Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells

Aleksandra Muratovska[a], Michael R. Eccles[b,*]

[a] *MRC-Dunn Human Nutrition Unit, Wellcome Trust/MRC building, Hills Rd, Cambridge CB2 2XY, UK*
[b] *Developmental Genetics Laboratory, Department of Pathology, Dunedin School of Medicine, University of Otago, P.O. Box 913, Dunedin, New Zealand*

Received 6 November 2003; revised 10 December 2003; accepted 11 December 2003

First published online 13 January 2004

Abstract The efficient delivery of short interfering RNAs (siRNAs) into cells provides a powerful approach to study cellular functions. SiRNAs were coupled to the membrane permeant peptides (MPPs) penetratin and transportan to improve their uptake by cells. Thiol-containing siRNAs corresponding to luciferase, or green fluorescent protein (*GFP*) transgenes, were synthesized and conjugated to penetratin or transportan via a disulfide bond that is labile in the reducing environment of the cytoplasm. These MPP-siRNAs efficiently reduced transient and stable expression of reporter transgenes in several mammalian cell types in a high proportion of cells, and demonstrated equivalent or better delivery characteristics than cationic liposomes with fewer manipulations.

Key words: Short interfering RNA; Penetratin; Transportan; Transfection; Gene silencing

1. Introduction

Recently, a naturally occurring process was discovered in fungi and plants that involves sequence-specific post-transcriptional silencing of gene expression [1]. Subsequently this process was shown to occur in bacterial and animal cells and the term RNA interference (RNAi) was coined for this phenomenon [1,2]. The silencing 'triggers' for RNAi are long double-stranded RNA (dsRNA) molecules complementary to mRNA sequences that are introduced into cells either experimentally or are derived from endogenous sources such as viruses, transgenes or cellular genes (transposons) [2]. These dsRNA molecules are processed into discrete short interfering RNA (siRNA) fragments that are 21–25 nucleotides in length by an enzyme known as Dicer [3]. The siRNAs are subsequently presented to a 500 kDa ribonuclear protein complex known as the RNA-induced silencing complex (RISC) comprised of nucleases, helicases and RNA-dependent polymerases to target specific mRNAs for destruction.

Long dsRNA sequences may activate the interferon response and 2-5A antisense pathways, but generally cause non-specific mRNA degradation in cells [4], whereas siRNAs induce sequence-specific degradation of only targeted mRNAs [3]. Tuschl and coworkers have shown that 21-nucleotide duplexes corresponding to a luciferase transgene introduced in different cell types cause specific inhibition of the luciferase gene expression [5]. Large dsRNAs in turn cause complete arrest of gene expression within the cells masking any sequence-specific effects.

SiRNAs are polyanions and unassisted permeation across lipid bilayers is negligible. DsRNA can be delivered to *Caenorhabditis elegans* by feeding or soaking [6], however in mammalian cells siRNAs are typically delivered using cationic liposomes [5]. Although the use of liposomes has shown some success, the major disadvantages of this delivery method for nucleic acids are that a number of cell types (examples include dendritic and HUVEC endothelial cells) cannot be transfected using liposomes at an efficiency that yields a significant biological effect [7,8], and in each experiment several manipulations of the cells are required. The development of a method for direct targeting of siRNA molecules from the extracellular environment to the cytoplasm may significantly enhance research using siRNAs to silence genes in cultured and somatic cells.

Membrane permeant peptides (MPPs) are suitable candidates for the delivery of relatively large molecules, such as nucleic acids, peptides, proteins and even paramagnetic particles up to 200 nm in diameter, to cells [9–12]. These short amphipathic peptides have been shown to translocate across lipid bilayers in an energy-independent manner [9]. Here we describe a conjugate for directly targeting siRNAs to the cytoplasm of cells, with delivery across the plasma membrane using the MPPs, penetratin or transportan. We report the synthesis of disulfide-linked MPP-siRNA and demonstrate the ability of these compounds to enter cells and influence transiently and stably expressed transgenes in cells.

