

# Enhanced Intrapulmonary Delivery of Anticancer siRNA for Lung Cancer Therapy Using Cationic Ethylphosphocholine-based Nanolipoplexes

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Here, we report a cationic nanolipoplex as a pulmonary cellular delivery system for small-interfering RNA (siRNA). Six nanoliposomes differing in cationic lipids were formulated and screened *in vitro* and *in vivo* for cellular delivery functions in lung cells/tissues. Although the six nanoliposomes showed similar siRNA delivery efficiency *in vitro*, they exhibited significant differences in pulmonary cellular delivery functions *in vivo*. Among the various nanoliposomes, cationic dioleoyl-sn-glycero-3-ethylphosphocholine and cholesterol (ECL)-based nanoliposomes showed the highest pulmonary cellular delivery *in vivo* and the lowest cytotoxicity *in vitro*. The delivery efficiency of fluorescent siRNA in ECL nanoliposomes was 26.2-fold higher than that of naked siRNA *in vivo*. Treatment with Mcl1 (myeloid cell leukemia sequence 1)-specific siRNA (siMcl1) using ECL nanolipoplexes reduced target expression in B16F10 cell lines, whereas control, luciferase-specific siGL2 in ECL nanolipoplexes did not. In metastatic lung cancer mouse models induced by B16F10 or Lewis lung carcinoma (LLC) cells, intratracheal administration of siMcl1 in ECL nanolipoplexes significantly silenced Mcl1 mRNA and protein levels in lung tissue. Reduced formation of melanoma tumor nodules was observed in the lung. These results demonstrate the utility of ECL nanoliposomes for pulmonary delivery of therapeutic siRNA for the treatment of lung cancers and potentially for other respiratory diseases.

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## INTRODUCTION

Despite the initial hope of small-interfering RNA (siRNA) as a next-generation therapeutic modality, progression of siRNA therapeutics to clinical trials has been halting.<sup>1,2</sup> One of the biggest challenges for progress in the siRNA field is the strong dependence of siRNA efficacy on effective intracellular delivery systems.<sup>3–5</sup> Since siRNA is processed and binds to specific mRNAs in the cytoplasm, the intracellular delivery of siRNA is a prerequisite for

the silencing of target genes. However, the relatively large size and negative charges of siRNA makes it impossible for siRNA to diffuse through the cell membrane from extracellular spaces.

The lung has been an attractive target organ for siRNA-based therapy.<sup>6</sup> Intravenous administration of functionalized lipopolyamine was reported to provide knockdown of a target gene in the lung tissue of normal mice.<sup>7</sup> However, upon systemic administration, siRNA may be rapidly degraded and cleared from the bloodstream, leading to inefficient delivery to target cells.<sup>8</sup> Compared with systemic delivery, the direct localized administration of siRNA *via* the pulmonary route may allow higher retention of siRNA in lung tissues and reduce systemic toxicity.<sup>9</sup> Reflecting the several advantages of pulmonary delivery over systemic administration, two of the four siRNA drugs currently in Phase II clinical trials are delivered intranasally or by inhalation.<sup>8</sup>

Despite these advances, there remains a need for the development of effective nanocarriers of siRNA for direct pulmonary delivery. A recent study reported that delivery is a crucial barrier against the effective silencing of target genes by intratracheally administered naked siRNA.<sup>10</sup> Several nanocarriers have been studied for localized lung delivery of siRNA. For example, a poly (ester amine) polymer has been used for aerosolized siRNA delivery in mice,<sup>11</sup> and a fatty acid-modified polyethylenimine derivative was recently studied for intratracheal administration of siRNA in mice.<sup>12</sup>

In this study, we screened various cationic nanoliposomes for delivery efficiency *in vitro* and *in vivo* after intratracheal administration in mice. For cationic nanoliposomes, we tested 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-3-trimethylammonium propane (DOTMA), and 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EDOPC) as cationic lipid components. DOTAP and DOTMA have been used previously as cationic lipid components of cationic liposomes or nanoparticles for siRNA delivery. For example, DOTAP-modified cationic poly (DL-lactide-co-glycolide acid) nanoparticles have been used in an inhalable dry powder formulation of siRNA,<sup>13</sup> and DOTAP-based cationic liposomes have been reported as a system for delivering siRNA to lung cancer cells.<sup>14</sup> DOTMA-based cationic liposomes have been used to increase the cellular delivery

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**Table 1** Molar compositions and particle sizes of cationic liposomes ( $n = 4$ )

| Components of cationic liposomes | Molar ratio | Particle size (nm) |             |
|----------------------------------|-------------|--------------------|-------------|
|                                  |             | (-) siRNA          | (+) siRNA   |
| EDOPC:Chol:DOPE (ECL)            | 8:5:2       | 175.0 ± 1.9        | 188.1 ± 3.9 |
| DOTMA:Chol:DOPE (DMCL)           | 8:5:2       | 190.8 ± 2.0        | 204.1 ± 2.9 |
| DOTAP:Chol:DOPE (DTCL)           | 8:5:2       | 178.1 ± 1.2        | 193.0 ± 4.1 |
| EDOPC:DC-Chol:DOPE (EDL)         | 8:5:2       | 174.3 ± 3.7        | 187.7 ± 4.1 |
| DOTMA:DC-Chol:DOPE (DMDL)        | 8:5:2       | 191.7 ± 1.7        | 199.8 ± 4.0 |
| DOTAP:DC-Chol:DOPE (DTDL)        | 8:5:2       | 178.6 ± 3.8        | 203.0 ± 4.2 |

**Abbreviations:** Chol, cholesterol; DC-Chol, 3β-[N-(N',N'-dimethylamino ethane)-carbamoyl]cholesterol; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DOTMA, 1,2-di-O-octadecyl-3-trimethylammonium propane; EDOPC, dioleoyl-sn-glycero-3-ethylphosphocholine.

of siRNA to human airway epithelial cells, and mouse neuroblastoma cells *in vitro*.<sup>15</sup> Compared with DOTAP and DOTMA, EDOPC is a relatively new cationic lipid, and is less studied as a carrier of nucleic acid therapeutics. Although it has been studied for transfection of human umbilical vein endothelial cells with plasmid DNA,<sup>16</sup> and *in vivo* systemic delivery of plasmid DNA in mice,<sup>17</sup> there are still few reports on the application of EDOPC-based nanoparticles for the delivery of siRNA.

Moreover, given the high mortality of lung cancers worldwide,<sup>18</sup> we tested the efficacy of anticancer siRNA in metastasized lung cancer models. Here, we report that *in vitro* and *in vivo* pulmonary cellular delivery functions of various cationic nanoliposomes are uncorrelated. Using Mcl1 (myeloid cell leukemia sequence 1)-specific siRNA (siMcl1) as an anticancer siRNA, we demonstrate the potential of EDOPC-based cationic nanolipoplexes as an effective *in vivo* siRNA delivery system that shows target silencing and anticancer activity in mouse metastasized lung cancer models.

