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The gene-silencing effect of siRNA in cationic lipoplexes is enhanced by incorporating pDNA in the complex

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Abstract

Efficient delivery is a key issue in translating interference RNA technology into a feasible therapy. The efficiency of carrier systems used for this technology is commonly tested by co-transfection, i.e. simultaneous transfection with an exogenous gene and with the siRNA. Two approaches can be distinguished: (1) with the two transfectants in the same carrier complex (siRNA/pDNA/carrier) and (2) with the two transfectants in different carrier complexes (pDNA/carrier and siRNA/carrier). The process to prepare the nucleic acid(s)–carrier complexes and the transfection procedure may affect the effectiveness of the gene-silencing process. In this study, two preparation methods were compared, namely the co-preparation of an siRNA/pDNA/liposome lipoplex (Method I) and the separate preparation of an siRNA/liposome lipoplex and a pDNA/liposome lipoplex (Method II). siRNA in the lipoplex produced by Method I showed a stronger gene-silencing effect than that in the lipoplexes prepared by Method II. There was no significant difference between the two methods in the amount of siRNA delivered to cells. Cellular entry and intracellular trafficking of siRNA/pDNA/liposome lipoplex is likely to differ from those of the separate lipoplexes. When in Method II non-transcriptional pDNA was included in the complex with siRNA, the gene-silencing effect was significantly enhanced. If and to what extent the experimental design is suitable to quantify RNA interference remains to be demonstrated.
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1. Introduction

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing produced by double-strand RNAs. This is a multiple-step process that involves the generation of 21–23-nt small interfering RNA (siRNA) and results in the degradation of the homologous RNA (Elbashir et al., 2001). RNAi has enormous potential not only as an invaluable tool in biological research and drug development but also as a possible approach to the *in vivo* inactivation of gene products linked to human disease and pathology. The primary obstacle in translating RNAi technology from an effective research tool into a feasible therapeutic strategy remains the efficient delivery of siRNA to the targeted cell *in vivo*. It is well recognized that non-viral vectors not only produce simpler transfection devices than viruses but they also provide a safer alternative than viral vectors, especially because of low host immunogenicity (Lundstrom and Boulikas, 2003). Among the non-viral vectors, several cationic liposomes have been reported to effectively transfer of siRNAs *in vitro* and *in vivo* (Sorensen et al., 2003; Sioud and Sorensen, 2003, 2004; Spagnou et al., 2004; Yano et al., 2004; Bitko et al., 2005; Landen et al., 2005; Morrissey et al., 2005; Nogawa et al., 2005; Khoury et al., 2006). So far, however, the achievements in terms of clinical outcome are very limited. This lack of success may be blamed in part to the wide-spread misconception that all nucleic acids are alike and to a general lack of notion that different delivery systems may deliver RNA and DNA to different intracellular pathways and thus bring about different transfection efficiencies.

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The gene-silencing effect (Paddison et al., 2002; Xu et al., 2003, 2004; Muratovska and Eccles, 2004) of siRNA as well as the ability of cationic liposomes to deliver siRNA into cells (Spagnou et al., 2004), are preferably assessed as knockdown of exogenous rather than of endogenous gene expression. In our study, simultaneous transfection with the target gene pDNA and the gene-silencing siRNA was accomplished by means of cationic liposomes. The co-transfection was achieved by two different approaches: Method I in which pDNA and siRNA were in the same carrier complex, and Method II in which a mixture of pDNA/carrier complex and siRNA/carrier complex was used for transfection. The physicochemical properties of the three complexes are likely to be significantly different, because of the substantial chemical and structural differences between pDNA and siRNA. This, in turn, is likely to lead to essential differences in the interaction of the complexes with cells, their cellular uptake and the intracellular distribution and ultimate fate of siRNA and pDNA, which will largely determine the effectiveness of the gene silencing effect.

We developed TFL-3, a cationic liposome composed of the cationic lipid, DC-6-14, with the helper lipids dioleoylphosphatidylethanolamine (DOPE) and cholesterol (CHOL), for pDNA delivery (Kikuchi et al., 1999). TFL-3 has shown high *in vitro* transfection efficiency in serum-containing media (Nguyen et al., 2003; Li et al., 2004; Nguyen et al., 2005) and effective *in vivo* gene transfection activity in a murine lung metastasis model (Li et al., 2005). Based on these features, TFL-3 may prove to be a profitable carrier not only for pDNA but also for siRNA, although systematic investigations with respect to siRNA transfer have not yet been performed.