2. Materials and methods

2.1. Oligonucleotides

Four sets of complementary siRNAs were purchased from Dharmacon Research (Lafayette, CO, USA). SiRNAs were designed to target the *GL2* luciferase mRNA (acc. no. X65324), corresponding to the coding region 153–173 relative to the first nucleotide of the start codon (5′-rCrGrUrArCrGrCrGrGrArArUrArCrUrUrCrGrATT-3′ and 5′-rUrCrGrArArGrUrArUrUrCCrGrCrGrUrArCrGTT-3′) and the green fluorescent protein (*GFP*) mRNA (acc. no. U55762), corresponding to the coding region 540–565 (5′-rArCrUrArCrCrArGrCrArGrArArCrArCrCrCrCTT-3′ and 5′-rGrGrGrGrUrGrUrUrCrUrGrCrUrGrGrUrArGrUTT-3′). A pair of *GL2* siRNAs and

*Corresponding author. Fax: (64)-3-479 7136.
E-mail addresses: af@mrc-dunn.cam.ac.uk (A. Muratovska), meccles@otago.ac.nz (M.R. Eccles).

Abbreviations: MPP, membrane permeant peptide; siRNA, short interfering RNA; GFP, green fluorescent protein; dsRNA, double-stranded RNA

0014-5793/04/$30.00
doi:10.1016/S0014-5793(03)01505-9

GFP siRNAs were chemically modified with a thiol group at the 5′ end of one RNA strand and a 5′ Cy3 on the complementary strand.

2.2. Solid phase peptide synthesis

Peptide synthesis for penetratin (CRQIKIWFQNRRMKWKK) and transportan (CLIKKALAALAKLNIKLLYGASNLTWG) was carried out on a 0.25 mmol scale (manual synthesis) starting from either an Fmoc (9-fluorenylmethoxycarbonyl)-lysine WANG resin, or an Fmoc-glycine WANG resin (substitution value 0.75 mmol/g resin) (Auspep, Parkville, Australia). All Fmoc amino acids in 4-fold excess were assembled using 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium (HBTU) and diisopropylethylamine (DIPEA) as coupling reagents (Auspep). The Fmoc groups protecting the amine groups on amino acids were removed using 50% piperidine in dimethylformamide (DMF) [13]. Both penetratin and transportan were synthesized with a cysteine residue at the N-terminus. Both peptides were cleaved from their resins by trifluoroacetic acid (TFA) and scavengers (TFA:H_2O:phenol:triisopropylsilane, 88:5:5:2) under nitrogen at 0°C for 1 h [13]. The products were precipitated with diethyl ether, dissolved in 50% acetonitrile, lyophilized and redissolved in water. The crude peptides were then purified by reverse phase-high performance liquid chromatography (RP-HPLC) using a semi-preparative column (Vydac C_{18}, 300 Å, 10×250 mm) and the peptide purity was checked using an analytical column (Vydac C_4, 300 Å, 4.6×250 mm) (Auspep). A linear gradient starting with 0.1% TFA in water and finishing with 90% acetonitrile in water and 0.1% TFA was run over 30 min. The peptide peaks were detected by absorbance at 280 nm, collected, lyophilized and dissolved in water for further use.

2.3. Synthesis of siRNA-MPP conjugates

For annealing of siRNAs, 35 μM of single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES–KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h incubation at 37°C. Annealed siRNAs were introduced into cells using Lipofectamine 2000 (Invitrogen, CA, USA). The annealed siRNAs containing thiol groups were desalted by incubating the hybridization mix for 7 min on ice in a pre-set 1% agarose in 100 mM glucose well in an Eppendorf tube (by leaving a 100 μl tip in the molten agarose mix and allowing it to set). The desalted siRNAs were supplemented with 1 vol reaction buffer (10 mM HEPES, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0) to adjust the final concentration of the siRNAs to 17.5 μM. Equimolar amounts of siRNAs, MPPs (penetratin or transportan) and the thiol oxidant diamide (Sigma, USA) were mixed and incubated for 1 h at 40°C. The MPP-siRNA/diamide solution was then mixed with culture media and incubated immediately with cells. MPP-DNA conjugates prepared as described above were >95% pure with an 80% yield following HPLC purification and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (not shown).

