoresis and bands representing full-length siRNA molecules were quantitated. (b) PEI complexation leads to cellular delivery of bioactive siRNAs also in the presence of FCS. Clonal SKOV-3 ovarian carcinoma cell lines with stable constitutive luciferase expression were generated by transfection with a recombinant luciferase expression vector (pGL3-Basic plasmid (Promega, Madison, WI, USA) with an inserted human NF-kB promoter driving luciferase expression). Stable mass-integrants were selected using G418 at 1 mg/ml prior to the generation of clonal cell lines by limited dilution. For luciferase targeting experiments, the optimized siRNA pGL3 was used with the unrelated siRNA pGL2 serving as negative control (Dharmacon, Lafayette, CO, USA). For transfections using TransIT-TKO (Mirus, Madison, WI, USA), conditions previously determined as optimal in our lab for high plasmid transfection efficacies in 24-well plates were used: 1.125 μl of the transfection reagent was diluted in 20 μl serum-free IMDM medium and the mixture was incubated for 10 min prior to addition of a solution of 10 pmol siRNAs+0.25 μg unrelated plasmid DNA and a final incubation for 60 min. PEI-siRNA complexes were prepared as described above. Cells were transfected at ~70% confluency by addition of the PEI-complexed siRNAs or the TransIT-TKO-siRNA mixture in the presence or absence of 2% FCS as indicated. The determination of luciferase activity was performed at the time points indicated using the luciferase assay kit from Promega (Mannheim, Germany) according to the manufacturer's protocol. While in the absence of serum, standard transfection conditions (eg, TransIT-TKO, upper left) result in the robust downregulation of endogenous luciferase expression, siRNA bioactivity is lost in the presence of serum (upper right). Upon PEI complexation, however, luciferase siRNAs display targeting activity for several days in transiently (lower left) or stably luciferase expressing cells (lower right) also upon transfection in the presence of serum.*

ior signal-transducing, antiapoptotic and cell growth-stimulating capabilities.[17] HER-2 overexpression has been observed in a wide variety of human cancers and cancer cell lines, and has been generally linked to an unfavorable prognosis and more aggressive malignant behavior of tumors (eg, Slamon *et al*[18]). The humanized monoclonal anti-HER-2 antibody trastuzumab (Herceptin (®)), has been approved for the adjuvant treatment of advanced breast and ovarian cancer (see McKeage and Perry[19] for a review; Bookman *et al*[20]) and several low molecular weight inhibitors are being developed and/or in clinical studies emphasizing the clinical relevance of HER-2 targeting. In a recent study in different tumor cell lines, synthetic HER-2 siRNAs were shown *in vitro* to reduce HER-2 expression resulting in growth inhibition or apoptosis and upregulation of HLA class I expression.[21] In another work, HER-2 silencing upon retrovirus-mediated siRNA transfer led to slower proliferation, increased apoptosis and changes in cell cycle-associated and pro-/anti-angiogenic factors in breast and ovarian cancer cell lines *in vitro*. Upon subcutaneous (s.c.) injection of these *in vitro* pretreated cells, decreased tumor growth was observed.[22]