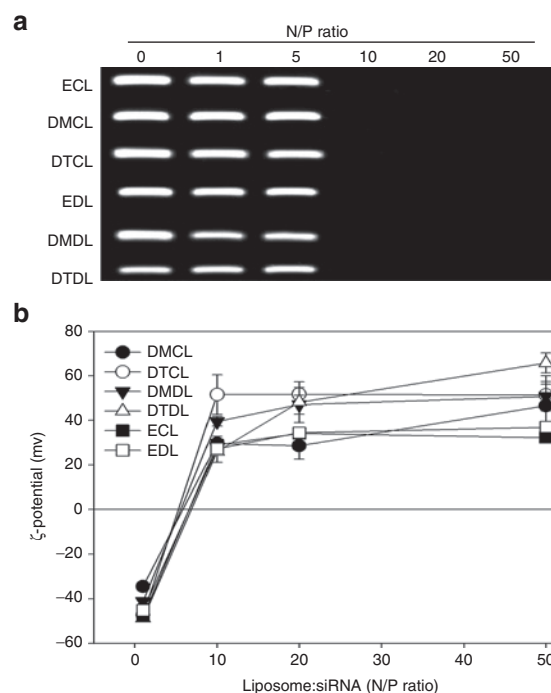
## RESULTS

### Characterization of cationic nanolipoplexes

The formation of nanolipoplexes between cationic nanoliposomes and siRNA was confirmed by gel retardation assays and size measurement. Regardless of nanoliposome compositions described in **Table 1**, retarded mobility of siRNA on gels was observed at N/P (nitrogen-to-phosphorus) ratios of 10:1 and above (**Figure 1a**). Upon complexation with siRNA, a slight increase in size was observed for all cationic nanoliposomes (**Table 1**). The extent of the mean size increase in nanolipoplexes relative to nanoliposomes was <25 nm. Regardless of cationic nanoliposome compositions, the zeta potential values of nanolipoplexes were >20 mV from the N/P ratio of 10:1 (**Figure 1b**). However, further increase of N/P ratios did not substantially enhance the zeta potential values as compared with the N/P ratio of 10:1.

### *In vitro* cellular uptake of fluorescent double-stranded RNA in cationic nanolipoplexes

The *in vitro* cellular uptake of fluorescent double-stranded RNA (dsRNA) did not significantly differ among various nanolipoplexes (**Figure 2a**). All nanoliposomes formulated in this study showed cellular uptake of fluorescent RNA similar to that of the commercial transfection agent, L2K. Fluorescence-activated cell

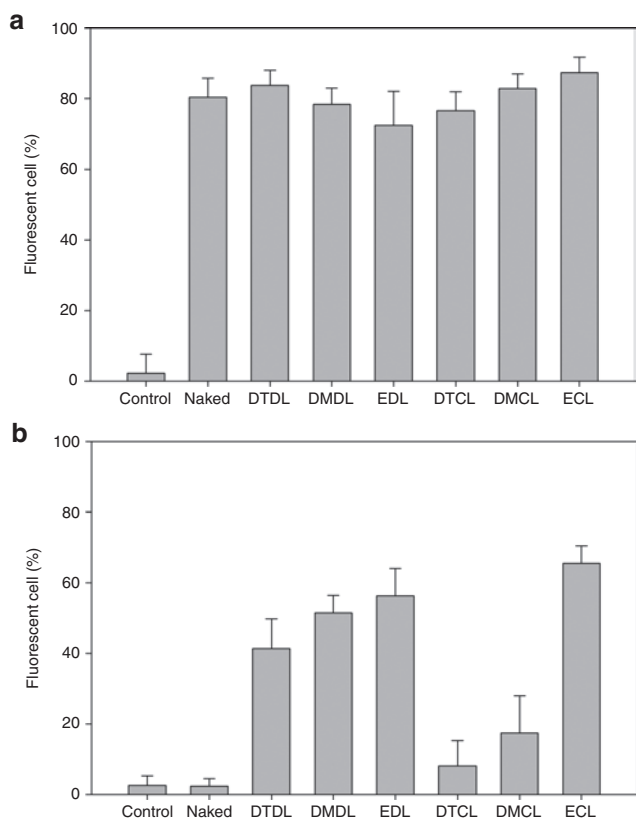


**Figure 1** Gel retardation behavior and zeta potentials of siRNA-containing nanolipoplexes. Cationic nanoliposomes were complexed with siRNA at different N/P ratio. **(a)** For gel retardation assays, siRNA in naked form or in various nanolipoplexes was electrophoresed on 1.5% (wt/vol) agarose gels. siRNA migration was visualized by ethidium bromide staining. **(b)** Zeta ( $\zeta$ ) potentials of siRNA complexed with cationic nanoliposomes at various N/P ratios are presented ( $n = 4$ ). N/P ratio, nitrogen-to-phosphorus ratio; siRNA, small-interfering RNA.

sorting analysis of B16F10 cells showed that the use of 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) in the nanoliposomal formulations (DTDL, DMDL, and EDL) did not significantly alter the cellular delivery functions of fluorescent dsRNA compared with nanoliposome formulations using Chol (DTCL, DMCL, and ECL). Moreover, the types of cationic lipids (*i.e.*, DOTAP in DTCL, DOTMA in DMCL, and EDOPC in ECL) did not significantly affect the *in vitro* cellular uptake of fluorescent dsRNA.

### *In vivo* pulmonary cellular uptake of fluorescent dsRNA in cationic nanolipoplexes

Unlike *in vitro* intracellular delivery, *in vivo* pulmonary cellular uptake of fluorescent dsRNA was significantly dependent on the lipid composition of nanolipoplexes (**Figure 2b**). Among DC-Chol-based nanoliposomes, EDL yielded a higher mean value of fluorescent-positive cell population compared with DTDL and DMDL. However, there was no significant difference among the DC-Chol-based cationic nanoliposomes in fluorescent dsRNA delivery functions *in vivo*. In contrast, Chol-based cationic nanoliposomes showed distinct differences in the *in vivo* pulmonary cellular delivery function of fluorescent dsRNA depending on the cationic lipid used in the nanolipoplexes. The use of EDOPC in ECL produced 8.0- and 3.2-fold higher levels of pulmonary cellular uptake of fluorescent dsRNA compared with DOTAP in DTCL and DOTMA in DMCL, respectively. Fluorescent dsRNA



**Figure 2** *In vitro* and *in vivo* cellular uptake of fluorescent dsRNA in nanolipplexes. Various cationic nanoliposomes were complexed with fluorescent marker-labeled dsRNA at an N/P ratio of 20:1. **(a)** B16F10 cells were treated with various nanolipplexes containing fluorescent dsRNA. After 24 hours, fluorescent dsRNA uptake was quantified by flow cytometry ( $n = 4$ ). Untreated cells were used as a control. **(b)** Fluorescent dsRNA, naked or in nanolipplexes, was administered to BALB/c mice by intratracheal injection. Lung tissues were extracted 4 hours post-dose, and the fluorescence of lung cells was measured by flow cytometry ( $n = 3$ ). dsRNA, double-stranded RNA; N/P ratio, nitrogen-to-phosphorus ratio.

complexed with ECL produced 26.2-fold higher *in vivo* delivery than did the naked form. Representative flow cytometry data are presented in [Figure 3a](#).

### ***In vivo* lung distribution of siRNA**

Although [Figures 2b](#) and [3a](#) show significantly higher pulmonary cellular uptake of fluorescent dsRNA in ECL nanolipplexes relative to the free form, imaging of lung tissue, including extracellular spaces, did not reveal significant differences ([Figure 3b](#)). Molecular imaging analyses of whole lungs showed a similar increase in fluorescence intensity in groups treated with fluorescent dsRNA in free or ECL nanolipplexes compared with the untreated control group ([Figure 3c](#)).

### **Cytotoxicity of various cationic nanolipplexes**

Although the *in vitro* cellular delivery of fluorescent dsRNA was not significantly different among cationic nanoliposome formulations, cytotoxicity was highly affected by the composition of cationic nanoliposomes ([Figure 4](#)). Cytotoxicity of luciferase-specific siGL2 complexed with various nanoliposomes was measured in B16F10 cells over 2 days. The cytotoxicity of siGL2/ECL

nanolipplexes was similar to that of siGL2/L2K complexes. After 2 days of treatment, the viability of cells treated with siGL2/ECL nanolipplexes was  $64.9 \pm 7.5\%$ , which is comparable to that of siGL2/L2K-treated cells ( $65.9 \pm 2.6\%$ ). Among the nanolipplexes prepared in this study, siGL2/ECL nanolipplexes had the least effect on cell viability, followed by siGL2/DTCL and siGL2/EDL nanolipplexes. Compared to cells treated with siGL2/DMCL nanolipplexes, those treated with siGL2/ECL nanolipplexes showed 3.2-fold higher viability after 48 hours of treatment.

