and Yusuke Nakamura, unpublished data), *MUC1* probably has a role specifically in DGC, contrary to *PSCA* whose association was revealed in both DGC and IGC (Table 2). This difference might derive from the difference in their pathogenesis.

Perspective

It is expected that identification of GC susceptibility genes will contribute to the development of a new approach in GC prevention in the future. The risk genotypes of the two genes identified by the previous Japanese GWAS classified the majority of Japanese people into a high-risk group (Fig. 1). This finding is supported by HapMap Project data on 11 ethnic populations, which show, for example, that the Japanese population has the lowest frequency of the protective genotype (C/C) of rs2294008 in the 11 populations (Supporting Information Table S1). This offers a good starting point for the development of a personalized DGC prevention, because it means that we could add other layers of risk factors to capture even higher DGC risk subgroups without too much size diminution and restriction of the target population. Adding non-genetic risk factors for a further stratification is of particular interest, because they could be modifiable, while the genetic predisposition presents a fixed and basic risk probability of each individual. It should also be noted that the Japanese GWAS on GC was initiated almost 10 years ago. Since then, more powerful platforms for efficient SNP typing including next-generation sequencers have been developed, and more numerous DNA samples have been accumulated for a GWAS by several groups in Japan, including those involved in prospective cohort studies. Other novel GC susceptibility genes and their interactions with non-genetic risk factors can be identified by conducting a GWAS with a large number of the samples and the latest typing platforms, especially in a nested case-control design based on a molecular epidemiological or "genome" cohort. Such a systematic approach will contribute not only to GC prevention but also to the development of new GC therapeutics by unveiling novel molecular pathways involved in GC carcinogenesis and should be one of the urgent agenda items in medical research in light of the overwhelming social burden of cancer death in Japan and the world.

Acknowledgments

This review is based on research grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan (JST grant), Grants-in-Aid for Scientific Research (KAKENHI) by the Japan Society for the Promotion of Science (No. 23501327), and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio, Grant No. 10–41).

Disclosure Statement

The authors have no conflict of interest.

References

1 Brenner H, Rothenbacher D, Arndt V. Epidemiology of gastric cancer. In: Verma M, eds. *Methods of Molecular Biology, Cancer Epidemiology*, vol. **472**. New Jersey: Humana Press, 2009; 467–77.
2 Yasui W, Sentani K, Motoshita J, Nakayama H. Molecular pathobiology of gastric cancer. *Scand J Surg* 2006; **95**: 225–31.
3 Pilpilidis I, Kountouras J, Zavos C, Katsinelos P. Upper gastrointestinal carcinogenesis: *H. pylori* and stem cell cross-talk. *J Surg Res* 2011; **166**: 255–64.
4 Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; **12**: 354–62.
5 Fock KM, Ang TL. Epidemiology of *Helicobacter pylori* infection and gastric cancer in Asia. *J Gastroenterol Hepatol* 2010; **25**: 479–86.
6 Helicobacter and Cancer CG. Gastric cancer and *Helicobacter pylori*: a combined analysis of 12 case control studies nested within prospective cohorts. *Gut* 2001; **49**: 347–53.
7 Gibson G. Rare and common variants: twenty arguments. *Nat Rev Genet* 2012; **13**: 135–45.
8 Palmer LJ, Cardon LR. Shaking the tree: mapping complex disease genes with linkage disequilibrium. *Lancet* 2005; **366**: 1223–34.
9 Hirakawa M, Tanaka T, Hashimoto Y, Kuroda M, Takagi T, Nakamura Y. JSNP: a database of common gene variations in the Japanese population. *Nucleic Acids Res* 2002; **30**: 158–62.
