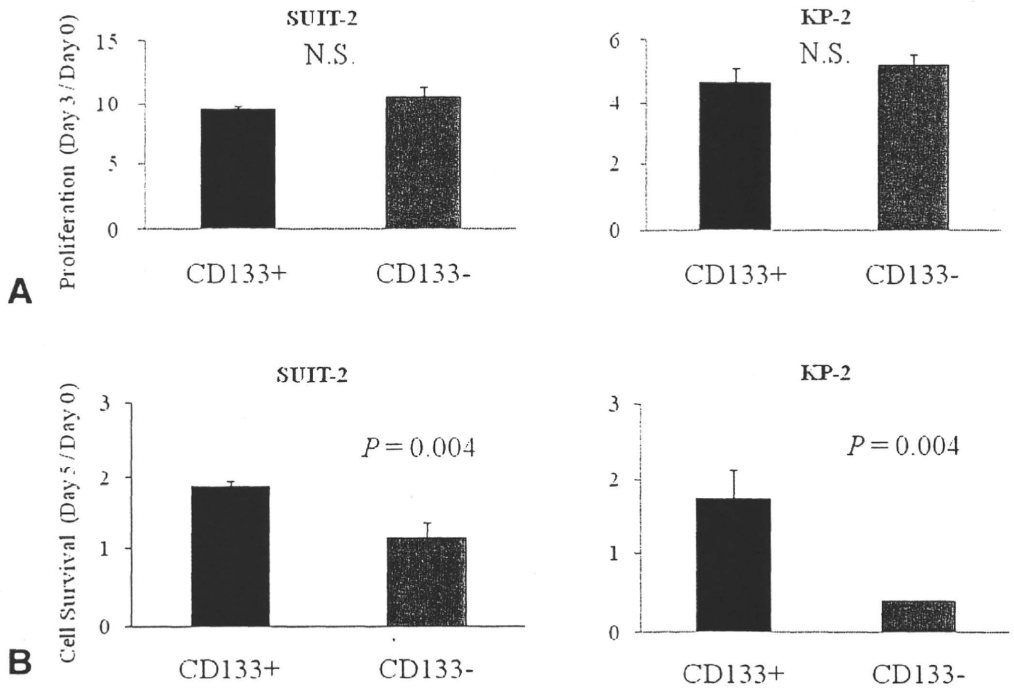

Figure 2. Cell proliferation and cell survival of pancreatic cancer cells are shown. The propidium iodide assay was used to compare cell proliferation and cell survival between $CD133^+$ and $CD133^-$ cells. Two kinds of pancreatic cancer cell lines, SUIT-2 and KP-2, were used and sorted by CD133 expression. (A) In anchorage-dependent conditions, there was no significant difference in proliferation between $CD133^+$ and $CD133^-$ cells. (B) In anchorage-independent conditions (floating culture), cell survival of $CD133^+$ cells was significantly increased compared with $CD133^-$ cells in both SUIT-2 and KP-2 cells (P=.004). Each value represents the mean ± standard deviation of triplicate measurements. N.S. indicates not significant.

effective (Fig. 5A), and no morphological differences between these transfected cells were observed.

Down-regulation of CXCR4 significantly decreased cell migration and invasion only in $CD133^+$ cells cocultured with pancreatic stromal cells (CAF-3) (Fig. 5B-E). These data suggest that the CXCR4/SDF-1 pathway strongly affects the migration and invasion ability of $CD133^+$ cells. We also evaluated several invasion-related genes, and found that $CD133^+$ cells expressed slightly increased levels of FOXM1, MMP-9, and VEGF-A mRNA (data not shown). These results suggest that $CD133^+$ cells may have several pathways influencing cell invasion in addition to the CXCR4/SDF-1 axis.

DISCUSSION

In the present study, we have evaluated the biological function of $CD133^+$ cells in pancreatic cancer, and obtained the first data regarding the specific relationship between $CD133^+$ cells and primary pancreatic stromal cells. We found that CD133 expression was increased in pancreatic cancer tissue compared with normal pancreatic tissues as well as in cancer cell lines compared with normal pancreatic epithelial cells, and that $CD133^+$ was a marker of high proliferative potential in floating cultures, migration, and invasion. In particular, migration and invasion of $CD133^+$ cells cocultured with primary pancreatic stromal cells was greatly enhanced.

Recently, CD133 expression has been shown to be a prognostic marker for poor survival in patients with colon cancer,[23,24] and brain tumors.[25,26] Maeda et al.[27] also reported that CD133 expression is correlated with lymph node metastasis, vascular endothelial growth factor-C expression, and poor prognosis in pancreatic cancer. As well as these previous studies, our data also suggest that $CD133^+$ cells are involved in pancreatic tumor progression, through processes such as invasion and migration.

In a further step, we evaluated the relationship between $CD133^+$ cancer cells and stromal cells, focusing on the CXCR4/SDF-1 axis. Previously, some studies reported that CXCR4/SDF-1 contributed to tumor progression. The CXCR4/SDF-1 axis promotes migration and invasion in breast cancer.[28] We have also previously reported that DNA methylation influenced CXCR4 expression in pancreatic cancer.[29] It is possible that these findings provide new insights into the role of CXCR4/

