3.2.2. microRNA/Anti-microRNA Delivery for Lung Cancer Therapy

Given the significant roles that miRNAs play in multiple pathways of lung carcinogenesis, increasing efforts are dedicated to the research and development of miRNA-based therapies, including restoring functions of tumor suppressive miRNAs or inhibiting oncogenic miRNAs. The development of miRNA-based therapies for lung cancer is growing prosperously with the help of new RNAi technologies. Compared to siRNA-based therapies, which are already in clinical trials, miRNAs are less toxic and have the potential to target multiple genes. The difficulty associated with miRNA delivery is mainly equal to that of siRNAs. The critical problems for the development of this therapy are effective delivery into target sites, potency of the therapy, and elimination of off-target effects [137].

There are two strategies as the therapeutic applications of miRNAs for lung cancer [138]. One strategy is miRNA replacement therapy, which involves the re-introduction of a tumor suppressor miRNA mimic to restore a loss of the function. MiRNA mimics are synthetic RNA duplexes designed to mimic the endogenous functions of miRNAs with chemical modifications for stability and cellular uptake. The concept of miRNA replacement therapy is most exemplified by the *let-7* miRNA. *let-7* is a tumor-suppressor miRNA in non-small-cell lung cancer that inversely correlates with the expression of the RAS oncoprotein, a key cancer gene [139]. Intranasal administration of *let-7* mimic into mouse models of lung cancer significantly reduced tumor growth, suggesting that miRNA replacement therapy is indeed promising [106,140,141]. Another miRNA that shows the value of miRNA replacement is provided by miR-34a [107,142]. Local and/or systemic delivery of a synthetic miR-34a mimic led to accumulation of miR-34a in the tumor tissue and inhibition of lung tumor growth. Lately, Ling *et al.* also showed that tumor suppressor miR-22 exhibited anti-lung cancer activity through post-transcriptional regulation of ErbB3 [143]. Thus, therapeutic miRNA mimics have a powerful potential by attacking multiple genes relevant to several diseases. However, it is necessary to pay attention to the potential toxicity in normal tissues under conditions in which the therapeutic delivery of miRNA mimics will lead to an accumulation of exogenous miRNAs in normal cells [138]. Although the assumptions are well founded, there is still insufficient evidence for toxicity caused by miRNA mimics. Indeed, several *in vivo* studies failed to reveal side effects caused by the miRNA mimics and suggested that delivery of miRNA mimics to normal tissues was well tolerated [107,141]. It will be important to research miRNA mimic-induced effects in normal cells and to carefully assess toxicity before using them in clinical practice.

The second strategy is directed toward a gain of function and aims to inhibit oncomiRs by using anti-miRNAs. Chemical modifications, such as 2'-*O*-methyl-group and locked nucleic acid (LNA), would increase oligo stability against nucleases [144]. Antisense oligonucleotides contained in these modifications are termed antagomirs or "LNA-antimiRs" [144,145]. They are oligonucleotides with sequences complementary to the endogenous miRNA and inhibit the specific miRNA function. An LNA-antimiR against miR-122 has been shown to effectively silence miR-122 in non-human primates [145], and the findings support the potential of these compounds as a new class of therapeutics. Moreover, it has also been reported that anti-miR-150 delivered into lung tumor xenografts in mice led to inhibited tumor growth [146]. Relative to studies on miRNA mimics, studies with antisense oligonucleotides have shown effective evidence with naked oligonucleotides. This illustrates the potential of chemical modifications of oligonucleotides to improve their stability, resistance to RNase, and pharmacologic

properties. Therefore, inhibition of miRNA function by chemically modified antimiR oligonucleotides has become an important and widely used approach. Recent data from the first phase II study in patients with chronic HCV infection treated with the LNA-modified antimiR-122 showed that this compound was well tolerated and provided continuing viral suppression.

An increasing number of studies have examined the therapeutic potential of miRNAs. Recently, the evidence of roles for miRNAs in determining drug resistance has emerged [147]. Cytotoxic and molecular target drugs have been widely used in the treatment of advanced lung cancer; unfortunately, many cases are still refractory to chemotherapy. In this situation, combining miRNA mimics or antimiR with chemotherapy may potentiate the efficacy of the cancer treatment in the future. In addition, miRNAs related with cancer stem cells may significantly broaden the field of miRNA-based therapy and suggest that miRNAs can be potential tools to kill cancer cells associated with therapy resistance, recurrence, and metastasis [108,148]. Hence, the main challenge is the successful delivery and chemical modifications of the therapeutic miRNAs to the target tissue without harming normal tissues.

4. Conclusions and Prospects

RNAi-based approaches provide a promising therapeutic modality for the treatment of various lung diseases. One of the greatest challenges in RNAi-based therapy continues to be the delivery method of the therapeutic siRNAs and miRNAs to the target cells. Pulmonary delivery applications are very attractive, since they tend to be non-invasive, are locally restricted, and can be administered by the patient. A realistic therapeutic intervention, such as aerosolization, can enhance drug delivery to the site of action and decrease systemic exposure of the patient to the therapy, thereby reducing off-target effects. The advancement of pulmonary siRNA delivery to the clinic illustrates that RNAi-based therapy holds a central place in the future treatment of lung diseases. On the other hand, miRNAs have the opportunity to target multiple genes in a fine-tuned manner, and the miRNA-based therapy will provide an attractive anti-tumor and anti-inflammatory approach for various lung diseases. In particular, anti-miRNA therapy by chemically modified antimiR oligonucleotides has become a potential therapy for lung diseases because the oligonucleotides can be successfully delivered without delivery vectors. Increased evidence has indicated that miRNAs fulfill causative roles in a variety of lung diseases and have prompted investigations into their potential as therapeutic targets. Further understanding of the detailed mechanisms of RNAi-based therapy and investigations of more effective delivery methods are required for future development. These novel approaches could open new avenues for various lung diseases and improve the clinical outcome of the patients.

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Conflict of Interest

The authors declare that there are not conflicts of interest to report.

References

1. Kim, D.H.; Rossi, J.J. Strategies for silencing human disease using RNA interference. *Nat. Rev. Genet.* **2007**, *8*, 173–184.
2. Bumcrot, D.; Manoharan, M.; Koteliansky, V.; Sah, D.W. RNAi therapeutics: A potential new class of pharmaceutical drugs. *Nat. Chem. Biol.* **2006**, *2*, 711–719.
3. Giladi, H.; Ketzinel-Gilad, M.; Rivkin, L.; Felig, Y.; Nussbaum, O.; Galun, E. Small interfering RNA inhibits hepatitis B virus replication in mice. *Mol. Ther.* **2003**, *8*, 769–776.
4. Xia, H.; Mao, Q.; Eliason, S.L.; Harper, S.Q.; Martins, I.H.; Orr, H.T.; Paulson, H.L.; Yang, L.; Kotin, R.M.; Davidson, B.L. RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nat. Med.* **2004**, *10*, 816–820.
5. Judge, A.D.; Robbins, M.; Tavakoli, I.; Levi, J.; Hu, L.; Fronda, A.; Ambegia, E.; McClintock, K.; MacLachlan, I. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J. Clin. Invest.* **2009**, *119*, 661–673.
6. Chernolovskaya, E.L.; Zenkova, M.A. Chemical modification of siRNA. *Curr Opin Mol. Ther.* **2010**, *12*, 158–167.
7. Schlee, M.; Hornung, V.; Hartmann, G. siRNA and isRNA: Two edges of one sword. *Mol. Ther.* **2006**, *14*, 463–470.
8. Li, S.D.; Huang, L. Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. *Mol. Pharm.* **2006**, *3*, 579–588.
9. Xu, C.X.; Jere, D.; Jin, H.; Chang, S.H.; Chung, Y.S.; Shin, J.Y.; Kim, J.E.; Park, S.J.; Lee, Y.H.; Chae, C.H.; *et al.* Poly(ester amine)-mediated, aerosol-delivered Akt1 small interfering RNA suppresses lung tumorigenesis. *Am. J. Respir. Crit. Care Med.* **2008**, *178*, 60–73.
10. Jere, D.; Xu, C.X.; Arote, R.; Yun, C.H.; Cho, M.H.; Cho, C.S. Poly(beta-amino ester) as a carrier for si/shRNA delivery in lung cancer cells. *Biomaterials* **2008**, *29*, 2535–2547.
11. Ren, X.L.; Xu, Y.M.; Bao, W.; Fu, H.J.; Wu, C.G.; Zhao, Y.; Li, Z.K.; Zhang, J.; Li, S.Q.; Chen, W.Q.; *et al.* Inhibition of non-small cell lung cancer cell proliferation and tumor growth by vector-based small interfering RNAs targeting HER2/neu. *Cancer Lett.* **2009**, *281*, 134–143.
12. Zamora-Avila, D.E.; Zapata-Benavides, P.; Franco-Molina, M.A.; Saavedra-Alonso, S.; Trejo-Avila, L.M.; Resendez-Perez, D.; Mendez-Vazquez, J.L.; Isaias-Badillo, J.; Rodriguez-Padilla, C. WT1 gene silencing by aerosol delivery of PEI-RNAi complexes inhibits B16-F10 lung metastases growth. *Cancer Gene Ther.* **2009**, *16*, 892–899.
13. Ge, Q.; Filip, L.; Bai, A.; Nguyen, T.; Eisen, H.N.; Chen, J. Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 8676–8681.
14. Li, B.J.; Tang, Q.; Cheng, D.; Qin, C.; Xie, F.Y.; Wei, Q.; Xu, J.; Liu, Y.; Zheng, B.J.; Woodle, M.C.; *et al.* Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat. Med.* **2005**, *11*, 944–951.

15. Rosas-Taraco, A.G.; Higgins, D.M.; Sanchez-Campillo, J.; Lee, E.J.; Orme, I.M.; Gonzalez-Juarrero, M. Intrapulmonary delivery of XCL1-targeting small interfering RNA in mice chronically infected with Mycobacterium tuberculosis. *Am. J. Respir. Cell Mol. Biol.* **2009**, *41*, 136–145.
16. Fulton, A.; Peters, S.T.; Perkins, G.A.; Jarosinski, K.W.; Damiani, A.; Brosnahan, M.; Buckles, E.L.; Osterrieder, N.; Van de Walle, G.R. Effective treatment of respiratory alphaherpesvirus infection using RNA interference. *PLoS One* **2009**, *4*, 5.
17. DeVincenzo, J.; Lambkin-Williams, R.; Wilkinson, T.; Cehelsky, J.; Nochur, S.; Walsh, E.; Meyers, R.; Gollob, J.; Vaishnaw, A. A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 8800–8805.
18. Lee, C.C.; Chiang, B.L. RNA interference: new therapeutics in allergic diseases. *Curr. Gene Ther.* **2008**, *8*, 236–246.
19. Seguin, R.M.; Ferrari, N. Emerging oligonucleotide therapies for asthma and chronic obstructive pulmonary disease. *Expert Opin. Investig. Drugs* **2009**, *18*, 1505–1517.
20. Senoo, T.; Hattori, N.; Tanimoto, T.; Furonaka, M.; Ishikawa, N.; Fujitaka, K.; Haruta, Y.; Murai, H.; Yokoyama, A.; Kohno, N. Suppression of plasminogen activator inhibitor-1 by RNA interference attenuates pulmonary fibrosis. *Thorax* **2010**, *65*, 334–340.
21. D'Alessandro-Gabazza, C.N.; Kobayashi, T.; Boveda-Ruiz, D.; Takagi, T.; Toda, M.; Gil-Bernabe, P.; Miyake, Y.; Yasukawa, A.; Matsuda, Y.; Suzuki, N.; *et al.* Development and preclinical efficacy of novel transforming growth factor-beta1 short interfering RNAs for pulmonary fibrosis. *Am. J. Respir. Cell. Mol. Biol.* **2012**, *46*, 397–406.
22. Lam, J.K.; Liang, W.; Chan, H.K. Pulmonary delivery of therapeutic siRNA. *Adv. Drug Deliv. Rev.*. **2012**, *64*, 1–15.
23. Agu, R.U.; Ugwoke, M.I.; Armand, M.; Kinget, R.; Verbeke, N. The lung as a route for systemic delivery of therapeutic proteins and peptides. *Respir. Res.* **2001**, *2*, 198–209.
24. Jensen, D.M.; Cun, D.; Maltesen, M.J.; Frokjaer, S.; Nielsen, H.M.; Foged, C. Spray drying of siRNA-containing PLGA nanoparticles intended for inhalation. *J. Control. Release* **2010**, *142*, 138–145.
25. Jensen, D.K.; Jensen, L.B.; Koocheki, S.; Bengtson, L.; Cun, D.; Nielsen, H.M.; Foged, C. Design of an inhalable dry powder formulation of DOTAP-modified PLGA nanoparticles loaded with siRNA. *J. Control. Release* **2012**, *157*, 141–148.
26. Perl, M.; Chung, C.S.; Lomas-Neira, J.; Rachel, T.M.; Biffl, W.L.; Cioffi, W.G.; Ayala, A. Silencing of Fas, but not caspase-8, in lung epithelial cells ameliorates pulmonary apoptosis, inflammation, and neutrophil influx after hemorrhagic shock and sepsis. *Am. J. Pathol.* **2005**, *167*, 1545–1559.
27. Lomas-Neira, J.L.; Chung, C.S.; Wesche, D.E.; Perl, M.; Ayala, A. In vivo gene silencing (with siRNA) of pulmonary expression of MIP-2 *versus* KC results in divergent effects on hemorrhage-induced, neutrophil-mediated septic acute lung injury. *J. Leukoc Biol* **2005**, *77*, 846–853.