In this study, we addressed two issues: (1) the potential of TFL-3 to transfer siRNA and (2) the suitability of the co-transfection method to determine the RNAi effect and the efficacy of carriers to transfer siRNA into cells. Lipoplexes were prepared from pDNA, siRNA and TFL-3 by two different methods. Method I involved the preparation of one single complex, siRNA/pDNA/TFL-3 lipoplex, and Method II the preparation of two separate complexes, siRNA/TFL-3 and pDNA/TFL-3. The lipoplexes were used to transfect B16BL6 cells, a murine melanoma, with the firefly luciferase gene and to assess the gene-silencing effect of siRNA on the expression of the exogenous gene.

2. Materials and methods

2.1. Preparation of siRNAs

A siRNA for firefly luciferase (sense sequence, 5′-CUUACGCUGAGUACUUCGATT-3′; anti-sense sequence, 5′-UCGAAGUACUCAGCGUAAGTT-3′) and an unrelated siRNA (sense sequence, 5′-AGCUUCAUAAGGCGCAUGCTT-3′; anti-sense sequence, 5′-GCAUGCGCCUUAUGAAGCUTT-3′) (Elbashir et al., 2001) were chemically synthesized and purified by means of HPLC by Hokkaido Systems Sciences (Hokkaido, Japan). A siRNA duplex (50 μM) was prepared by mixing complementary antisense-stranded RNA and sense-stranded RNA in TE buffer (10 μM Tris–HCl, 1 μM EDTA, pH 8.0, DNase and RNase free grade) (Nippon Gene, Tokyo, Japan) at equal molar concentrations. The mixture was incubated in boiling water for 1 min and cooled slowly to room temperature. The quality of the siRNA duplexes siRNA was checked by 15% PAGE. The prepared siRNA duplexes were stored at −80 °C.

2.2. Preparation of lipoplex

A cationic lipid mixture, TFL-3, composed of DC-6-14/DOPE/CHOL (1/0.75/0.75 mol/mol) was a generous gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). TFL-3 liposomes were prepared at a concentration of 2.4 mM by addition of ddH_2O (milliQ) to the lyophilized lipid mixture under vortexing, to give unilamellar liposomes. Two luciferase pDNAs (*Photinus* (firefly) luciferase, pGL-3 and *Renilla* (sea pansy) luciferase, pRL-TK) were purchased from Promega (WI, USA). A mixture of a reporter gene and a control gene (1 μg/μl) was prepared by mixing 45 μl of pGL3 solution (1 μg/μl in TE buffer) and 5 μl of pRL-TK (1 μg/μl in TE buffer). A stock solution of siRNA was serially diluted with TE buffer (DNase and RNase free grade, pH 8.0) to obtain concentrations of 5000, 2500, 1250, 625, 312.5, 156.25 and 78.125 nM, respectively.

2.2.1. Co-preparation of the siRNA/pDNA/TFL-3 lipoplex (Method I)

Five microliter of pDNAs (pGL-3 and pRL-TK) solution (1 μg/μl) were mixed with 100 μl of the diluted siRNA (78.125, 156.25, 312.5, 625, 1250 nM). The final volume of the mixture was adjusted to 2.5 ml with OPTIMEM I. To the mixtures of pDNA and siRNA 2.5 ml of TFL-3 (20 μM) was added, diluted by OPTIMEM I. The final mixture was allowed to stand for 20 min at room temperature.

2.2.2. Separate preparation of siRNA/TFL-3 and pDNA/TFL-3 lipoplexes (Method II)

For the siRNA/TFL-3 complex, 100 μl of the diluted siRNA (312.5, 625, 1250, 2500, 5000 nM) was further diluted with 1150 μl of OPTIMEM I. The siRNA solution was mixed with 1250 μl of TFL-3 (20 μM) in OPTIMEM I. For the pDNA/TFL-3 lipoplex, 1250 μl of pDNAs (1 μg/1 μl) were mixed with equal volume of TFL-3 (20 μM) in OPTIMEM I. The mixtures were allowed to stand for 20 min at room temperature.