We generated three synthetic siRNAs against different regions of the HER-2 receptor, which displayed comparable efficiencies and were combined in the subsequent experiments. Treatment of SKOV-3 ovarian carcinoma cells *in vitro* with a single dose of 10 pmol PEI-complexed siRNAs resulted in a $\sim$50% downregulation of HER-2 mRNA already after 24 h. This specific reduction of HER-2 mRNA lasted for at least 72 h and, more importantly, was observed also in the presence of FCS in the culture medium (Figure 2a). Western blotting of cell lysates confirmed the Northern blot results and showed an even more pronounced 65–75% reduction of HER-2 protein levels at days 2 and 3 after treatment (Figure 2b). Concomitanty, p42/44 activation (phosphorylation), which has been shown recently to be reduced upon ribozyme-mediated HER-2 downregulation in SKOV-3 cells,[23] was decreased indicating alterations of molecules downstream in the HER-2 signaling pathways upon siRNA-mediated HER-2 targeting (Figure 2b). The biological relevance of this robust HER-2 downregulation was demonstrated in soft agar assays. In previous studies,[24] colony formation of stably ribozyme-transfected SKOV-3 cells was decreased dependent on the reduction of HER-2 levels. While SKOV-3 cells treated with a PEI-complexed nonspecific control siRNA showed high numbers of large colonies >90 μm, a single treatment with HER-2-specific, PEI-complexed siRNAs led to a $\sim$50% reduction of colony formation (Figure 2c). It is particularly noteworthy that, despite the fact that cells were treated with PEI-complexed siRNAs only once prior to embedding into the agar and were then cultivated for 2–3 weeks without any further treatment, this strong effect on colony formation was observed. The decreased capability of treated cells to form colonies in a soft agar assay is comparable to previous observations in stably HER-2 ribozyme-transfected SKOV-3 cells. While HER-2 depletion also leads to induction of apoptosis, in these studies clonal SKOV-3 cell lines with only $\sim$20% residual HER-2 levels still grew on plastic as well as in soft agar assays.[24] Hence, the reduced colony formation observed here may indicate a combination of a long-lasting intracellular effect, that is, biological half-life of the PEI-delivered siRNAs as well as induction of apoptosis.

In proliferation assays, we furthermore investigated the toxicity of PEI and PEI-complexed siRNAs on SKOV-3 cells. Under standard treatment conditions (10 pmol siRNA/well of a 24-well plate), no toxicity or growth-inhibitory effect of complexed or noncomplexed siRNA, or of the corresponding amount of free PEI, was observed after 2 days (Figure 2d, black bars). The same was true when the cells were treated with three-fold higher amounts (Figure 2d, gray bars) indicating that the dosages used in our experiments were well below any toxic levels. Only a 10-fold excess showed some (PEI/siRNA complex) or considerable (PEI alone) toxicity (Figure 2d, dark gray bars).

Finally, by confocal microscopy we studied the half-life/intracellular fate of fluorescently labeled JetPEI upon transfection of cells with PEI/siRNA complexes. Large and numerous fluorescent dots probably corresponding to endocytotic vesicles were observed in the entire cytoplasm already at early time points (30 or 60 min after addition of the complex to the cells; ie 0 min post-transfection in Figure 2e). When after 1 h the transfection mixture was replaced by normal medium, strong fluorescent signals in the cytoplasm, but never in the nucleus, were still observed for another 30 and 60 min (Figure 2e, middle panels). At 2 h (Figure 2e, right) or 3 h (not shown) post-transfection, however, only very weak fluorescence was detected. Compared to previous studies,[25] we observed a very similar staining pattern while the time-course appears to be faster in our experiments.

Recently, it has been shown that under certain conditions the intratumoral injection of siRNAs displays antitumoral effects.[26] The ultimate goal, however, is the therapeutic use of RNAi through systemic application of a specific RNAi-inducing agent *in vivo*. Therefore, we examined if PEI-complexed siRNAs are stable also under *in vivo* conditions and if intact siRNA molecules reach organs/tissues distant from the site of injection. SKOV-3 cells were subcutaneously injected into the flanks of athymic nude mice and, when tumors reached a size of $\sim 4 \times 4$ mm, a single dose of ^{32}P-labeled siRNA was injected intraperitoneally. At different time points, the mice were sacrificed and total RNA from various tissues was isolated. As expected, in the case of noncomplexed siRNAs gel electrophoresis of the tissue RNA and subsequent autoradiography of the blotted gel showed no bands indicating the rapid and complete degradation of the radiolabeled siRNA (Figure 3, upper panel, −PEI). In contrast, upon PEI complexation intact siRNA was detected in several tissues already after 30 min as well as after 4 h (Figure 3, +PEI). Since blood was completely negative for ^{32}P-labeled siRNA, the bands represent intact siRNA, which is not located in the residual blood but indeed taken up by the tissue. Notably, compared to other (excretory) organs, particularly strong signals were observed in the tumors probably indicating a preferential uptake due to the EPR (enhanced permeability and retention) effect in addition to high vascularization (see Maeda *et al*[27] for a review). As expected, almost no siRNA was detected in brain indicating that the complex does not cross the blood–brain barrier. The i.p. injection not only proved to be a very simple and reproducible way of application of PEI-complexed siRNAs, but also led to a depot effect of complex residing in the