28. Merkel, O.M.; Beyerle, A.; Librizzi, D.; Pfestroff, A.; Behr, T.M.; Sproat, B.; Barth, P.J.; Kissel, T. Nonviral siRNA delivery to the lung: investigation of PEG-PEI polyplexes and their *in vivo* performance. *Mol. Pharm.* **2009**, *6*, 1246–1260.
29. Garbuzenko, O.B.; Saad, M.; Betigeri, S.; Zhang, M.; Vetcher, A.A.; Soldatenkov, V.A.; Reimer, D.C.; Pozharov, V.P.; Minko, T. Intratracheal *versus* intravenous liposomal delivery of siRNA, antisense oligonucleotides and anticancer drug. *Pharm. Res.* **2009**, *26*, 382–394.
30. Wang, J.C.; Lai, S.; Guo, X.; Zhang, X.; de Crombrugghe, B.; Sonnylal, S.; Arnett, F.C.; Zhou, X. Attenuation of fibrosis *in vitro* and *in vivo* with SPARC siRNA. *Arthritis Res. Ther.* **2010**, doi: 10.1186/ar2973.
31. Gutbier, B.; Kube, S.M.; Reppe, K.; Santel, A.; Lange, C.; Kaufmann, J.; Suttorp, N.; Witzenrath, M. RNAi-mediated suppression of constitutive pulmonary gene expression by small interfering RNA in mice. *Pulm. Pharmacol. Ther.* **2010**, *23*, 334–344.
32. Driscoll, K.E.; Costa, D.L.; Hatch, G.; Henderson, R.; Oberdorster, G.; Salem, H.; Schlesinger, R.B. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. *Toxicol. Sci.* **2000**, *55*, 24–35.
33. Bivas-Benita, M.; Zwier, R.; Junginger, H.E.; Borchard, G. Non-invasive pulmonary aerosol delivery in mice by the endotracheal route. *Eur. J. Pharm. Biopharm.* **2005**, *61*, 214–218.
34. Lu, D.; Hickey, A.J. Pulmonary vaccine delivery. *Expert Rev. Vaccines* **2007**, *6*, 213–226.
35. Bitko, V.; Musiyenko, A.; Shulyayeva, O.; Barik, S. Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* **2005**, *11*, 50–55.
36. Massaro, D.; Massaro, G.D.; Clerch, L.B. Noninvasive delivery of small inhibitory RNA and other reagents to pulmonary alveoli in mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2004**, *287*, L1066–L1070.
37. Hickey, A.J.; Garcia-Contreras, L. Immunological and toxicological implications of short-term studies in animals of pharmaceutical aerosol delivery to the lungs: relevance to humans. *Crit. Rev. Ther. Drug Carrier. Syst.* **2001**, *18*, 387–431.
38. DeVincenzo, J.; Cehelsky, J.E.; Alvarez, R.; Elbashir, S.; Harborth, J.; Toudjarska, I.; Nechev, L.; Murugaiah, V.; Van Vliet, A.; Vaishnaw, A.K.; *et al.* Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). *Antiviral. Res.* **2008**, *77*, 225–231.
39. Mastrandrea, L.D.; Quattrin, T. Clinical evaluation of inhaled insulin. *Adv. Drug Deliv. Rev.* **2006**, *58*, 1061–1075.
40. Bai, S.; Gupta, V.; Ahsan, F. Inhalable lactose-based dry powder formulations of low molecular weight heparin. *J. Aerosol. Med. Pulm. Drug Deliv.* **2010**, *23*, 97–104.
41. Sanders, N.; Rudolph, C.; Braeckmans, K.; De Smedt, S.C.; Demeester, J. Extracellular barriers in respiratory gene therapy. *Adv. Drug Deliv. Rev.* **2009**, *61*, 115–127.
42. Roy, I.; Vij, N. Nanodelivery in airway diseases: Challenges and therapeutic applications. *Nanomedicine* **2010**, *6*, 237–244.
43. Groneberg, D.A.; Eynott, P.R.; Lim, S.; Oates, T.; Wu, R.; Carlstedt, I.; Roberts, P.; McCann, B.; Nicholson, A.G.; Harrison, B.D.; *et al.* Expression of respiratory mucins in fatal status asthmaticus and mild asthma. *Histopathology* **2002**, *40*, 367–373.

44. Jeffery, P.K. Remodeling in asthma and chronic obstructive lung disease. *Am. J. Respir. Crit. Care Med.* **2001,** *164*, S28–S38.
45. Lebhardt, T.; Roesler, S.; Beck-Broichsitter, M.; Kissel, T.; Polymeric nanocarriers for drug delivery to the lung. *J. Drug Deliv. Sci. Technol.* **2010**, *20*, 171–180.
46. Akhtar, S.; Benter, I.F. Nonviral delivery of synthetic siRNAs *in vivo*. *J. Clin. Invest.* **2007**, *117*, 3623–3632.
47. Goula, D.; Becker, N.; Lemkine, G.F.; Normandie, P.; Rodrigues, J.; Mantero, S.; Levi, G.; Demeneix, B.A. Rapid crossing of the pulmonary endothelial barrier by polyethylenimine/DNA complexes. *Gene Ther.* **2000**, *7*, 499–504.
48. Wang, J.; Lu, Z.; Wientjes, M.G.; Au, J.L. Delivery of siRNA therapeutics: Barriers and carriers. *AAPS J.* **2010**, *12*, 492–503.
49. Endoh, T.; Ohtsuki, T. Cellular siRNA delivery using cell-penetrating peptides modified for endosomal escape. *Adv. Drug Deliv. Rev.* **2009**, *61*, 704–709.
50. Novobrantseva, T.I.; Akinc, A.; Borodovsky, A.; de Fougerolles, A. Delivering silence: advancements in developing siRNA therapeutics. *Curr. Opin. Drug Discov. Devel.* **2008**, *11*, 217–224.
51. Shen, C.; Buck, A.K.; Liu, X.; Winkler, M.; Reske, S.N. Gene silencing by adenovirus-delivered siRNA. *FEBS Lett.* **2003**, *539*, 111–114.
52. Narvaiza, I.; Aparicio, O.; Vera, M.; Razquin, N.; Bortolanza, S.; Prieto, J.; Fortes, P. Effect of adenovirus-mediated RNA interference on endogenous microRNAs in a mouse model of multidrug resistance protein 2 gene silencing. *J. Virol.* **2006**, *80*, 12236–12247.
53. Guo, X.; Wang, W.; Hu, J.; Feng, K.; Pan, Y.; Zhang, L.; Feng, Y. Lentivirus-mediated RNAi knockdown of NUPR1 inhibits human nonsmall cell lung cancer growth *in vitro* and *in vivo*. *Anat. Rec.* **2012**, *295*, 2114–2121.
54. Scanlon, K.J. Cancer gene therapy: Challenges and opportunities. *Anticancer Res.* **2004**, *24*, 501–504.
55. Teichler Zallen, D. US gene therapy in crisis. *Trends Genet.* **2000**, *16*, 272–275.
56. Musiyenko, A.; Bitko, V.; Barik, S. RNAi-dependent and -independent antiviral phenotypes of chromosomally integrated shRNA clones: role of VASP in respiratory syncytial virus growth. *J. Mol. Med.* **2007**, *85*, 745–752.
57. Thomas, M.; Lu, J.J.; Chen, J.; Klibanov, A.M. Non-viral siRNA delivery to the lung. *Adv. Drug Deliv. Rev.* **2007**, *59*, 124–133.
58. Tseng, Y.C.; Mozumdar, S.; Huang, L. Lipid-based systemic delivery of siRNA. *Adv. Drug Deliv. Rev.* **2009**, *61*, 721–731.
59. Tompkins, S.M.; Lo, C.Y.; Tumpey, T.M.; Epstein, S.L. Protection against lethal influenza virus challenge by RNA interference *in vivo*. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 8682–8686.
60. Han, S.W.; Roman, J. Fibronectin induces cell proliferation and inhibits apoptosis in human bronchial epithelial cells: pro-oncogenic effects mediated by PI3-kinase and NF-kappa B. *Oncogene* **2006**, *25*, 4341–4349.
61. Thomas, M.; Lu, J.J.; Ge, Q.; Zhang, C.; Chen, J.; Klibanov, A.M. Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 5679–5684.

