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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 plastica 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 activ-

ity 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'-CCAUUACCUAUUAGCU AAA t-3' and 5'-UUUAGCAUUAAGGUAUUGGtt-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

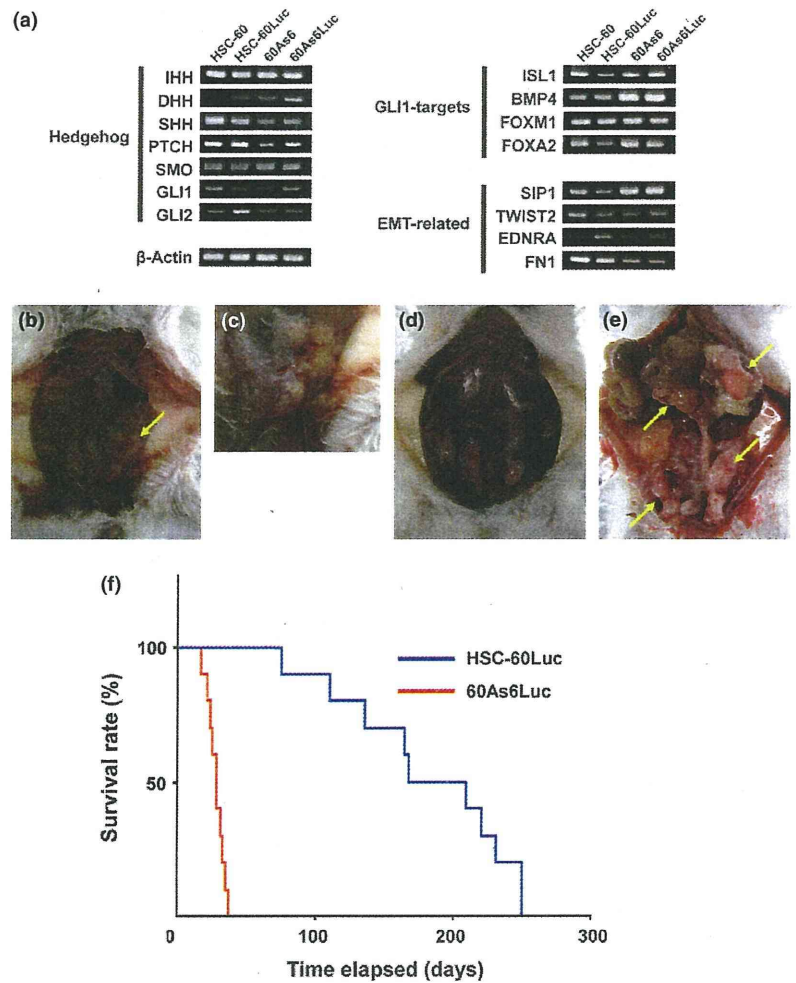


Fig. 1. Characteristics of HSC-60 and 60As6 cells. (a) mRNA expression of hedgehog- and epithelial-mesenchymal transition (EMT)-related gene in HSC-60 and 60As6. Shown are results of reverse transcription-polymerase chain reaction (RT-PCR) of hedgehog ligands (*IHH*, *DHH*, and *SHH*), a receptor (*PTCH*), 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. $n = 10$; $P < 0.0001$.