After preparation, all lipoplexes were checked for the presence of free pDNA and siRNA by agarose electrophoresis (0.8% and 2%, respectively). Throughout this study, the amounts of cationic liposomes for transfection were kept constant to avoid saturation or competitive inhibition of the delivery of siRNA and pDNA by the liposomes.

2.3. Mean diameter and zeta potential of the lipoplexes

The mean diameter and the zeta potential of the lipoplexes were determined using a laser particle analyzer and a laser electrophoresis zeta potential analyzer device, NICOMP 380 (Particle Sizing System, CA, USA). A 5% dextrose solution was used to dilute the samples.

2.4. Cell culture and transfection procedure

B16BL6, a murine melanoma cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) containing 10 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a 5% CO_2 air incubator at 37 °C. The cells were maintained in exponential growth. Two luciferase plasmids (*Photinus* (firefly) luciferase, pGL-3 and *Renilla* (sea pansy) luciferase, pRL-TK) were used as reporter and control genes, respectively, by a reported transfection protocol with modifications (Elbashir et al., 2001). The cells were seeded in 24-well plates at a density of 5.0×10^4 cells/well 24 h before the transfection was carried out. Prior to transfection, the medium was removed. The cells were co-transfected with pDNA/siRNA/TFL-3 lipoplex or with the mixture of pDNA/TFL-3 lipoplex and siRNA/TFL-3 lipoplex in OPTIMEM I (500 μl/well). The siRNA concentrations for transfection were 1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 nM. The transfection medium was exchanged for culture medium (500 μl) after 4 h. All experiments were performed in triplicate and repeated at last twice.

2.5. Dual-luciferase reporter assay

Cells were harvested 20 h after transfection and lysed by passive lysis buffer (100 μl/well), according to the instruction of the Dual-Luciferase Reporter Assay System (Promega) with modifications. The luciferase activities of the samples were measured using a luminometer (BLR-301, Aloka, Tokyo, Japan), with a delay time of 2 s and an integrate time of 10 s. The volumes of sample (20 μl), luciferase assay reagent II (100 μl), and Stop & Glo Reagent (100 μl) were reduced to half the amount when the activity of the reporter or control gene saturated the luminometer. The inhibitory effects generated by siRNA were expressed as normalized ratios between the activities of reporter (firefly) luciferase gene and control (sea pansy) luciferase gene (Elbashir et al., 2001; Xu et al., 2003).

2.6. Effect of non-transcriptional pDNA on the gene-silencing effect of siRNA

Non-transcriptional pDNA, pGEM-luc, was purchased from Promega (WI, USA). pDNA (pGL-3 and pRL-TK, 0.5 μg)/TFL-3 lipoplex (2.5 nmol) was prepared in OPTIMEM I as described above. siRNA/pDNA (pGEM-luc)/TFL-3 lipoplex was also prepared in OPTIMEM I as described above. For preparation of the siRNA/pDNA/TFL-3 lipoplex, siRNA was kept at 25 nM and pDNA (pGEM-luc) was varied from 0.0625 to 0.5 μg. The cells were co-transfected as described above and the dual-luciferase assay was performed.

2.7. Amount of siRNA transferred into the cells by transfection

Lipoplexes containing fluorescence (FAM)-labeled siRNA, custom synthesized by Hokkaido System Science, were transfected as described above. At 4 h after transfection, the cells were washed twice with ice-cold PBS and treated with 100 μl/well of a lysis buffer (ice-cold PBS containing 2% (w/v) Triton-X 100, 1 μl of protease inhibitor (×100 protease inhibitor cocktail (50 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 15 μM aprotinin (from bovine lung), 100 μM E-64, 100 μM leupeptin hemisulfate salt, 50 μM EDTA, Nacalai Tesque, Kyoto, Japan))) on ice for 1 h. The cell lysate was collected and centrifuged (20,000 × *g*, 5 min, 4 °C) to give a clear supernatant. The fluorescence of samples was determined using a fluorometer (F-4500, Hitachi, Tokyo, Japan). The protein content in the samples was determined with the DC protein assay kit (Bio-Rad Laboratories, CA, USA). The data are expressed as the fluorescence/mg of protein.