62. Sioud, M.; Sorensen, D.R. Cationic liposome-mediated delivery of siRNAs in adult mice. *Biochem. Biophys. Res. Commun.* **2003**, *312*, 1220–1225.
63. Wu, S.Y.; McMillan, N.A. Lipidic systems for *in vivo* siRNA delivery. *AAPS J.* **2009**, *11*, 639–652.
64. Jackson, A.L.; Bartz, S.R.; Schelter, J.; Kobayashi, S.V.; Burchard, J.; Mao, M.; Li, B.; Cavet, G.; Linsley, P.S. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **2003**, *21*, 635–637.
65. Bridge, A.J.; Pebernard, S.; Ducraux, A.; Nicoulaz, A.L.; Iggo, R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.* **2003**, *34*, 263–264.
66. Heidel, J.D.; Hu, S.; Liu, X.F.; Triche, T.J.; Davis, M.E. Lack of interferon response in animals to naked siRNAs. *Nat. Biotechnol.* **2004**, *22*, 1579–1582.
67. Urban-Klein, B.; Werth, S.; Abuharbeid, S.; Czubayko, F.; Aigner, A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA *in vivo*. *Gene Ther.* **2005**, *12*, 461–466.
68. Pal, A.; Ahmad, A.; Khan, S.; Sakabe, I.; Zhang, C.; Kasid, U.N.; Ahmad, I. Systemic delivery of RafsiRNA using cationic cardiolipin liposomes silences RAF-1 expression and inhibits tumor growth in xenograft model of human prostate cancer. *Int. J. Oncol.* **2005**, *26*, 1087–1091.
69. Nielsen, E.J.; Nielsen, J.M.; Becker, D.; Karlas, A.; Prakash, H.; Glud, S.Z.; Merrison, J.; Besenbacher, F.; Meyer, T.F.; Kjems, J.; Howard, K.A. Pulmonary gene silencing in transgenic EGFP mice using aerosolised chitosan/siRNA nanoparticles. *Pharm. Res.* **2010**, *27*, 2520–2527.
70. Akinc, A.; Zumbuehl, A.; Goldberg, M.; Leshchiner, E.S.; Busini, V.; Hossain, N.; Bacallado, S.A.; Nguyen, D.N.; Fuller, J.; Alvarez, R.; *et al.* A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat. Biotechnol.* **2008**, *26*, 561–569.
71. Cosio, M.G.; Saetta, M.; Agusti, A. Immunologic aspects of chronic obstructive pulmonary disease. *New Engl. J. Med.* **2009**, *360*, 2445–2454.
72. Xu, L.; Anchordoquy, T. Drug delivery trends in clinical trials and translational medicine: challenges and opportunities in the delivery of nucleic acid-based therapeutics. *J. Pharm. Sci.* **2011**, *100*, 38–52.
73. Shen, J.; Samul, R.; Silva, R.L.; Akiyama, H.; Liu, H.; Saishin, Y.; Hackett, S.F.; Zinnen, S.; Kossen, K.; Fosnaugh, K.; *et al.* Suppression of ocular neovascularization with siRNA targeting VEGF receptor 1. *Gene Ther.* **2006**, *13*, 225–234.
74. The US National Institutes of Health. ClinicalTrials.gov. Available on line: http://www.clinicaltrials.gov/ (accessed on 30 November 2012).
75. Falsey, A.R.; Hennessey, P.A.; Formica, M.A.; Cox, C.; Walsh, E.E. Respiratory syncytial virus infection in elderly and high-risk adults. *New Engl. J. Med.* **2005**, *352*, 1749–1759.
76. Zamora, M.R.; Budev, M.; Rolfe, M.; Gottlieb, J.; Humar, A.; Devincenzo, J.; Vaishnaw, A.; Cehelsky, J.; Albert, G.; Nochur, S.; *et al.* RNA interference therapy in lung transplant patients infected with respiratory syncytial virus. *Am. J. Respir. Crit. Care Med.* **2011**, *183*, 531–538.
77. Dong, A.Q.; Kong, M.J.; Ma, Z.Y.; Qian, J.F.; Xu, X.H. Down-regulation of IGF-IR using small, interfering, hairpin RNA (siRNA) inhibits growth of human lung cancer cell line A549 *in vitro* and in nude mice. *Cell Biol. Int.* **2007**, *31*, 500–507.

78. Xia, H.; Yu, C.H.; Zhang, Y.; Yu, J.; Li, J.; Zhang, W.; Zhang, B.; Li, Y.; Guo, N. EZH2 silencing with RNAi enhances irradiation-induced inhibition of human lung cancer growth *in vitro* and *in vivo*. *Oncol. Lett.* **2012**, *4*, 135–140.
79. Aleku, M.; Schulz, P.; Keil, O.; Santel, A.; Schaeper, U.; Dieckhoff, B.; Janke, O.; Endruschat, J.; Durieux, B.; Roder, N.; *et al.* Atu027, a liposomal small interfering RNA formulation targeting protein kinase N3, inhibits cancer progression. *Cancer Res.* **2008**, *68*, 9788–9798.
80. Leenders, F.; Mopert, K.; Schmiedeknecht, A.; Santel, A.; Czauderna, F.; Aleku, M.; Penschuck, S.; Dames, S.; Sternberger, M.; Rohl, T.; *et al.* PKN3 is required for malignant prostate cell growth downstream of activated PI 3-kinase. *EMBO J.* **2004**, *23*, 3303–3313.
81. Katso, R.; Okkenhaug, K.; Ahmadi, K.; White, S.; Timms, J.; Waterfield, M.D. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu. Rev. Cell Dev. Biol.* **2001**, *17*, 615–675.
82. Hao, S.; Baltimore, D. The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules. *Nat. Immunol.* **2009**, *10*, 281–288.
83. Falk, J.A.; Minai, O.A.; Mosenifar, Z. Inhaled and systemic corticosteroids in chronic obstructive pulmonary disease. *Proc. Am. Thorac. Soc.* **2008**, *5*, 506–512.
84. Tsuji, T.; Aoshiba, K.; Nagai, A. Cigarette smoke induces senescence in alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **2004**, *31*, 643–649.
85. Tuder, R.M.; Kern, J.A.; Miller, Y.E. Senescence in chronic obstructive pulmonary disease. *Proc. Am. Thorac. Soc.* **2012**, *9*, 62–63.
86. Molfino, N.A. Genetics of COPD. *Chest* **2004**, *125*, 1929–1940.
87. Chen, Z.H.; Kim, H.P.; Ryter, S.W.; Choi, A.M. Identifying targets for COPD treatment through gene expression analyses. *Int. J. Chron. Obstruct. Pulmon. Dis.* **2008**, *3*, 359–370.
88. Edwards, M.R.; Bartlett, N.W.; Clarke, D.; Birrell, M.; Belvisi, M.; Johnston, S.L. Targeting the NF-kappaB pathway in asthma and chronic obstructive pulmonary disease. *Pharmacol. Ther.* **2009**, *121*, 1–13.
89. Shapiro, S.D.; Goldstein, N.M.; Houghton, A.M.; Kobayashi, D.K.; Kelley, D.; Belaaouaj, A. Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. *Am. J. Pathol.* **2003**, *163*, 2329–2335.
90. Yoshida, T.; Tuder, R.M. Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiol. Rev.* **2007**, *87*, 1047–1082.
91. Huang, S.L.; Su, C.H.; Chang, S.C. Tumor necrosis factor-alpha gene polymorphism in chronic bronchitis. *Am. J. Respir. Crit. Care Med.* **1997**, *156*, 1436–1439.
92. Stevenson, C.S.; Coote, K.; Webster, R.; Johnston, H.; Atherton, H.C.; Nicholls, A.; Giddings, J.; Sugar, R.; Jackson, A.; Press, N.J.; *et al.* Characterization of cigarette smoke-induced inflammatory and mucus hypersecretory changes in rat lung and the role of CXCR2 ligands in mediating this effect. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2005**, *288*, 29.
93. Stevenson, C.S.; Docx, C.; Webster, R.; Battram, C.; Hynx, D.; Giddings, J.; Cooper, P.R.; Chakravarty, P.; Rahman, I.; Marwick, J.A.; *et al.* Comprehensive gene expression profiling of rat lung reveals distinct acute and chronic responses to cigarette smoke inhalation. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2007**, *293*, L1183–L1193.

94. Aoshiba, K.; Yokohori, N.; Nagai, A. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am. J. Respir. Cell Mol. Biol.* **2003**, *28*, 555–562.
95. Kasahara, Y.; Tuder, R.M.; Taraseviciene-Stewart, L.; Le Cras, T.D.; Abman, S.; Hirth, P.K.; Waltenberger, J.; Voelkel, N.F. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J. Clin. Invest.* **2000**, *106*, 1311–1319.
96. Barnes, P.J.; Stockley, R.A. COPD: current therapeutic interventions and future approaches. *Eur. Respir. J.* **2005**, *25*, 1084–1106.
97. Banerjee, A.; Koziol-White, C.; Panettieri, R., Jr. p38 MAPK inhibitors, IKK2 inhibitors, and TNFalpha inhibitors in COPD. *Curr. Opin. Pharmacol.* **2012**, *12*, 287–292.
98. World Health Organization. Chronic respiratory disease—Asthma. Available online: http://www.who.int/respiratory/asthma/en/ (accessed on 30 November 2012).
99. Huang, H.Y.; Chiang, B.L. siRNA as a therapy for asthma. *Curr. Opin. Mol. Ther.* **2009**, *11*, 652–663.
100. Meinicke, H.; Darcan, Y.; Hamelmann, E. Targeting allergic airway diseases by siRNA: An option for the future? *Curr. Mol. Med.* **2009**, *9*, 483–494.
101. Popescu, F.D. Antisense- and RNA interference-based therapeutic strategies in allergy. *J. Cell Mol. Med.* **2005**, *9*, 840–853.
102. Stenton, G.R.; Kim, M.K.; Nohara, O.; Chen, C.F.; Hirji, N.; Wills, F.L.; Gilchrist, M.; Hwang, P.H.; Park, J.G.; Finlay, W.; *et al.* Aerosolized Syk antisense suppresses Syk expression, mediator release from macrophages, and pulmonary inflammation. *J. Immunol.* **2000**, *164*, 3790–3797.
103. Ulanova, M.; Puttagunta, L.; Marcet-Palacios, M.; Duszyk, M.; Steinhoff, U.; Duta, F.; Kim, M.K.; Indik, Z.K.; Schreiber, A.D.; Befus, A.D. Syk tyrosine kinase participates in beta1-integrin signaling and inflammatory responses in airway epithelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2005**, *288*, 19.
104. Winter, J.; Jung, S.; Keller, S.; Gregory, R.I.; Diederichs, S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat. Cell. Biol* **2009**, *11*, 228–234.
105. Pagdin, T.; Lavender, P. MicroRNAs in lung diseases. *Thorax* **2012**, *67*, 183–184.
106. Trang, P.; Medina, P.P.; Wiggins, J.F.; Ruffino, L.; Kelnar, K.; Omotola, M.; Homer, R.; Brown, D.; Bader, A.G.; Weidhaas, J.B.; *et al.* Regression of murine lung tumors by the let-7 microRNA. *Oncogene* **2010**, *29*, 1580–1587.
107. Wiggins, J.F.; Ruffino, L.; Kelnar, K.; Omotola, M.; Patrawala, L.; Brown, D.; Bader, A.G. Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. *Cancer Res.* **2010**, *70*, 5923–5930.
108. Liu, C.; Kelnar, K.; Liu, B.; Chen, X.; Calhoun-Davis, T.; Li, H.; Patrawala, L.; Yan, H.; Jeter, C.; Honorio, S.; *et al.* The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nat. Med.* **2011**, *17*, 211–215.
109. Van Rooij, E.; Sutherland, L.B.; Thatcher, J.E.; DiMaio, J.M.; Naseem, R.H.; Marshall, W.S.; Hill, J.A.; Olson, E.N. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13027–13032.
110. Lanford, R.E.; Hildebrandt-Eriksen, E.S.; Petri, A.; Persson, R.; Lindow, M.; Munk, M.E.; Kauppinen, S.; Orum, H. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* **2010**, *327*, 198–201.