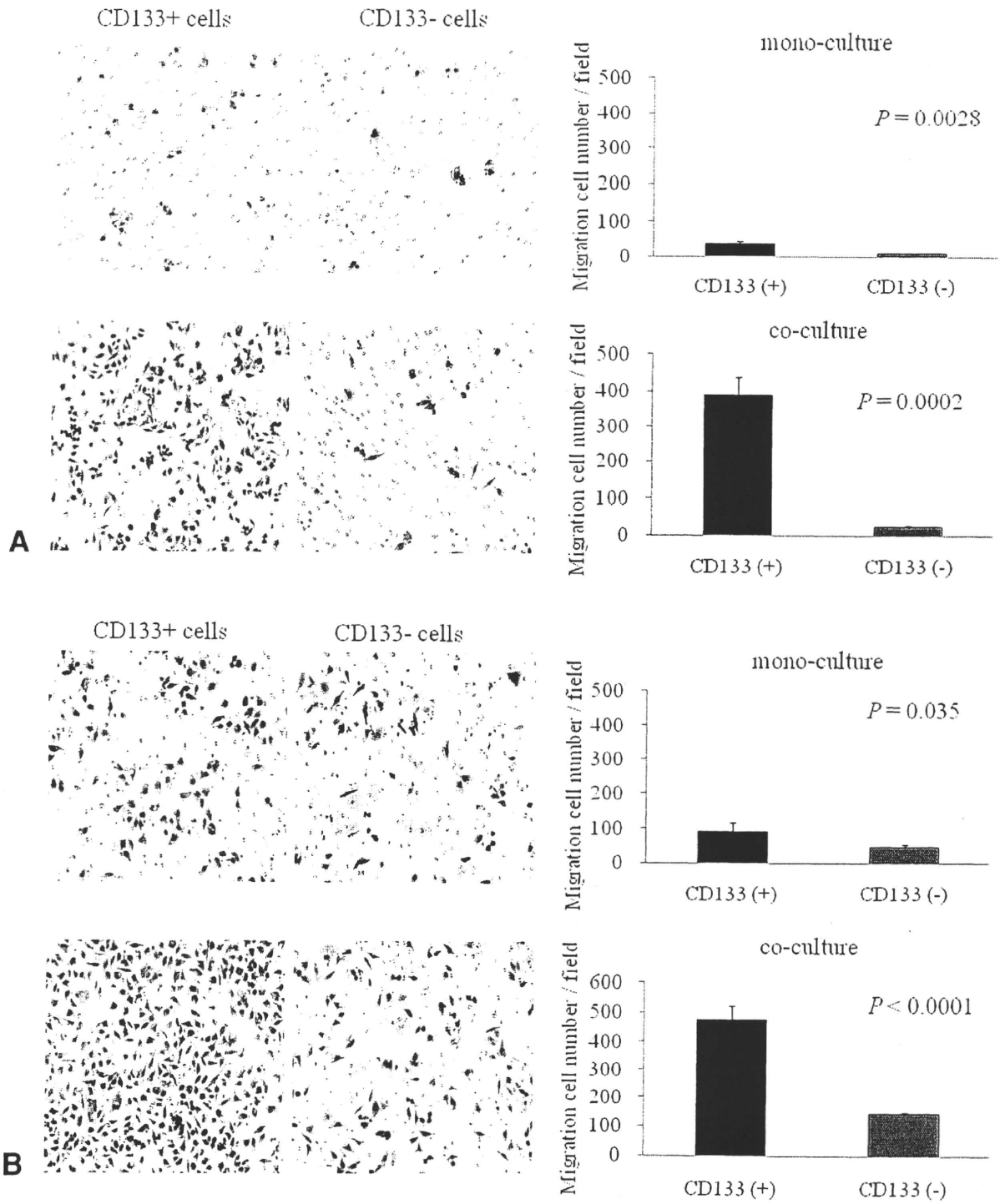

Figure 3. Cell migration and invasion of $CD133^+$ and $CD133^-$ cells in pancreatic cancer are shown. (A) Migration of SUIT-2 $CD133^+$ cells increased slightly compared with $CD133^-$ cells in monoculture conditions ($P=.0028$), and increased markedly when cocultured with pancreatic stromal cells ($P=.0002$). (B) Migration of KP-2 $CD133^+$ cells increased slightly compared with $CD133^-$ cells in monoculture conditions ($P=.035$), and increased markedly when cocultured with pancreatic stromal cells ($P < .0001$). (C, D) A comparison of invasive ability of $CD133^+$ and $CD133^-$ cells is shown. (C) The invasive ability of SUIT-2 $CD133^+$ cells increased slightly compared with $CD133^-$ cells in monoculture conditions ($P=.12$), and increased markedly when cocultured with pancreatic stromal cells ($P < .0001$). (D) The invasive ability of KP-2 $CD133^+$ cells increased slightly compared with $CD133^-$ cells in monoculture conditions ($P=.035$), and increased markedly when cocultured with pancreatic stromal cells ($P < .0001$).