2.4. Cell culture and transfections

Cells were grown as previously described [13]. Briefly, COS-7 cells, C166-GFP and EOMA-GFP cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated fetal calf serum (FCS) and CHO-AA8-Luc Tet-Off cells were grown in minimum essential medium (alpha modification) with 10% FCS (Gibco, USA). In addition to 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin, C166-GFP and EOMA-GFP cells were supplemented with 200 μg ml^{-1} G418 (Gibco) and CHO-AA8-Luc Tet-Off cells with 100 μg ml^{-1} G418 and 100 μg ml^{-1} hygromycin B (Gibco). 24 h before transfection at ~80% confluency, the cells were trypsinized, diluted 1:3 with fresh medium ($1–5\times10^5$ cells ml^{-1}) and transferred to 24-well plates (300 μl per well). Cotransfection of reporter plasmids and siRNAs in COS-7 cells was carried out by incubation with Lipofectamine 2000 in serum-free DMEM for 6 h, followed by replacement with fresh media containing 10% fetal bovine serum (FBS), as described by the manufacturer. The concentrations of the plasmids per well were 1.0 μg for pGL2 and 0.1 μg for pCB11 (Rluc expressed by the SV40 promoter) (Promega, USA). The siRNAs were supplemented in all cell lines at 25 nM concentrations by addition directly in the cell culture media, or by transfection with Lipofectamine 2000, as above. Penetratin-siRNAs and transportan-siRNAs were supplemented in 25 nM concentrations by addition directly to the cell culture media, or by transfection with Lipofectamine 2000, as above. COS-7 cells were also treated with siRNAs, which were directly added to the cell culture media 24 h after the transfection with transgenic reporter plasmids. Luciferase expression in COS-7 cells was assayed using a dual luciferase kit (Promega) to measure both firefly luciferase and Renilla luciferase activities (Rluc), allowing ease of comparison between transfection experiments. CHO-AA8-Luc Tet-Off cells were incubated with the siRNAs continuously for up to 3 days, and C166-GFP and EOMA-GFP cells were incubated with siRNAs continuously for up to 7 days. Gene silencing was monitored by Western blotting, flow cytometry and fluorescence microscopy.

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

SDS–PAGE and immunoblotting were carried out as previously described [13]. In each lane 20 μg of protein was loaded as quantitated using the bicinchoninic acid (BCA) assay. Quantitative densitometry to calculate the area under the peak, which was representative of the intensity of the band, was carried out on the original image using NIH image software.

2.6. Flow cytometry

C166-GFP and EOMA-GFP cells grown in 24-well plates were treated with *GFP* siRNAs delivered using Lipofectamine 2000, penetratin and transportan, for 1, 3 and 7 days after which the cells were prepared for flow cytometry as described [14].

2.7. Fluorescence microscopy

C166-GFP and EOMA-GFP cells grown in 35 mm dishes were treated with *GFP* siRNAs delivered using Lipofectamine 2000, penetratin and transportan for 1 day, 3 days and 7 days. Photographs of the cells were acquired using a Zeiss inverted confocal microscope with a Plan-Neofluar 40×/1.3 oil DIC objective and 488 nm LASER excitation, and Zeiss Imaging Software with equal exposure times.

3. Results

SiRNAs 21 nucleotides in length were designed to be complementary to a region of the *firefly luciferase* or *GFP* transgenes [1,5]. One set of the siRNAs was introduced into cells by liposomes and the other set of siRNAs was chemically modified with a free thiol group at the 5′ end of one strand so that it could react with a free thiol group from a cysteine amino acid on an MPP (Fig. 1).

Two experiments were carried out to test the efficiency of delivery of the MPP-siRNA on its own, or in the presence of Lipofectamine 2000. In the first experiment *firefly luciferase* (*GL2*) siRNAs or MPP-siRNAs were introduced into the cells together with transgenes during Lipofectamine 2000-mediated transfection, while in the second experiment siRNAs or MPP-siRNAs were introduced 24 h following transgene transfection. In control experiments cells were transfected with only the *firefly* or *Renilla luciferase* transgenes to determine the basal (exogenous) luciferase activity within the cells. The delivery of siRNAs linked to penetratin by transfection using Lipofectamine 2000 gave an approximately 75% decrease in luciferase activity, while transportan-linked siRNA resulted in a markedly smaller (~40%) decrease in luciferase activity (Fig. 2A). The luciferase activity was reduced approximately 92% in cells treated with siRNAs delivered using Lipofectamine (Fig. 2A). These results suggest that MPP-siRNAs may not be ideal substrates for cationic liposome-mediated transfection. SiRNAs unassisted by penetratin or Lipofectamine could not gain entrance to the cells and the luciferase activity remained the same as in cells transfected only with the transgenes. The activity of the Renilla luciferase remained constant in cells treated with or without the siRNAs, suggesting that the decrease of the firefly luciferase activity was due to the sequence-specific binding of the siRNAs to its mRNA.

