been revealed by gene expression profiling studies [44,45]. Interestingly, one of these reports has shown that, using a high concentration of siRNA for transfection, siRNAs against different target molecules cause a nonspecific induction (not knockdown) of a large number of common genes, including some apoptosis-related genes, for example, *BAK, bcl-2,* and *death-associating protein kinase* (*DAPK*), or stress-response genes [44]. It is possible that nonspecific induction of such molecules may contribute to the delay in tumor formation observed following inoculation of cells infected with Adv-siGL3B. Further studies might be needed to both determine and refine the specificity of siRNA transduced by expression vectors, including adenoviral vectors.

In the present study, we developed recombinant adenoviral vectors encoding siRNA against the survivin transcript and explored the impact of these novel vectors on three representative cancer cell lines. In all cell lines examined, a knockdown of survivin expression was confirmed following infection with these adenoviruses, which resulted in apoptotic cell death. Cells infected with these vectors nearly lost their tumorigenicity following inoculation into nude mice. Intratumoral injection with these vectors significantly suppressed tumor growth in a mouse xenograft model. This novel strategy may be a promising tool for cancer gene therapy.

Materials and Methods

Cell culture. The human embryonic kidney 293T cells were obtained from GenHunter (Nashville, TN, USA) and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 0.2% sodium bicarbonate, 2 mM glutamine, at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. The human cancer cell lines, HeLa, U251, and MCF-7, were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained under conditions recommended by this organization.

Plasmid vectors. Tandem-type siRNA expression vectors in this study were constructed as described previously [12]. In brief, a human U6 promoter-driven expression cassette for the antisense strand was amplified by PCR and inserted into a plasmid vector containing another U6 promoter-driven expression cassette for the sense strand, resulting in a plasmid vector harboring two separate U6 promoter-driven expression units for each RNA strand of siRNA. Sequences inserted immediately downstream of the U6 promoter were as follows (only sense sequence shown): psiGL3B [12] against firefly luciferase, 5′-GTGCGCTGCTGGTGCCAAC-3′; psiSurv(A) against survivin, 5′-GAGCCAAGAACAAAATTGC-3′; psiSurv(B) against survivin, 5′-GAAAGTGCGCCGTGCCATC-3′. pUC19, a control vector without a U6 promoter-driven expression cassette, was obtained from New England Biolabs (Beverly, MA, USA). Transfection of plasmid DNA was carried out by lipofection using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer.

Adenoviral vectors. All the recombinant adenoviral vectors used in this study were based on the E1- and E3-deleted serotype 5 adenovirus with a modified fiber F/RGD, harboring an integrin-binding RGD-motif within the HI loop of its knob protein [35]. In the first step, a 14,896-bp *Eco*RI fragment (including the right side of the adenoviral genome) of pWEAxKM-F/RGD [35] was joined with a 24,505-bp *Eco*RI fragment (including the left side of the adenoviral genome) of pI_{RI} [46], generating the cosmid vector pWEAx-F/RGD. Then, a 592-bp *Eco*RI–*Hin*dIII fragment, which included the tandemly arranged U6 promoter-driven siRNA expression cassette of psiGL3B, psiSurv(A), or psiSurv(B), was blunt-ended and inserted into the *Swa*I site of pWEAx-F/RGD according to the direction shown in Fig. 2, resulting in the cosmid vectors pWEAx-siGL3B-F/RGD, pWEAxsiSurv(A)-F/RGD, and pWEAxsiSurv(B)-F/RGD, respectively. For the generation of recombinant adenovirus, each cosmid was transfected into 293 cells by lipofection using Lipofectamine2000 reagent (Invitrogen). Plaques arising from the transfected 293 cells were isolated and evaluated by restriction enzyme digestion of the viral genome and sequencing of the expression units. The resultant adenoviral vectors, Ax-F/RGD (Adv-Null), a control adenovirus devoid of an expression cassette, and AxsiGL3B-F/RGD (Adv-siGL3B), AxsiSurv(A)-F/RGD (Adv-siSurv(A)), and AxsiSurv(B)-F/RGD (Adv-siSurv(B)), all carrying expression cassettes for siRNA against corresponding target transcripts, were expanded in 293 cells and purified by cesium chloride ultracentrifugation [47]. Purified viruses were dialyzed in phosphate-buffered saline (PBS) with 10% glycerol and stored at −70°C until use. To determine the viral concentration (pt/ml), the viral solution was incubated in 0.1% sodium dodecyl sulfate (SDS) and A_{260} measured [48]. The concentration was defined as pt/ml = $A_{260} \times (1.1 \times 10^{12})$. Before use, contamination with replication-competent viruses in the viral stock was ruled out by PCR analysis using primers specific for E1A, forward primer 5′-ATTACCGAAGAAATGGCCGC-3′, reverse primer 5′-CCCATTTAACACGCCATGCA-3′; E1B, forward primer 5′-CGGCTGCTGTTGCTTTTTG-3′, reverse primer 5′-GTATCTTCATCGCTAGAGCC-3′; and E2B (positive control), forward primer 5′-TCGTTTCTCAGCAGCTGTTG-3′, reverse primer 5′-CATCTGAACTCAAAGCGTGG-3′ [49]. Adenoviral infection was performed essentially as described previously [50]. All infection experiments were performed at 3000 pt/cell, a condition that enabled almost 100% transduction of transgenes into the cancer cells used in this study (data not shown). In a preliminary experiment, the transduction efficiency at 100 pt/cell was 96% for HeLa cells, 79% for U251 cells, and 56% for MCF-7 cells, determined using an EGFP-expressing adenoviral vector with F/RGD (data not shown).