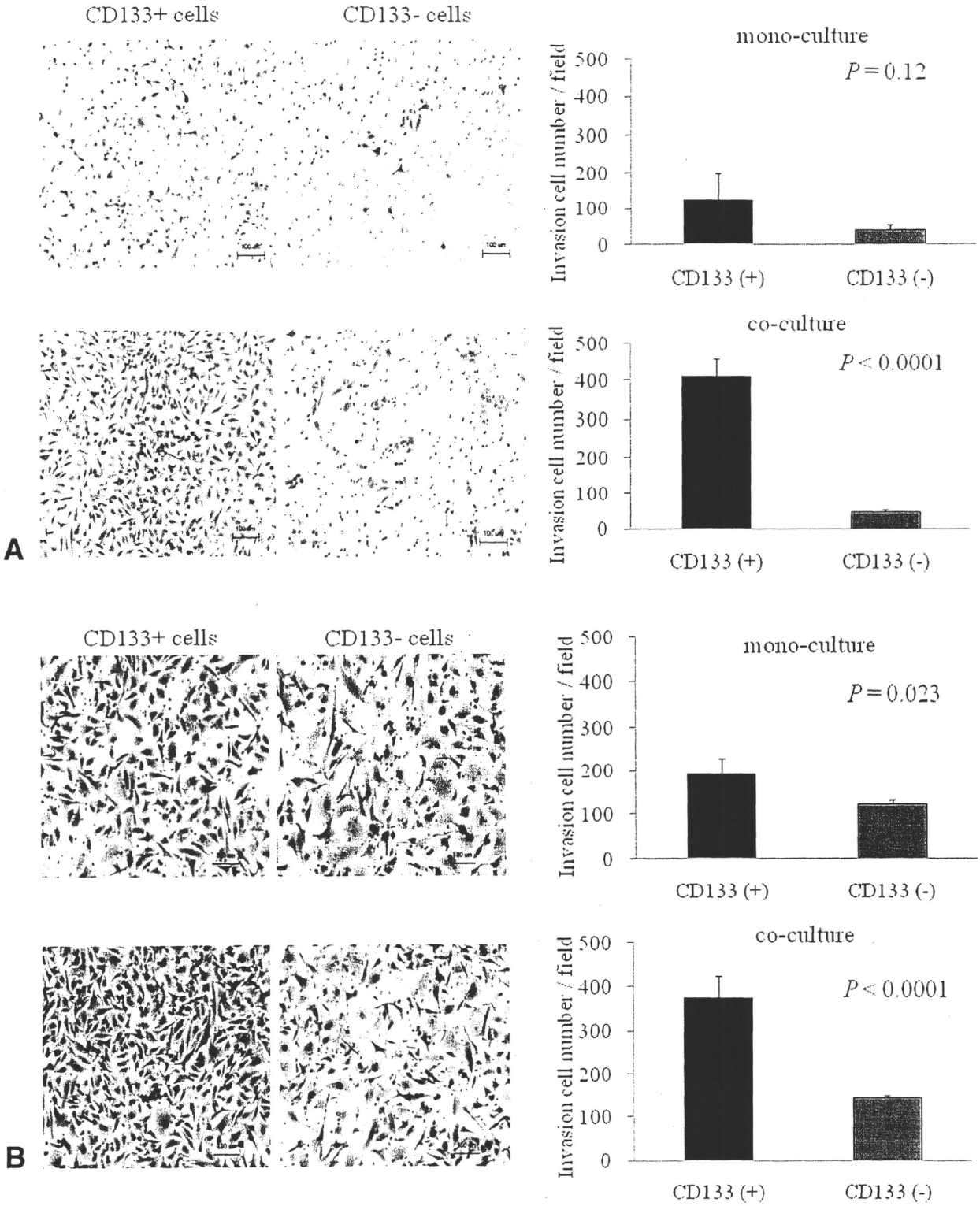

Figure 3. (*Continued*).

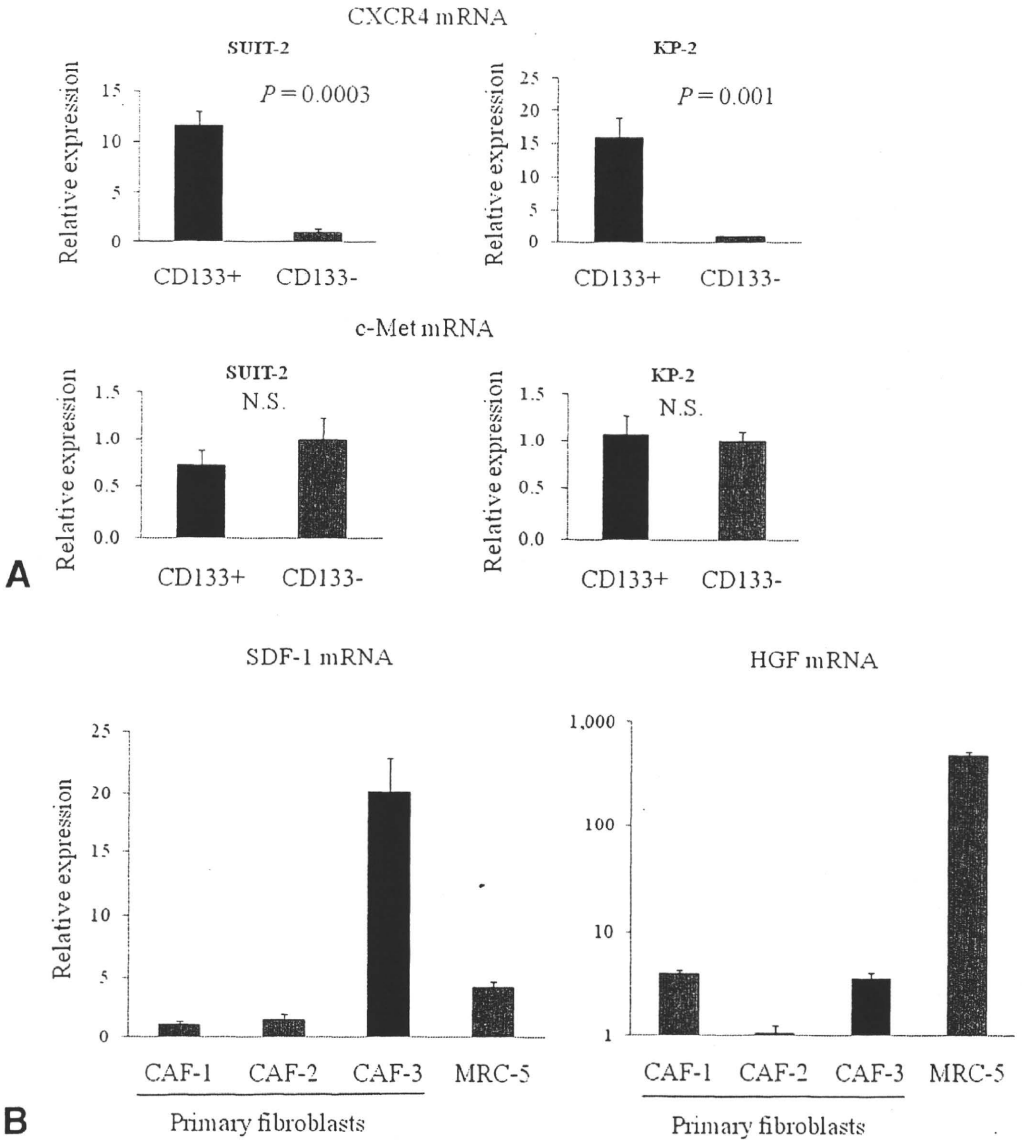

Figure 4. Levels of chemokine-related receptor-4 (CXCR4) and c-Met mRNA in pancreatic cancer are shown. (A) CXCR4 mRNA was assessed, and a significant difference between $CD133^+$ and $CD133^-$ cells in both SUIT-2 and KP-2 cells was found ($P = .0003$ and .001, respectively). However, there was no significant difference in c-Met mRNA expression. (B) Stromal cell-derived factor-1 (SDF-1) and hepatocyte growth factor (HGF) mRNA were expressed in the pancreatic stromal cells used in this study. N.S. indicates not significant.