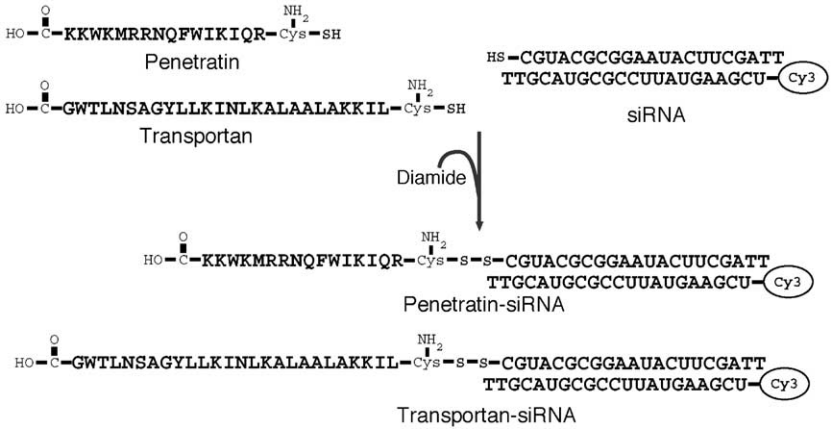

Fig. 1. Synthesis of disulfide-linked MPPs and siRNAs. MPPs and siRNAs were synthesized with a thiol group attached as described in Section 2. The single-stranded RNAs were annealed together to form a duplex and the duplexes mixed with MPP in equimolar amounts for the conjugation reaction. SiRNAs had a thiol group attached at the 5′ end of one strand, and a Cy3 fluorescent label attached at the 5′ end of the complementary strand to enable the identification of the siRNA within cells by fluorescence microscopy. The reaction between the thiol groups on the siRNA and the MPP was catalyzed by the oxidant diamide as shown.

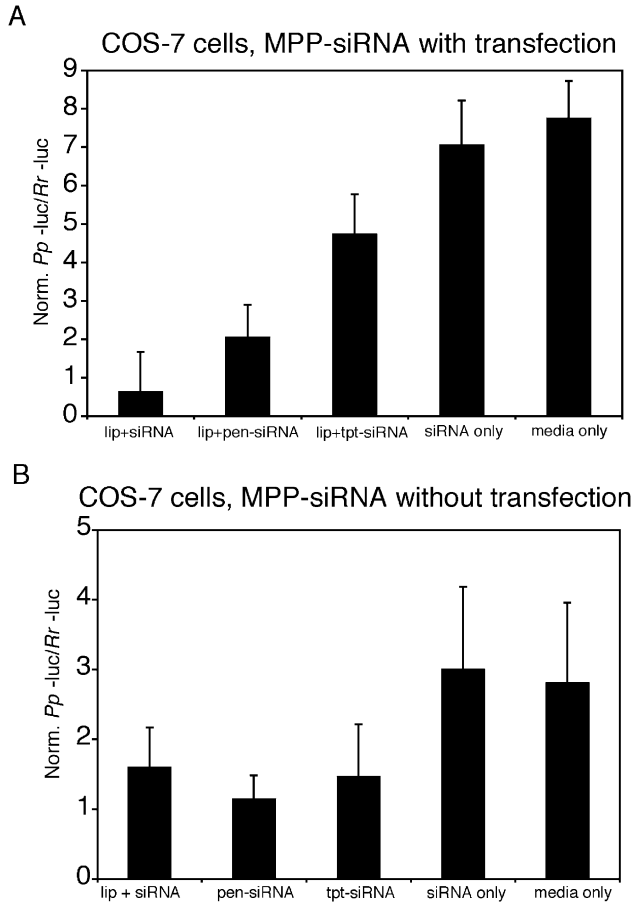

Fig. 2. Effects of siRNAs on luciferase expression in COS-7 cells. Experiments were performed with pGL2 control and pCB11 reporter plasmids. The data were averaged from three independent experiments and expressed as ratios of normalized target to control luciferase. *GL2* siRNAs were delivered by liposomes (using Lipofectamine 2000) or MPPs (penetratin and transportan). Control incubations were carried out with media only. A: COS-7 cells were cotransfected with siRNAs and reporter plasmids at the same time point. B: COS-7 cells were treated with siRNAs 24 h after transfection of the reporter plasmid.