111. van Rooij, E.; Sutherland, L.B.; Qi, X.; Richardson, J.A.; Hill, J.; Olson, E.N. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **2007**, *316*, 575–579.
112. Porrello, E.R.; Johnson, B.A.; Aurora, A.B.; Simpson, E.; Nam, Y.J.; Matkovich, S.J.; Dorn, G.W., II.; van Rooij, E.; Olson, E.N. MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes. *Circ. Res.* **2011**, *109*, 670–679.
113. Zhu, H.; Yang, Y.; Wang, Y.; Li, J.; Schiller, P.W.; Peng, T. MicroRNA-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating Sirt1. *Cardiovasc. Res.* **2011**, *92*, 75–84.
114. Williams, A.H.; Valdez, G.; Moresi, V.; Qi, X.; McAnally, J.; Elliott, J.L.; Bassel-Duby, R.; Sanes, J.R.; Olson, E.N. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science* **2009**, *326*, 1549–1554.
115. Patrick, D.M.; Zhang, C.C.; Tao, Y.; Yao, H.; Qi, X.; Schwartz, R.J.; Jun-Shen Huang, L.; Olson, E.N. Defective erythroid differentiation in miR-451 mutant mice mediated by 14–3-3zeta. *Genes Dev.* **2010**, *24*, 1614–1619.
116. Lewis, A.; Riddoch-Contreras, J.; Natanek, S.A.; Donaldson, A.; Man, W.D.; Moxham, J.; Hopkinson, N.S.; Polkey, M.I.; Kemp, P.R. Downregulation of the serum response factor/miR-1 axis in the quadriceps of patients with COPD. *Thorax* **2012**, *67*, 26–34.
117. Pottelberge, G.R.; Mestdagh, P.; Bracke, K.R.; Thas, O.; Durme, Y.M.; Joos, G.F.; Vandesompele, J.; Brusselle, G.G. MicroRNA expression in induced sputum of smokers and patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **2011**, *183*, 898–906.
118. Liu, G.; Friggeri, A.; Yang, Y.; Milosevic, J.; Ding, Q.; Thannickal, V.J.; Kaminski, N.; Abraham, E. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *J. Exp. Med.* **2010**, *207*, 1589–1597.
119. Pandit, K.V.; Corcoran, D.; Yousef, H.; Yarlagadda, M.; Tzouvelekis, A.; Gibson, K.F.; Konishi, K.; Yousem, S.A.; Singh, M.; Handley, D.; *et al.* Inhibition and role of let-7d in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **2010**, *182*, 220–229.
120. Cushing, L.; Kuang, P.P.; Qian, J.; Shao, F.; Wu, J.; Little, F.; Thannickal, V.J.; Cardoso, W.V.; Lu, J. miR-29 is a major regulator of genes associated with pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* **2011**, *45*, 287–294.
121. Yang, S.; Banerjee, S.; de Freitas, A.; Sanders, Y.Y.; Ding, Q.; Matalon, S.; Thannickal, V.J.; Abraham, E.; Liu, G. Participation of miR-200 in pulmonary fibrosis. *Am. J. Pathol.* **2012**, *180*, 484–493.
122. Rodriguez, A.; Vigorito, E.; Clare, S.; Warren, M.V.; Couttet, P.; Soond, D.R.; van Dongen, S.; Grocock, R.J.; Das, P.P.; Miska, E.A.; *et al.* Requirement of bic/microRNA-155 for normal immune function. *Science* **2007**, *316*, 608–611.
123. Lu, T.X.; Munitz, A.; Rothenberg, M.E. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. *J. Immunol.* **2009**, *182*, 4994–5002.
124. Mattes, J.; Collison, A.; Plank, M.; Phipps, S.; Foster, P.S. Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 18704–18709.

125. Chiba, Y.; Tanabe, M.; Goto, K.; Sakai, H.; Misawa, M. Down-regulation of miR-133a contributes to up-regulation of Rhoa in bronchial smooth muscle cells. *Am. J. Respir. Crit. Care Med.* **2009**, *180*, 713–719.
126. Ezzie, M.E.; Crawford, M.; Cho, J.H.; Orellana, R.; Zhang, S.; Gelinas, R.; Batte, K.; Yu, L.; Nuovo, G.; Galas, D.; *et al.* Gene expression networks in COPD: microRNA and mRNA regulation. *Thorax* **2012**, *67*, 122–131.
127. Sato, T.; Liu, X.; Nelson, A.; Nakanishi, M.; Kanaji, N.; Wang, X.; Kim, M.; Li, Y.; Sun, J.; Michalski, J.; *et al.* Reduced miR-146a increases prostaglandin E(2)in chronic obstructive pulmonary disease fibroblasts. *Am. J. Respir. Crit. Care Med.* **2010**, *182*, 1020–1029.
128. Pottier, N.; Maurin, T.; Chevalier, B.; Puissegur, M.P.; Lebrigand, K.; Robbe-Sermesant, K.; Bertero, T.; Lino Cardenas, C.L.; Courcot, E.; Rios, G.; *et al.* Identification of keratinocyte growth factor as a target of microRNA-155 in lung fibroblasts: Implication in epithelial-mesenchymal interactions. *PLoS One* **2009**, doi:10.1371/journal.pone.0006718.
129. Izzotti, A.; Calin, G.A.; Arrigo, P.; Steele, V.E.; Croce, C.M.; de Flora, S. Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. *FASEB J.* **2009**, *23*, 806–812.
130. Schembri, F.; Sridhar, S.; Perdomo, C.; Gustafson, A.M.; Zhang, X.; Ergun, A.; Lu, J.; Liu, G.; Zhang, X.; Bowers, J.; *et al.* MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 2319–2324.
131. Decramer, M.; Rennard, S.; Troosters, T.; Mapel, D.W.; Giardino, N.; Mannino, D.; Wouters, E.; Sethi, S.; Cooper, C.B. COPD as a lung disease with systemic consequences--clinical impact, mechanisms, and potential for early intervention. *COPD* **2008**, *5*, 235–256.
132. Gower, A.C.; Steiling, K.; Brothers, J.F., II.; Lenburg, M.E.; Spira, A. Transcriptomic studies of the airway field of injury associated with smoking-related lung disease. *Proc. Am. Thorac. Soc.* **2011**, *8*, 173–179.
133. Agusti, A.G. COPD, a multicomponent disease: implications for management. *Respir. Med.* **2005**, *99*, 670–682.
134. Locksley, R.M. Asthma and allergic inflammation. *Cell* **2010**, *140*, 777–783.
135. Williams, A.E.; Larner-Svensson, H.; Perry, M.M.; Campbell, G.A.; Herrick, S.E.; Adcock, I.M.; Erjefalt, J.S.; Chung, K.F.; Lindsay, M.A. MicroRNA expression profiling in mild asthmatic human airways and effect of corticosteroid therapy. *PLoS One* **2009**, doi:10.1371/journal.pone.0005889.
136. Oglesby, I.K.; McElvaney, N.G.; Greene, C.M. MicroRNAs in inflammatory lung disease--master regulators or target practice? *Respir. Res.* **2010**, *11*, 148.
137. Boudreau, R.L.; Martins, I.; Davidson, B.L. Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs *in vitro* and *in vivo*. *Mol. Ther.* **2009**, *17*, 169–175.
138. Bader, A.G.; Brown, D.; Winkler, M. The promise of microRNA replacement therapy. *Cancer Res.* **2010**, *70*, 7027–7030.
139. Johnson, S.M.; Grosshans, H.; Shingara, J.; Byrom, M.; Jarvis, R.; Cheng, A.; Labourier, E.; Reinert, K.L.; Brown, D.; Slack, F.J. RAS is regulated by the let-7 microRNA family. *Cell* **2005**, *120*, 635–647.

140. He, X.Y.; Chen, J.X.; Zhang, Z.; Li, C.L.; Peng, Q.L.; Peng, H.M. The let-7a microRNA protects from growth of lung carcinoma by suppression of k-Ras and c-Myc in nude mice. *J. Cancer Res. Clin. Oncol.* **2010**, *136*, 1023–1028.
141. Esquela-Kerscher, A.; Trang, P.; Wiggins, J.F.; Patrawala, L.; Cheng, A.; Ford, L.; Weidhaas, J.B.; Brown, D.; Bader, A.G.; Slack, F.J. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* **2008**, *7*, 759–764.
142. Chen, Y.; Zhu, X.; Zhang, X.; Liu, B.; Huang, L. Nanoparticles modified with tumor-targeting scFv deliver siRNA and miRNA for cancer therapy. *Mol. Ther.* **2010**, *18*, 1650–1656.
143. Ling, B.; Wang, G.X.; Long, G.; Qiu, J.H.; Hu, Z.L. Tumor suppressor miR-22 suppresses lung cancer cell progression through post-transcriptional regulation of ErbB3. *J. Cancer Res. Clin. Oncol.* **2012**, *138*, 1355–1361.
144. Krutzfeldt, J.; Rajewsky, N.; Braich, R.; Rajeev, K.G.; Tuschl, T.; Manoharan, M.; Stoffel, M. Silencing of microRNAs *in vivo* with "antagomirs". *Nature* **2005**, *438*, 685–689.
145. Elmen, J.; Lindow, M.; Schutz, S.; Lawrence, M.; Petri, A.; Obad, S.; Lindholm, M.; Hedtjarn, M.; Hansen, H.F.; Berger, U.; *et al.* LNA-mediated microRNA silencing in non-human primates. *Nature* **2008**, *452*, 896–899.
146. Li, Y.J.; Zhang, Y.X.; Wang, P.Y.; Chi, Y.L.; Zhang, C.; Ma, Y.; Lv, C.J.; Xie, S.Y. Regression of A549 lung cancer tumors by anti-miR-150 vector. *Oncol. Rep.* **2012**, *27*, 129–134.
147. Zheng, T.; Wang, J.; Chen, X.; Liu, L. Role of microRNA in anticancer drug resistance. *Int. J. Cancer* **2010**, *126*, 2–10.
148. Ji, Q.; Hao, X.; Zhang, M.; Tang, W.; Yang, M.; Li, L.; Xiang, D.; Desano, J.T.; Bommer, G.T.; Fan, D.; *et al.* MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. *PLoS One* **2009**, *4*, doi:10.1371/journal.pone.0006816.

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Intraperitoneal delivery of a small interfering RNA targeting *NEDD1* prolongs the survival of scirrhous gastric cancer model mice

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The prognosis of patients with advanced diffuse-type gastric cancer (GC), especially scirrhous gastric cancer (SGC) remains extremely poor. Peritoneal carcinomatosis is a frequent form of metastasis of SGC. With survival rates of patients with peritoneal metastasis at 3 and 5 years being only 9.8% and 0%, respectively, development of a new treatment is urgently crucial. For such development, the establishment of a therapeutic mouse model is required. Among the 11 GC cell lines we examined, HSC-60 showed the most well-preserved expression profiles of the Hedgehog and epithelial-mesenchymal transition pathways found in primary SGCs. After six cycles of harvest of ascitic tumor cells and their orthotopic inoculation in scid mice, a highly metastatic subclone of HSC-60, 60As6 was obtained, by means of which we successfully developed peritoneal metastasis model mice. The mice treated with small interfering (si) RNA targeting *NEDD1*, which encodes a gamma-tubulin ring complex-binding protein, by the atelocollagen-mediated delivery system showed a significantly prolonged survival. Our mouse model could thus be useful for the development of a new therapeutic modality. Intraperitoneal administration of siRNAs of targeted genes such as *NEDD1* could provide a new opportunity in the treatment of the peritoneal metastasis of SGC. (*Cancer Sci* 2013; 104: 214–222)

Gastric cancer (GC) is one of the leading causes of cancer-related death worldwide.[1,2] Histopathological research has long suggested that gastric cancer is not a single disease and recognizes two major categories: intestinal and diffuse.[3] Intestinal-type GC develops through some sequential stages including *Helicobacter pylori* (*H. pylori*)-associated gastritis, intestinal metaplasia (IM), and dysplasia. This type predominates in high-risk geographic areas, such as East Asia, showing a correlation with the prevalence there of *H. pylori* infection among elderly people. Diffuse-type GC, however, is more uniformly distributed geographically, is apparently unrelated to *H. pylori* prevalence and typically develops from *H. pylori*-free, morphologically normal gastric mucosa without atrophic gastritis, or IM. Unlike the decreasing incidence of the intestinal-type, the prevalence of the diffuse-type is reportedly increasing worldwide.[4] Although therapeutic results for GC have recently improved, the prognosis for patients with advanced diffuse-type GC, especially scirrhous gastric cancer (SGC, Borrmann's type IV carcinoma or the linitis plastic type) remains extremely poor. The 5-year overall survival rate of SGC is approximately 10%, and ranges from 18% to 29% even after curative surgery.[5–7] Histopathologically, SGC does not form glands; instead, it causes diffuse infiltration of a broad region of the gastric wall rather than a well-defined mass, resulting in a fibrous-like thickening of the wall. Such pathological features make an early clinical diagnosis of SGC difficult, and in approximately half of the cases, by the time the diagnosis is made, peritoneal dissemination has, unfortunately, already occurred.[5,8] Peritoneal dissemination, known to be a frequent form of metastasis and recurrence of SGC, serves as a major factor determining patient prognosis.[9] Currently, no effective therapy exists for this condition. For SGC patients with peritoneal metastasis, the survival rates at 3 and 5 years are only 9.8% and 0%, respectively, even if the patients received multidisciplinary treatment.[5]

It has been suggested that peritoneal dissemination is a consequence of free cancer cells that are shed from the serosa of the primary lesion and/or may leak out from the lymphatics to the peritoneal cavity; however, no detailed mechanism of peritoneal dissemination has been fully elucidated. In either situation, it is assumed that free cancer cells detached from a primary lesion must have a predilection for the peritoneum. Efficacious control of invisible free cancer cells in the peritoneal cavity should help suppress the progression of carcinomatous peritonitis, and could ultimately yield a survival benefit. Some investigators have reported good, but limited, outcomes with new treatment strategies for peritoneal dissemination, including systemic chemotherapy,[10] intraperitoneal (i.p.) chemotherapy and/or hyperthermia,[11] and peritonectomy.[12] Therefore, to improve patient outcome, the development of a new therapeutic strategy for peritoneal dissemination of SGC is urgently crucial.