SDF-1 interaction in tumor progression. Other studies have demonstrated that the CXCR4/SDF-1 axis is involved in tumor progression, influencing cell invasion, metastasis, and neoangiogenesis, in acute myeloid leukemia,[30] glioma,[31] breast cancer,[32] ovarian cancer,[33] and others. CXCR4 expression is associated with poor survival in colon cancer,[34] malignant melanoma,[35] and sarcoma.[36,37] In this study, we found that CD133 expression correlated with CXCR4 expression, and that $CD133^+$ cells exhibited markedly increased cell invasiveness compared with $CD133^-$ cells when cocultured with pancreatic stromal cells secreting SDF-1. The data suggest that $CD133^+$ cells increase tumor progression via the CXCR4/SDF-1 axis through tumor/stromal cell interaction in pancreatic cancer. There were no differences in the expression of c-Met mRNA between $CD133^+$ cells and $CD133^-$ cells (Fig. 4A), suggesting that the differences in stromal cell-enhanced invasion between $CD133^+$ cells and $CD133^-$ cells is not dependent on the differences in activation of c-Met/HGF pathway. However, there may be contribution of other signaling molecules, which were not examined here.

Although some studies have demonstrated that high expression levels of specific adenosine triphosphate-binding cassette drug transporters increase resistance of

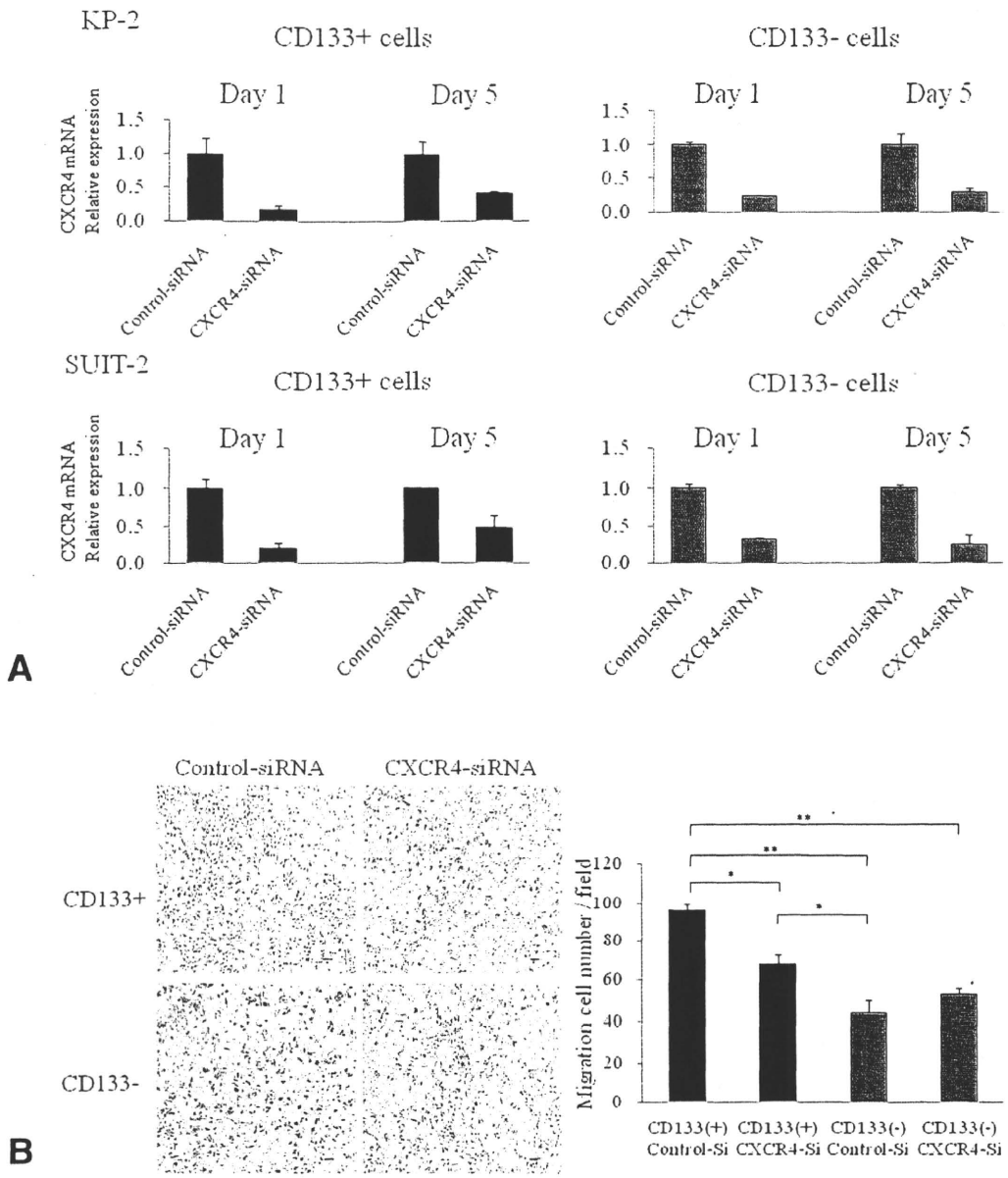

Figure 5. The effect of chemokine-related receptor-4 (CXCR4) on cell migration and Matrigel invasion in CD133$^+$ cells cocultured with pancreatic stromal cells is shown. (A) Levels of CXCR4 mRNA in CD133$^+$ and CD133$^-$ cells transfected with CXCR4-siRNA or control-siRNA at 24 hours (Day 1) and 120 hours (Day 5) in KP-2 and SUIT-2 cells are shown. Transfection with CXCR4-siRNA led to 0.3-fold lower levels of CXCR4 mRNA than the cells transfected with control-siRNA in both CD133$^+$ and CD133$^-$ cells. (B, C) Down-regulation of CXCR4 significantly decreased cell migration in CD133$^+$ cells cocultured with pancreatic stromal cells in KP-2 cells and SUIT-2 cells (*$P < .05$, **$P < .01$). (D, E) CXCR4 down-regulation decreased Matrigel invasion, especially in CD133$^+$ cells cocultured with pancreatic stromal cells in KP-2 cells and SUIT-2 cells(*$P < .05$, **$P < .01$, ***$P < .001$).