10 Teruhiko Y, Yoshimura K. Outline of disease gene hunting approaches in the Millennium Genome Project of Japan. *Proc Japan Acad* 2003; **79B**: 34–50.
11 Yoshida T, Ono H, Kuchiba A, Saeki N, Sakamoto H. Genome-wide germline analyses on cancer susceptibility and GeMDBJ database: gastric cancer as an example. *Cancer Sci* 2010; **101**: 1582–9.
12 Sakamoto H, Yoshimura K, Saeki N *et al.* Genetic variation in *PSCA* is associated with susceptibility to diffuse-type gastric cancer. *Nat Genet* 2008; **40**: 730–40.
13 Saeki N, Saito A, Choi IJ *et al.* A functional single nucleotide polymorphism in mucin 1, at chromosome 1q22, determines susceptibility to diffuse-type gastric cancer. *Gastroenterology* 2011; **140**: 892–902.
14 Wu C, Wang G, Yang M *et al.* Two genetic variants in prostate stem cell antigen and gastric cancer susceptibility in a Chinese population. *Mol Carcinog* 2009; **48**: 1131–8.
15 Matsuo K, Tajima K, Suzuki T *et al.* Association of prostate stem cell antigen gene polymorphisms with the risk of stomach cancer in Japanese. *Int J Cancer* 2009; **125**: 1961–4.
16 Lu Y, Chen J, Ding Y *et al.* Genetic variation of PSCA gene is associated with the risk of both diffuse- and intestinal-type gastric cancer in a Chinese population. *Int J Cancer* 2010; **127**: 2183–9.
17 Ou J, Li K, Ren H, Bai H, Zeng D, Zhang C. Association and haplotype analysis of prostate stem cell antigen with gastric cancer in Tibetans. *DNA Cell Biol* 2010; **29**: 319–23.
18 Lochhead P, Frank B, Hold GL *et al.* Genetic variation in the prostate stem cell antigen gene and upper gastrointestinal cancer in white individuals. *Gastroenterology* 2011; **140**: 435–41.
19 Zeng Z, Wu X, Chen F *et al.* Polymorphisms in prostate stem cell antigen gene rs2294008 increase gastric cancer risk in Chinese. *Mol Carcinog* 2011; **50**: 353–8.
20 Song HR, Kim HN, Piao JM *et al.* Association of a common genetic variant in prostate stem-cell antigen with gastric cancer susceptibility in a Korean population. *Mol Carcinog* 2011; **50**: 871–5.
21 Sala N, Muñoz X, Travier N *et al.* Prostate stem-cell antigen gene is associated with diffuse and intestinal gastric cancer in Caucasians: results from the EPIC-EURGAST study. *Int J Cancer* 2012; **130**: 2417–27.
22 Wu X, Ye Y, Kiemeney LA *et al.* Genetic variation in the prostate stem cell antigen gene *PSCA* confers susceptibility to urinary bladder cancer. *Nat Genet* 2009; **41**: 991–5.
23 Shi Y, Hu Z, Wu C *et al.* A genome-wide association study identifies new susceptibility loci for non-cardia gastric cancer at 3q13.31 and 5p13.1. *Nat Genet* 2011; **43**: 1215–18.
24 Abnet CC, Freedman ND, Hu N *et al.* A shared susceptibility locus in PLCE1 at 10q23 for gastric adenocarcinoma and esophageal squamous cell carcinoma. *Nat Genet* 2010; **42**: 764–7.
25 Reiter RE, Gu Z, Watabe T *et al.* Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. *Proc Natl Acad Sci USA* 1998; **95**: 1735–40.
26 Amara N, Palapattu GS, Schrage M *et al.* Prostate stem cell antigen is overexpressed in human transitional cell carcinoma. *Cancer Res* 2001; **61**: 4660–5.
27 Elsamman EM, Fukumori T, Tanimoto S *et al.* The expression of prostate stem cell antigen in human clear cell renal cell carcinoma: a quantitative reverse transcriptase-polymerase chain reaction analysis. *BJU Int* 2006; **98**: 668–73.
28 Feng HC, Tsao SW, Ngan HY *et al.* Overexpression of prostate stem cell antigen is associated with gestational trophoblastic neoplasia. *Histopathology* 2008; **52**: 167–74.
29 Cao D, Ji H, Ronnett BM. Expression of mesothelin, fascin, and prostate stem cell antigen in primary ovarian mucinous tumors and their utility in differentiating primary ovarian mucinous tumors from metastatic pancreatic mucinous carcinomas in the ovary. *Int J Gynecol Pathol* 2005; **24**: 67–2.
30 Argani P, Rosty C, Reiter RE *et al.* Discovery of new markers of cancer through serial analysis of gene expression: prostate stem cell antigen is overexpressed in pancreatic adenocarcinoma. *Cancer Res* 2001; **61**: 4320–44.