### ***In vitro* reduction of target gene expression**

Since siGL2/ECL nanolipplexes exhibited the lowest cytotoxicity ([Figure 4](#)), we tested the *in vitro* target gene reduction capability of siRNA using ECL nanoliposomes. As a functional siRNA, Mcl-1-specific siMcl-1 was complexed to ECL nanoliposomes. For comparison, luciferase-specific siGL2 was complexed to ECL nanoliposomes. Gel electrophoresis of reverse transcription-PCR (RT-PCR) products ([Figure 5a](#)) revealed that Mcl-1 mRNA levels were reduced after treatment of cells with siMcl-1 in ECL nanolipplexes or L2K lipoplexes. However, no decrease in Mcl-1 mRNA was observed after treatment of B16F10 cells with siGL2 complexed to ECL nanoliposomes or L2K.

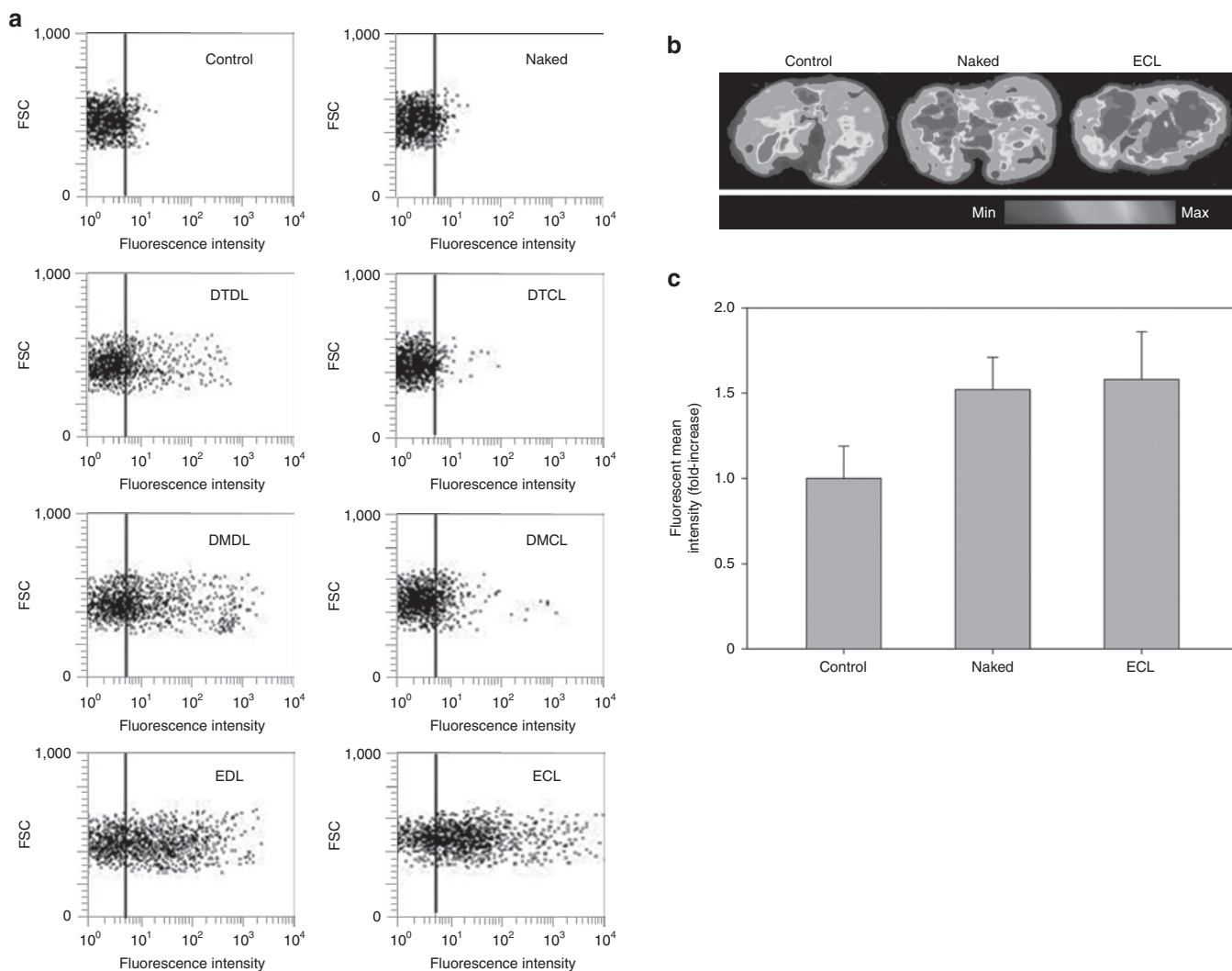
Real-time RT-PCR was used to quantify the extent of Mcl-1 mRNA downregulation after treatment with siMcl-1 in ECL nanolipplexes ([Figure 5b](#)). Mcl-1 mRNA expression levels following treatment of cells with nonfunctional siGL2 in L2K and ECL nanolipplexes were  $100.4 \pm 2.5$  and  $102.7 \pm 3.5\%$ , respectively. In contrast, following treatment of B16F10 cells with siMcl-1/ECL nanolipplexes, Mcl-1 mRNA expression levels were reduced to  $8.1 \pm 2.4\%$  of those observed in the group treated with siGL2/ECL nanolipplexes ( $P < 0.05$ ).

### ***In vivo* antitumor effects of siMcl-1 delivered in ECL nanolipplexes**

Intratracheal administration of siMcl-1 in ECL nanolipplexes inhibited the growth of B16F10 and Lewis lung carcinoma (LLC) cells in lung tissues. After intravenous injection of B16F10 or LLC on day 0, siRNA, naked or in ECL complexes, was intratracheally injected on days 5, 7, 9, and 11 ([Figure 6a](#)). Lung tissue was extracted on day 14 for evaluation of the metastasis and growth of intravenously administered B16F10 or LLC. Mice injected with B16F10, but not treated with any siRNA, were used as controls. Black colonies of metastasized B16F10 were observed in lung tissues of untreated groups ([Figure 6b](#)) and in groups treated with free siMcl-1 ([Figure 6c](#)), siGL2/ECL nanolipplexes ([Figure 6d](#)), or siMcl-1/ECL nanolipplexes ([Figure 6e](#)). However, the extent of blackish B16F10 tumor nodules was lowest in the group treated with siMcl-1/ECL nanolipplexes ([Figure 6e](#)).

### ***In vivo* silencing of target gene expression**

Silencing of Mcl-1 in lung tissues was observed at both mRNA and protein levels after delivery of siMcl-1 in ECL nanolipplexes ([Figure 7](#)). Quantitative real-time RT-PCR data ([Figure 7a](#)) revealed that relative Mcl-1 mRNA expression levels normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reduced to  $19.9 \pm 7.3$  and  $50.7 \pm 1.9\%$  with B16F10 and LLC-bearing lung tissues, respectively after intratracheal treatment



**Figure 3** *In vivo* lung retention and representative lung cellular uptake. Fluorescent dsRNA, naked or in nanolipoplexes, was administered to BALB/c mice by intratracheal injection. (a) Representative lung cell uptake patterns of fluorescent dsRNA in naked form or in various nanolipoplexes are presented. (b) Lung tissues were extracted 4 hours after intratracheal injection, and the retention of fluorescent dsRNA in the lung was visualized by molecular imaging. (c) The mean intensity of fluorescence in lung tissues was quantified and presented as fold-increase compared with untreated control ( $n = 4$ ). dsRNA, double-stranded RNA; FSC, forward scatter.

of mice with siMcl-1 in ECL nanolipoplexes compared with  $86.9 \pm 14.0$  and  $92.7 \pm 4.1$  % in mice treated with siGL2/ECL nanolipoplexes ( $P < 0.05$ ). Similar to mRNA expression levels, protein expression of Mcl-1 in lung tissues was reduced upon intratracheal administration of siMcl-1/ECL nanolipoplexes. In both lung cancer animal models induced by B16F10 (Figure 7b) or LLC (Figure 7c), western blot analyses showed notable silencing of Mcl-1 protein expression in the siMcl-1/ECL nanolipoplex-treated group, but not in other groups. There was no difference in  $\beta$ -actin protein levels among groups.