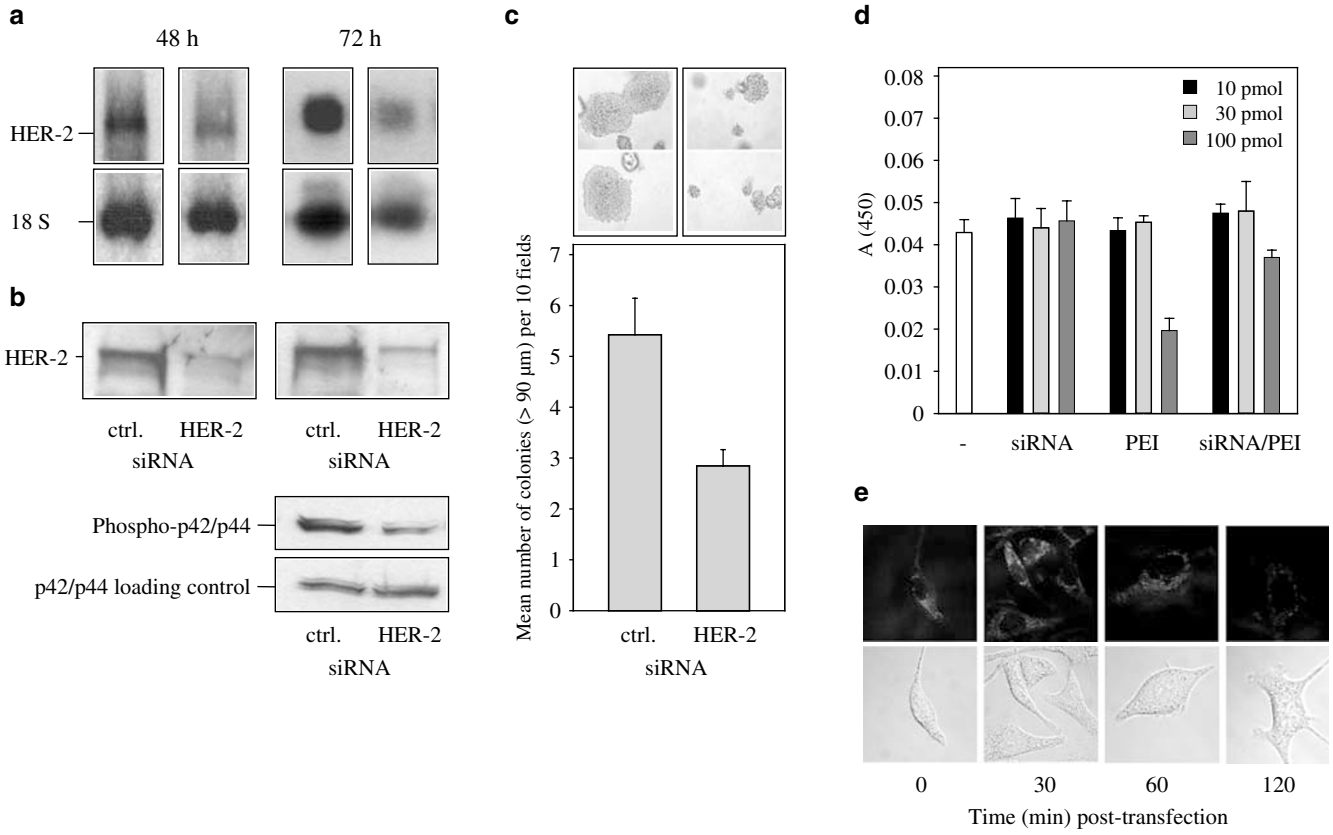

Figure 2 *Downregulation of HER-2 (*neu*/c-erbB-2) in SKOV-3 ovarian carcinoma cells upon a single treatment with PEI-complexed HER-2-specific siRNAs. For HER-2 targeting, three custom-designed siRNAs (CCUGGAACUCACCUACCUGdTdT/CAGGUAGGUGAGUUCCAGGdTdT, CUAC CUUUCUACGGACGUGdTdT/CACGUCCGUAGAAAGGUAGdTdT, GAUCCGGAAGUACACGAUGdTdT/CAUCGUGUACUUCCGGAUCdTdT) were chemically synthesized and annealed (Dharmacon), and the three duplexes were mixed at equimolar ratios. SKOV-3 cells were seeded into six-well plates at 2×10^6 cells/well and, at ~70% confluency, cells were transfected in serum-containing medium by addition of the PEI-siRNA complexes for 48 or 72 h as indicated. Total RNA from cells was isolated using the Tri reagent according to the manufacturer's protocol (Sigma, Taufkirchen, Germany), and 20 µg were separated, blotted, probed for HER-2 as described[32] and, to correct for variability in loading, reprobed with a ^{32}P-labeled 18S cDNA probe. Signals were visualized by autoradiography and quantitated by PhosphoImager analysis. Western blot analysis of equal amounts of cell lysates was performed as described.[23,33] (a) By Northern blotting, a ~50% reduction of HER-2 mRNA was observed after 48 or 72 h, which resulted in a 65–75% decrease of HER-2 protein levels as determined by Western blotting at the same time points (b). Concomitanty, p42/44 activation (phosphorylation) was decreased indicating alterations of molecules downstream in the HER-2 signaling pathways (b, lower panel). (c) In soft agar assays, which were performed as described previously,[34] cells received a single treatment with 0.6 nmol PEI-complexed HER-2 specific or nonspecific siRNA prior to embedding and were allowed to grow for 3 weeks. The reduction of HER-2 levels led to a ~50% decrease of colony formation demonstrating the biological feasibility of HER-2 targeting by PEI complexed siRNAs. (d) SKOV-3 cells were treated with the indicated amounts of complexed or noncomplexed siRNA, or the corresponding amount of free PEI, and after 48 h the numbers of viable cells were determined by WST-1 (Roche). Under standard treatment (10 pmol siRNA/well of a 24-well plate), as well as upon addition of three-fold higher amounts, no toxicity or growth-inhibitory effects were observed (d, black