© 2012 Japanese Cancer Association

31 Kawaguchi T, Sho M, Tojo T *et al.* Clinical significance of prostate stem cell antigen expression in non-small cell lung cancer. *Jpn J Clin Oncol* 2010; **40**: 319–26.
32 Geiger KD, Hendruschk S, Rieber EP *et al.* The prostate stem cell antigen represents a novel glioma-associated antigen. *Oncol Rep* 2011; **26**: 13–21.
33 Zhao Z, Ma W, Zeng G, Qi D, Ou L, Liang Y. Small-interference RNA-mediated silencing of prostate stem cell antigen attenuates growth, reduces migration and invasion of human prostate cancer PC-3M cells. *Urol Oncol* 2011. doi:10.1016/j.urolonc.2011.02.004.
34 Bahrenberg G, Brauers A, Joost HG, Jakse G. Reduced expression of PSCA, a member of the LY-6 family of cell surface antigens, in bladder, esophagus, and stomach tumors. *Biochem Biophys Res Commun* 2000; **275**: 783–8.
35 Ono H, Hiraoka N, Lee YS *et al.* Prostate stem cell antigen, a presumable organ-dependent tumor suppressor gene, is down-regulated in gallbladder carcinogenesis. *Genes Chromosom Cancer* 2012; **51**: 30–41.
36 Hattori T. Development of adenocarcinomas in the stomach. *Cancer* 1986; **57**: 1528–34.
37 Fu YP, Kohaar I, Rothman N *et al.* Common genetic variants in the PSCA gene influence gene expression and bladder cancer risk. *Proc Natl Acad Sci U S A.* 2012; **109**: 4974–9.
38 Tanikawa C, Urabe Y, Matsuo K *et al.* A genome-wide association study identifies two susceptibility loci for duodenal ulcer in the Japanese population. *Nat Genet* 2012; **44**: 430–4.
39 Cho SJ, Choi IJ, Kim CG *et al.* Risk factors associated with gastric cancer in patients with a duodenal ulcer. *Helicobacter* 2010; **15**: 516–23.
40 Malfertheiner P. The intriguing relationship of *Helicobacter pylori* infection and acid secretion in peptic ulcer disease and gastric cancer. *Dig Dis* 2011; **29**: 459–64.
41 Ubukata H, Nagata H, Tabuchi T, Konishi S, Kasuga T, Tabuchi T. Why is the coexistence of gastric cancer and duodenal ulcer rare? Examination of factors related to both gastric cancer and duodenal ulcer *Gastric Cancer* 2011; **14**: 4–12.
42 Saeki N, Gu J, Yoshida T, Wu X. Prostate stem cell antigen: a Jekyll and Hyde molecule? *Clin Cancer Res* 2010; **16**: 3533–8.
43 Sharom FJ, Radeva G. GPI-anchored protein cleavage in the regulation of transmembrane signals. *Subcell Biochem* 2004; **37**: 285–315.
44 Bafna S, Kaur S, Batra SK. Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. *Oncogene* 2010; **29**: 2893–904.
45 Gendler SJ. MUC1, the renaissance molecule. *J Mammary Gland Biol Neoplasia* 2001; **6**: 339–53.
46 Carson DD. The cytoplasmic tail of MUC1: a very busy place. *Sci Signal* 2008; **1**: pe35.
47 Behrens ME, Grandgenett PM, Bailey JM *et al.* The reactive tumor microenvironment: MUC1 signaling directly reprograms transcription of CTGF. *Oncogene* 2010; **29**: 5667–77.
48 Lee HS, Lee HK, Kim HS, Yang HK, Kim YI, Kim WH. MUC1, MUC2, MUC5AC, and MUC6 expressions in gastric carcinomas: their roles as prognostic indicators. *Cancer* 2001; **92**: 1427–34.
49 Wang JY, Chang CT, Hsieh JS *et al.* Role of MUC1 and MUC5AC expressions as prognostic indicators in gastric carcinomas. *J Surg Oncol* 2003; **83**: 253–60.
50 Hikita ST, Kosik KS, Clegg DO, Bamdad C. MUC1* mediates the growth of human pluripotent stem cells. *PLoS ONE* 2008; **3**: e3312.
51 Carvalho F, Seruca R, David L *et al.* *MUC1* gene polymorphism and gastric cancer - an epidemiological study. *Glycoconj J* 1997; **14**: 107–11.
52 Silva F, Carvalho F, Peixoto A *et al.* *MUC1* gene polymorphism in the gastric carcinogenesis pathway. *Eur J Hum Genet* 2001; **9**: 548–52.
53 Xu Q, Yuan Y, Sun LP *et al.* Risk of gastric cancer is associated with the *MUC1* 568 A/G polymorphism. *Int J Oncol* 2009; **35**: 1313–20.
54 Jia Y, Persson C, Hou L *et al.* A comprehensive analysis of common genetic variation in *MUC1, MUC5AC, MUC6* genes and risk of stomach cancer. *Cancer Causes Control* 2010; **21**: 313–21.
55 Ng W, Loh AX, Teixeira AS, Pereira SP, Swallow DM. Genetic regulation of MUC1 alternative splicing in human tissues. *Br J Cancer* 2008; **99**: 978–85.
56 Kufe DW. Mucins in cancer: function, prognosis and therapy. *Nat Rev Cancer* 2009; **9**: 874–85.
57 Meyer TE, Verwoert GC, Hwang SJ *et al.* Genome-wide association studies of serum magnesium, potassium, and sodium concentrations identify six loci influencing serum magnesium levels. *PLoS Genet* 2010; **6**: e1001045.
58 Pasternak K, Przyszlak W. Magnesium in stomach cancer. *Magnes Res* 1999; **12**: 139–43.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Genotype and allele frequencies of two single nucleotide polymorphisms, rs2294008 and rs4072037, in 11 ethnic populations.

doi: 10.1111/cas.12042
© 2012 Japanese Cancer Association

Cancer Science
The official journal of the Japanese Cancer Association

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

Takeshi Fujita,[1,2] Kazuyoshi Yanagihara,[1] Fumitaka Takeshita,[3] Kazuhiko Aoyagi,[1] Takao Nishimura,[1] Misato Takigahira,[4] Fumiko Chiwaki,[1] Takeo Fukagawa,[5] Hitoshi Katai,[5] Takahiro Ochiya,[3] Hiromi Sakamoto,[1] Hiroyuki Konno,[2] Teruhiko Yoshida[1] and Hiroki Sasaki[1,6]

[1]Division of Genetics, National Cancer Center Research Institute, Tokyo; [2]Second Department of Surgery, Hamamatsu University School of Medicine, Shizuoka; [3]Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo; [4]Shien Lab, [5]Department of Surgery, National Cancer Center Hospital, Tokyo, Japan

(Received July 6, 2012/Revised October 22, 2012/Accepted October 22, 2012/Accepted manuscript online October 27, 2012/Article first published online December 13, 2012)

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 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′-ttGCUUCACAAUUACACUUAC-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′-AUUGCCUUGCUCUCUCAGCtt-3′ (SI00300146, Qiagen, Valencia, CA, USA) and *MSX2* siRNA, 5′-CCAUUACCUAUAUGCUAAAt t-3′ and 5′-UUUAGCAUAUAGGUAAUGGtt-3′(SI00038031, 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