CD133 cancer stem cells to chemotherapeutic agents in hepatocellular carcinoma[15] and brain tumors,[16] we found no difference in chemoresistance between CD133$^+$ cells and CD133$^-$ cells (data not shown). Collectively, our data suggest that CD133$^+$ cells possess more aggressive behavior, such as increased cell proliferation, migration, and invasion, especially when cocultured with pancreatic stromal cells. The targeting therapy for the interaction between CD133$^+$ cancer cells and stromal cells may be a new approach to the treatment of pancreatic cancer.

CONFLICT OF INTEREST DISCLOSURES

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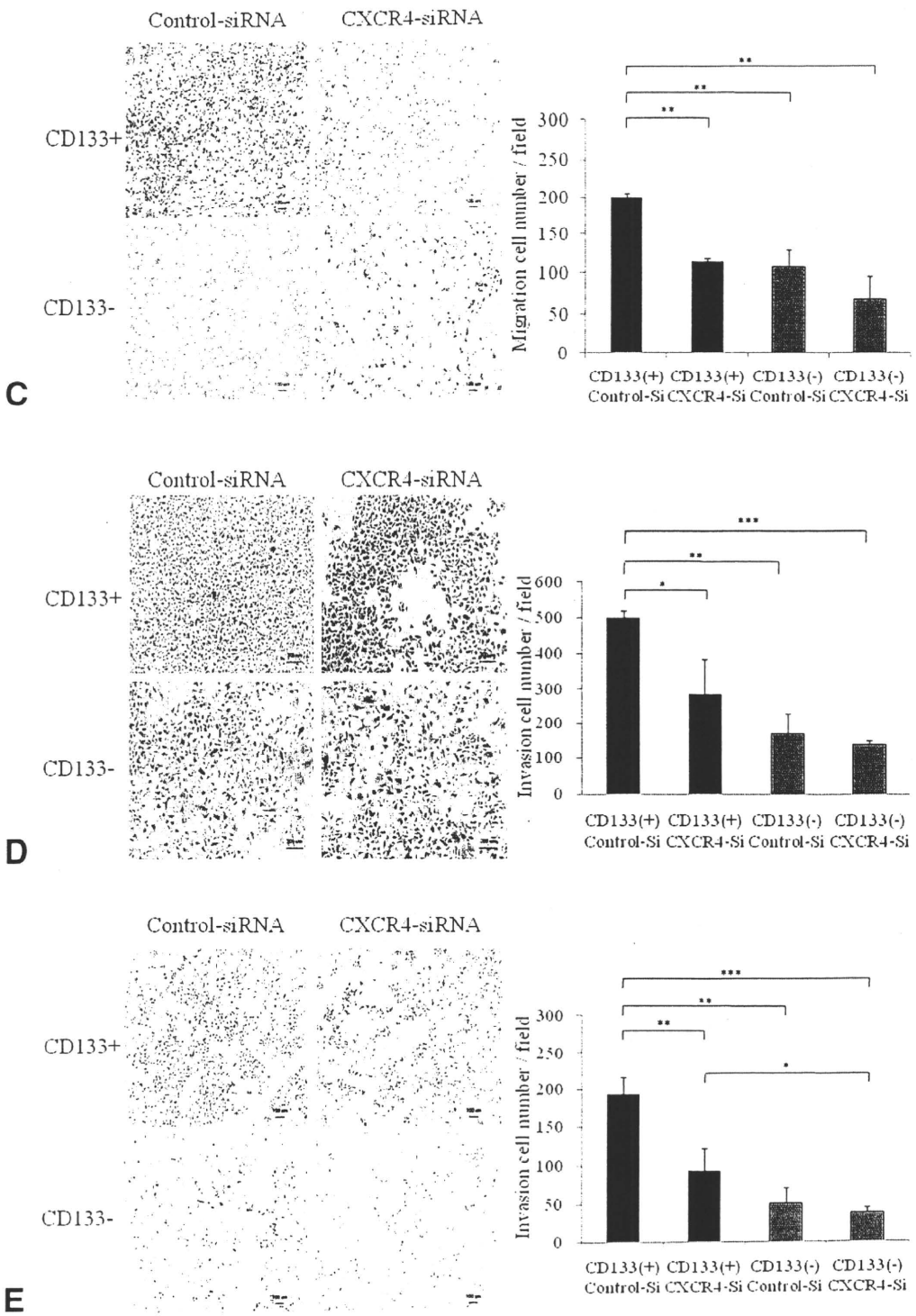

Figure 5. (*Continued*).