and grey bars) indicating that the dosages used in our experiments were well below any toxic levels. Only a 10-fold excess showed some (PEI/siRNA complex) or considerable (PEI alone) growth inhibition indicating toxicity (dark grey bars). (e) Confocal microscopy of cells transfected with 10 pmol siRNA complexed with fluorescently labeled PEI. Cells were plated on glass coverslips 24 h prior to the transfection with FluoF-PEI/siRNA complexes for 60 min under standard conditions, and were analyzed without fixation at 0–120 min after transfection as indicated in the figure. Excitation of the cells containing FluoF-PEI was achieved at 488 nm and the resulting fluorescence emission was observed using a 505 nm longpass filter. While the nucleus was negative, fluorescent vesicles were observed in the cytoplasm already at early time points and for 1 h post-transfection.*

peritoneum for several hours (not shown). Furthermore, since PEIs at higher concentrations tend to show toxicity in the lung, it is important that in our experimental setting siRNA amounts were generally low in the lung, which corresponded well with the absence of microscopically visible changes in lung histology (not shown) and the absence of visible side effects of PEI/siRNA treatment.

Previously, by ribozyme-targeting we have demonstrated that the stable reduction of HER-2 expression in SKOV-3 cells reduces subcutaneous tumor growth in an athymic nude mouse model indicating that HER-2 expression is rate-limiting for tumor growth *in vivo*.[28,29] Therefore, we used this model to test if the systemic application of HER-2-specific, PEI-complexed siRNAs results in the growth inhibition of established s.c. tumors. SKOV-3 cells were subcutaneously injected into the flanks of athymic nude mice, and when tumors reached a size of ~10 mm^2, animals were treated every 2–3 days with 0.6 nmol PEI-complexed HER-2 siRNAs or a PEI-complexed unrelated siRNA as negative control. As shown in Figure 4A, tumors in mice treated with an unrelated PEI-complexed siRNA grew very well reaching a mean size of >80 mm^2 after 2 weeks. The treatment with the HER-2-specific siRNAs, however, resulted in a significantly reduced tumor growth. Differences were obvious already after ~1 week of treatment and statistical significance was reached at day 12. Also,