2.8. Confocal microscopy

The cells were transfected with lipoplexes containing FAM-labeled siRNA and rhodamine-labeled TFL-3 (supplied by Daiichi Pharmaceutical). For preparation of the siRNA/pDNA/TFL-3 lipoplex, FAM-labeled siRNA (25 pmol), pDNA (2 μg) and rhodamine-labeled TFL-3 (5 nmol) were mixed, and the total volume was adjusted to 250 μl with OPTIMEM I. For preparation of the siRNA/TFL-3 lipoplex, FAM-labeled siRNA (25 pmol) and rhodamine-labeled TFL-3 (2.5 nmol) were mixed, and the total volume was adjusted to 250 μl with OPTIMEM I. For preparation of the pDNA/TFL-3 lipoplex, pDNA (2 μg) and rhodamine-labeled TFL-3 (2.5 nmol) were mixed, and the total volume was adjusted to 250 μl with OPTIMEM I. At 4 h after transfection, the cells were washed twice with ice-cold PBS. Then the cells were immediately examined using a Zeiss LSM5 inverted confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) without fixation. FAM-siRNA was imaged using a 488 nm filter for excitation and a 510–530 nm filter for emission, respectively. Rhodamine-labeled TFL-3 was imaged using a 540 nm filter for excitation and a 650 nm filter for emission, respectively.

2.9. Statistics

All values are expressed as the mean ± S.D. Statistical analysis was performed with a two-tailed unpaired *t*-test using GraphPad InStat software (GraphPad Software, CA, USA). The level of significance was set at $p < 0.05$.

3. Results

3.1. Characterization of lipoplexes

After preparation, all lipoplexes (siRNA/pDNA/TFL-3, siRNA/TFL-3 and pDNA/TFL-3) were checked for free nucleic acids by an agarose electrophoresis. No free siRNA and/or pDNA were detected (not shown). The mean diameters and zeta potentials of all lipoplexes were analyzed (Table 1). The mean particle sizes of siRNA/pDNA/TFL-3 lipoplexes produced by the co-preparation method (Method I) increased with

Table 1
Characterization of the lipoplexes

siRNA/pDNA/TFL-3			Diameter (nm)	Zeta potential (mV)
siRNA (μM)	pDNA (μg/ml)	TFL-3 (μM)		
Co-preparation (Method I)				
12.5	2	20	305.3	+29.26
25	2	20	374.2	+29.07
50	2	20	392.4	+29.44

siRNA/TFL-3		Diameter (nm)	Zeta potential (mV)
siRNA (μM)	TFL-3 (μM)		
Separate preparation (Method II)			
12.5	10	301.9	+36.59
25	10	279.9	+37.13
50	10	307.7	+30.35

pDNA/TFL-3		Diameter (nm)	Zeta potential (mV)
pDNA (μg/ml)	TFL-3 (μM)		
Separate preparation (Method II)			
20	10	317.5	+27.54

TFL-3 (μM)	Diameter (nm)	Zeta potential (mV)
Control		
10	266.5	+37.49

Mixture of pGL-3 and pRL-TK (9:1, w/w) was used as pDNA. siRNA for *Renilla* (firefly) luciferase was used.

increasing amount of siRNA, while zeta potential values did not change. The mean particle size of the siRNA/TFL-3 complexes produced by separate preparation (Method II) did not change regardless of the amount of siRNA, while the zeta potential values of these lipoplexes decreased with increasing amounts of siRNA. The mean diameter of TFL-3 liposomes increased by complexing with pDNA (separate preparation according to Method II), while the zeta potential value decreased.

3.1.1. Suppression of Renilla (firefly) luciferase expression in B16BL6 cells

The ratio of firefly and sea pansy luciferase activities was determined after co-transfection (Fig. 1). The siRNA in the lipoplexes produced by co-preparation (Method I) was far more effective than the siRNA in the separately prepared lipoplexes (Method II). Even at a concentration as low as 1.5625 nM, the siRNA in the co-prepared lipoplexes induced more than 80% inhibition. To obtain a similar inhibition with the separately prepared lipoplexes, more than 100 nM of siRNA was required. The gene-silencing effect of siRNA in the lipoplexes produced by co-preparation was 60-fold higher than that in the lipoplexes produced by separate preparation. It is to be noted that the activities of sea pansy luciferase (control) were similar throughout the experiments (Table 2). Furthermore, unrelated siRNA did not show any significant gene-silencing effect at any of the applied concentrations regardless of the preparation method (data not shown).