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REFERENCES

1. Hirata K, Egawa S, Kimura Y, et al. Current status of surgery for pancreatic cancer. *Dig Surg.* 2007;24:137-147.
2. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. *CA Cancer J Clin.* 2003;53:5-26.
3. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature.* 2001;414:105-111.
4. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1997;3:730-737.
5. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res.* 2003;63:5821-5828.
6. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature.* 2004;432:396-401.
7. Hemmati HD, Nakano I, Lazareff JA, et al. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci U S A.* 2003;100:15178-15183.
8. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature.* 2007;445:106-110.
9. Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature.* 2007;445:111-115.
10. Yin S, Li J, Hu C, et al. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. *Int J Cancer.* 2007;120:1444-1450.
11. Ma S, Chan KW, Hu L, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology.* 2007;132:2542-2556.
12. Richardson GD, Robson CN, Lang SH, et al. CD133, a novel marker for human prostatic epithelial stem cells. *J Cell Sci.* 2004;117:3539-3545.
13. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res.* 2007;67:1030-1037.
14. Hermann PC, Huber SL, Herrler T, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell.* 2007;1:313-323.
15. Ma S, Lee TK, Zheng BJ, Chan KW, Guan XY. CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. *Oncogene.* 2008;27:1749-1758.
16. Liu G, Yuan X, Zeng Z, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer.* 2006;5:67.
17. Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature.* 2006;444:756-760.
18. Phillips TM, McBride WH, Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. *J Natl Cancer Inst.* 2006;98:1777-1785.
19. Vonlaufen A, Joshi S, Qu C, et al. Pancreatic stellate cells: partners in crime with pancreatic cancer cells. *Cancer Res.* 2008;68:2085-2093.
20. Ohuchida K, Mizumoto K, Murakami M, et al. Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor-stromal interactions. *Cancer Res.* 2004;64:3215-3222.
21. Ohuchida K, Mizumoto K, Ishikawa N, et al. The role of S100A6 in pancreatic cancer development and its clinical implication as a diagnostic marker and therapeutic target. *Clin Cancer Res.* 2005;11:7785-7793.
22. Zhang L, Mizumoto K, Sato N, et al. Quantitative determination of apoptotic death in cultured human pancreatic cancer cells by propidium iodide and digitonin. *Cancer Lett.* 1999;142:129-137.
23. Horst D, Kriegl L, Engel J, et al. CD133 expression is an independent prognostic marker for low survival in colorectal cancer. *Br J Cancer.* 2008;99:1285-1289.
24. Kojima M, Ishii G, Atsumi N, Fujii S, Saito N, Ochiai A. Immunohistochemical detection of CD133 expression in colorectal cancer: a clinicopathological study. *Cancer Sci.* 2008;99:1578-1583.
25. Zeppernick F, Ahmadi R, Campos B, et al. Stem cell marker CD133 affects clinical outcome in glioma patients. *Clin Cancer Res.* 2008;14:123-129.
26. Beier D, Wischhusen J, Dietmaier W, et al. CD133 expression and cancer stem cells predict prognosis in high-grade oligodendroglial tumors. *Brain Pathol.* 2008;18:370-377.
27. Maeda S, Shinchi H, Kurahara H, et al. CD133 expression is correlated with lymph node metastasis and vascular endothelial growth factor-C expression in pancreatic cancer. *Br J Cancer.* 2008;98:1389-1397.
28. Kang H, Mansel RE, Jiang WG. Genetic manipulation of stromal cell-derived factor-1 attests the pivotal role of the autocrine SDF-1-CXCR4 pathway in the aggressiveness of breast cancer cells. *Int J Oncol.* 2005;26:1429-1434.
29. Sato N, Matsubayashi H, Fukushima N, Goggins M. The chemokine receptor CXCR4 is regulated by DNA methylation in pancreatic cancer. *Cancer Biol Ther.* 2005;4:70-76.
30. Kalinkovich A, Tavor S, Avigdor A, et al. Functional CXCR4-expressing microparticles and SDF-1 correlate with circulating acute myelogenous leukemia cells. *Cancer Res.* 2006;66:11013-11020.
31. Ehtesham M, Winston JA, Kabos P, Thompson RC. CXCR4 expression mediates glioma cell invasiveness. *Oncogene.* 2006;25:2801-2806.
32. Wendt MK, Cooper AN, Dwinell MB. Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells. *Oncogene.* 2008;27:1461-1471.
33. Kryczek I, Lange A, Mottram P, et al. CXCL12 and vascular endothelial growth factor synergistically induce neoangiogenesis in human ovarian cancers. *Cancer Res.* 2005;65:465-472.
34. Kim J, Takeuchi H, Lam ST, et al. Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. *J Clin Oncol.* 2005;23:2744-2753.
35. Scala S, Ottaiano A, Ascierto PA, et al. Expression of CXCR4 predicts poor prognosis in patients with malignant melanoma. *Clin Cancer Res.* 2005;11:1835-1841.
36. Laverdiere C, Hoang BH, Yang R, et al. Messenger RNA expression levels of CXCR4 correlate with metastatic behavior and outcome in patients with osteosarcoma. *Clin Cancer Res.* 2005;11:2561-2567.
37. Oda Y, Tateishi N, Matono H, et al. Chemokine receptor CXCR4 expression is correlated with VEGF expression and poor survival in soft-tissue sarcoma. *Int J Cancer.* 2009;124:1852-1859.

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Combination with low-dose gemcitabine and hTERT-promoter-dependent conditionally replicative adenovirus enhances cytotoxicity through their crosstalk mechanisms in pancreatic cancer

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ABSTRACT

To overcome the limited clinical efficacy of conditionally replicative adenoviruses (CRAds), we investigated the effects of combination therapy with gemcitabine (GEM) and the hTERT-promoter-dependent CRAd (hTERT-CRAd), Ad5/3hTERTE1. This combination therapy exhibited enhanced cytotoxic effects on pancreatic cancer both *in vitro* and *in vivo*. Furthermore, we revealed that this enhancement effect was due to the multiple bidirectional interactions between hTERT-CRAd and GEM. The GEM-sensitizing effect of E1 expression derived from hTERT-CRAd, and the enhancement effect by GEM on hTERT promoter activity which led to the increase of adenovirus E1 and viral infectivity. This combination therapy may be a promising therapeutic approach for pancreatic cancer.

1. Introduction

Pancreatic cancer is among the most lethal of all common gastrointestinal cancers, with an overall 5 year survival rate of approximately 3–5% [1]. Gemcitabine (GEM) is a deoxycytidine analog, and is widely accepted as the first-line treatment for patients with advanced pancreatic cancer [2]. However, the median overall survival of GEM-treated patients with advanced pancreatic cancer is still only 5–6 months [3]. To improve the prognosis of patients with pancreatic cancer, GEM-based therapy combined with radiotherapy or other chemotherapeutic agents has been used, but with a few exceptions, no improvements in the overall survival of patients with advanced pancreatic cancer have been reported [3,4].