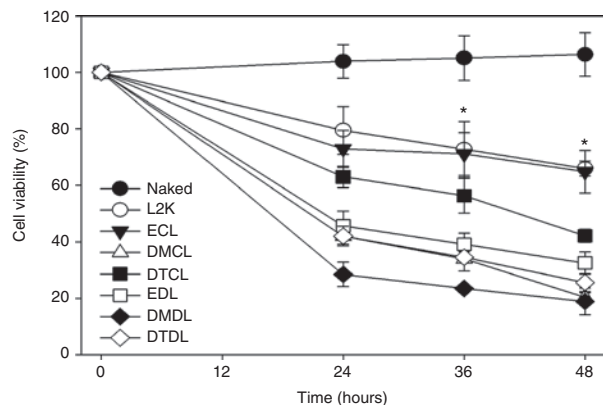
## DISCUSSION

In this study, we demonstrated that *in vitro* siRNA delivery efficiencies do not closely correspond with *in vivo* pulmonary siRNA delivery efficiencies. Moreover, we showed that the *in vivo* efficiency of siRNA delivery to pulmonary cells was significantly affected by the type of cationic lipids and co-lipids used, with ECL

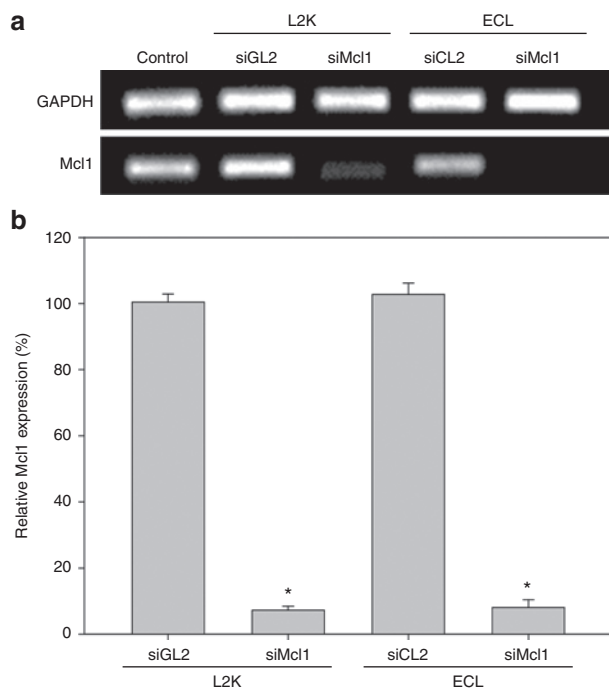
nanolipoplexes providing the highest *in vivo* siRNA delivery efficiency. Importantly, intratracheal administration of siMcl1/ECL nanolipoplexes silenced Mcl1 at mRNA and protein levels and inhibited the growth of metastasized lung cancers in mice.

In addition to cationic lipids, the cationic nanoliposomes described here were prepared using 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and DC-Chol or Chol as co-lipids. All nanoliposomes contained DOPE as a fusogenic lipid component, regardless of whether DC-Chol or Chol were used. Exploiting this fusogenic function, researchers have used DOPE as a helper lipid for liposomes based on DOTAP<sup>15</sup> and DOTMA.<sup>19</sup> DC-Chol, a derivative of Chol, differs structurally from Chol in that it contains a cationic moiety. A recent study reported the use of DC-Chol and DOPE-based cationic liposomes for the delivery of plasmid DNA and siRNA.<sup>20</sup>

A comparative analysis revealed a substantial discrepancy between *in vitro* and *in vivo* cellular uptake. Although fluorescent

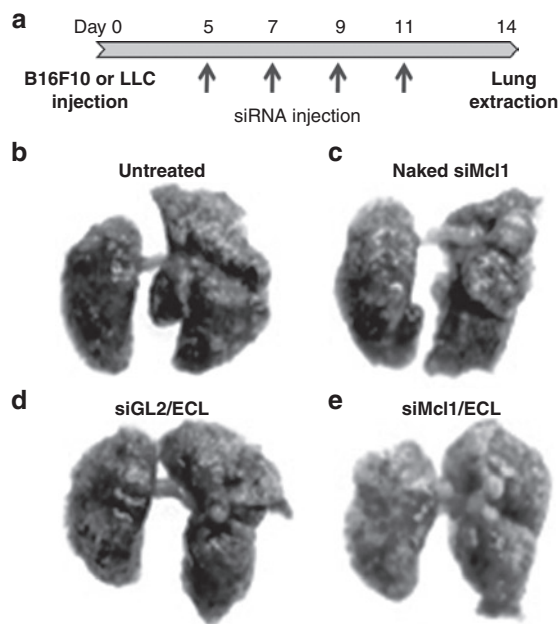


**Figure 4** *In vitro* cytotoxicity of nanolipoplexes in the lung cancer cell line, B16F10. B16F10 cells were treated with nanolipoplexes of siGL2 with L2K or various cationic nanoliposomes. After incubating for 24, 36, and 48 hours, cell viability was measured by MTT assay ( $n = 4$ ). \*Significantly higher ( $P < 0.05$ ) compared with DMCL-, DTCL-, EDL-, DMDL-, and DTDL-treated groups (ANOVA and Student–Newman–Keuls). ANOVA, analysis of variance; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide.



**Figure 5** *In vitro* reduction of target mRNA expression by ECL nanolipoplexes. B16F10 cells were treated with siGL2 or siMcl-1 complexed with L2K or ECL. (a) After 24 hours, target mRNA was amplified by RT-PCR, and products were electrophoresed on 1% agarose gels. (b) Target mRNA levels, normalized to those of GAPDH, were quantified by quantitative real-time RT-PCR. \*Significantly reduced ( $P < 0.05$ ) compared with control, siGL2-treated groups. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR.

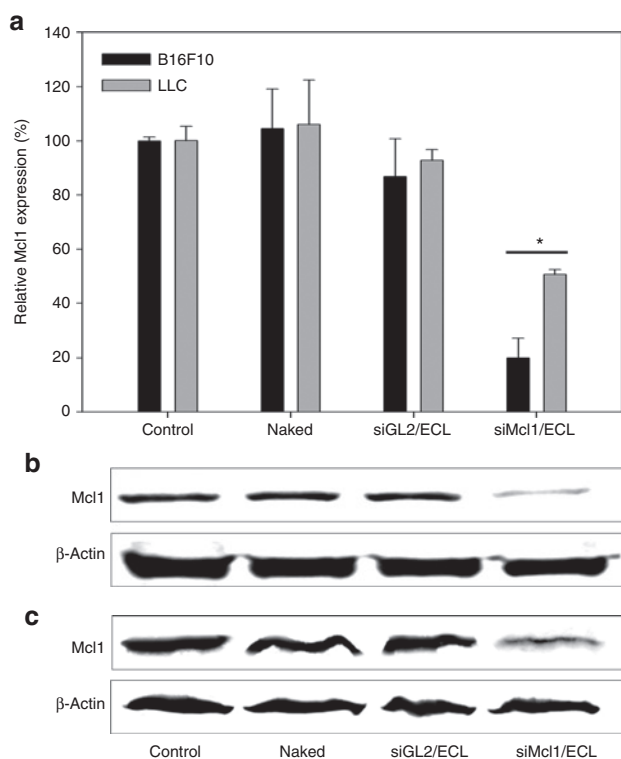
dsRNA delivery efficiency was similar for all nanoliposomes *in vitro*, pulmonary cell dsRNA delivery functions differed for different nanoliposomes *in vivo*. These results suggest a caveat in extrapolating from *in vitro* cellular uptake data in screens for *in vivo* siRNA delivery nanocarriers. The mechanisms underlying the higher *in vivo* siRNA delivery efficiency of ECL nanolipoplexes