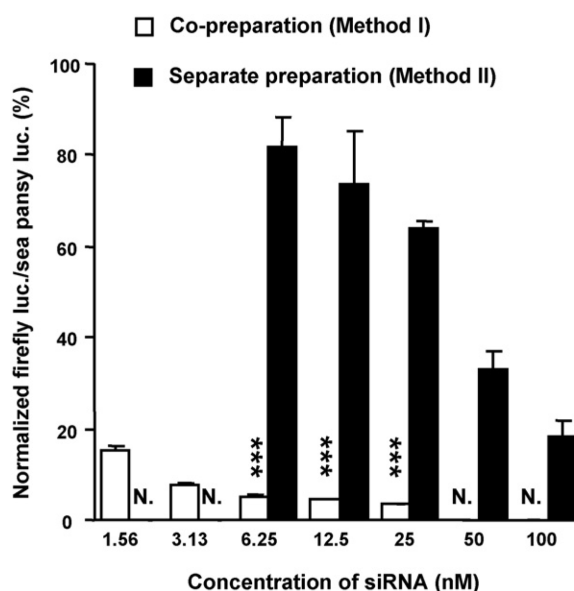

Fig. 1. Effect of the lipoplex preparation method (Method I or Method II) on the gene-silencing potential of transfected siRNA. Results presented are from three independent experiments. $^{***}p < 0.005$; N., not determined.

3.2. Amount of siRNA associated with the cells

To evaluate whether intracellular uptake of siRNA was related to the induced gene-silencing effect, the amount of siRNA transferred into the cells was determined by using FAM-labeled siRNA. The amount of siRNA associated with the cells increased with increasing the siRNA concentration (Fig. 2). Although the amount of siRNA transferred by lipoplexes produced by co-preparation was larger than that transferred by separately prepared lipoplexes, there was no significant difference in the amount of transferred siRNA.

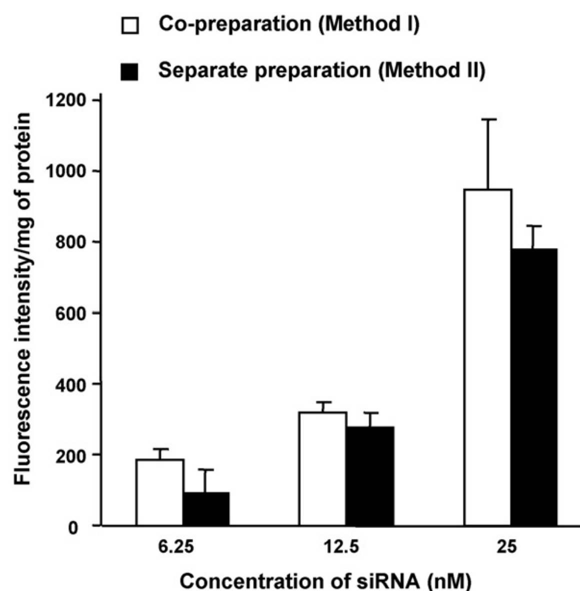

Fig. 2. Amount of siRNA associated with the cells following transfection. Cells were incubated with lipoplexes containing a fluorescently labeled siRNA. After 4 h the cells were lysed and fluorescence in the lysates was quantified. For details see Section 2. Results presented are from three independent experiments.

Table 2
Firefly and sea pansy luciferase activities in B16BL6 cells

	siRNA concentration							
	0 nM (Control)	1.56 nM	3.13 nM	6.25 nM	12.5 nM	25 nM	50 nM	100 nM
Co-preparation (Method I)								
Firefly luciferase activity (cpm)	266137.3 ± 41567.5	37204.3 ± 1214.6	19677.5 ± 1974.9	15127.3 ± 1869.0	12394.3 ± 1175.7	9367. 7 ± 2710.1	N.	N.
Sea pansy luciferase activity (cpm)	4187.8 ± 491.4	4256.3 ± 198.1	4583.3 ± 321.9	5270.0 ± 349.8	4562.7 ± 299.2	3823.0 ± 1039.9	N.	N.
Separate preparation (Method II)								
Firefly luciferase activity (cpm)	256359.2 ± 29810.1	N.	N.	180235.5 ± 13314.2	155066.8 ± 16901.1	144200.5 ± 19648.0	44268.8 ± 4925.7	25888.0 ± 2866.2
Sea pansy luciferase activity (cpm)	5141.4 ± 728.5	N.	N.	5621.8 ± 518.5	5867.0 ± 772.4	5893.3 ± 215.9	4216.5 ± 380.9	3919.8 ± 343.6