In this study, we developed peritoneal metastasis model mice of SGC and an atelocollagen-mediated delivery system for i.p. administration of small interfering (si) RNA, and also reported that the i.p. delivery of an siRNA targeting *NEDD1*, which functions in the metaphase regulation of the cell cycle, was able to regress the tumor and prolong, without toxicity, the survival of the mice.

Materials and Methods

Tissue samples. Gastric cancer and non-cancerous tissues were provided by the National Cancer Center Hospital (Tokyo, Japan) after obtaining informed consent from each patient and approval by the Center's Ethics Committee. Tissue specimens were immediately frozen with liquid nitrogen after surgical extraction, and stored at −80°C until use.

Cell lines and culture. A human scirrhous gastric cancer cell line, HSC-60 was established by a collaborator using the

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procedure as described.[13] A highly peritoneal-seeding cell line, 60As6 was established from HSC-60 using orthotopic tissue implantation into scid mice as briefly follows: the xenografted tumor of HSC-60 cells was transplanted into the gastric wall of a scid mouse. We repeated six cycles of harvest of ascitic tumor cells and the orthotopic inoculation of these cells, in turn, into the animals to establish a highly metastatic 60As6 cell line. These two cell lines were maintained in an RPMI1640 medium supplemented with 10% FCS. In this study, we also used luciferase- or green fluorescence protein (GFP)-expressing transfectants. Another 11 GC-derived cell lines (HSC-39, HSC-43, HSC-44, HSC-58, HSC-59, HSC-60, KATOIII, MKN7, MKN28, MKN74, and HSC-57) were also maintained in the same way. Of them, seven HSC cell lines were established by a collaborator using the procedure as described,[13] and four other cell lines were obtained from American Type Culture Collection.

***In vivo* photon counting analysis.** To establish transfectants expressing the luciferase gene, plasmid vectors carrying the firefly luciferase gene named pLuc/Neo and a transfection reagent, LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) were used in accordance with the manufacturer's instructions. Stable transfectants were selected in geneticin (500 μg/mL; Invitrogen) and bioluminescence was used to screen the transfected clones for luciferase gene expression using the IVIS system (Xenogen, Alameda, CA, USA). *In vivo* photon counting and optical imaging to detect luciferase activity in the mice were conducted on the IVIS system as described previously.[14] Animal protocols were approved by the committee for Ethics of Animal Experimentation and were in accordance with the Guideline for Animal Experiments at the National Cancer Center.

siRNA preparation. The sequence of *NEDD1* siRNA was 5′-CGAAGUGUUAAUGUGAAUGtt-3′ and 3′-ttGCUUCACA AUUACACUUAC-5′ (Ambion, Austin, TX, USA). Non-specific control siRNA duplex and luciferase GL3 siRNA duplex were purchased from Dharmacon (Lafayette, CO, USA). *ELK1* siRNA, 5′-GCUGAGAGAGCAAGGCAAUtt-3′and 5′-AUUGC CUUGCUCUCUCAGCtt-3′ (SI00300146, Qiagen, Valencia, CA, USA) and *MSX2* siRNA, 5′-CCAUUACCUAUAUGCU AAAt t-3′ and 5′-UUUAGCAUAUAGGUAAUGGtt-3′(SI0003 8031, Qiagen) were used. For *in vitro* studies, 5×10^4 cells were seeded per 6-well culture dish. When cells had grown to approximately 80% confluency, a mixture of 3 μg siRNA and 5 μL DharmaFECT (Dharmacon) was added to the medium in each dish.

Therapeutic studies with *NEDD1* siRNA. Intraperitoneal (i.p.) injection of 60As6Luc cells resuspended in 1 mL PBS was conducted in 6-week-old female C.B17/Icr-scid (scid/scid) mice, followed by i.p. inoculation of various siRNA/atelocollagen complexes. Atelocollagen is a highly purified type I collagen of calf dermis with pepsin treatment (Koken, Tokyo, Japan). The siRNA/atelocollagen complexes were prepared as follows: an equal volume of atelocollagen (pH 7.4) and an

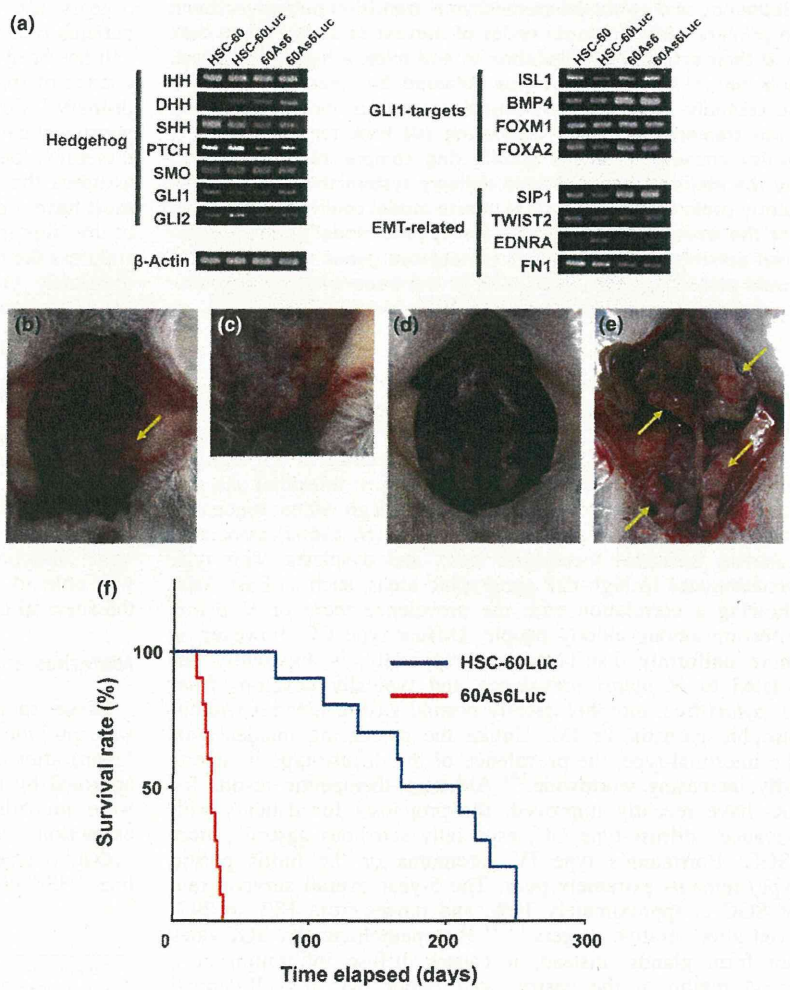

Fig. 1. *Characteristics of HSC-60 and 60As6 cells.* (a) mRNA expression of hedgehog- and epithelial-mesenchymal transition (EMT)-related gene in HSC-60 and 60As6. Shown are results of reverse transcription-polymerase chain reaction (RT-PCR) of hedgehog ligands (*IHH*, *DHH*, and *SHH*), a receptor (*PTCH*), a modulator (*SMO*), two primary target transcriptional factors (*GLI1* and *GLI2*), four authentic GLI1-targets (*ISL1*, *BMP4*, *FOXM1*, and *FOXA2*), two EMT regulators (*SIP1* and *TWIST2*), two EMT-related molecules (*FN1* and *EDNRA*), and a control (*β-Actin*). (b, c) Macroscopic appearance of the peritoneal dissemination and survival of scid mice after intraperitoneal injection of HSC-60Luc and 60As6Luc. A few peritoneal nodules are observed 15 weeks after i.p injection of HSC-60Luc. Yellow arrow: tumor nodule. (d, e) Carcinomatous peritonitis forming multiple tumor nodules observed 2 weeks after i.p. injection of 60As6. Abdominal distension because of bloody ascites was evident. Yellow arrow: tumor nodule. (f) Survival of HSC-60Luc- and 60As6Luc-tumor-bearing mice. Each median survival time is 28 days and 167 days, respectively. $n = 10$; $P < 0.0001$.

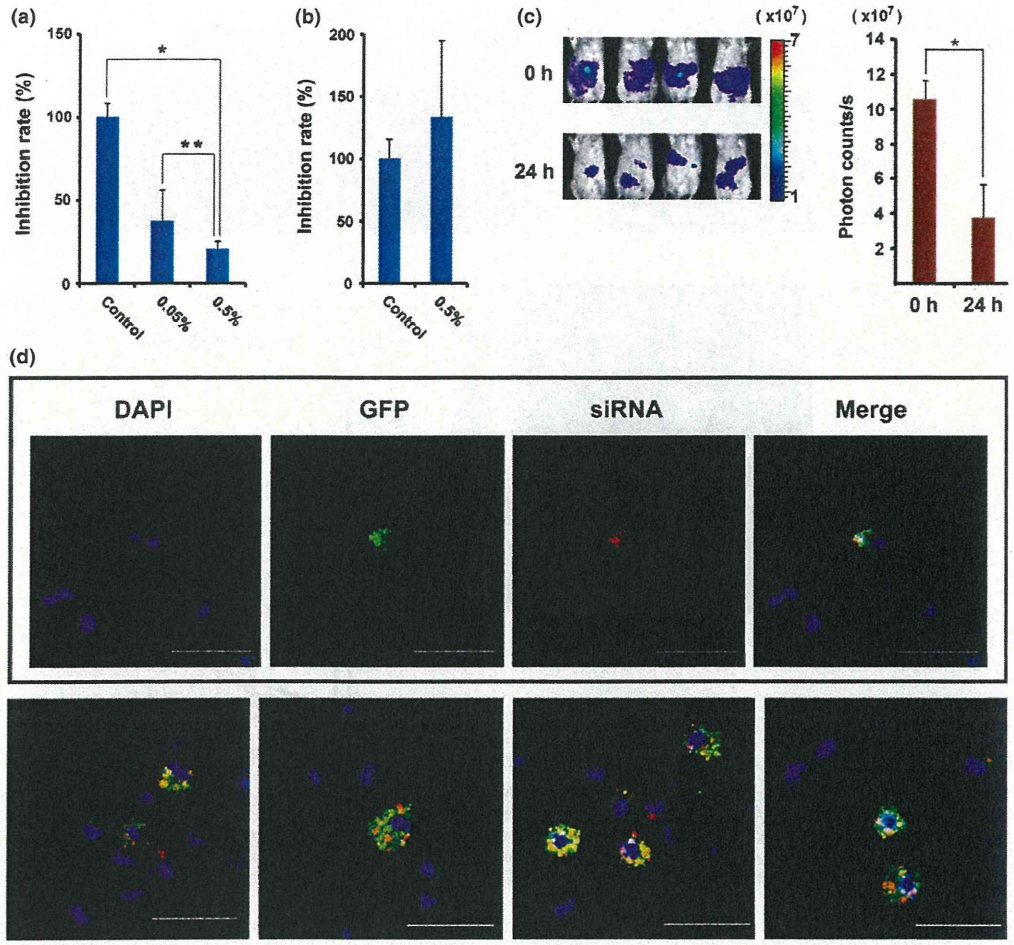

Fig. 2. Evaluation of an atelocollagen-mediated siRNA delivery system by measuring the luciferase activity after i.p injection of luciferase siRNA. (a) Inhibition rates of photon counts 48 h after injection are compared between 0.05% and 0.5% atelocollagen/luciferase siRNA/DharmaFECT1. $*P = 0.006$; $**P = 0.012$. (b) Inhibition rates of photon counts 48 h after injection are compared between 0.5% atelocollagen/control siRNA and 0.5% atelocollagen/luciferase siRNA. No significant inhibition is observed in a luciferase siRNA complex containing no DharmaFECT1. (c) Reduction of luciferase activity 24 h after injection with 0.5% atelocollagen/luciferase siRNA/DharmaFECT1 is visualized in four representative mice (left). Significance of the reduction is also shown (right). $*P < 0.0001$. Color bar indicates $\times 10^7$ photon/s. (d) Delivery evidence of siRNA to cancer cells in the peritoneal cavity. Most green fluorescent protein (GFP)-expressing 60As6 cells (green) incorporate fluorescence-labeled siRNA (red). Bar, 50 μm.

siRNA solution were mixed by rotation at 4°C for 20 min. Before i.p. inoculation, 10 μL of DharmaFECT1 was added to the complex. The final mixture was 1 mL containing 50 μg siRNA, 10 μL DharmaFECT1 and 0.5% atelocollagen. For obtaining delivery evidence of siRNA to cancer cells in the peritoneal cavity, we used a fluorescence-labeled human siGLO LaminA/C Control siRNA (Thermo Fisher Scientific, Rockford, IL, USA).