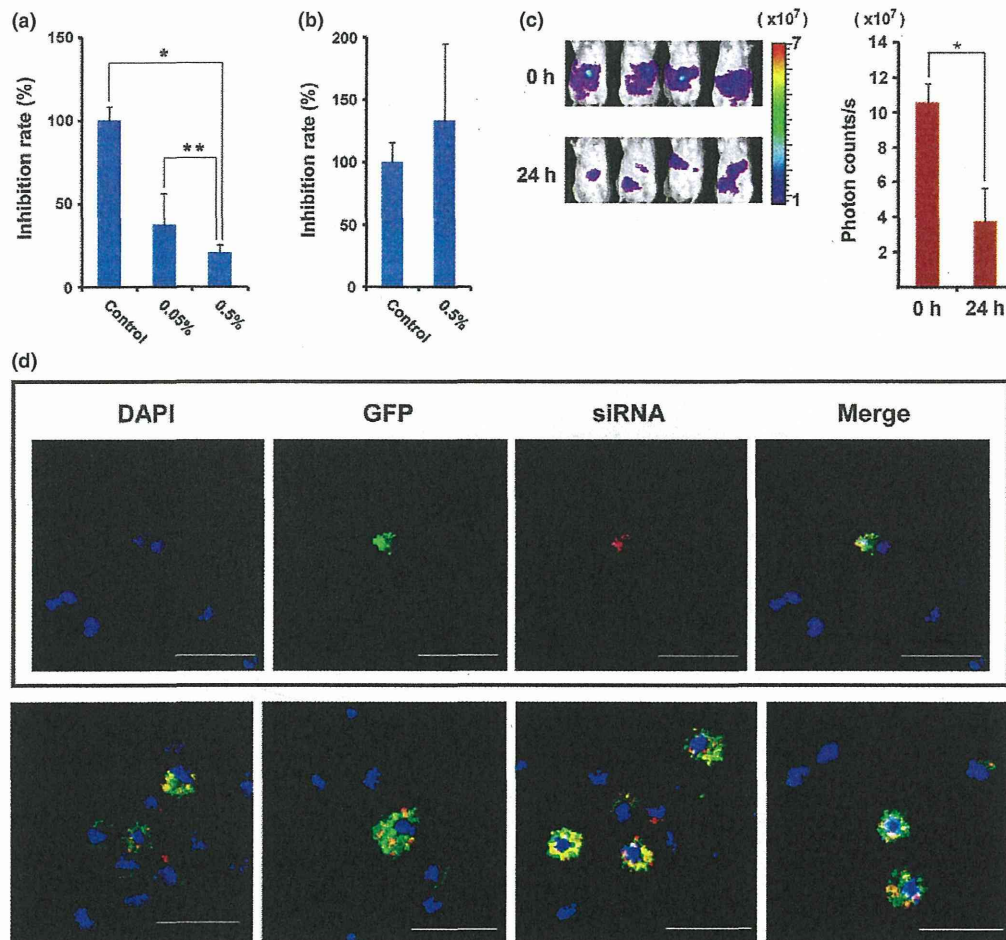


Fig. 2. Evaluation of an atelocollagen-mediated siRNA delivery system by measuring the luciferase activity after i.p. injection of luciferase siRNA. (a) Inhibition rates of photon counts 48 h after injection are compared between 0.05% and 0.5% atelocollagen/luciferase siRNA/DharmaFECT1. * $P = 0.006$; ** $P = 0.012$. (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 60A6 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*, *FNI*, *EDNRA*, and *ACTB* (β -Actin) was carried out. For *NEDD1*, 5'-TTCTGTCACGTGCTGGAGTTG-3' and 5'-TGTGTTGCCAGAACTCCC-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 \pm SE, and analyzed using the unpaired *t*-test. Survival curves were calculated according to the Kaplan–Meier method. Differences

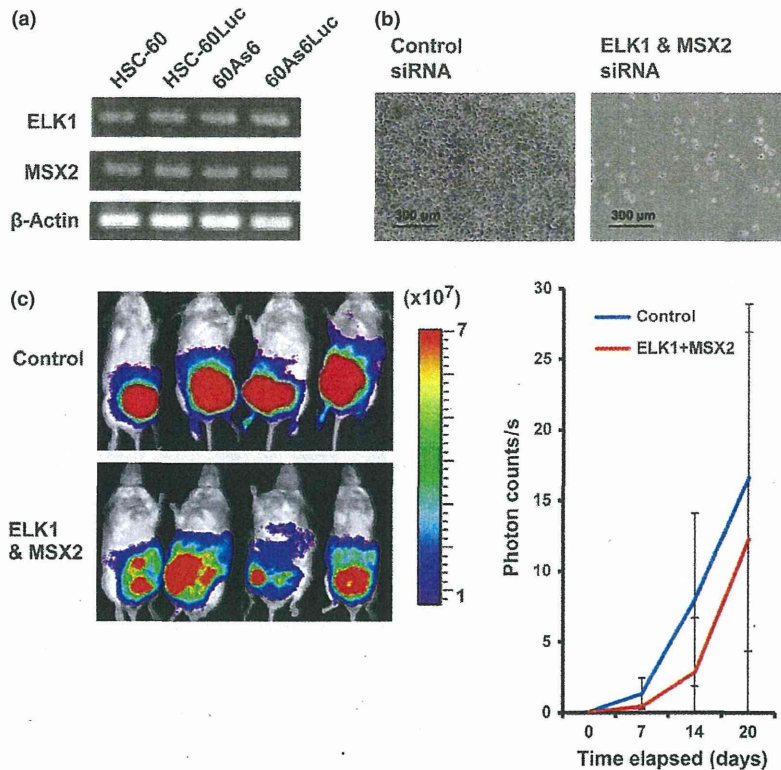


Fig. 3. The expression of cancer-specific hedgehog targets *ELK1* and *MSX2* in HSC-60 and 60As6 and the effect of silencing of *ELK1* and *MSX2* on *in vitro* and *in vivo* cell growth of 60As6. (a) RT-PCR of *ELK1* and *MSX2* in HSC-60 and 60As6. (b) Cell growth inhibition of 60As6 cells 2 days after double transfection of *ELK1* and *MSX2* siRNA 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 μ g luciferase