Conditionally replicative adenoviruses (CRAds) have been used as a potent new approach for chemotherapy-resistant solid cancers, including pancreatic cancer [5]. Ad5/3hTERTE1 is a human telomerase reverse transcriptase (hTERT)-promoter-dependent CRAd (hTERT-CRAd), with tumor-specific promoters to control the essential elements of viral replication, such as E1A and E1B. hTERT is the core component of the telomerase, which is physiologically inactive in normal cells, but is highly active in 85–90% of malignant human cells [6–8]. Therefore, this virus can infect, replicate and propagate selectively in hTERT-positive malignant cells [9]. In the field of adenovirus (Ad) cancer therapy, Ad serotype 5 (Ad5) has been used as a

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promising Ad vector, but efficiency is sometimes limited because the expression of its binding receptor, coxsackievirus and adenovirus receptor (CAR) [10], is highly variable and often low in certain cancer cells [11,12]. To overcome this limitation of infectivity, we previously established a chimeric Ad5 vector, Ad5/3, and reported the anti-tumor effects of Ad5/3hTERTE1 in non-small cell lung cancer cell lines [13]. The chimeric fiber consists of the tail and shaft of Ad5 in combination with the knob of Ad3 [14] and binds to the Ad3 receptor, CD46 [15], which has been shown to be overexpressed in some human cancer cells [16], including pancreatic cancer cells [17].

Although CRAds have demonstrated clinical efficacy in cancer treatment [18], their efficacy is still low. Kirn [19] reported that single agent efficacy using dl1520 (ONYX-015), a replication-selective adenovirus, was limited with local tumor regression rates of 0–14%. To improve the efficacy of treatment with CRAds, several researchers have recently evaluated combination treatments with chemotherapeutic agents and various types of CRAds, which produced promising results [20–22]. Two recent reports showed that adenovirus E1 derived from hTERT-CRAd enhanced sensitivity to GEM in lung and pancreatic cancer cells [23,24]. Shieh et al. demonstrated that etoposide enhanced telomerase-dependent adenovirus-mediated gene therapy in bladder tumor [25], and we also reported that etoposide increased telomerase activity in pancreatic cancer [26]. These data suggest that chemotherapeutic agents such as etoposide activate the hTERT promoter. Therefore, we hypothesized that GEM also activated the hTERT promoter and combination therapy with GEM and hTERT-CRAd might provide enhanced antitumor effects because GEM-induced activity of the hTERT promoter may lead to an increase in E1 expression, thereby enhancing sensitivity to GEM.

In the present study, we evaluated the effects of a combination treatment with GEM and Ad5/3hTERTE1, at low doses, in pancreatic cancer *in vitro* and *in vivo*. As well as the mechanism of the GEM-sensitizing effect induced by hTERT-CRAd, we examined the low dose GEM-induced change of hTERT promoter activation and E1 expression, and viral infectivity. These results showed that GEM enhanced Ad5/3hTERTE1 infectivity and that GEM enhanced E1 expression derived from hTERT-CRAd and the enhanced E1 expression additionally increased E1-induced sensitivity to GEM, suggesting that combination therapy with hTERT-CRAd and GEM is a promising and reasonable approach for pancreatic cancer therapy.

2. Materials and methods

2.1. Cells and reagents

Four human pancreatic cancer cell lines were used: SUIT-2 and KP-2 (a generous gift from Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan) and NOR-P1 (established in our laboratory) [27]. One primary culture of pancreatic fibroblasts derived from patients with invasive pancreatic cancer was used (established in our laboratory) [28]. Cells were cultured in DMEM medium supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml) and 10% fetal bovine serum (FBS) at 37 °C in a humidified 90% air and 10% CO_2 environment. GEM (2′,2′-difluorodeoxycytidine) was kindly provided by Eli Lilly and Company (Indianapolis, IN, USA).

2.2. Construction of recombinant adenovirus

The luciferase gene driven by the hTERT promoter was recombined with an E1 (early region 1)-deleted adenoviral backbone vector expressing a chimeric fiber with a serotype 3 adenoviral knob, named pAd5/3Easy1, to generate pAd5/3hTERTLuc. Ad5/3hTERTE1 was also constructed as described previously [13,14,29]. Viruses were propagated in HEK 293 cells. Adenovirus titer was determined by plaque assays on HEK 293 cells. The multiplicity of infection (MOI) was defined as the ratio between the total number of plaque forming units (pfus) used in a particular infection and the total number of cells to be infected.

2.3. Treatment with adenovirus in combination with GEM

Cells were cultured on plates in DMEM supplemented with 10% FBS overnight. They were infected with Ad5/3hTERTE1 or Ad5/3hTERTLuc for 1 h and the culture medium was replaced with fresh medium. GEM was dissolved in phosphate buffered saline (PBS) and added to the fresh medium at the indicated concentrations. The culture media were not replaced after GEM treatment.

2.4. Cell viability assay

Cell viability was evaluated by measuring the fluorescence intensity of propidium iodide (PI) as described previously [30]. Cells were counted using a particle distribution counter, PDA-500 (Sysmex, Kobe, Japan). Cells were plated at 2×10^4 cells/well in 24-well tissue culture plates (Becton Dickinson Labware, Bedford, MA, USA) and cultured overnight. The cells were infected with Ad5/3hTERTE1 with or without GEM. PI (30 μM) and digitonin (600 μM) were added to each well to label all nuclei. The fluorescence intensity, corresponding to total cells, was measured with a CytoFluor II multi-well plate reader (PerSeptive Biosystems Inc., Framingham, MA, USA) using 530 nm excitation and 645 nm emission filters. Cell viability was defined as the ratio between the fluorescence intensity at a specific point and that measured at the beginning of the experiment. All experiments were performed in triplicate wells and repeated at least three times.

2.5. Quantitative analysis of E1A and CD46 mRNA levels by one-step quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cultured cells using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) with DNaseI (Roche Diagnostics) treatment, according to the manufacturer's instructions. We designed specific primers as follows: *E1A* forward, 5′-AAC CAG TTG CCG TGA GAG TTG-3′ and reverse primer, 5′-CTC GTT AAG

CAA GTC CTC GAT ACA-3′; *CD46* forward, 5′-GGA TTG TTG CGT CCC ATA TC-3′ and reverse primer, 5′-GCG GAA GAC GCT GTT ATT TC-3′; *18S rRNA* forward, 5′-GTA ACC CGT TGA ACC CCA TT-3′ and reverse primer, 5′-CCA TCC AAT CGG TAG TAG CCG-3′. We performed BLAST searches to ensure the specificity of these primers. The qRT-PCR was performed using a QuantiTect SYBR Green Reverse Transcription-PCR kit (Qiagen KK, Tokyo, Japan) and a Chrom4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) Each sample was run in triplicate and the expression of each gene was presented as the ratio between the expression of each target gene mRNA and that of *18S* rRNA.