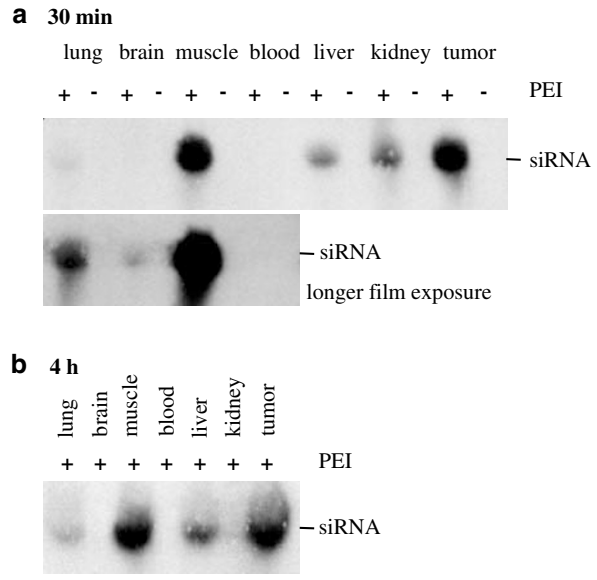

Figure 3 *In vivo application of PEI-complexed siRNAs. 2.5×10^6 SKOV-3 ovarian carcinoma cells per site were injected subcutaneously into athymic nude mice (nu/nu), and tumors were allowed to grow until they reached a size of $\sim 4 \times 4$ mm after 5 days. ^{32}P-labeled siRNAs, complexed (+) or not complexed (−) with PEI, were injected i.p. into tumor-bearing mice, and after 30 min (upper panels) or 4 h (lower panel) total RNA from various organs and tissue homogenates was prepared as described above and subjected to agarose gel electrophoresis prior to blotting and autoradiography. The bands represent intact ^{32}P-labeled siRNA, which for several hours is mainly found in tumor and muscle as well as in the liver and, time-dependently, in the kidney. Only little siRNA amounts are detected in the lung and traces in the brain.*

analysis of the tumors revealed a ∼50% reduction of HER-2 mRNA levels in the group treated with the PEI-complexed, HER-2-specific siRNAs (Figure 4B, left), which is consistent with the *in vitro* effects. To determine HER-2 protein levels, tumor sections were immunohistochemically stained and evaluated for HER-2 membrane staining. While the HER-2 staining was heterogeneous in all tumor sections independent of the treatment regimen, the blinded rating of the whole sections for staining intensity (no staining = 0 to very strong staining = 3) and abundance of stained areas revealed that 63% of the tumors of the control group were above the cutoff (defined as the mean of the scores of all sections) while this number dropped to 29% in the treatment group (Figure 4C). To test if HER-2-specific siRNAs exert an inhibitory effect on tumor growth also without prior PEI complexation, the experiment was repeated with i.p. injection of naked compared to PEI-complexed HER-2 siRNA (Figure 4D). The comparison of curves A in Figure 4A and D shows that the injection of naked siRNA resulted in no reduction of tumor growth since in both experiments the tumors show the same growth kinetics reaching a mean tumor size of 70–80 mm^2 at day 14. Again, PEI-complexed, HER-2-specific siRNAs resulted in a significantly reduced tumor growth (Figure 4D). In this experiment, the siRNA/PEI effect was even stronger, which may be due to a slightly earlier (∼2 days) onset of the treatment after established tumors were visible. From these data, we conclude that PEI significantly improves the *in vivo* efficiency of siRNAs.