**Figure 6** Anticancer effect of siMcl-1 delivered in ECL nanolipoplexes. siGL2 or siMcl-1 (0.21 mg/kg), naked or in ECL nanolipoplexes, was sprayed into the pulmonary area of B16F10 or LLC-metastasized BALB/c mice. (a) Intratracheal injections were administered every other day on four occasions beginning on day 5 after B16F10 inoculation. Three days after the final intratracheal injection with (c) naked siMcl1, (d) siGL2/ECL, or with (e) siMcl1/ECL nanolipoplexes, mice were killed and lung tissues were extracted. (b) For control group, mice were inoculated with B16F10, but not treated with siRNA. On day 14, mice were killed and lung tissues were extracted. LLC, Lewis lung carcinoma; siRNA, small-interfering RNA.

compared with other nanolipoplexes are not yet understood (Figure 2b). However, enhanced transfection of human umbilical vein endothelial cells has previously been observed with plasmid DNA in EDOPC-based.<sup>17</sup> Moreover, EDOPC is known to be serum compatible, enhancing the transfection efficacies of plasmid DNA regardless of the presence of serum.<sup>21,22</sup> Given these previous findings, it is possible that ECL might stabilize nanolipoplexes in the bloodstream due to its serum compatibility and confer increased binding to the surface of pulmonary endothelial cells, thereby facilitating the uptake of complexed siRNA into the cells.

In addition to the lack of correlation between *in vitro* and *in vivo* fluorescent dsRNA delivery efficiencies, there was a lack of correlation between pulmonary cellular delivery efficiency (Figure 3a) and whole lung tissue delivery, as determined by molecular imaging (Figure 3c). Fluorescent dsRNA has been used as a model for evaluating the cellular uptake of various siRNA nanocarriers.<sup>23</sup> Although, we observed more than a 20-fold increase in fluorescent dsRNA delivery in ECL nanolipoplexes compared with the naked form, there was no difference in fluorescence intensity in whole lung tissue images. The discrepancy between *in vivo* cellular level and whole tissue level data might reflect the presence of naked fluorescent dsRNA in the extracellular spaces of lung tissues, which yields a fluorescence signal on molecular imaging comparable to that of nanolipoplexed fluorescent dsRNA inside pulmonary cells. These results emphasize the importance of measuring cellular uptake levels of siRNA *in vivo* in evaluations of nanocarriers, in



**Figure 7** *In vivo* silencing of Mcl-1 by ECL nanolipplexes. siGL2 or siMcl-1 (0.21 mg/kg), naked or in ECL nanolipplexes, was sprayed into the pulmonary area of B16F10 or LLC-metastasized BALB/c mice. Intratracheal injections were administered every other day on four occasions beginning on day 5 after tumor inoculation. (a) On day 14, Mcl-1 mRNA and protein levels in lung tissues were analyzed by quantitative real-time RT-PCR and western blotting for lung cancer models induced by (b) B16F10 and (c) LLC, respectively ( $n = 4$ ). \*Significantly reduced ( $P < 0.05$ ) compared with other groups. LLC, Lewis lung carcinoma; RT-PCR, reverse transcription-PCR.

addition to molecular imaging of whole tissues or the entire body. The *in vivo* tumor distribution of polymeric micelles with or without folate ligand modification has also been investigated at tumor tissue and tumor cell levels.<sup>24</sup> In this previous study, the tumor tissue distribution of polymeric micelles was found to be similar regardless of folate ligand modification. Despite a similar distribution at the whole tissue level, polymeric micelles with folate ligand were taken up by the cells, whereas plain polymeric micelles were found in the extracellular spaces. This study provides additional support for the interpretation of the discrepancy between whole tissue distribution and target cell-level uptake, presented above.

ECL nanolipplexes not only showed the highest *in vivo* pulmonary cellular delivery of fluorescent dsRNA, they also exhibited the lowest cytotoxicity in B16F10 cells. In Figure 4, we aimed to compare the cytotoxicity of all cationic nanoliposomes *per se*, not of siRNA. We thus used nanolipplexes formed by nonfunctional luciferase GL2 siRNA, rather than cytotoxic siMcl-1.<sup>23</sup> The cytotoxicity of cationic nanocarriers has been reported to reflect interaction of the cationic component with the mitochondrial membrane, activation of the caspase 3 pathway, and induction of mitochondrially mediated apoptosis.<sup>25,26</sup> The dependence of cytotoxicity on the cationic lipids might be explained in part by the degradation kinetics of these lipids in endosomal environments and the toxicity of

their cellular degradation products. Unlike chemically synthesized cationic lipids such as DOTA and DOTAP, EDOPC is a derivative of the natural lipid phosphatidylcholine with a simple modification.<sup>27</sup> Because of structural similarity to natural phosphatidylcholine, EDOPC is considered to be more readily metabolizable in the body. It has been reported that EDOPC is readily hydrolyzed by phospholipase A2, and metabolized with a half-life of a few days in cells.<sup>21</sup> Moreover, the greater viability of cell treated with ECL relative to those treated with EDL (Figure 4) may be explained by the additional presence of the cationic lipid DC-Chol in EDL rather than the natural Chol present in ECL.

As an anticancer siRNA, siMcl1 was complexed to ECL nanoliposomes for the *in vivo* treatment of lung cancer. For the *in vivo* study, we compared the anticancer activity of siMcl1 in free or ECL nanolipplexes, not with L2K complexes. L2K is commercially supplied for *in vitro* transfection of nucleic acids for cell culture. For *in vivo* experiments, L2K needs to be complexed with siRNA sufficient for intratracheal administration dose (0.21 mg/kg). However, the L2K at its currently supplied concentration could not form nanolipplexes with siRNA at the concentrations for *in vivo* injection. Mcl1 has been reported to be an antiapoptotic protein involved in the proliferation and survival of lung cancer cells.<sup>28,29</sup> Moreover, overexpression of Mcl1 has been found in lung cancer tissues.<sup>30</sup> Thus, silencing of the Mcl1 protein by siMcl1 is predicted to promote the apoptosis of cancer cells. Consistent with this, siMcl1 has recently been reported to enhance the apoptosis of various solid tumor cells, including lung cancer cells.<sup>31</sup> In addition, intratumoral treatment with siMcl1 complexed to a tocopherol derivative of oligochitosan-based nanoparticles was shown to inhibit the growth of KB tumors xenografted in mice.<sup>23</sup> Finally, the co-delivery of siMcl1 with a histone deacetylase inhibitor using a cationic nanoliposome was demonstrated to provide synergistic anticancer activity after intravenous administration.<sup>32</sup>

Using lung metastasis after intravenous injection of B16F10 cells into mice as a lung cancer model, we found that intratracheal administration of siMcl1/ECL nanolipplexes reduced the formation of B16F10 tumor nodules in lung tissue. Previous studies have used this approach to establish an *in vivo* lung metastasis animal model.<sup>33,34</sup> This lung cancer model has significant advantages as an orthotopic disease model in that it may mimic the pathogenesis of metastatic lung cancer in humans. Intratracheal nebulization with a microsyringe was used for lung delivery of siMcl1,<sup>35</sup> an administration method that may more closely mimic a clinically suitable aerosol dosage form than the intranasal route.