RT-PCR analysis. Total RNA from transfected cells was isolated using the RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed at 42°C for 60 min using oligo$(dT)_{12-18}$ (Amersham, Little Chalfont, UK). The primers for PCR amplification used in this study were as follows: survivin, forward primer 5′-ACGAGCCAGACTTGGCCCAGTGTT-3′, reverse primer 5′-TCAATCCATGGCAGCCAGCTGCTC-3′; GAPDH (internal control), forward primer 5′-CCACCCATGGCAAATTCCATGGCA-3′, reverse primer 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. The PCR conditions were 96°C for 0.5 min, 65°C for 0.5 min, and 72°C for 1 min, over 24 cycles. PCR products were visualized with ethidium bromide on 2% agarose gels.

Immunoblot analysis. Total cell lysates from transfected cells were extracted with lysis buffer containing 10 mM Tris–HCl (pH 8.0), 0.2% NP-40, 1 mM EDTA, and 2% SDS. Immunoblot analysis was then carried out essentially as described previously [50]. The primary antibody was mouse anti-survivin monoclonal antibody (2802; Cell Signaling Technology, Beverly, MA, USA), rabbit anti-XIAP polyclonal antibody (X-2503; Sigma, St. Louis, MO, USA), rabbit anti-caspase-3 polyclonal antibody (9662; Cell Signaling Technology), or mouse anti-β-actin monoclonal antibody (A-5441; Sigma). The secondary antibody was either horseradish peroxidase-conjugated rabbit anti-mouse IgG+A+M (H+L) (Zymed Laboratories, San Francisco, CA, USA), in the case of samples treated with primary antibodies to survivin or β-actin, or donkey anti-rabbit IgG, peroxidase-linked species-specific $F(ab')_2$ fragment (Amersham), in the case of samples treated with primary antibodies to caspase-3 or XIAP. Chemiluminescence detection was carried out using the ECL Kit (Amersham) according to the manufacturer's instructions.

Cell cycle analysis. Cell cycle analysis was performed on cells following propidium iodide incorporation as described previously [50]. Briefly, cells were removed by trypsinization 6 days after adenoviral infection, washed

Copyright © The American Society of Gene Therapy

in PBS, and fixed with 70% ethanol. Fixed samples were centrifuged, treated with RNase (0.25 mg/ml), and resuspended in propidium iodide (50 μg/ml). Propidium iodide-stained cells were analyzed by a FACS Calibur (Becton–Dickinson, San Jose, CA, USA) flow cytometer.

Colony forming assay. Twenty-four hours after adenoviral infection, cells were seeded onto six-well culture plates at a concentration of 10^2 or 10^3 cells per well and were cultured for 12 days. Following removal of the medium, the wells were rinsed twice with PBS. Glutaraldehyde (1.25%) in PBS was added to each well and the plates were incubated for 30 min at room temperature to allow for cell fixation. After two rinses with distilled water, 0.05% methylene blue solution was added to each well and plates were incubated for 30 min at room temperature to facilitate staining of the colonies. After two rinses with distilled water, the plates were dried and photographed. For quantification of cell growth, stained colonies were solubilized by adding 1 ml of 0.33 N HCl solution per well. Following sampling of 100 μl of the resultant solution, the A_{655} was determined.