The second experiment tested whether MPP-siRNAs could specifically decrease luciferase activity when added to the cell culture media (i.e. the MPP-siRNAs were not transfected using Lipofectamine 2000) 24 h after transfection of the luciferase transgene. Penetratin-siRNA and transportan-siRNA conjugates were able to enter the cells without transfection using Lipofectamine 2000, and efficiently decreased luciferase activity (Fig. 2B). Similar levels of luciferase activity were observed with siRNAs delivered by Lipofectamine and MPP-siRNAs. Unmodified siRNAs did not decrease luciferase activity.

The ability of the MPP-conjugated siRNAs to silence endogenous gene expression in cells was then investigated in Chinese hamster ovary (CHO-AA8-Luc Tet-Off) cells that stably expressed luciferase. Lipofectamine-delivered siRNAs decreased luciferase activity by 36% 48 h after the treatment (Fig. 3). However, the basal luciferase activity returned to normal levels 24 h later. Penetratin- and transportan-delivered siRNAs decreased the luciferase levels by 53 and 63% respectively, within the initial 24 h after treatment and this decrease remained stable for up to 3 days (Fig. 3). These results suggest that transfection of CHO-AA8-Luc Tet-Off cells with siRNAs using Lipofectamine 2000 results in a significantly lower efficiency of transfection than when siRNAs are delivered as MPP-siRNAs. Unassisted delivery of siRNAs and mock treatment of cells with media did not alter the basal luciferase activity within the cells.

To further test the specificity and efficiency of the silencing effects of MPP-linked siRNAs, C166-GFP and EOMA-GFP cells stably expressing *GFP* were transfected with *GFP* siRNAs using Lipofectamine or treated with penetratin-siRNAs and transportan-siRNAs. Silencing of *GFP* was monitored for 1, 3 and 7 days after the treatment.

Fluorescence microscopy was used initially to show the general decrease in fluorescence of cells treated with siRNAs delivered by Lipofectamine and MPP (Fig. 4A–D). The fluorescence of cells treated with media only was unchanged over 7 days. The intensity of the cell fluorescence decreased dramatically with prolonged exposure to siRNAs, which was most evident after 7 days of siRNA treatment (Fig. 4B, C).

Western blots were then used to show the qualitative decrease of GFP following treatment of C166-GFP and EOMA-