Formation of tumor nodules was lowest in the siMcl1/ECL nanocomplex-treated group, consistent with the highest uptake of siMcl1 by pulmonary cells *in vivo* (Figure 2b) and silencing of the antiapoptotic Mcl1 in lung tissue at both mRNA and protein levels in this group. Previously, siRNA against SARS coronavirus was intratracheally administered to rhesus macaques.<sup>36</sup> In this latter study, siRNA was delivered in the naked form using 5% glucose in distilled water as a carrier solution. A recent report on the uptake and efficacy of naked siRNA *via* intratracheal administration in mice<sup>10</sup> suggested that delivery remains a key obstacle to the efficacy of topically administered, naked oligonucleotide in the lung, supporting the importance of effective delivery systems for localized delivery of siRNA.

Several possibilities may exist to explain the highest siRNA delivery efficiencies of ECL nanolipoplexes in the lung tissues. One possibility is the enhanced serum stability of ECL as compared with other cationic liposomes. EDOPC, a cationic derivative of dioleoylphosphatidylcholine, has a structure similar to natural membrane lipid component phosphatidylcholine.<sup>37</sup> EDOPC is known to have a phase transition temperature very similar to the natural phosphatidylcholine, and be quite insensitive to ionic strength.<sup>37</sup> The low sensitivity of EDOPC to ionic strength in biological environment may confer the stability of ECL *in vivo*, and contribute to the higher interaction with lung epithelial cells. Previously, the serum stability of EDOPC-based lipoplexes with plasmid DNA was reported. In the study, the incubation of EDOPC and plasmid DNA complexes in nearly pure serum did not reduce the transfection efficiencies.<sup>21</sup> These previous studies support that the high *in vivo* efficiencies of ECL might be contributed in part by the serum stability of EDOPC. Moreover, we cannot exclude the possibility that EDOPC may enhance the interaction with lung cells after intratracheal administration. The biodistribution study in mice following the intravenous administration of plasmid DNA with EDOPC-based nanoparticles revealed the discrete expression of plasmid DNA in lung tissues.<sup>17</sup>

Our results suggest the importance of cationic lipids and co-lipids for selection of *in vivo* siRNA delivery. The effects of cationic lipid structure on siRNA transfection efficiencies have been previously reported. The head groups of cationic lipids were shown to influence the *in vivo* siRNA delivery efficiencies. The small molecular dissimilarity in the ionizable cationic lipids resulted in significant differences in the gene-silencing potencies of siRNA in mouse.<sup>38</sup> The head groups affecting the pKa values of cationic lipids may modulate the extent of positive charges at physiological pH, and consequently influence the adsorption of plasma protein, and biodistribution behavior.<sup>39</sup> Moreover, the extent of unsaturation in hydrocarbon chains of cationic lipids may affect the siRNA delivery efficiencies. The high degree of unsaturated bonds in hydrocarbon chains of cationic lipid is known to facilitate siRNA delivery.<sup>40</sup> In addition to the cationic lipids, the co-lipids were reported to play an important role in the siRNA delivery efficiencies. Among various co-lipids, DOPE has been widely used as a co-lipid for cationic liposome-mediated transfection of siRNA and plasmid DNA.<sup>41</sup> The role of DOPE is not fully understood, but a recent study reported that DOPE might affect the structural transition of cationic liposomes at the acid pH of late endosomes in the cells.<sup>42</sup> Moreover, DOPE in cationic liposomes has been reported to induce the formation of hexagonal phase, facilitating the release of nucleic acids from the complexes in endosomes.<sup>43</sup> Although, we used DOPE as a co-lipid in this study, the inclusion of polyunsaturated fatty acids such as linoleic acid and linolenic acid in lipid nanoparticles were shown to further enhance the *in vivo* gene-silencing efficiencies.<sup>44</sup>

In this study, we specifically demonstrated the anticancer therapeutic effect of siMcl1 in ECL nanolipoplexes in a B16F10-metastasized lung tumor model. But the effective pulmonary cellular delivery efficiency of siRNA and the *in vivo* silencing of target protein in lung tissue suggest the utility of ECL nanoliposomes for inhalation dosage forms of other siRNA and nucleic acid therapeutics. It is anticipated that inhalable siRNA will be applied to various lung diseases, including cystic fibrosis, inflammatory conditions, infectious diseases, and cancers.<sup>6</sup> Because

ECL nanoliposomes provide a means for delivering pathogenic protein-specific siRNAs to lung cells in aerosol dosage forms, they offer an approach for targeting malignant proteins whose overexpression contributes to the pathology of various lung diseases.

## Conclusion

In conclusion, our results suggest the importance of *in vivo* screening in evaluating siRNA nanocarriers and *in vivo* cellular level uptake studies in differentiating between extracellular and intracellular delivery of nanocarriers. Moreover, ECL nanolipoplexes might be applied in future for intrapulmonary delivery of siRNA to treat various lung diseases caused by the overexpression of pathogenic proteins.

## MATERIALS AND METHODS

**Preparation of cationic nanoliposomes.** Cationic nanoliposomes were prepared using the lipid-film hydration method as previously described.<sup>32</sup> Chol was purchased from the Sigma-Aldrich (St Louis, MO), and the lipids EDOPC, DOTAP, DOTMA, DC-Chol, and DOPE were obtained from Avanti Polar Lipids (Birmingham, AL, USA). These lipids, dissolved in chloroform, were mixed at different compositions and evaporated using a rotary evaporator to eliminate the organic solvent. For nanoliposome preparation, EDOPC, Chol, and DOPE (15  $\mu$ mol total lipids) were mixed at a molar ratio of 8:5:2 (Table 1). In other nanoliposome formulations, DOTAP or DOTMA were used instead of EDOPC, and DC-Chol was used in place of Chol. Thin lipid films were hydrated with 1 ml of 20 mmol/l HEPES (pH 7.4), and the resulting nanoliposomes were extruded three times through 0.2  $\mu$ m polycarbonate membrane filters (Isopore; Millipore, Billerica, MA) using an Extruder (Northern Lipids, Vancouver, British Columbia, Canada). After complexation of various cationic nanoliposomes with siRNA, the sizes and zeta potentials of nanolipoplexes were determined using an ELS-8000 instrument (Photal, Osaka, Japan).

**Cell culture and siRNA uptake.** The murine melanoma cell line B16F10 and murine LLC cells were purchased from the American Type Culture Collection (Manassas, VA). Both cells were maintained in Dulbecco's modified Eagle's medium at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. All media were enriched with 10% fetal bovine serum (HyClone, Logan, UT) and 1% of each penicillin and streptomycin (Sigma-Aldrich). Cellular uptake of siRNA was determined in B16F10 cells seeded onto 24-well plates 1 day before treatment. After replacing medium with fresh (300  $\mu$ l/well), fluorescently labeled dsRNA (Block-iT; Invitrogen, Carlsbad, CA) at a concentration of 50 nmol/l was mixed with nanoliposomes at an N/P ratio of 20:1, or with Lipofectamine 2000 (L2K; Invitrogen), according to the manufacturer's instructions. The resulting nanolipoplexes were added to the cells and incubated at 37°C for 24 hours. Cells were harvested, washed three times with phosphate-buffered saline (PBS), and evaluated by flow cytometry using a BD FACSCalibur system and Cell Quest Pro analysis software (BD Biosciences, San Jose, CA).

**Cytotoxicity assay.** The cytotoxicity of cationic nanoliposomes was monitored using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. B16F10 cells were seeded onto 48-well plates at a density of  $1 \times 10^4$  cells/well and allowed to attach for 1 day, after which the medium was replaced with 200  $\mu$ l of fresh culture medium. Luciferase-specific GL2 siRNA (siGL2; ST Pharm., Seoul, Korea), which is nonfunctional in mammals, was complexed with various cationic nanoliposomes at an N/P ratio of 20:1, or with L2K according to the manufacturer's instruction. siGL2-containing nanolipoplexes were added to cells at an siGL2 concentration of 50 nmol/l. After incubation for various time periods, cells were treated with 20  $\mu$ l of a 5 mg/ml MTT solution for 2 hours. Untreated cells were used as a control. The culture medium was then removed, and 200  $\mu$ l of a 0.04 N HCl/isopropanol solution was added. The viability of

cells was measured at a wavelength of 570 nm using a microplate reader (Sunrise; TECAN, Männedorf, Switzerland). The viability of cells in each group was expressed relative to that of untreated cells as a percentage.