The luciferase activities were determined according to the method described in Section 2. Results presented are from three independent experiments. N., not determined.

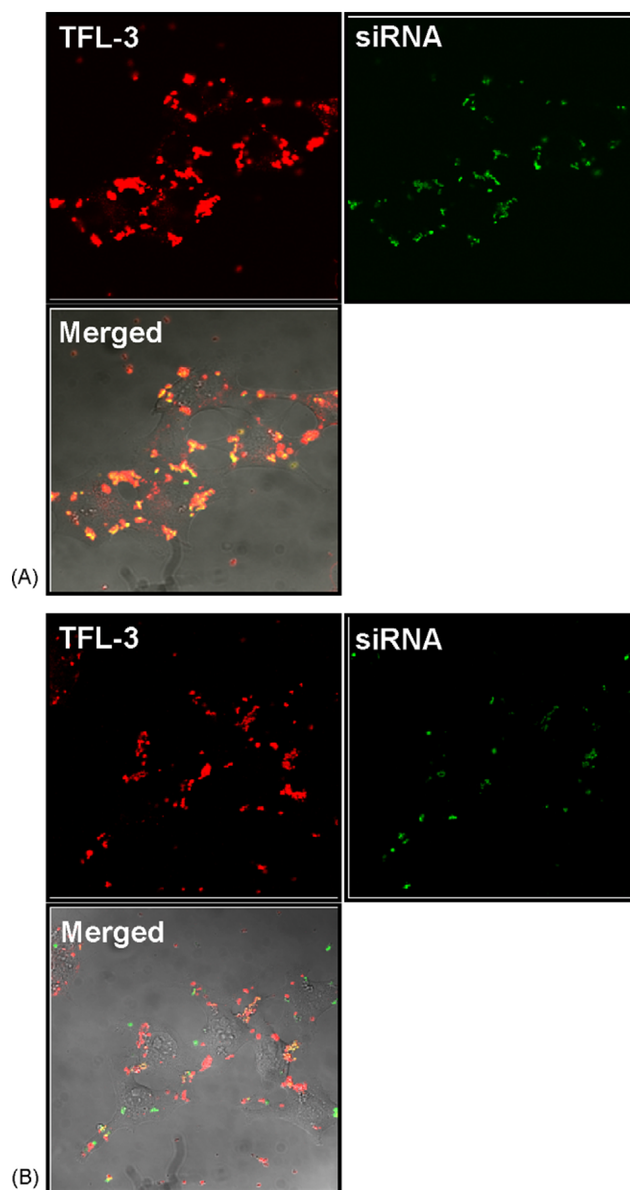

Fig. 3. Intracellular distribution of the lipoplexes produced by the (A) co-preparation method and (B) separate preparation method. Cells were incubated for 4 h with lipoplexes containing rhodamine-labeled TFL and FAM-labeled siRNA and immediately thereafter observed by confocal microscopy. Rhodamine, red; FAM, green.

3.3. Intracellular distribution of lipoplexes

The intracellular trafficking of the lipoplexes containing FAM-labeled siRNA, pDNAs and rhodamine-labeled TFL-3 was examined by means of confocal microscopy. Pictures were taken immediately after the transfection was terminated. Fig. 3A shows the intracellular distribution of lipoplexes (FAM-siRNA/pDNA/rhodamine-TFL-3) produced by co-preparation (Method I). Co-localization of siRNA and TFL-3 (merging green and red producing yellow) was observed. Fig. 3B shows the intracellular distribution of lipoplexes (FAM-siRNA/TFL-3 and pDNA/rhodamine-TFL-3) produced by separate preparation (Method II). siRNA (green) and TFL-3 (red) can be distin-