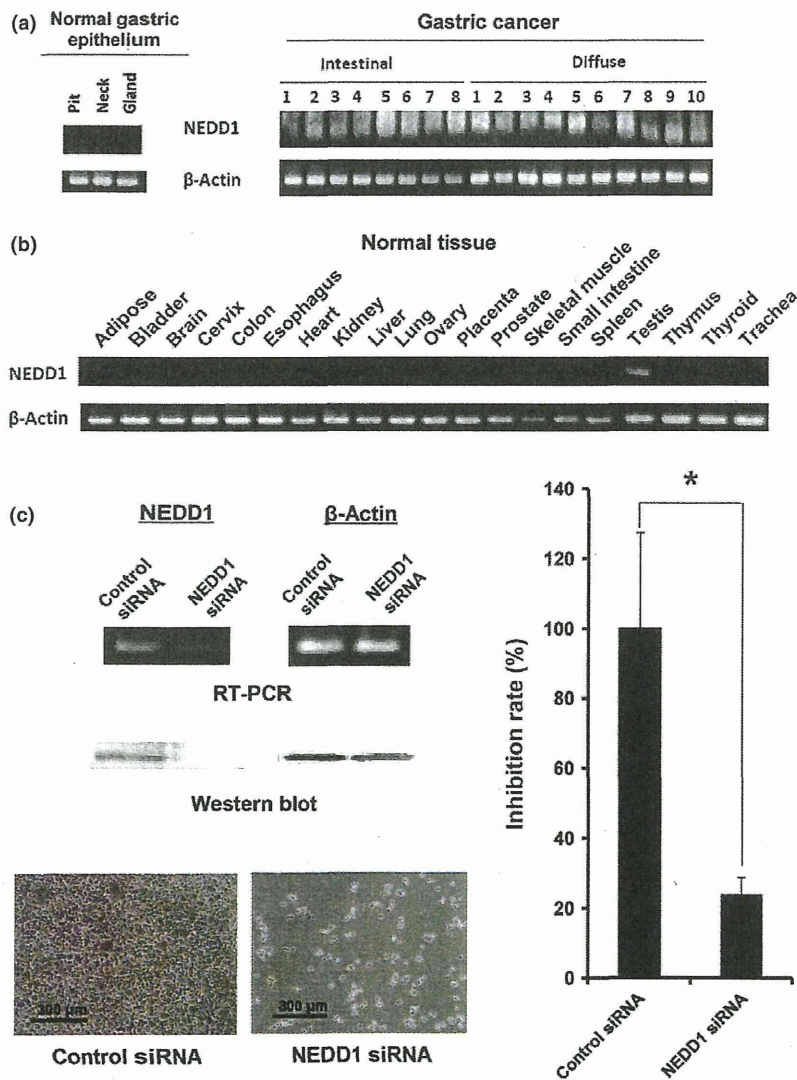


Fig. 4. The expression and silencing of *NEDD1*. (a) *NEDD1* mRNA expression in three regions of normal gastric mucosa (pit, neck, and gland) and 18 primary GCs containing eight intestinal-type and 10 diffuse-type. (b) *NEDD1* mRNA expression in 20 other organs of the human body. (c) Reverse transcription-polymerase 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 μ g 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 μ g 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 μ g 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