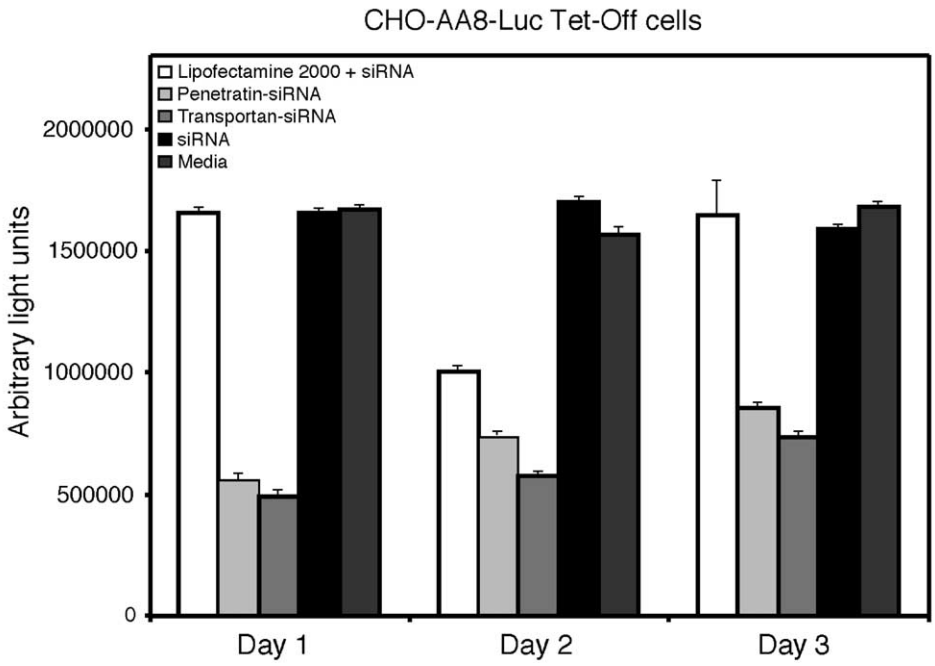

Fig. 3. Effect of siRNAs delivered in CHO-AA8-Luc Tet-Off cells by liposomes and MPP. CHO-AA8-Luc Tet-Off cells stably expressing luciferase were treated with 25 nM concentrations of siRNAs delivered by transfection with Lipofectamine 2000 for 6 h, or by incubating in media containing penetratin-siRNAs or transportan-siRNAs for up to 3 days. Luciferase activity was assayed every 24 h and plotted in arbitrary luminescence units. Control incubations were carried out with siRNAs alone and media only.

GFP with siRNAs delivered by Lipofectamine, or MPPs. Cells treated with media only showed abundant GFP in the absence of RNAi (Fig. 4E). The silencing of GFP expression was more pronounced in the EOMA-GFP cell line, where treatment with MPP-siRNAs resulted in an average of 86–97% decrease in protein expression. In the C166-GFP cell line the amount of GFP was silenced by an average of 67–80%, which was less than in EOMA-GFP cells, presumably due to the presence of multiple *GFP* transgenes in the stably transfected cell line.

As before EOMA-GFP cells were treated with siRNAs delivered by Lipofectamine and MPPs and the delivery efficiencies were compared by quantitating GFP expression using flow cytometry. Cells transfected with siRNAs using Lipofectamine 2000 decreased the cell fluorescence by 65%, implying that 65% of cells were transfected after 7 days, while penetratin and transportan siRNA resulted in 73 and 80% decrease (implying 73–80% transfection efficiency) in cell fluorescence, respectively (Fig. 4F).

4. Discussion

Here we show that siRNAs can be delivered directly to the cytoplasm when conjugated to MPPs, such as penetratin or transportan. SiRNAs delivered in this way are capable of silencing the expression of either transfected or endogenous genes. The salient features of this system are as follows: (i) the peptide facilitates transport across the plasma membrane and the siRNA-MPPs are freely translocated into the cytoplasm, (ii) the disulfide bond is reduced in the cytoplasm, releasing the bioactive siRNA to cause sequence-specific mRNA degradation, and (iii) the uptake of the conjugate is rapid and occurs directly through the membrane without the need for classical receptor-mediated uptake or endo- or pinocytosis. While we have not characterized whether the siRNAs remain intact after delivery to the cell, it is possible that the two strands of the siRNA partially dissociate during delivery of the conjugate to the cytoplasm, although this would not necessarily affect the function of the siRNA, as single-strand antisense siRNAs are able to silence endogenous gene expression in cells [15].

The data obtained from the experiments involving transfection of COS-7 cells with MPP-siRNAs using Lipofectamine 2000 indicate that a lower percentage of luciferase transgene silencing had occurred when MPP-siRNAs were transfected into COS-7 cells compared to cells transfected with siRNAs, suggesting that the transfection of MPP-siRNAs was less efficient than with siRNAs. One explanation for this may be that during transfection with Lipofectamine, the delivery is affected by the overall charge of the MPP-siRNA molecule, which is more positive than the siRNA itself, because MPPs are positively charged. A negative overall charge is required for the efficient transfection of nucleic acids with cationic liposomes. On the other hand, the efficiency of silencing of luciferase activity in COS-7 cells following the incubation of cells with MPP-siRNAs present in the media was similar to that involving Lipofectamine 2000-mediated transfection of siRNAs, indicating that MPP-siRNAs were just as effective as Lipofectamine-transfected siRNAs in silencing luciferase in COS-7 cells. In CHO-AA8-Luc Tet-Off cells, however, the percentage of luciferase activity silenced on each of the 3 days following incubation of the cells with MPP-siRNAs was markedly superior to the silencing following transfection with Lipofectamine 2000, suggesting that MPP-siRNAs enter CHO-AA8 cells more efficiently than Lipofectamine-transfected siRNAs. Following treatment with MPP-siRNAs, or transfection with siRNAs, the expression of *GFP* in EOMA-GFP and C166-GFP mouse fibroblast cell lines was silenced