**RT-PCR and quantitative real-time RT-PCR.** Knockdown of mRNA by siRNA was evaluated by RT-PCR and quantitative real-time RT-PCR. For *in vitro* knockdown studies, siMcl1 or siGL2 (at a concentration of 50 nmol/l) was complexed with ECL nanoliposomes at an N/P ratio of 20:1, and the resulting nanolipoplexes were applied to cells seeded onto a 24-well plate. After 24 hours, total RNA was isolated using the TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using AccuPower RT PreMix (Bioneer, Daejeon, Korea). For *in vivo* knockdown studies, free siRNA or siRNA complexed in ECL nanolipoplexes was intratracheally administered (see below), and total RNA was isolated from the collected lung tissues and reverse transcribed into cDNA. The primers for murine Mcl1 were 5'-GCATGCTCCGGAAACTGGACATTA-3' (sense) and 5'-CTTTGTTT GACAAGCCAGTCCCGT-3' (antisense). RT-PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

Quantitative real-time RT-PCR was performed in 20 µl glass capillaries using a LightCycler 2.0 instrument with LightCycler FastStart, DNA Master PLUS SYBR Green I reagents; data were analyzed using the LightCycler software program (Roche Diagnostics, Mannheim, Germany). Thermocycling parameters consisted of a hot start at 95°C for 10 minutes followed by 45 cycles of 95°C for 10 seconds, 57°C for 20 seconds, and 72°C for 20 seconds. A melting curve analysis was performed to confirm the specificity of the PCR products after the amplification step. The level of Mcl1 mRNA expression was normalized to that of the housekeeping gene, GAPDH.

**Flow cytometry and molecular imaging.** *In vivo* uptake of siRNA by lung tissue was tested by flow cytometry and molecular imaging. Six weeks old female BALB/c mice were purchased from Daehan Biolink (Seungnam, Korea). All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Seoul National University. siRNA delivery efficiency in various nanoliposomes was monitored after administering nanoliposomes complexed with Block-iT siRNA at an N/P ratio of 20:1 via the intratracheal route. After 4 hours, mice were killed and lung tissue samples were collected and homogenized in 1 ml PBS using a 40 µm pore diameter Cell Strainer (SPL Life Sciences, Pocheon, Korea) followed by centrifugation at 700g for 5 minutes. Pellets were suspended and incubated at room temperature for 5 minutes in 1 ml of red blood cell lysis buffer (0.165 mol/l NH<sub>4</sub>Cl and 0.1266 mmol/l ethylenediaminetetraacetic acid) and centrifuged at 700g for 5 minutes. After washing three times with PBS, the suspended cells were analyzed using flow cytometry. For molecular imaging, extracted lung tissues were evaluated with a LAS 1000 image analyzer (FUJIFILM, Tokyo, Japan). Fluorescence intensity was analyzed using Image Gauge Analyzer Software (FUJIFILM).

**In vivo silencing by siRNA in nanolipoplexes.** The *in vivo* tumor model was prepared by injecting 6-week-old female BALB/c mice with 1 × 10<sup>6</sup> B16F10 or 2 × 10<sup>6</sup> murine lung carcinoma LLC cells in 200 µl PBS via the tail vein. The siRNA for specific silencing of Mcl1 (siMcl1) was purchased from Bioneer. Five days after inoculation, mice were given initial intratracheal injections of siMcl1 or siGL2 in nanocomplexes at a dose of 0.21 mg/kg using a MicroSprayer (Penn-century, Wyndmoor, PA). Mice were administered with nanolipoplexes four times every other day and killed on day 15. Lung tissue samples were collected and prepared as cell suspensions as described above. The *in vivo* silencing of target mRNA and protein by siMcl1 was analyzed using quantitative real-time RT-PCR (as described above) and western blot analysis, respectively.

**Western blot analysis.** *In vivo* silencing of target protein expression by siMcl1 was evaluated by western blotting as previously described.<sup>45</sup> RNA interference effects at the protein level were evaluated by first homogenizing

extracted lung tissues in cell lysis buffer (0.05% Triton X-100 and 2 mmol/l ethylenediaminetetraacetic acid in 0.1 mol/l Tris-HCl) followed by centrifugation at 13,000g for 15 minutes. Extracted total proteins were quantified using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instruction, and separated by SDS-PAGE on 10% polyacrylamide gels. After transferring proteins onto polyvinylidene difluoride membranes, western blotting was performed using specific antibodies to Mcl1 (1:1,000, ab32087; Abcam, Cambridge, UK) and β-actin (1:2,500, sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA). Bands were visualized using an alkaline phosphatase-conjugated anti-IgG antibody (Santa Cruz Biotechnology).

**Statistics.** Analysis of variance was used to analyze experimental data with application of a post hoc Student–Newman–Keuls test for pairwise comparisons. All statistical analyses were done using SigmaStat software (version 3.5, Systat Software, Richmond, CA); a *P* value < 0.05 was considered significant.

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## REFERENCES

- Chen, SH and Zhaori, G (2011). Potential clinical applications of siRNA technique: benefits and limitations. *Eur J Clin Invest* **41**: 221–232.
- Burnett, JC, Rossi, JJ and Tiemann, K (2011). Current progress of siRNA/shRNA therapeutics in clinical trials. *Biotechnol J* **6**: 1130–1146.
- Oh, YK and Park, TG (2009). siRNA delivery systems for cancer treatment. *Adv Drug Deliv Rev* **61**: 850–862.
- Wang, J, Lu, Z, Wientjes, MG and Au, JL (2010). Delivery of siRNA therapeutics: barriers and carriers. *AAPS J* **12**: 492–503.
- Shim, MS and Kwon, YJ (2010). Efficient and targeted delivery of siRNA *in vivo*. *FEBS J* **277**: 4814–4827.
- Durcan, N, Murphy, C and Cryan, SA (2008). Inhalable siRNA: potential as a therapeutic agent in the lungs. *Mol Pharm* **5**: 559–566.
- Polach, KJ, Matar, M, Rice, J, Slobodkin, G, Sparks, J, Congo, R *et al.* (2012). Delivery of siRNA to the mouse lung via a functionalized lipopolyamine. *Mol Ther* **20**: 91–100.
- Merkel, OM and Kissel, T (2012). Nonviral pulmonary delivery of siRNA. *Acc Chem Res* **45**: 961–970.
- Lam, JK, Liang, W and Chan, HK (2012). Pulmonary delivery of therapeutic siRNA. *Adv Drug Deliv Rev* **64**: 1–15.
- Moschos, SA, Frick, M, Taylor, B, Turmpenny, P, Graves, H, Spink, KG *et al.* (2011). Uptake, efficacy, and systemic distribution of naked, inhaled short interfering RNA (siRNA) and locked nucleic acid (LNA) antisense. *Mol Ther* **19**: 2163–2168.
- Xu, CX, Jere, D, Jin, H, Chang, SH, Chung, YS, Shin, JY *et al.* (2008). Poly(ester amine)-mediated, aerosol-delivered Akt1 small interfering RNA suppresses lung tumorigenesis. *Am J Respir Crit Care Med* **178**: 60–73.
- Beyerle, A, Braun, A, Merkel, O, Koch, F, Kissel, T and Stoeger, T (2011). Comparative *in vivo* study of poly(ethylene imine)/siRNA complexes for pulmonary delivery in mice. *J Control Release* **151**: 51–56.
- Jensen, DK, Jensen, LB, Koocheki, S, Bengtson, L, Cun, D, Nielsen, HM *et al.* (2012). Design of an inhalable dry powder formulation of DOTAP-modified PLGA nanoparticles loaded with siRNA. *J Control Release* **157**: 141–148.
- Taetz, S, Bochof, A, Surace, C, Arpicco, S, Renoir, JM, Schaefer, UF *et al.* (2009). Hyaluronic acid-modified DOTAP/DOPE liposomes for the targeted delivery of anti-telomerase siRNA to CD44-expressing lung cancer cells. *Oligonucleotides* **19**: 103–116.
- Tagalakis, AD, He, L, Saraiva, L, Gustafsson, KT and Hart, SL (2011). Receptor-targeted liposome-peptide nanocomplexes for siRNA delivery. *Biomaterials* **32**: 6302–6315.
- Matsumura, JS, Kim, R, Shively, VP, MacDonald, RC and Pearce, WH (1999). Characterization of vascular gene transfer using a novel cationic lipid. *J Surg Res* **85**: 339–345.
- Mahato, RI, Anwer, K, Tagliaferri, F, Meaney, C, Leonard, P, Wadhwa, MS *et al.* (1998). Biodistribution and gene expression of lipid/plasmid complexes after systemic administration. *Hum Gene Ther* **9**: 2083–2099.
- Mok, TS (2011). Personalized medicine in lung cancer: what we need to know. *Nat Rev Clin Oncol* **8**: 661–668.
- Writer, M, Hurley, CA, Sarkar, S, Copeman, DM, Wong, JB, Odlyha, M *et al.* (2006). Analysis and optimization of the cationic lipid component of a lipid/peptide vector formulation for enhanced transfection *in vitro* and *in vivo*. *J Liposome Res* **16**: 373–389.
- Zhang, Y, Li, H, Sun, J, Gao, J, Liu, W, Li, B *et al.* (2010). DC-Chol/DOPE cationic liposomes: a comparative study of the influence factors on plasmid pDNA and siRNA gene delivery. *Int J Pharm* **390**: 198–207.
- MacDonald, RC, Rakhmanova, VA, Choi, KL, Rosenzweig, HS and Lahiri, MK (1999). O-ethylphosphatidylcholine: A metabolizable cationic phospholipid which is a serum-compatible DNA transfection agent. *J Pharm Sci* **88**: 896–904.