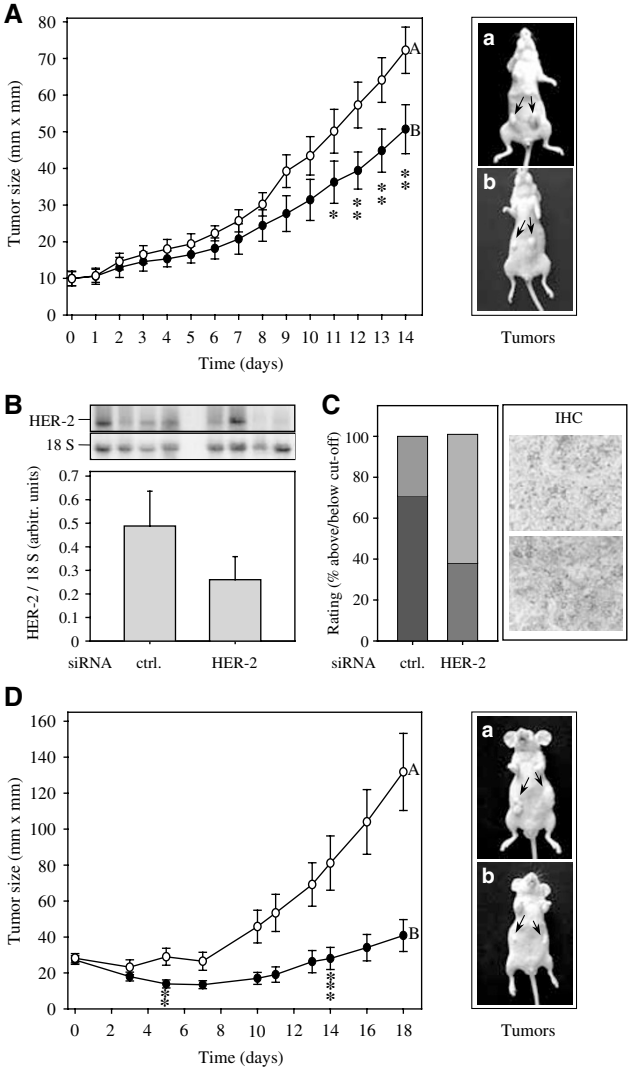

Figure 4 *Systemic treatment of mice with PEI-complexed HER-2-specific siRNAs leads to reduced growth of s.c. SKOV-3 tumor xenografts due to decreased HER-2 expression. Subcutaneous tumor xenografts in athymic nude mice were generated as described above. (A) Mice were injected i.p. with 0.6 nmol nonspecific (A, open circles) or HER-2 specific (B, closed circles) PEI-complexed siRNAs 2–3 times per week and tumor sizes were evaluated daily from the product of the perpendicular diameters of the tumors. Differences in tumor growth were visible after ∼1 week and reached statistical significance at day 11. Mean ± s.e. of the mean (s.e.m.) is depicted. Student's unpaired t-test was used for comparisons between data sets (*<0.05, **<0.03), and examples of tumors (arrows) at day 14 are shown in panel a right. (B) After 2 weeks, tumors were removed, and Northern blotting revealed a ∼50% reduction of HER-2 mRNA levels in the treatment group. (C) These findings were confirmed by immunohistochemical analysis of the tumor sections, which was performed as previously described [35] using mouse monoclonal anti-HER2 antibodies, 1:400 (Ab 17, Neomarkers, Fremont, CA, USA). A marked reduction of HER-2 staining intensity and abundance upon treatment with PEI-complexed HER-2-specific siRNAs was observed. (D) I.p. injection of naked HER-2-specific siRNAs fails to exert an inhibitory effect on tumor growth (open circles; compare curves A in A and D) while, again, PEI-complexation of HER-2 siRNA results in a significantly reduced tumor growth. In this experiment, the siRNA/PEI effect was stronger as compared to panel (A) reaching significance already at day 5, which may be due to a slightly earlier onset of the treatment after established tumors were visible. Mean ± s.e.m. is depicted. Student's unpaired t-test was used for comparisons between data sets (**<0.03, ***<0.01), and examples of tumors (arrows) at day 18 are shown on the right.*

So far, only very few studies describe the *in vivo* use of siRNAs using either very high siRNA amounts (~4 nmol) injected close to the target organ[30] or showing rather poor tissue penetration and little effects,[31] or relying on the direct injection of the siRNAs into the tumor region.[26] In this paper, we present a novel system providing simultaneously the protection and efficient exogenous delivery of any siRNA *in vivo* upon simple systemic application and without any chemical modification. The effects observed in our *in vivo* tumor model upon targeting of the HER-2 receptor proves that PEI complexation of siRNAs offers an avenue for the development of highly efficient, specific and safe agents for therapeutical applications.

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