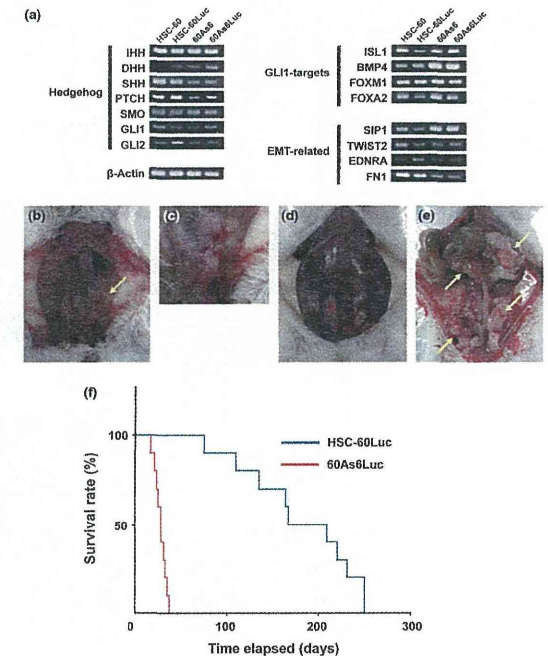

Fig. 1. Characteristics of HSC-60 and 60As6 cells. (a) mRNA expression of hedgehog- and epithelial-mesenchymal transition (EMT)-related gene in HSC-60 and 60As6. Shown are results of reverse transcription-polymerase chain reaction (RT-PCR) of hedgehog ligands (*IHH*, *DHH*, and *SHH*), a receptor (*PTCH*),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

[6]To whom correspondence should be addressed.
E-mail: hksasaki@ncc.go.jp

doi: 10.1111/cas.12054
© 2012 Japanese Cancer Association

© 2012 Japanese Cancer Association

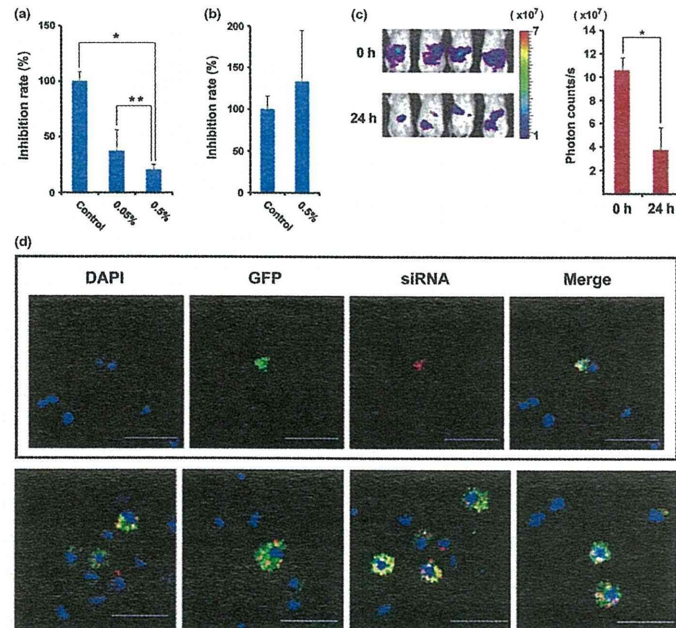

Fig. 2. Evaluation of an atelocollagen-mediated siRNA delivery system by measuring the luciferase activity after i.p injection of luciferase siRNA. (a) Inhibition rates of photon counts 48 h after injection are compared between 0.05% and 0.5% atelocollagen/luciferase siRNA/DharmaFECT1. $*P = 0.006$; $**P = 0.012$. (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 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.

doi: 10.1111/cas.12054
© 2012 Japanese Cancer Association

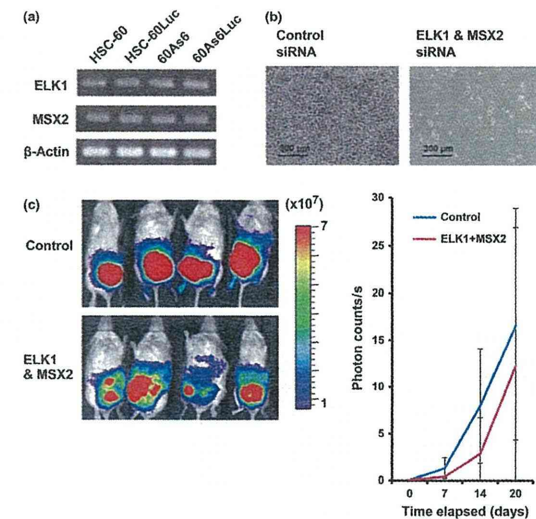

Fig. 3. The expression of cancer-specific hedgehog targets *ELK1* and *MSX2* in HSC-60 and 60As6 and the effect of silencing of *ELK1and MSX2* on *in vitro* and *in vivo* cell growth of 60As6. (a) RT-PCR of *ELK1* and *MSX2* in HSC-60 and 60As6. (b) Cell growth inhibition of 60As6 cells 2 days after 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.

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

© 2012 Japanese Cancer Association

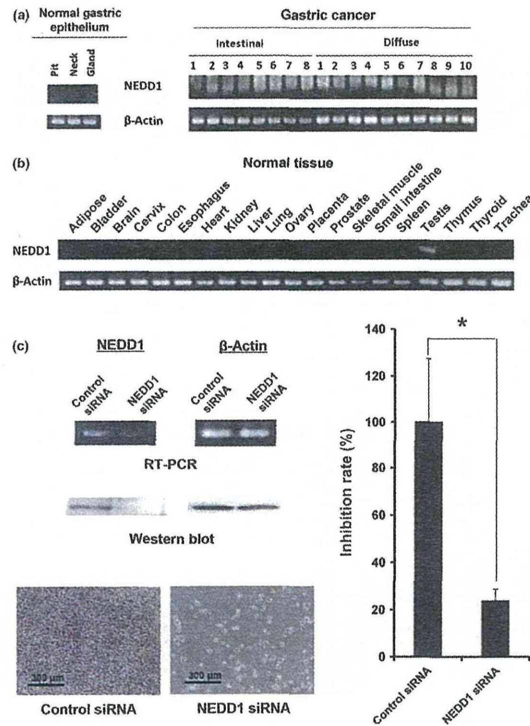

Fig. 4. The expression and silencing of *NEDD1*. (a) *NEDD1* mRNA expression in three regions of normal gastric mucosa (pit, neck, and gland) and 18 primary GCs containing eight intestinal-type and 10 diffuse-type. (b) *NEDD1* mRNA expression in 20 other organs of the human body. (c) Reverse transcription-polymerase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