Laser microdissection, RNA extraction and RT-PCR. In the gastric corpus, the epithelium consists of three tubular units from surface to base: a pit region containing mucus-secreting pit cells, an isthmus/neck region containing stem cells, and a gland region containing chief and parietal cells.[15] We prepared each region (pit, neck, and gland) as follows: the cryostat sections (8 μm) of frozen tissues were microdissected with a Pixcell II LCM system (Arcturus Engineering, Mountain View, CA, USA). Total RNA was isolated by suspending the cells in an ISOGEN lysis buffer (Nippon Gene, Toyama, Japan) followed by precipitation with isopropanol. The mRNA was amplified by an efficient method of high-fidelity mRNA amplification.[16,17] Other normal and gastric cancer tissues were provided by our hospital between 2003 and 2004 after obtaining informed consent from each patient and approval by the Institutional Ethics Committee. Tissue specimens were snap-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was isolated by suspending the cells in an ISOGEN lysis buffer followed by precipitation with isopropanol. As described in our previous report,[18] semi-quantitative RT-PCR within linear range by performing 25–35 cycles for *IHH, DHH, SHH, GLI1, GLI2, PTCH, SMO, SIP1, TWIST2, ISL1, BMP4, FOXM1, FOXA2, FN1, EDNRA,* and *ACTB* (*β-Actin*) was carried out. For *NEDD1*, 5′-TTCTGTCACTGCT GGAGTTG-3′ and 5′-TGTGTTGCCAGAAACTTCCC-3′ were used as primers.

Western blot analysis. The proteins (20 μg) were separated on a 10% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA). The blots were incubated overnight with mouse monoclonal anti-human Nedd1 antibody (Abcam, Cambridge, MA, USA).

Statistical analysis. All data were expressed as the mean ± SE, and analyzed using the unpaired *t*-test. Survival curves were calculated according to the Kaplan–Meier method. Differences

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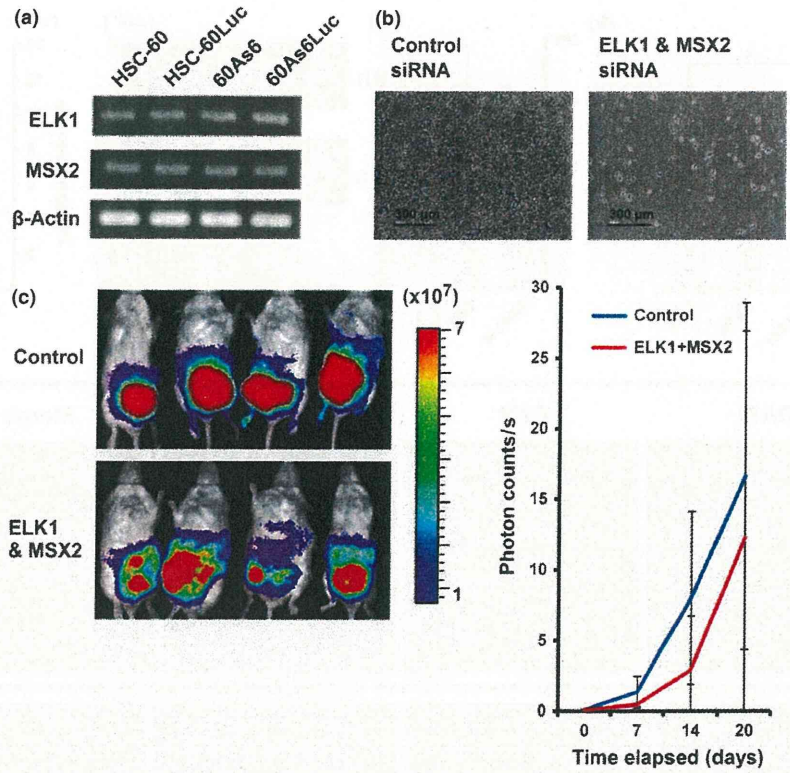

Fig. 3. The expression of cancer-specific hedgehog targets *ELK1* and *MSX2* in HSC-60 and 60As6 and the effect of silencing of *ELK1and MSX2* on *in vitro* and *in vivo* cell growth of 60As6. (a) RT-PCR of *ELK1* and *MSX2* in HSC-60 and 60As6. (b) Cell growth inhibition of 60As6 cells 2 days after double transfection of *ELK1* and *MSX2* siRNA. (c) Each of four mice treated with *ELK1 and MSX2* siRNA or control siRNA is visualized 27 days after inoculation of 60As6Luc by the IVIS system (left). Color bar indicates $\times 10^7$ photon/s. Results of the time course experiments on tumor growth inhibition by the double treatment are shown (right). Although small effects on the growth inhibition are observed, no significance is shown.

between survival curves were examined with the log rank test. The accepted level of significance was $P < 0.05$. SPSS (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Establishment of a highly metastatic cell line 60As6 from a parental cell line HSC-60. We previously reported that the hedgehog signal is more active in diffuse-type gastric cancer (GC) including scirrhous GC (SGC) than in the intestinal-type GC and recently reported that crossroad between hedgehog and epithelial-mesenchymal transition (EMT) signals is present in the diffuse-type.[17,18] Among 11 GC-derived cell lines (HSC-39, HSC-43, HSC-44, HSC-58, HSC-59, HSC-60, KATOIII, MKN7, MKN28, MKN74, and HSC-57), HSC-60 was found to most-closely mimic the diffuse-type GC phenotype in mRNA expression of hedgehog- and EMT-related genes.[18] However, HSC-60 cells often formed only a single tumor nodule in the peritoneal cavity and no ascites in scid mice despite intraperitoneal (i.p.) implantation of many cells (more than 1×10^6 cells) (data not shown). Therefore, we established a highly peritoneal-seeding cell line, 60As6, from this parental cell line, HSC-60, by six cycles of isolating ascitic tumor cells and orthotopic inoculation of these cells as described in our previous report,[13] and we next obtained transfectants (HSC-60Luc and 60As6Luc) containing the luciferase gene for *in vivo* imaging in animal experiments. Reverse transcription-PCR showed that the expression of the above-mentioned hedgehog and EMT signaling genes in HSC-60 cells was maintained in the 60As6 cells (Fig. 1a). The doubling time of these two cell lines was comparable (30 h in HSC-60 and 31 h in 60As6). The peritoneal dissemination and the survival rates of scid mice after i.p. implantation of HSC-60 and 60As6 are shown (Fig. 1b–f). None of the HSC-60-tumor-bearing mice developed ascites (Fig. 1b,c), and the median mice survival time was 167 days after implantation of 5×10^6 HSC-60Luc cells (Fig. 1f). On the other hand, implantation of 5×10^6 60As6Luc cells resulted in the formation of remarkably bloody ascites approximately 14 days later (Fig. 1d), and the median survival time was 28 days (Fig. 1f). In the 60As6-tumor-bearing mice, peritoneal dissemination was often seen in the omentum, mesenterium, parietal peritoneum, diaphragm, and so on (Fig. 1e).

Development of an siRNA delivery system into peritoneal metastatic tumor cells. The atelocollagen-mediated gene delivery system was originally developed by a collaborator.[19] In mice, this delivery system has been reported to be useful for gene delivery into some body sites including metastatic tumors and also for systemic gene delivery;[20] however, its application into a peritoneal metastatic tumor has not been reported. Previous studies indicated that a low concentration (0.05%) of atelocollagen was effective for systemic siRNA delivery, whereas a high concentration (0.5%) was useful for an intratumor siRNA delivery,[20] and also indicated that a transfection reagent, DharmaFECT1 accelerated an atelocollagen-mediated siRNA delivery.[20] We first investigated an optimal atelocollagen concentration for an i.p. siRNA delivery into tumor cells by measuring luciferase activity. Those between a 50 μg luciferase

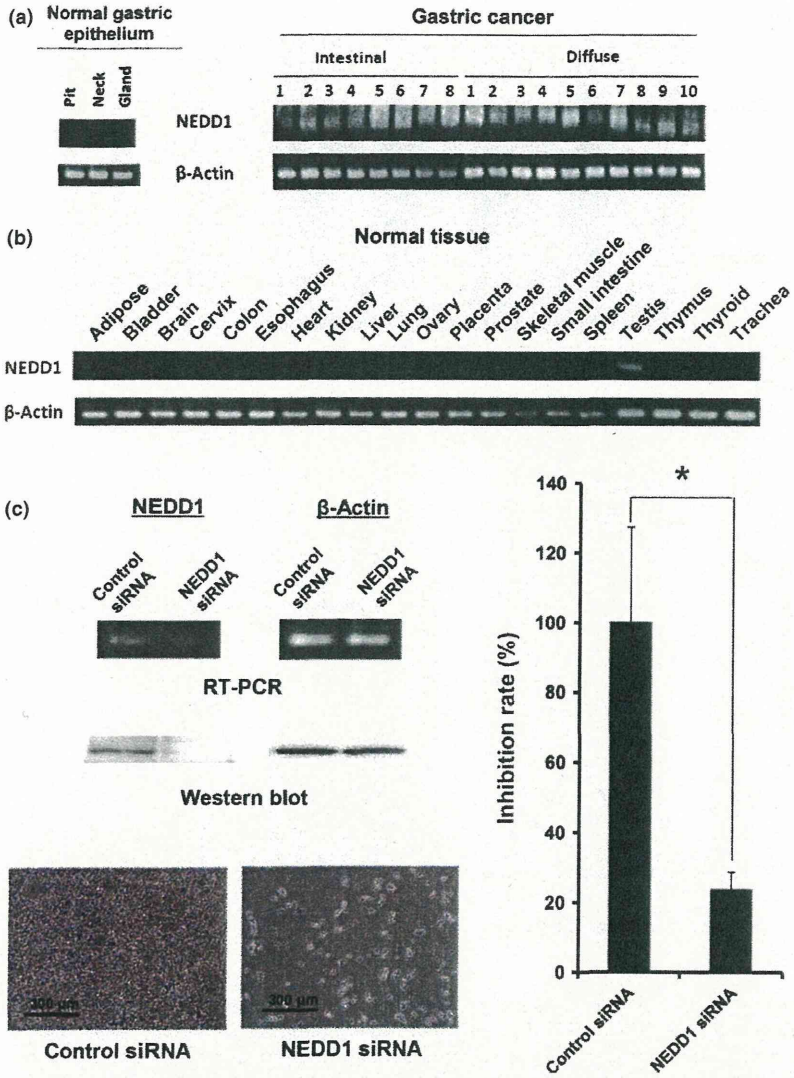

Fig. 4. The expression and silencing of *NEDD1*. (a) *NEDD1* mRNA expression in three regions of normal gastric mucosa (pit, neck, and gland) and 18 primary GCs containing eight intestinal-type and 10 diffuse-type. (b) *NEDD1* mRNA expression in 20 other organs of the human body. (c) Reverse transcription-polymerase chain reaction (RT-PCR) and western blot results for *NEDD1* gene silencing (upper left) and representative photos for growth inhibition (lower left) of 60As6 cells 2 days after treatment of *NEDD1* siRNA are shown. The cell growth inhibition rate 5 days after siRNA transfection is also shown (*P = 0.029) (right).