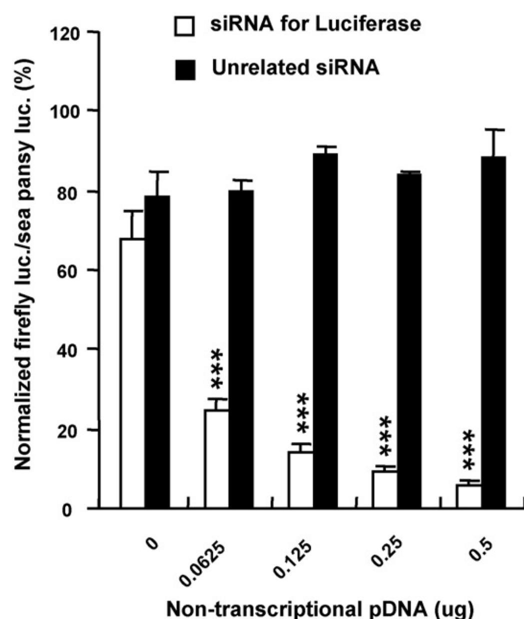

Fig. 4. Effect of the presence of non-transcriptional pDNA in the lipoplex on the gene-silencing potential of siRNA. See Section 2 for details. Results presented are from three independent experiments. $^{***}p < 0.005$.

guished as individual spots in the cells. It should be noted that the amount of rhodamine-labeled TFL-3 for the preparation of lipoplexes with siRNA and pDNA was twice as large as that for the preparation of lipoplexes with pDNA. The fluorescence intensity of siRNA/pDNA/rhodamine-labeled TFL-3 lipoplexes will therefore be higher than that of pDNA/rhodamine-labeled TFL-3.

3.4. Induction of the gene-silencing effect of siRNA by a non-transcriptional pDNA

From the results of the confocal microscopy, we assumed that pDNA in the lipoplexes produced by co-preparation (Method I) has somehow the ability to bring the siRNA to an optimal position in the cytoplasm allowing it to induce a higher gene-silencing effect. To test this, we prepared lipoplexes containing a non-transcriptional pDNA and siRNA for *Renilla* (firefly) luciferase and incubated them with cells after transfection with pDNAs/TFL-3 lipoplexes. Inhibition of gene expression increased with the amount of non-transcriptional pDNA (Fig. 4). It is noteworthy that the sea pansy luciferase activities (control) were similar throughout the experiments (data not shown).

4. Discussion

Gene silencing by siRNA can be assayed by studying its inhibitory effect on the expression of an exogenous gene co-transfected into the cell simultaneously with the siRNA. In this study, we compared two principally different approaches for this co-transfection, either by accommodating the pDNA in the same complex together with the siRNA (Method I), or by incorporating the pDNA and the siRNA in two separate complexes (Method II).

The siRNA lipoplexes produced by Method I showed a much stronger reporter-gene silencing effect than those produced by Method II (Fig. 1). This observation clearly calls for caution when drawing conclusions concerning the activity of synthesized siRNAs and/or the effectiveness of the delivery system used. It may be necessary to first establish a reliable evaluation method. Aiming at an endogenous gene or a stably expressed exogenous gene rather than at a transiently expressed gene would probably provide more reliable results.

During transfection with lipoplexes produced by Method II, one lipoplex might competitively inhibit the cellular uptake of the other. The consequently lower amount of transferred siRNA might be insufficient to silence the larger exogenous gene expression. In addition, lower target gene-expression due to lower cellular uptake of pDNA might lead to an over-estimation of the gene-silencing effect by transferred siRNA. We observed, however, that the expression of the control gene (*Renilla* (sea pansy) luciferase) as well as the amount of siRNA associated with the cells were nearly constant, irrespective of the preparation method (Fig. 2). This strongly suggests that the lower gene-silencing effect of lipoplexes prepared by Method II cannot be attributed to mutually competitive inhibition of the cellular uptake of pDNA or siRNA.