doi: 10.1111/cas.12054
© 2012 Japanese Cancer Association

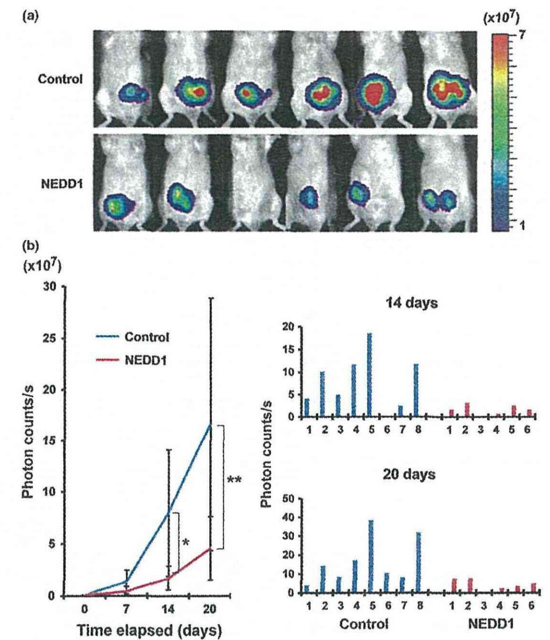

Fig. 5. Inhibition of tumor cell growth in the peritoneal cavity by atelocollagen-mediated *NEDD1* siRNA delivery. (a) Each of six mice treated with *NEDD1* siRNA or vehicle alone as control is visualized at 21 days after inoculation of 60As6Luc. Color bar indicates $\times 10^7$ photon/s. (b) Results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

© 2012 Japanese Cancer Association

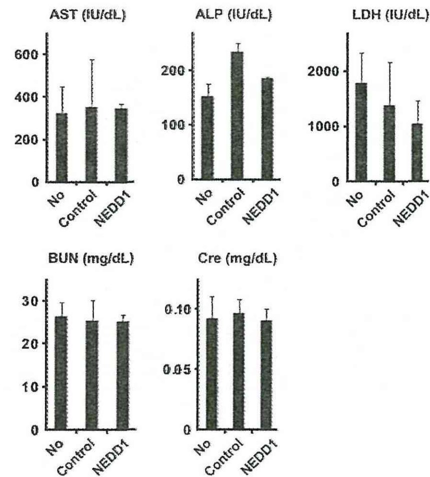

Fig. 6. Evaluation of selected serum chemistries 3 weeks after i.p. administration of *NEDD1* siRNA. No significant difference in the activity of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) and in levels of blood urea nitrogen (BUN) and Cre is observed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

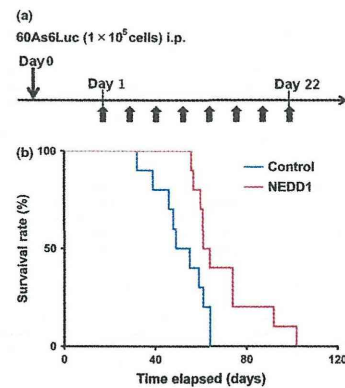

Fig. 7. Effect on survival of mice treated with *NEDD1* siRNA. (a) Schedule of i.p. administration of *NEDD1* siRNA 1 day after inoculation of 1×10^5 60As6Luc cells. (b) The i.p. administration of *NEDD1* siRNA significantly prolongs mice survival ($P = 0.012$).

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 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

This work was supported by of the National Institute of Biomedical Innovation (for the Advanced Research for Medical Products Mining Programme), The Ministry of Health, Labour and Welfare of Japan (for the Third Comprehensive 10-Year Strategy for Cancer Control), and The Princess Takamatsu Cancer Research Fund. Drs T Fujita and T Nishimura are the recipients of a Research Resident Fellowship from the Foundation of Promotion of Cancer Research in Japan.

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 Bormann 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