GL3 siRNA/0.05%atelocollagen/DharmaFECT1 complex and a 50 μg luciferase GL3 siRNA/0.5%atelocollagen/DharmaFECT1 complex 48 h after i.p. injection were compared in scid mice that had 1×10^6 60As6Luc cells introduced into the peritoneal cavity. Both of the two complexes clearly reduced the luciferase activity compared with an untransfected control (Fig. 2a), and the 0.5% atelocollagen complex rather than the 0.05% complex reduced it significantly (Fig. 2a). However, a 50 μg luciferase GL3 siRNA/0.5%atelocollagen only complex did not inhibit luciferase activity (Fig. 2b). The reduction of the luciferase activity 24 h after injection of a 50 μg luciferase GL3 siRNA/0.5%atelocollagen/DharmaFECT1 complex was visualized in four scid mice (Fig. 2c, right), and the significant reduction of the photon counts was shown (Fig. 2c, left).

By this method, we also obtained delivery evidence of fluorescence-labeled siRNA to tumor cells in scid mice that had 1×10^6 GFP-expressing 60As6 cells introduced into the peritoneal cavity. As shown in Figure 2(d), most GFP-expressing 60As6 cells (89.6%, 69/77 cells in eight fields), which were recovered from the peritoneal cavity 72 h after injection, incorporated fluorescence-labeled siRNA. These results demonstrated that siRNA was able to be delivered into peritoneal metastatic tumor cells by using both 0.5% atelocollagen and DharmaFECT1.

***In vitro* and *in vivo* effects of siRNA treatment of two diffuse-type GC-specific hedgehog targets, *ELK1* and *MSX2* on highly metastatic 60As6 cells.** As mentioned in the first part of the Results, we previously reported hedgehog signal activation in diffuse-type GC including a scirrhous-type,[17] and identified two cancer-specific hedgehog targets, *ELK1* and *MSX2*.[18] Treatment of each siRNA of *ELK1* and *MSX2* induced growth inhibition (53% and 41% respectively), of HSC-60 cells. Reverse transcription-PCR confirmed that expression of *ELK1* and *MSX2* in HSC-60 cells was maintained in 60As6 cells (Fig. 3a). Double transfection of these two siRNA strongly inhibited *in vitro* cell growth of 60As6 (Fig. 3b) as well as HSC-60.[18]

Before starting *in vivo* tumor growth inhibition studies, we accessed the tumor formation ability of 60As6 cells in scid mice. Tumor formation rates in serial i.p. injection of 1×10^4, 1×10^5, 5×10^5, 1×10^6, and 5×10^6 cells into 10–27 mice showed 0% (0/10), 76% (13/17), 100% (20/20), 100% (27/27, and 100% (15/15), respectively. By i.p. injection of more than 5×10^5 cells, all of the subject mice formed multiple tumors. In 1×10^6 or 5×10^6 cells, tumors were formed

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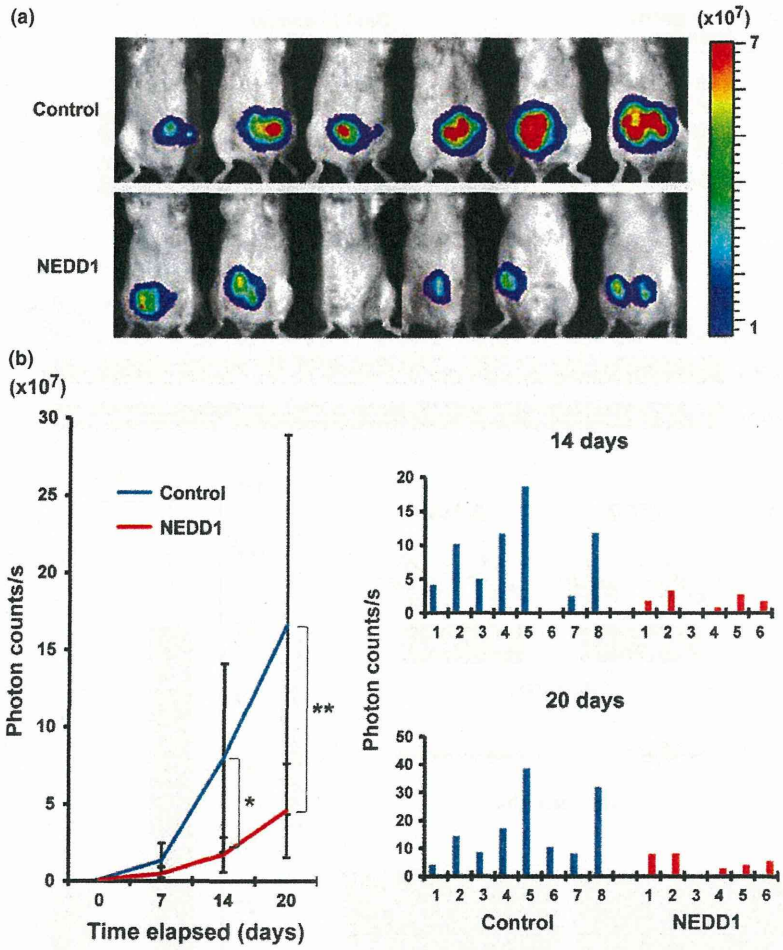

Fig. 5. Inhibition of tumor cell growth in the peritoneal cavity by atelocollagen-mediated *NEDD1* siRNA delivery. (a) Each of six mice treated with *NEDD1* siRNA or vehicle alone as control is visualized at 21 days after inoculation of 60As6Luc. Color bar indicates $\times 10^7$ photon/s. (b) Results of quantitative photon-counting analysis three times a week for 3 weeks are shown after inoculation of 60As6Luc with or without the *NEDD1* siRNA treatment. This experiment was repeated three times, and similar results were observed (*P = 0.04; **P = 0.034) (left). Photon counts 14 and 20 days after inoculation in each mouse are also indicated (right), because results among animal experiments often are variable.

within 2 weeks, and the tumor-bearing mice died rapidly (median survival time: approximately 30 days). These cases were thought to be quite different from peritoneal recurrence in humans, which is known to develop from occult or minimal tumor cells.[21] On the other hand, by injection of 5×10^5 cells, tumors were formed within 4 weeks, and a median survival time was approximately 50 days (data not shown). To extend our *in vitro* studies (Fig. 3b), 10 scid mice were inoculated with 5×10^5 60As6Luc cells, and each five of those 10 mice was injected with a 25 μg *ELK1 and* 25 μg *MSX2* siRNA/0.5% atelocollagen/DharmaFECT1 complex or a 0.5% atelocollagen/DharmaFECT1 complex as control five times every 3 days for 15 days. After exclusion of one pair of un-inoculated mice, optical imaging of the luciferase activity by use of the IVIS system at 27 days after administration was shown (Fig. 3c, left). Results of the time course experiments (at 0, 7, 14, 20 days) of tumor growth inhibition were shown by the quantification of photon counts (Fig. 3c, right). Although small effects on tumor growth inhibition by the double treatment of *ELK1* and *MSX2* siRNAs were observed, no significance was shown. Accordingly, no significant difference on mouse survival was found (data not shown). Therefore we next searched for other powerful targets for prolonging survival by intraperitoneal delivery of a single siRNA.

***NEDD1* siRNA inhibited *in vitro* cell growth of a highly metastatic 60As6 cell line.** Taxanes, which bind microtubules and inhibit tumor cell division in the metaphase of the cell cycle, are anti-tumor reagents for GC patients with peritoneal recurrence, because a significant pharmacokinetic advantage associated with i.p. delivery was predicted by their large bulky structure and known hepatic metabolism.[22] However, this drug has many adverse reactions including bone marrow suppression, alopecia, and neuropathy.[23] Therefore, we investigated target genes, which are involved in the metaphase regulation, from our previously reported GC-related genes.[18] In the previous study, we obtained gene expression profiles of 18 intestinal-type GCs and 12 diffuse-type GCs, and six non-cancerous tissues, and approximately 60 genes were found to be expressed aberrantly in more than 80% of GCs. Among them, only the *NEDD1* gene was known to be involved in the metaphase regulation. Therefore, we selected this gene as a new target for evaluating the therapeutic effect of an atelocollagen-mediated siRNA delivery on the peritoneal metastasis model mice established here. The *NEDD1* gene encodes a protein that binds to the gamma-tubulin ring complex, a multiprotein complex at the centrosome and at the mitotic spindle that mediates the nucleation of microtubules.[24] First, we showed *NEDD1* mRNA expression in three regions (pit, neck, and gland) of normal gastric mucosa prepared by laser-captured microdissection[17,18] in 18 primary gastric cancer tissues (Fig. 4a) and in 20 normal organs of the human body (Fig. 3b). *NEDD1* mRNA was found to be highly expressed in all primary lesions of gastric cancer by RT-PCR, while no or low expression was observed in normal organs except for the testis. The expression pattern of *NEDD1* was similar to that of the testis-tumor antigen gene. Next, we examined whether

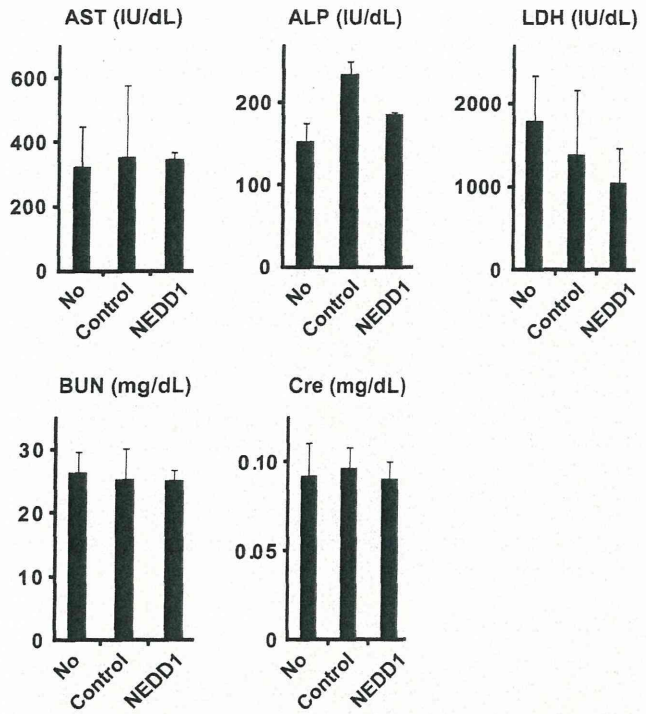

Fig. 6. Evaluation of selected serum chemistries 3 weeks after i.p. administration of *NEDD1* siRNA. No significant difference in the activity of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) and in levels of blood urea nitrogen (BUN) and Cre is observed among the no-treatment group (No), control siRNA-treated group (Control) and *NEDD1* siRNA-treated group (NEDD1) (n = 9).