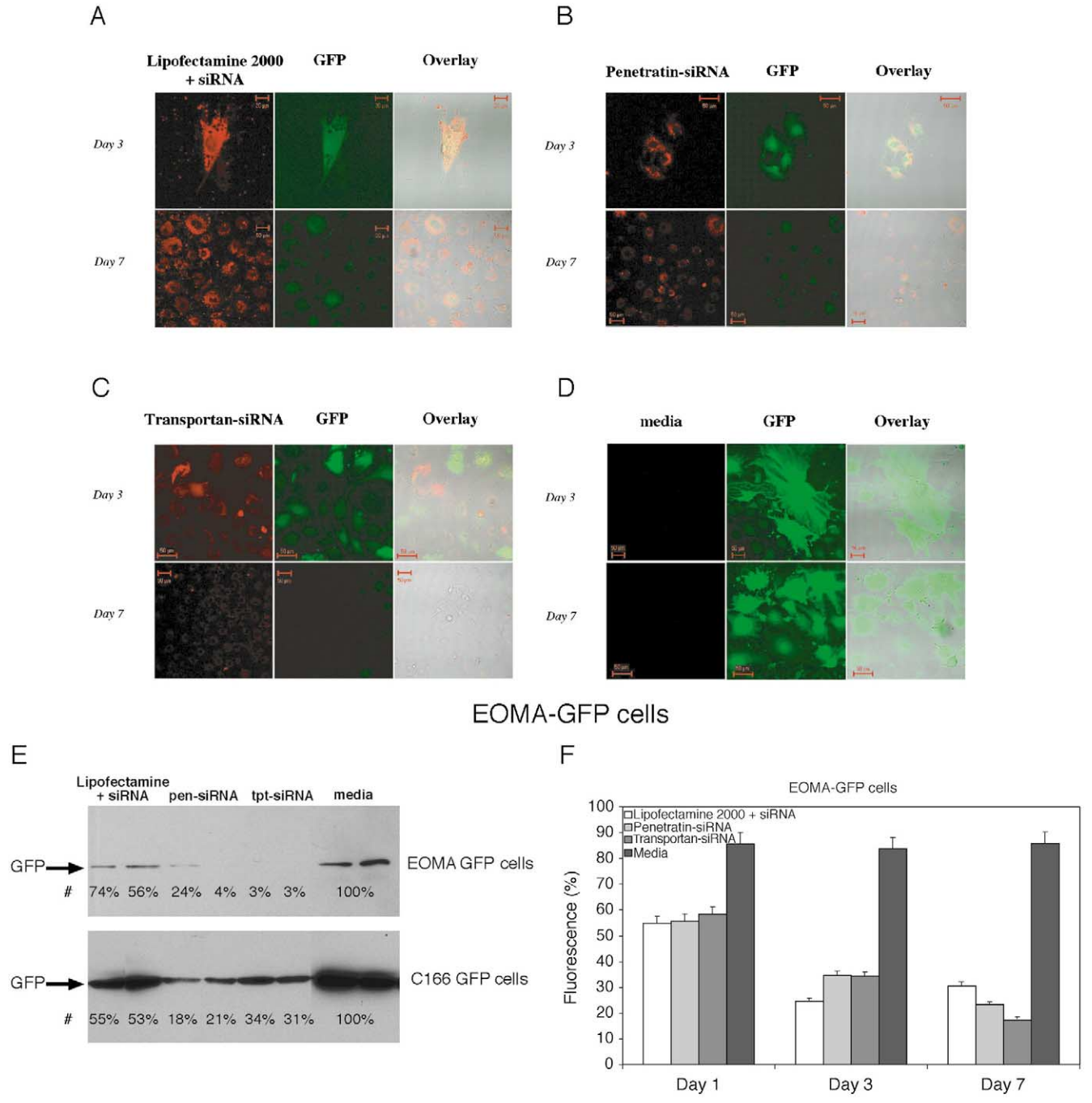

Fig. 4. Silencing of *GFP* in mouse fibroblast cell lines stably expressing GFP. C166-GFP and EOMA-GFP cell lines were treated with 25 nM *GFP* siRNAs delivered by liposomes or MPP. A–D: Double fluorescence staining of EOMA-GFP cells treated with siRNAs delivered using Lipofectamine (A), penetratin (B), transportan (C), or media only (D), as a control. siRNAs were labeled with the red fluorophore Cy3, and green fluorescence from GFP was also visualized using confocal fluorescence microscopy after 3 and 7 days. E: Western blot of C166-GFP and EOMA-GFP cells transfected with *GFP* siRNA duplex using Lipofectamine 2000, or treated with penetratin-siRNAs, transportan-siRNAs or with media only. Densitometric quantitation of the amount of GFP detected by Western blot for each lane as a percentage of the average intensity of the media control is shown for each cell line (#). RNAi of *GFP* was carried out for 7 days during which GFP protein expression was detected with a GFP-specific antibody. Only results from day 7 are shown. F: The fluorescence of EOMA-GFP cells treated with or without siRNAs was measured after day 1, day 3 and day 7 by flow cytometry.