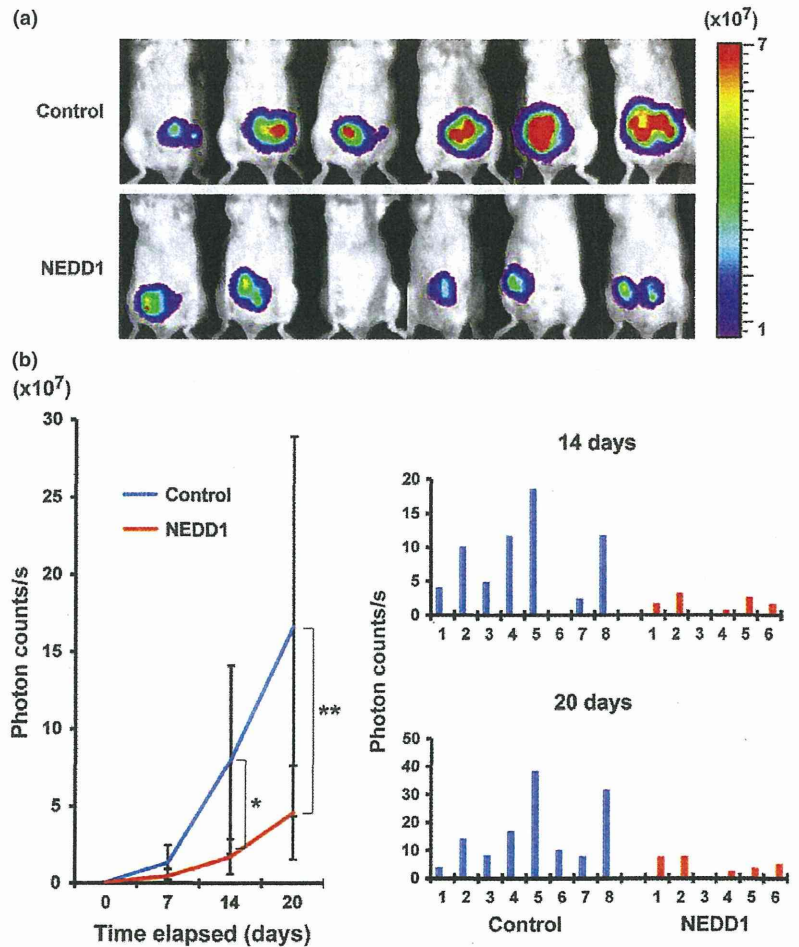


Fig. 5. Inhibition of tumor cell growth in the peritoneal cavity by atelocollagen-mediated *NEDD1* siRNA delivery. (a) Each of six mice treated with *NEDD1* siRNA or vehicle alone as control is visualized at 21 days after inoculation of 60As6Luc. Color bar indicates $\times 10^7$ photon/s. (b) Results 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 μ g *ELK1* and 25 μ g *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 recur-

rence, 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 multi-protein 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

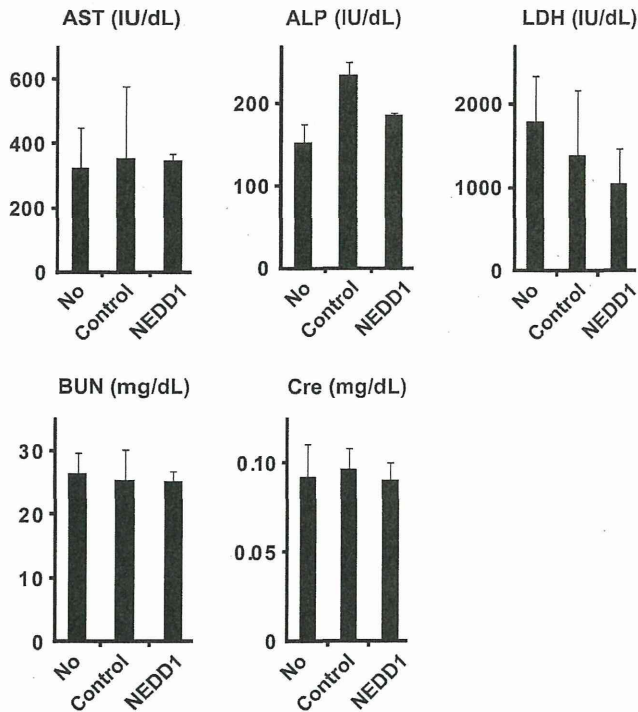


Fig. 6. Evaluation of selected serum chemistries 3 weeks after i.p. administration of *NEDD1* siRNA. No significant difference in the activity of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) and in levels of blood urea nitrogen (BUN) and Cre is observed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

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 μ g *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

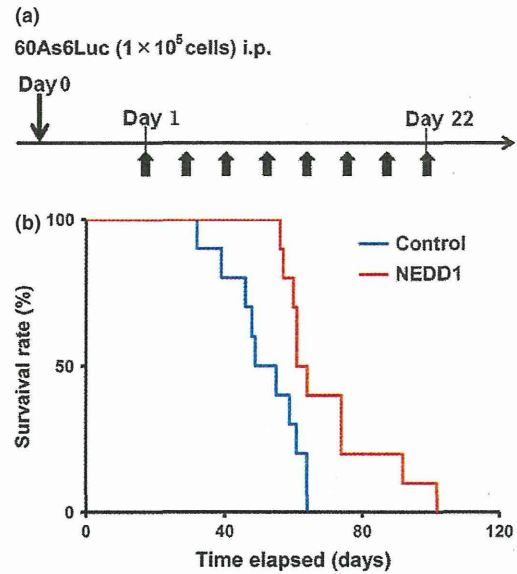


Fig. 7. Effect on survival of mice treated with *NEDD1* siRNA. (a) Schedule of i.p. administration of *NEDD1* siRNA 1 day after inoculation of 1×10^5 60As6Luc cells. (b) The i.p. administration of *NEDD1* siRNA significantly prolongs mice survival ($P = 0.012$).

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 an 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