Animal experiments. All mice ware fed *ad libitum* and received human care in compliance with the institution's guidelines for the care and use of laboratory animals in research. Five-week-old female BALB/cAnNCjr-nu/nu mice were purchased from Japan Charles River (Yokohama, Japan). In tumorigenicity experiments, HeLa cells were infected with Adv-Null, Adv-siGL3B, or Adv-siSurv(B) at 3000 pt/cell. Twenty-four hours later, 5×10^6 infected cells were suspended in 50 μl of PBS and injected subcutaneously into the right flank of each mouse. The tumor diameter was measured, and the volume (product of 0.4 × largest diameter × smallest diameter × smallest diameter) was calculated. Mice were humanely killed following development of a tumor larger than 2000 mm^3 or a tumor harboring an ulcer. In *in vivo* treatment experiments, 5×10^6 U251 cells were suspended in 100 μl of PBS and injected subcutaneously into the right flank of each mouse. When the tumor size reached about 5 to 7 mm in diameter, six mice in each group received the first intratumoral injection of Adv-Null, Adv-siGL3B, or Adv-siSurv(B), each at 10^{10} pt/tumor. The volume of tumors was recorded as mentioned above from the first injection (day 0). Intratumoral injections of the adenoviral vectors at the same dose were repeated as indicated in Fig. 7.

Statistical analysis. Statistical comparison of the data was performed using a Student *t* test. A *P* value of less than 0.05 was considered to be significant.

Acknowledgments

We thank Aiko Kuroishi, Hanae Inoue, and Yuko Tani for technical assistance. This work was supported in part by a Grant-in-Aid for JSPS Fellows to H.U. and grants to H.H. from the Ministry of Education, Science, and Culture of Japan and from the Terumo Foundation, Kanagawa, Japan.

RECEIVED FOR PUBLICATION SEPTEMBER 9, 2003; ACCEPTED MAY 4, 2004.