with greater efficiency using MPP-siRNAs than transfection with siRNAs when examined by Western blot, which is a measure of the absolute level of GFP expression. In examining EOMA-GFP cells by fluorescence-activated cell sorter (FACS) to identify the percentage of cells receiving the MPP-siRNA, the numbers of cells exhibiting *GFP* silencing was similar to or slightly better than the number receiving Lipofectamine-transfected siRNAs, suggesting that in these cells both MPP-siRNA and Lipofectamine-delivered siRNA underwent similar transfection efficiencies.

In conclusion, constitutively expressed luciferase and GFP genes were successfully silenced in our experiments in a high proportion of cells of different types with the penetratin- or transportan-siRNA conjugates. Although there is an initial cost associated with synthesizing MPPs, the siRNAs bearing a thiol group were only marginally more expensive than standard siRNAs. Moreover, once MPPs had been generated the cost of producing MPP-siRNAs was minimal, and therefore MPPs could be a powerful and relatively inexpensive tool for effective delivery of siRNAs to multiple cell types, and may complement the use of transfection with cationic liposomes, especially where multiple manipulations of the cells are to be avoided such as in high-throughput strategies. Our observation that CHO-AA8 cells were more efficiently treated with MPP-siRNAs than with siRNAs transfected using Lipofectamine 2000 suggests that other cell types not efficiently targeted with standard transfection protocols may also be suitable for specific inhibition of gene expression using MPP-siRNAs. This may open the way to study gene function by modulating naturally occurring processes in mammalian cells, ultimately leading to the development of screens to analyze cellular functions, or to new gene-specific therapeutic agents for treating disease.

Acknowledgements: The authors thank Dr. Jacqueline Wilce for the assistance with the peptide synthesis and Oliver Rackham for the CHO-AA8-Luc Tet-Off cells. This work was funded by the Cancer Society of New Zealand, a Royal Society of New Zealand James Cook Fellowship (MRE), and MedTech Partners Inc.

References

[1] Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Nature 391, 806–811.
[2] Tuschl, T. (2001) Chembiochemistry 2, 239–245.
[3] Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R. and Hannon, G.J. (2001) Science 293, 1146–1150.
[4] Kumar, M. and Carmichael, G.G. (1998) Microbiol. Mol. Biol. Rev. 62, 1415–1434.
[5] Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Nature 411, 494–498.
[6] Bosher, J.M. and Labouesse, M. (2000) Nat. Cell Biol. 2, E31–E36.
[7] Awasthi, S. and Cox, R.A. (2003) BioTechniques 35, 602–604.
[8] Krotz, F., Sohn, H.Y., Gloe, T., Plank, C. and Pohl, U. (2003) J. Vasc. Res. 40, 425–434.
[9] Hallbrink, M., Floren, A., Elmquist, A., Pooga, M., Bartfai, T. and Langel, U. (2001) Biochim. Biophys. Acta 1515, 101–109.
[10] Josephson, L., Tung, C.H., Moore, A. and Weissleder, R. (1999) Bioconjug. Chem. 10, 186–191.
[11] Lindgren, M., Hallbrink, M., Prochiantz, A. and Langel, U. (2000) Trends Pharmacol. Sci. 21, 99–103.
[12] Schwarze, S.R. and Dowdy, S.F. (2000) Trends Pharmacol. Sci. 21, 45–48.
[13] Sole, N.A. and Baranay, G. (1992) J. Org. Chem. 57, 5399–5403.
[14] Muratovska, A., Zhou, C., He, S., Goodyer, P. and Eccles, M.R. (2003) Oncogene 22, 7989–7997.
[15] Holen, T., Amarzguioui, M., Babaie, E. and Prydz, H. (2003) Nucleic Acids Res. 31, 2401–2407.