doi: 10.1111/cas.12054

© 2012 Japanese Cancer Association

© 2012 Japanese Cancer Association

21 Mori K, Suzuki T, Uozaki H *et al.* Detection of minimal gastric cancer cells in peritoneal washings by focused microarray analysis with multiple markers: clinical implications. *Ann Surg Oncol* 2007; **14**: 1694–702.
22 Markman M, Brady MF, Spirtos NM, Hanjani P, Rubin SC. Phase II trial of intraperitoneal paclitaxel in carcinoma of the ovary, tube, and peritoneum: a gynecologic oncology group study. *J Clin Oncol* 1998; **16**: 2620–4.
23 Nishiyama M, Wada S. Docetaxel: its role in current and future treatments for advanced gastric cancer. *Gastric Cancer* 2009; **12**: 132–41.
24 Haren L, Remy MH, Bazin I, Callebaut I, Wright M, Merdes A. NEDD1-dependent recruitment of the gamma-tubulin ring complex to the centrosome is necessary for centriole duplication and spindle assembly. *J Cell Biol* 2006; **172**: 505–15.
25 Inoue M, Matsumoto S, Saito H, Tsujitani S, Ikeguchi M. Intraperitoneal administration of a small interfering RNA targeting nuclear factor-kappa B with paclitaxel successfully prolongs the survival of xenograft model mice with peritoneal metastasis of gastric cancer. *Int J Cancer* 2008; **123**: 2696–701.
26 Takeshita F, Patrawala L, Osaki M *et al.* Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes. *Mol Ther* 2010; **18**: 181–7.
27 Ueda T, Volinia S, Okumura H *et al.* Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *Lancet Oncol* 2010; **11**: 136–46.
28 Yanagihara K, Tsumuraya M. Transforming growth factor β1 induces apoptotic cell death in cultured human gastric carcinoma cells. *Cancer Res* 1992; **52**: 4042–5.
29 Saeki N, Kim DH, Usui T *et al.* GASDERMIN, suppressed frequently in gastric cancer, is a target of LMO1 in TGF-β-dependent apoptotic signalling. *Oncogene* 2007; **26**: 6488–98.

doi: 10.1111/cas.12054
© 2012 Japanese Cancer Association

Molecular and Cellular Biology

Regulatory Nexus of Synthesis and Degradation Deciphers Cellular Nrf2 Expression Levels

Takafumi Suzuki, Tatsuhiro Shibata, Kai Takaya, Kouya Shiraishi, Takashi Kohno, Hideo Kunitoh, Koji Tsuta, Koh Furuta, Koichi Goto, Fumie Hosoda, Hiromi Sakamoto, Hozumi Motohashi and Masayuki Yamamoto
Mol. Cell. Biol. 2013, 33(12):2402. DOI: 10.1128/MCB.00065-13.
Published Ahead of Print 9 April 2013.

Updated information and services can be found at:
http://mcb.asm.org/content/33/12/2402

These include:

SUPPLEMENTAL MATERIAL — Supplemental material

REFERENCES — This article cites 43 articles, 18 of which can be accessed free at: http://mcb.asm.org/content/33/12/2402#ref-list-1

CONTENT ALERTS — Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to to another ASM Journal go to: http://journals.asm.org/site/subscriptions/

Journals.ASM.org

Downloaded from http://mcb.asm.org/ on May 6, 2014 by KOKURITSU GAN CENTER

MCB
Journals.ASM.org

Regulatory Nexus of Synthesis and Degradation Deciphers Cellular Nrf2 Expression Levels

Takafumi Suzuki,[a] Tatsuhiro Shibata,[b] Kai Takaya,[a] Kouya Shiraishi,[c] Takashi Kohno,[c] Hideo Kunitoh,[d] Koji Tsuta,[e] Koh Furuta,[e] Koichi Goto,[f] Fumie Hosoda,[b] Hiromi Sakamoto,[g] Hozumi Motohashi,[h] Masayuki Yamamoto[a,i]

Department of Medical Biochemistry[a] and Center for Radioisotope Sciences,[h] Tohoku University Graduate School of Medicine, and Tohoku Medical-Megabank Organization,[i] Sendai, Japan; Divisions of Cancer Genomics,[b] Genome Biology,[c] and Genetics,[g] National Cancer Center Research Institute, and Divisions of Pathology and Clinical Laboratories, National Cancer Center Hospital,[e] Tokyo, Japan; Department of Respiratory Medicine, Mitsui Memorial Hospital, Tokyo, Japan[d]; Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa City, Chiba, Japan[f]

Transcription factor Nrf2 (NF-E2-related factor 2) is essential for oxidative and electrophilic stress responses. While it has been well characterized that Nrf2 activity is tightly regulated at the protein level through proteasomal degradation via Keap1 (Kelch-like ECH-associated protein 1)-mediated ubiquitination, not much attention has been paid to the supply side of Nrf2, especially regulation of *Nrf2* gene transcription. Here we report that manipulation of *Nrf2* transcription is effective in changing the final Nrf2 protein level and activity of cellular defense against oxidative stress even in the presence of Keap1 and under efficient Nrf2 degradation, determined using genetically engineered mouse models. In excellent agreement with this finding, we found that minor A/A homozygotes of a single nucleotide polymorphism (SNP) in the human *NRF2* upstream promoter region (rs6721961) exhibited significantly diminished *NRF2* gene expression and, consequently, an increased risk of lung cancer, especially those who had ever smoked. Our results support the notion that in addition to control over proteasomal degradation and derepression from degradation/repression, the transcriptional level of the *Nrf2* gene acts as another important regulatory point to define cellular Nrf2 levels. These results thus verify the critical importance of human SNPs that influence the levels of transcription of the *NRF2* gene for future personalized medicine.