References

1. Fire, A., *et al.* (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391:** 806–811.
2. Sharp, P. A. (2001). RNA interference—2001. *Genes Dev.* **15:** 485–490.
3. Hutvagner, G., and Zamore, P. D. (2002). RNAi: nature abhors a double-strand. *Curr. Opin. Genet. Dev.* **12:** 225–232.
4. Hannon, G. J. (2002). RNA interference. *Nature* **418:** 244–251.
5. Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409:** 363–366.
6. Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404:** 293–296.
7. Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., and Hannon, G. J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293:** 1146–1150.
8. Elbashir, S. M., *et al.* (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:** 494–498.
9. Manche, L., Green, S. R., Schmedt, C., and Mathews, M. B. (1992). Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol. Cell. Biol.* **12:** 5238–5248.
10. Minks, M. A., West, D. K., Benvin, S., and Baglioni, C. (1979). Structural requirements of double-stranded RNA for the activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. *J. Biol. Chem.* **254:** 10180–10183.
11. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296:** 550–553.
12. Miyagishi, M., and Taira, K. (2002). U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol.* **20:** 497–500.
13. Lee, N. S., *et al.* (2002). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat. Biotechnol.* **20:** 500–505.
14. Paul, C. P., Good, P. D., Winer, I., and Engelke, D. R. (2002). Effective expression of small interfering RNA in human cells. *Nat. Biotechnol.* **20:** 505–508.
15. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* **16:** 948–958.
16. Sui, G., *et al.* (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99:** 5515–5520.
17. Yu, J. Y., DeRuiter, S. L., and Turner, D. L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99:** 6047–6052.
18. McManus, M. T., Petersen, C. P., Haines, B. B., Chen, J., and Sharp, P. A. (2002). Gene silencing using micro-RNA designed hairpins. *RNA* **8:** 842–850.
19. Kawasaki, H., and Taira, K. (2003). Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. *Nucleic Acids Res.* **31:** 700–707.
20. Crook, N. E., Clem, R. J., and Miller, L. K. (1993). An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol.* **67:** 2168–2174.
21. Liston, P., *et al.* (1996). Suppresion of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* **379:** 349–353.
22. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* **388:** 300–304.
23. Takahashi, R., *et al.* (1998). A single BIR domain of XIAP sufficient for inhibiting caspases. *J. Biol. Chem.* **273:** 7787–7790.
24. Ambrosini, G., Adida, C., and Altieri, D. C. (1997). A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med.* **3:** 917–921.
25. Reed, J. C., and Bischoff, J. R. (2000). BIRinging chromosomes through cell division—and survivin' the experience. *Cell* **102:** 545–548.
26. Li, F., *et al.* (1998). Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* **396:** 580–584.
27. Tamm, I., *et al.* (1998). IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res.* **58:** 5315–5320.
28. Kobayashi, K., Hatano, M., Otaki, M., Ogasawara, T., and Tokuhisa, T. (1999). Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. *Proc. Natl. Acad. Sci. USA* **96:** 1457–1462.
29. Lu, C. D., Altieri, D. C., and Tanigawa, N. (1998). Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. *Cancer Res.* **58:** 1808–1812.
30. Mesri, M., Wall, N. R., Li, J., Kim, R. W., and Altieri, D. C. (2001). Cancer gene therapy using a survivin mutant adenovirus. *J. Clin. Invest.* **108:** 981–990.
31. Ambrosini, G., Adida, C., Sirugo, G., and Altieri, D. C. (1998). Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J. Biol. Chem.* **273:** 11177–11182.
32. Olie, R. A., *et al.* (2000). A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. *Cancer Res.* **60:** 2805–2809.
33. Pennati, M., *et al.* (2002). Ribozyme-mediated attenuation of survivin expression sensitizes human melanoma cells to cisplatin-induced apoptosis. *J. Clin. Invest.* **109:** 285–286.
34. Choi, K. S., Lee, T. H., and Jung, M. H. (2003). Ribozyme-mediated cleavage of the human survivin mRNA and inhibition of antiapoptotic function of survivin in MCF-7 cells. *Cancer Gene Ther.* **10:** 87–95.
35. Nakamura, T., Sato, K., and Hamada, H. (2002). Effective gene transfer to human melanomas via integrin-targeted adenoviral vectors. *Hum. Gene Ther.* **13:** 613–626.
36. Velculescu, V. E., *et al.* (1999). Analysis of human transcriptomes. *Nat. Genet.* **23:** 387–388.
37. Riedl, S. J., *et al.* (2001). Structural basis for the inhibition of caspase-3 by XIAP. *Cell* **104:** 791–800.
38. Shi, Y. (2000). Survivin structure: crystal unclear. *Nat. Struct. Biol.* **7:** 620–623.
39. Marusawa, H., *et al.* (2003). HBXIP functions as a cofactor of survivin in apoptosis suppression. *EMBO J.* **22:** 2729–2740.
40. Song, Z., Yao, X., and Wu, M. (2003). Direct interaction between survivin and Smac/

Copyright © The American Society of Gene Therapy

DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. *J. Biol. Chem.* **278:** 23130–23140.
41. Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *EMBO J.* **20:** 6877–6888.
42. Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P., and Sharp, P. A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev.* **13:** 3191–3197.
43. Hutvagner, G., and Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297:** 2056–2060.
44. Semizarov, D., *et al.* (2003). Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl. Acad. Sci. USA* **100:** 6347–6352.
45. Jackson, A. L., *et al.* (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **21:** 635–637.
46. Yoshida, Y., *et al.* (1998). Generation of fiber-mutant recombinant adenoviruses for gene therapy of malignant glioma. *Hum. Gene Ther.* **9:** 2503–2515.
47. Kanegae, Y., *et al.* (1995). Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic Acids Res.* **23:** 3816–3821.
48. Nyberg-Hoffman, C., Shabram, P., Li, W., Giroux, D., and Aguilar-Cordova, E. (1997). Sensitivity and reproducibility in adenoviral infectious titer determination. *Nat. Med.* **3:** 808–811.
49. Zhang, W. W., Koch, P. E., and Roth, J. A. (1995). Detection of wild-type contamination in a recombinant adenoviral preparation by PCR. *Biotechniques* **18:** 444–447.
50. Uchida, H., *et al.* (2003). 5-Fluouracil efficiently enhanced apoptosis induced by adenovirus-mediated transfer of caspase-8 in DLD-1 colon cancer cells. *J. Gene Med.* **5:** 287–299.

Copyright © The American Society of Gene Therapy