22. Wang, L and MacDonald, RC (2007). Synergistic effect between components of mixtures of cationic amphipaths in transfection of primary endothelial cells. *Mol Pharm* **4**: 615–623.
23. Noh, SM, Han, SE, Shim, G, Lee, KE, Kim, CW, Han, SS *et al.* (2011). Tocopheryl oligochitosan-based self assembling oligomersomes for siRNA delivery. *Biomaterials* **32**: 849–857.
24. Bae, Y, Nishiyama, N and Kataoka, K (2007). *In vivo* antitumor activity of the folate-conjugated pH-sensitive polymeric micelle selectively releasing adriamycin in the intracellular acidic compartments. *Bioconjug Chem* **18**: 1131–1139.
25. Moghimi, SM, Symonds, P, Murray, JC, Hunter, AC, Debska, G and Szewczyk, A (2005). A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. *Mol Ther* **11**: 990–995.
26. Hunter, AC and Moghimi, SM (2010). Cationic carriers of genetic material and cell death: a mitochondrial tale. *Biochim Biophys Acta* **1797**: 1203–1209.
27. Wang, L and MacDonald, RC (2011). Cationic phospholiposomes: efficient delivery vehicles of anticancer derivatives of ATP to multiple myeloma cells. *J Liposome Res* **21**: 306–314.
28. Song, L, Coppola, D, Livingston, S, Cress, D and Haura, EB (2005). Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. *Cancer Biol Ther* **4**: 267–276.
29. Simonin, K, Brotin, E, Dufort, S, Dutoit, S, Goux, D, N'diaye, M *et al.* (2009). Mcl-1 is an important determinant of the apoptotic response to the BH3-mimetic molecule HA14-1 in cisplatin-resistant ovarian carcinoma cells. *Mol Cancer Ther* **8**: 3162–3170.
30. Luo, L, Zhang, T, Liu, H, Lv, T, Yuan, D, Yao, Y *et al.* (2012). MiR-101 and Mcl-1 in non-small-cell lung cancer: expression profile and clinical significance. *Med Oncol* **29**: 1681–1686.
31. Zhou, W, Hu, J, Tang, H, Wang, D, Huang, X, He, C *et al.* (2011). Small interfering RNA targeting mcl-1 enhances proteasome inhibitor-induced apoptosis in various solid malignant tumors. *BMC Cancer* **11**: 485.
32. Shim, G, Han, SE, Yu, YH, Lee, S, Lee, HY, Kim, K *et al.* (2011). Trilysinoyl oleylamide-based cationic liposomes for systemic co-delivery of siRNA and an anticancer drug. *J Control Release* **155**: 60–66.
33. Yonenaga, N, Kenjo, E, Asai, T, Tsuruta, A, Shimizu, K, Dewa, T *et al.* (2012). RGD-based active targeting of novel polycation liposomes bearing siRNA for cancer treatment. *J Control Release* **160**: 177–181.
34. Zhou, H, Roy, S, Cochran, E, Zouaoui, R, Chu, CL, Duffner, J *et al.* (2011). M402, a novel heparan sulfate mimetic, targets multiple pathways implicated in tumor progression and metastasis. *PLoS ONE* **6**: e21106.
35. Chandenier, J, Bernard, S, Montharu, J, Bailly, E, Fetissov, F, de Monte, M *et al.* (2009). The utility of a nebulised intra-tracheal rat model of invasive pulmonary aspergillosis. *Mycoses* **52**: 239–245.
36. Li, BJ, Tang, Q, Cheng, D, Qin, C, Xie, FY, Wei, Q *et al.* (2005). Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat Med* **11**: 944–951.
37. MacDonald, RC, Ashley, GW, Shida, MM, Rakhmanova, VA, Tarahovsky, YS, Pantazatos, DP *et al.* (1999). Physical and biological properties of cationic triesters of phosphatidylcholine. *Biophys J* **77**: 2612–2629.
38. Semple, SC, Akinc, A, Chen, J, Sandhu, AP, Mui, BL, Cho, CK *et al.* (2010). Rational design of cationic lipids for siRNA delivery. *Nat Biotechnol* **28**: 172–176.
39. Akinc, A, Zumbuehl, A, Goldberg, M, Leshchiner, ES, Busini, V, Hossain, N *et al.* (2008). A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat Biotechnol* **26**: 561–569.
40. Heyes, J, Palmer, L, Bremner, K and MacLachlan, I (2005). Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids. *J Control Release* **107**: 276–287.
41. Aissaoui, A, Chami, M, Hussein, M and Miller, AD (2011). Efficient topical delivery of plasmid DNA to lung *in vivo* mediated by putative triggered, PEGylated pDNA nanoparticles. *J Control Release* **154**: 275–284.
42. Mochizuki, S, Kanegae, N, Nishina, K, Kamikawa, Y, Koikawa, K, Masunaga, H *et al.* (2013). The role of the helper lipid dioleoylphosphatidylethanolamine (DOPE) for DNA transfection cooperating with a cationic lipid bearing ethylenediamine. *Biochim Biophys Acta* **1828**: 412–418.
43. Zuhorn, IS, Bakowsky, U, Polushkin, E, Visser, WH, Stuart, MC, Engberts, JB *et al.* (2005). Nonbilayer phase of lipoplex-membrane mixture determines endosomal escape of genetic cargo and transfection efficiency. *Mol Ther* **11**: 801–810.
44. Yu, B, Hsu, SH, Zhou, C, Wang, X, Terp, MC, Wu, Y *et al.* (2012). Lipid nanoparticles for hepatic delivery of small interfering RNA. *Biomaterials* **33**: 5924–5934.
45. Chang, RS, Suh, MS, Kim, S, Shim, G, Lee, S, Han, SS *et al.* (2011). Cationic drug-derived nanoparticles for multifunctional delivery of anticancer siRNA. *Biomaterials* **32**: 9785–9795.