The *Nrf2* (NF-E2-related factor 2; or *Nfe2l2*) gene encodes a basic leucine zipper-type transcription factor that belongs to the CNC (cap'n'collar) family (1). Nrf2 displays its transactivation activity through dimerization with one of the small Maf (sMaf) proteins, and the Nrf2-sMaf heterodimer recognizes a specific DNA sequence known as the antioxidant (ARE)/electrophile response element (EpRE) (2, 3). Downstream target genes of Nrf2 include enzymes that act in detoxifying and antioxidative stress responses, enzymes related to glutathione synthesis, and transporters, which together constitute a network to facilitate the cellular adaptation to oxidative and xenobiotic stresses (4, 43). Studies with the *Nrf2* gene knockout ($Nrf2^{-/-}$) mouse clearly demonstrate that Nrf2 deficiency attenuates the response to oxidative and electrophilic stresses (5, 6), resulting in high-level susceptibility to a variety of toxic chemicals and carcinogens (7–9). Similarly, Nrf2-deficient mice are prone to the initiation of carcinogenesis, demonstrating that Nrf2 contributes to cancer chemoprevention (10–12). Conversely, large numbers of cancer cells express high levels of Nrf2, and this fact indicates that cancer cells hijack and exploit Nrf2 activity for their malignant growth (13–15).

One of the important characteristics of Nrf2 is the inducible nature of its function in response to oxidative and electrophilic stresses (16). Under homeostatic and stress-free conditions, cellular Nrf2 abundance is maintained at a very low level, as the ubiquitin E3 ligase complex composed of Keap1 (Kelch-like ECH-associated protein 1) and cullin 3 specifically promotes ubiquitination and proteasomal degradation of Nrf2 (16, 44). Notably, Keap1 acts as a sensor for electrophilic and oxidative stresses by using reactive cysteine residues within the protein (17). Exposure to electrophiles or reactive oxygen species hampers Keap1 activity, reducing Nrf2 ubiquitination and leading to the stabilization and nuclear translocation/accumulation of Nrf2 (17). Subsequently, the expression of a battery of Nrf2 target genes is induced for cytoprotection against these insults. Thus, cellular Nrf2 activity is induced by a derepression mechanism utilizing the proteasomal protein degradation machinery (4).

Multiple lines of evidence support the mechanism of Nrf2 derepression from proteasomal degradation, which accounts for the inducible expression of Nrf2 target genes. On the contrary, changes in the supply side of Nrf2 seem to be less significant under these stress conditions than the derepression/accumulation mechanism of the Nrf2 protein (18). Thus, not much attention has been paid to the contribution of transcriptional regulation of the *Nrf2* gene to the accumulation of Nrf2 protein and inducible expression of its target genes. However, several lines of evidence suggest the importance of the transcriptional regulation of the *Nrf2* gene. For instance, the *Nrf2* mRNA level was found to increase approximately 2-fold 6 h after treatment of an electrophile in murine keratinocytes (19).

A promoter single nucleotide polymorphism (SNP) of the mouse *Nrf2* gene was found to be tightly linked to the sensitivity/

Received 16 January 2013 **Returned for modification** 31 January 2013
Accepted 1 April 2013
Published ahead of print 9 April 2013
Address correspondence to Masayuki Yamamoto, masiyamamoto@med.tohoku.ac.jp.
Takafumi Suzuki and Tatsuhiro Shibata contributed equally to this article.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/MCB.00065-13.
Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/MCB.00065-13

Downloaded from http://mcb.a...org/ on May 6, 201... KOKURITSU GAN CENTER

resistance of various inbred mouse lines to the toxicity of high concentrations (95%) of oxygen (8). Similarly, a variant of the *NRF2* gene in the upstream promoter region (rs6721961) (20) is associated with susceptibility to acute lung injury in humans (21). This human SNP is located in the middle of the ARE motif and weakens the affinity of NRF2 binding to the ARE. This regulatory SNP (rSNP) appears to disrupt the positive-feedback regulation of *NRF2* expression by NRF2 itself (21). Other consequences of this *NRF2* rSNP have also been reported, including the risk of venous thromboembolism (22), reduced vital capacity (23), and an impaired forearm vasodilator response (24).

However, it remains to be determined how significantly the *Nrf2* transcript level affects Nrf2 definitive activity *in vivo*. This is the most critical issue for the future use of this and related *NRF2* SNPs in risk assessment and personalized medicine. Therefore, to address this critical issue, we have exploited genetically engineered mouse models. Our present results unequivocally demonstrate the importance of the level of the Nrf2 supply in both the presence and absence of Keap1-mediated protein degradation regulation. In addition, in order to clarify how significantly the reduction of the *NRF2* mRNA level is linked to the pathogenesis of human diseases, we explored whether the *NRF2* rSNP rs6721961 contributes to the increased risk of non-small-cell lung carcinomas. We compared the incidence of each genotype of the *NRF2* rSNP in a lung cancer population and a control population. We also measured the endogenous expression of *NRF2* in immortalized lymphocytes. We found that the rSNP genotype indeed affects the *NRF2* mRNA level in peripheral lymphocytes and also brings about an increased risk of non-small-cell lung cancers. These results strongly argue that transcription of the *NRF2* gene is an important regulatory point for cellular NRF2 activity.

MATERIALS AND METHODS

Mice. $Nrf2^{-/-}$ and Keap1 gene knockout ($Keap1^{-/-}$) mice were produced and characterized as described previously (6, 25). Transgene construct *KRD*-Nrf2 was generated by subcloning the Flag-hemagglutinin (HA)-tagged mouse Nrf2 cDNA into the vector harboring a 5.7-kb *Keap1* gene regulatory domain (*KRD*) (26). Transgenic mice were generated as described previously (26). Four independent lines were established for *KRD*-Nrf2. All compound mutant mice examined in this study were from a mixed genetic background, with contributions from 129Sv/J, C57BL/6J, and ICR strains. For hematoxylin-eosin (H&E) staining, the esophagi of P10 pups or adult mice were fixed in 3.7% formalin and embedded in paraffin.