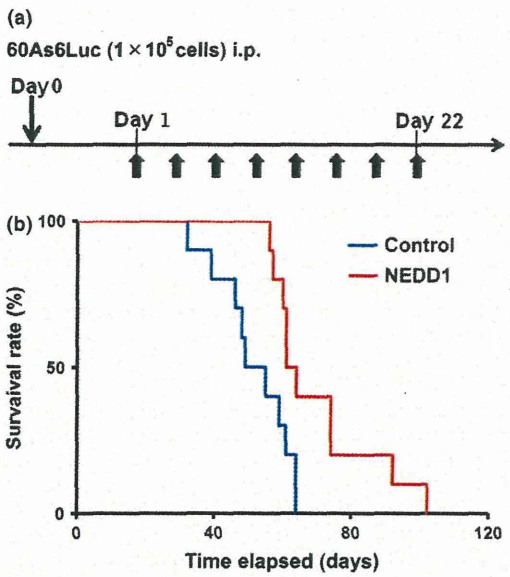

Fig. 7. Effect on survival of mice treated with *NEDD1* siRNA. (a) Schedule of i.p. administration of *NEDD1* siRNA 1 day after inoculation of 1×10^5 60As6Luc cells. (b) The i.p. administration of *NEDD1* siRNA significantly prolongs mice survival (P = 0.012).

NEDD1 knockdown inhibits the growth of highly metastatic 60As6 cells *in vitro*. RT-PCR and Western blot analyses revealed that Nedd1 protein expression was diminished efficiently by treatment of the *NEDD1* siRNA (Fig. 4c, upper left). In accordance with a decrease of *NEDD1* mRNA, 60As6 cell growth was eminently inhibited by treatment of *NEDD1* siRNA compared with the control siRNA (Fig. 4c, right). Representative photos of the cells are also shown (Fig. 4c, lower left).

***In vivo* inhibition of peritoneal metastasis in the mouse xenograft model by i.p. administration of *NEDD1* siRNA.** To extend our *in vitro* studies (Fig. 4), 12 scid mice were inoculated with 5×10^5 60As6Luc cells, and each of six of those 12 mice was injected with a 50 μg *NEDD1*siRNA/0.5% atelocollagen /DharmaFECT1 complex or a 0.5% atelocollagen/DharmaFECT1 complex as control five times every 3 days for 15 days. Optical imaging to detect luciferase activity in the mice was performed by using the IVIS system to evaluate tumor progression three times a week for 3 weeks. Quantitative photon-counting analysis of disseminated 60As6 cells revealed effective and significant inhibition of the tumor growth in the mice treated with *NEDD1* siRNA (Fig. 5).

Evaluation of i.p. administration safety of *NEDD1* siRNA and mice survival rates. To assess the safety of i.p. administration of a *NEDD1*siRNA/atelocollagen/DharmaFECT1 complex in liver and kidney function, we compared the activities of three enzymes (aspartate aminotransferase [AST], lactate dehydrogenase [LDH], alkaline phosphatase [ALP]) and the levels of blood urea nitrogen (BUN) and Cre in the serum of each group (no treatment, control siRNA, and *NEDD1*siRNA group; n = 9) 3 weeks after treatment. No significant toxicity was detected in the mice treated with *NEDD1*siRNA (Fig. 6). In addition, no difference in the activity or glossiness of hair was also observed among the three groups (data not shown). To yield survival benefits, 20 scid mice were inoculated with 1×10^5 60As6Luc cells and, 3 weeks after inoculation, each of 10 of those 20 mice was injected with *NEDD1* siRNA or control siRNA eight times every 3 days for 22 days (Fig. 7a). The survival rates of these 20 mice are shown in Figure 7(b). Although *NEDD1* siRNA administration stopped at 22 days after inoculation of 60As6Luc, mice treated with *NEDD1* siRNA survived longer than the control mice with a significance (*NEDD1* siRNA: the median survival time was 61 ± 2 days; control siRNA: the median survival time was 49 ± 6 days, P = 0.0115).

Discussion

For gene therapy, one of the most dramatic events of the past 5 years in this field has been the discovery of RNA interference (RNAi). The success of cancer therapeutic use of RNAi relies on the development of safe and efficacious delivery systems that introduce siRNA and shRNA expression vectors into target tumor cells. However, such delivery systems into peritoneal metastatic tumor cells have not been established well. The atelocollagen-mediated gene delivery system was originally developed for a adenovirus vector by a collaborator.[19] An atelocollagen is a highly purified type I collagen of calf dermis with pepsin treatment, which allows nuclease resistance, prolonged release of genes and reduction of cellular immune responses. In mice, this delivery system has been reported to be useful for gene delivery into some body sites including metastatic tumors and also for systemic gene delivery.[20] As mentioned in the introduction, diffuse-type GCs including scirrhous GC frequently show peritoneal dissemination even if tumor cells are circulating systemically. Therefore, in this type of GCs, peritoneal metastasis control is urgently crucial for improving the quality of life and patient outcome. Here we provided peritoneal metastasis model mice and an effective delivery system for i.p. administration of

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siRNA. Figure 2 showed that the 0.5% atelocollagen/DharmaFECT1/siRNA complex rather than the 0.05% atelocollagen /DharmaFECT1/siRNA complex reduced luciferase activity and that the DharmaFECT1 free complex did not reduce it. To date, a collaborator usually uses DharmaFECT1 in the atelocollagen-mediated systemic gene delivery by i.v. administration, because this reagent improves it (Takeshita F, unpublished observation, 2010).

In peritoneal metastasis model mice, the i.p. administration of *NEDD1* siRNA was able to inhibit tumor growth and prolong survival even without any side effects (Figs 5,7). If targets such as *NEDD1* function in the cell cycle regulation, the slow gene release arising from protection from nucleases by atelocollagen may provide an advantage for long acting and for reducing the number of administrations. As shown in Figure 7, we administered the *NEDD1* siRNA complex five times every 3 days for 15 days in this study; however, that number may possibly be reduced. In another report for intraperitoneal administration of siRNA targeting nuclear factor-κB with only DharmaFECT1, the siRNA prolonged the survival of mice only by the administration of paclitaxel, whereas the siRNA/DharmaFECT1 complex alone could not succeed.[25] Taken together, the atelocollagen/DharmaFECT1/siRNA complex is very useful for gene delivery to the peritoneal cavity.

In the mouse model used, the number of tumor cells (1×10^5 cells) implanted into the mouse peritoneal cavity was estimated to be still very large compared with the number of tumor cells in the peritoneal cavity in human GC patients with cytology positive, who often showed peritoneal metastasis within 2 years. Therefore, the present i.p. delivery system of siRNA has a great potential for treatment of such GC patients.

Although *in vitro* cell growth inhibition was observed by the double siRNA treatment of *ELK1* and *MSX2* (Fig. 3b), no significant difference on *in vivo* tumor growth and mouse survival was found (Fig. 3c and data not shown). Investigation of other hedgehog components (SMO, GLIs, ISL1, BMP4, FOXM1, and FOXA2) and EMT-regulators (SIP1/ZEB2, TWIST2) remains for a future study, because cross-talk between hedgehog and EMT signals is specific to diffuse-type GC.[18]

Atelocollagen has also been reported to efficiently deliver microRNA.[26] Recently, genome-wide microRNA expression profiles of 353 GC samples have shown that some microRNAs including let-7b, miR-214, and miR-433 are expressed aberrantly and correlate with tumorigenesis, progression, and prognosis of diffuse-type GC.[27] Thus, these microRNAs may be candidates for SGC therapy. In addition, transforming growth factor-β (TGF-β) has been reported to induce apoptosis of a subset of diffuse-type GCs whose receptor is not inactivated.[28,29] Therefore, adenovirus-mediated TGF-β or the downstream targets such as Gasdermin/GasderminA delivery also have great potential for SGC therapy.

In conclusion, we developed a novel i.p. delivery system of siRNA to disseminated tumor cells in the peritoneal cavity that successfully prolongs the survival of model mice. The present mouse model is for an adjuvant therapy after surgical resection. The ability of atelocollagen/DharmaFECT complex is keeping siRNA from nucleases, leading slow gene release and reducing the amount of administration that results in effective eradication of residual tumor cells in the peritoneal cavity.

Thus, considering other potential targets of the diffuse-type GCs, this system is a highly flexible therapeutic platform for the treatment of peritoneal dissemination.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

References

1 Ries LAG, Eisner MP, Kosary CL *et al. SEER Cancer Statistics Review, 1973–1998*, Bethesda, MD: National Cancer Institute, 2001.
2 Maxwell Parkin D. Global cancer statistics in the year 2000. *Lancet Oncol* 2001; **2**: 533–43.
3 Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31–49.
4 Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; **12**: 354–62.
5 Nakamura R, Saikawa Y, Wada N *et al.* Retrospective analysis of prognosis for scirrhous-type gastric cancer: one institution's experience. *Int J Clin Oncol* 2007; **12**: 291–4.
6 Maehara Y, Moriguchi S, Orita H *et al.* Lower survival rate for patients with carcinoma of the stomach of Borrmann type IV after gastric resection. *Surg Gynecol Obstet* 1992; **175**: 13–6.
7 Arveux P, Faivre J, Boutron MC *et al.* Prognosis of gastric carcinoma after curative surgery: a population-based study using multivariate crude and relative survival analysis. *Dig Dis Sci* 1992; **37**: 757–63.
8 Kunisaki C, Shimada H, Nomura M *et al.* Therapeutic strategy for scirrhous type gastric cancer. *Hepatogastroenterology* 2005; **52**: 314–8.
9 Kitamura K, Beppu R, Anai H *et al.* Clinicopathologic study of patients with Borrmann type IV gastric carcinoma. *J Surg Oncol* 1995; **58**: 112–7.
10 Sugarbaker PH, Yonemura Y. Clinical pathway for the management of resectable gastric cancer with peritoneal seeding: best palliation with a ray of hope for cure. *Oncology* 2000; **58**: 96–107.
11 Fujimoto S, Takahashi M, Kobayashi K *et al.* Relation between clinical and histologic outcome of intraperitoneal hyperthermic perfusion for patients with gastric cancer and peritoneal metastasis. *Oncology* 1993; **50**: 338–43.
12 Yonemura Y, Kawamura T, Bandou S, Takahashi S, Sawa T, Matsuki N. Treatment of peritoneal dissemination from gastric cancer by peritonectomy and chemohyperthermic peritoneal perfusion. *Br J Surg* 2005; **92**: 370–5.
13 Yanagihara K, Takigahira M, Tanaka H *et al.* Development and biological analysis of peritoneal metastasis mouse models for human scirrhous stomach cancer. *Cancer Sci* 2005; **96**: 323–32.
14 Yanagihara K, Takigahira M, Takeshita F *et al.* A photon counting technique for quantitatively evaluating progression of peritoneal tumor dissemination. *Cancer Res* 2006; **66**: 7532–9.
15 Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. *Anat Rec* 1993; **236**: 259–340.
16 Aoyagi K, Tatsuta T, Nishigaki M *et al.* A faithful method for PCR-mediated global mRNA amplification and its integration into microarray analysis on laser-captured cells. *Biochem Biophys Res Commun* 2003; **300**: 915–20.
17 Fukaya M, Isohata N, Ohta H *et al.* Hedgehog signal activation in gastric pit cell and in diffuse-type gastric cancer. *Gastroenterology* 2006; **131**: 14–29.
18 Ohta H, Aoyagi K, Fukaya M *et al.* Cross talk between hedgehog and epithelial-mesenchymal transition pathways in gastric pit cells and in diffuse-type gastric cancer. *Br J Cancer* 2009; **100**: 389–98.
19 Ochiya T, Takahama Y, Nagahara S *et al.* New delivery system for plasmid DNA *in vivo* using atelocollagen as a carrier material: the minipellet. *Nat Med* 1999; **5**: 707–10.
20 Takeshita F, Minakuchi Y, Nagahara S *et al.* Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen *in vivo*. *Proc Natl Acad Sci USA* 2005; **102**: 12 177–82