Although pDNA and siRNA both have anionic phosphodiester backbones with identical negative charge/nucleotide ratios and should therefore readily interact electrostatically with cationic liposomes to form lipoplexes, they are very different from each other in terms of molecular weight and molecular topography. This may bring along potentially important consequences. The resulting lipoplexes, i.e. complexes with only siRNA or only pDNA or complexes with both siRNA and pDNA, are each likely to display unique physicochemical properties and, consequently, to lead to different types of interaction with the cells. In fact, in the case of pDNA, it has been reported that larger lipoplexes tend to give better transfection results than smaller lipoplexes (Kreiss et al., 1999; Ross and Hui, 1999; Simberg et al., 2001; Almofti et al., 2003). In contrast to the pDNA delivery, no difference in gene-silencing potential was observed between smaller lipoplexes (50–100 nm) and larger lipoplexes (200–600 nm) (Spagnou et al., 2004). In our experiments we observed no significant differences between complexes prepared by the two different methods in terms of particle diameter and zeta potential (Table 1), presumably due to the small amounts of siRNA and pDNA that were used to form the lipoplexes. We therefore believe that it is also not a difference in physicochemical properties of the lipoplexes that is the major cause of the different gene-silencing effects of siRNA observed in our study. This view is supported by the constant values of the control exogenous gene expression levels we observed and by the similar amounts of siRNA associated with the cells, irrespective of the preparation method.

It is generally assumed that the cellular entry and intracellular trafficking of siRNA are the key processes in determining the efficiency of gene-silencing effects with exogenous siRNA following transfection (Hammond et al., 2000; Martinez et al.,

2002; Schwarz et al., 2002). Our confocal microscopy study demonstrated that the siRNA delivered by siRNA/pDNA/TFL-3 lipoplexes co-localized in the cell with TFL-3 (Fig. 3A), implying that siRNA also co-localized with pDNA. By contrast, the observation that the siRNA delivered by siRNA/TFL-3 lipoplexes did not co-localize with pDNA/rhodamine-labeled TFL-3 lipoplexes (Fig. 3B) indicated that siRNA and pDNA localize separately after entry in the cells. These observations may indicate that the cellular entry and the intracellular trafficking of the siRNA/pDNA/TFL-3 lipoplexes differ from that of other lipoplexes (siRNA/TFL-3 and pDNA/TFL-3). We recently reported that the cellular entry of pDNA/TFL-3 lipoplex occurs via the endocytosis pathway (Almofti et al., 2003). The mechanisms of siRNA entry into the cells and their intracellular trafficking mediated by the TFL-3 have not yet been established, however. Miller and co-workers (Keller et al., 2003; Spagnou et al., 2004) demonstrated that intracellular uptake and localization of siRNA and pDNA complexed in their carrier system (CDAN (N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine)/DOPE (dioleoylphosphatidylethanolamine) liposomes) differ substantially between siRNA lipoplexes and pDNA lipoplexes. Uptake and intracellular trafficking of siRNA/pDNA/TFL-3 lipoplex likewise may differ from those of siRNA/TFL-3 and pDNA/TFL-3 lipoplexes.

Although we do not yet understand the underlying mechanism, we clearly demonstrated that pDNAs, also if non-transcriptional, can increase the gene-silencing effect of the associated siRNA provided that it is present with the siRNA in the same lipoplex (Figs. 1 and 4). Recently, Schiffelers et al. (2004) reported similar findings in *in vivo* experiments. They compared co-injection (pDNA and siRNA injected simultaneously) with sequential injections (siRNA injected 2 h after pDNA) with respect to exogenous-gene-silencing potential siRNA-RGD-PEI-PEG-nanocomplex, targeted to tumor neovasculature expressing integrins, *in vivo*. siRNA was efficiently delivered by the nanocomplex using either injection protocol, but the induced gene-silencing effect was lower with the sequential injection protocol than with the co-injection protocol. The pDNA in the same lipoplex with siRNA may guide the siRNA to the correct position to function in the cells. This may open up new perspectives for gene therapy by means of pDNA and siRNA. The silencing of (aberrant) genes linked to human disorders and at the same time expressing genes producing a therapeutic protein with specific functions in the same cells may synergistically increase the efficacy of the gene therapy, when lipoplexes containing both a pDNA encoding functional gene and a gene-specific siRNA are injected. To achieve this ambitious goal suitably targeted carrier systems will have to be developed which are capable of delivering their nucleic acid cargos to target tissues or cells.

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