Cell culture. Peritoneal macrophages were isolated as described previously (5). Whole-cell extracts were prepared in a lysis buffer (26) and subjected to immunoblot analysis using anti-Nrf2 (27), anti-Flag (Sigma-Aldrich), anti-HA (Roche), and anti-α-tubulin (Sigma-Aldrich) antibodies. Cell viability after 1-chloro-2,4-dinitrobenzene (CDNB) treatment was determined using a Cell Counting Kit-8 (Dojin Laboratories) according to the manufacturer's protocol. Diethyl maleate (DEM) and CDNB were purchased from Wako Pure Chemicals. Menadione and benzyl isothiocyanate (BITC) were purchased from Sigma-Aldrich.

Real-time PCR. Total RNA was prepared from forestomachs or macrophages using an Isogen RNA extraction kit (Nippon Gene) or from immortalized lymphocytes using an RNeasy kit (Qiagen). The cDNAs were synthesized from the total RNA using SuperScript III reverse transcriptase (Invitrogen by Life Technology). Real-time quantitative PCR was performed using an ABI 7300 (Applied Biosystems by Life Technology) or LightCycler 480 (Roche) system. Primer and probe sequences are available upon request.

Study participants. All lung cancer cases and controls were Japanese. These cases received treatments at the National Cancer Center Hospitals (NCCH), Japan, from 2000 to 2008. All surgically collected lung cancer specimens were pathologically examined by at least two board-certified pathologists in the NCCH. Histological diagnosis is based on the WHO classification of lung tumors (28). The controls were volunteers enrolled at the NCCH and at Keio University, located in Tokyo, Japan, with the following inclusion criteria: they could not have lung or other cancers or a history of cancer. All cases and controls, all of whom provided informed consent, were consecutively included in this study without any exclusion criteria. This study was approved by the institutional review boards of the National Cancer Center. Smoking habit was expressed by the number of pack-years, which was defined as the number of cigarette packs smoked daily multiplied by the number of years of smoking. Those who had never smoked (never smokers) were defined as individuals for whom the number of pack-years was 0. Those who had ever smoked (ever smokers) were defined as individuals for whom the number of pack-years was >0 and included both former and current smokers.

SNP analysis. Genomic DNA was extracted from whole blood from lung cancer cases and controls enrolled in the NCCH. Genomic DNA was extracted from Epstein-Barr virus-transformed B lymphocytes derived from whole blood collected from volunteers enrolled at Keio University. Genomic DNA was extracted using a blood maxikit or a QIAamp DNA minikit (Qiagen). The genotypes of NRF2 rSNP rs6721961 (referred to here as NRF2 rSNP-617) were determined by TaqMan SNP genotyping assays (Applied Biosystems by Life Technology).

Detection of somatic *EGFR* and *KRAS* mutations in lung tumors. Tumor samples were obtained at the time of surgery, rapidly frozen in liquid nitrogen, and stored at −80°C. Genomic DNA from the tissues was extracted using a QIAamp DNA minikit (Qiagen). Somatic mutations in the *EGFR* and *KRAS* genes were examined by high-resolution melting analysis (HRMA) as previously described (29).

Statistical analysis. Odds ratios (ORs) and 95% confidence intervals (CIs) for lung adenocarcinoma (ADC) risk were calculated after adjusting for gender, age ($\leq$49, 50 to 59, 60 to 69, and $\geq$70 years), and smoking (never smoker versus ever smoker) by unconditional logistic regression analysis. These analyses were performed using JMP (version 8.0) software (SAS Institute Inc., Cary, NC).

RESULTS

Reflection of *Nrf2* gene dosage on cellular Nrf2 activity. To clarify how significantly reduction of *Nrf2* synthesis affects Nrf2 activity *in vivo*, we decided to exploit genetically engineered mouse models and examine whether transcriptional regulation of the *Nrf2* gene makes a substantial contribution to Nrf2 activity. Because Keap1 represses Nrf2 activity by accelerating the proteasomal degradation of the Nrf2 protein, a *Keap1*-null background provides an ideal model to analyze the gene dosage effect of *Nrf2*. Importantly, in this model system we can ignore the influence of Nrf2 degradation provoked by the Keap1-based ubiquitination of Nrf2. Indeed, *Keap1* gene knockout results in the constitutive accumulation of Nrf2, and the *Keap1*-null background is lethal in pups due to severe hyperkeratosis of the upper digestive tract (25). These phenotypes of the *Keap1*-null mice can be restored by simultaneous deletion of the *Nrf2* gene, indicating that the *Keap1*-null phenotype is attributable to the hyperactivation of Nrf2 (25).

When we deleted the *Nrf2* gene heterozygously in Keap1-deficient (i.e., $Keap1^{-/-}$::$Nrf2^{+/-}$) mice, we found a partial rescue of the severe phenotype of *Keap1*-null mice in the compound mutant mice. In contrast to the *Keap1*-null ($Keap1^{-/-}$::$Nrf2^{+/+}$) mice, the $Keap1^{-/-}$::$Nrf2^{+/-}$ mice survived to adulthood, as was the case for *Keap1*-null mice with the complete knockout of Nrf2 ($Keap1^{-/-}$::$Nrf2^{-/-}$). This indicates that deletion of a single allele

Downloaded from http://mcb.asm.org/ on May 6, 2014 by KOKURITSU GAN CENTER