2.6. Quantitative analysis of hexon DNA levels using quantitative PCR

Total DNA was extracted with a DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan). We designed specific primers as follows: *hexon* forward, 5′-TGC CTT TAC GCC ACC TTC TTC-3′ and reverse primer, 5′-CGG GTA TAG GGT AGA GCA TGT TG-3′. Quantitative PCR was done using a SYBR Premix Ex Taq (TaKaRa Bio Inc., Japan) and Chrom4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each sample was run in triplicate and the relative expression of the *hexon* gene was given as the ratio between the expression of *hexon* gene DNA and that of *18S* DNA.

2.7. In vitro analysis of hTERT promoter activation

The activity of the hTERT promoter in an Ad context was analyzed by luciferase assay as reported previously [13]. Briefly, cells were transfected with 2 μl pAd5/3hTERTLuc using a Cell Line Nucleofector kit V (Amaxa Biosystems) and were cultured in triplicate in 6-well plates at a density of 1×10^5 cells/well overnight, then treated with GEM. The cells were harvested and treated with 100 μl lysis buffer (Promega, Madison, WI, USA) on the indicated days. A luciferase assay was performed using a Luciferase Assay System according to the manufacturer's instructions (Promega, Madison, WI, USA) and a GENE LIGHT 55A luminometer (Microtec Nition, Tokyo, Japan) was used for quantitation of the luminescence. Luciferase activities were normalized to the protein concentration in the cell lysate, which was determined using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

2.8. Analysis of viral infectivity

The viral infectivity was determined by the amount of virus *hexon* DNA in infected cells. SUIT-2 cells (1×10^5) were seeded in 60 mm dishes and treated with 1 nM GEM at 1, 6, 12, 24, 48 and 72 h before Ad5/3hTERTLuc infection at an MOI of 10. At 48 h post-infection, the infected SUIT-2 cells were rinsed, the virus *hexon* DNA was extracted from the cells and qPCR was performed.

2.9. Treatment with Ad5/3hTERTE1 in combination with GEM in vivo

Six-week-old female nude mice (BALB/c nu/nu) were obtained from Japan SLC (Hamamatsu, Japan). Single-cell suspensions (5×10^6 SUIT-2 cells/200 μl) were injected subcutaneously into the backs of mice. Seven days later (day 0), mice were administered nothing (control), 5×10^7 pfu Ad5/3hTERTE1 around the periphery of tumors (p.t.) (Ad5/3hTERTE1 only), GEM 10 mg/kg into the peritoneum (i.p.) (GEM only) or both Ad5/3hTERTE1 and GEM (combination), followed by weekly injections. Five mice were used in each group. The size of the tumors was measured weekly, starting on day 0, and the tumor volume was calculated according to the following formula: tumor volume = $ab^2/2$, (a: the longest diameter, b: the shortest diameter).

2.10. In vivo analysis of E1A mRN

Six-week-old female nude mice were subcutaneously injected in their backs with 5×10^6 SUIT-2 cells/200 μl. Six mice were used in each group. Seven days later (day 0), they were administered 5×10^7 pfu of Ad5/3hTERTE1 p.t. and 0, 10, 20 or 40 mg/kg GEM i.p. Seven days after administration, mice were sacrificed and tumors excised. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). Quantitative analysis of *E1A* mRNA was performed as described above. All animal procedures were approved by the Ethics Committee of Kyushu University.

2.11. Statistical analysis

Statistical significance was evaluated using the nonparametric Mann–Whitney U test. Statistical significance was defined as $P < 0.01$ or $P < 0.05$, based on a two-tailed test.

3. Results

3.1. Combination treatment with GEM and Ad5/3hTERTE1 enhances the cytotoxic effect in pancreatic cancer cell lines in vitro

To investigate the cytotoxic effect of GEM and Ad5/3hTERTE1, we measured their inhibitory effects on the viability of telomerase-positive pancreatic cancer cells (SUIT-2, KP-2, MIA PaCa-2 and NOR-P1) [31] using PI [30]. Firstly, we investigated the cytotoxic effects of GEM or Ad5/3hTERTE1 treatment alone, 5 days after treatment. Above a certain concentration, both GEM and Ad5/3hTERTE1 inhibited the viability of all pancreatic cancer cell lines in a dose-dependent manner. However, no inhibition was observed at concentrations below 1 nM for SUIT-2, 10 nM for KP-2, 5 nM for MIA PaCa-2 and 10 nM for NOR-P1 of GEM, and below a MOI of 0.05 for SUIT-2, 0.1 for KP-2, 0.1 for MIA PaCa-2 and 0.01 for NOR-P1of Ad5/3hTERTE1 (data not shown). We then measured the effects of combined treatment with GEM and Ad5/3hTERTE1 at doses which did not affect cell viability when administered alone (SUIT-2: GEM 1 nM, Ad5/3hTERTE1 0.01 MOI; KP-2: GEM 10 nM, Ad5/3hTERTE1 0.1 MOI; MIA PaCa-2: GEM 1 nM, Ad5/3hTERTE1 0.1 MOI; NOR-P1: GEM 10 nM, Ad5/3hTERTE1 0.01 MOI). As shown in Fig. 1A–D, combination treatment significantly inhibited cell viability in all four pancreatic cancer cell lines 5 days following treatment. This combination effect was not seen in primary-cultured normal pancreatic fibroblasts, which had no hTERT expression (Fig. 1E). SUIT-2 cell showed the highest therapeutic gain in the four pancreatic cancer cell lines and we used mainly SUIT-2 cells in the following experiments.

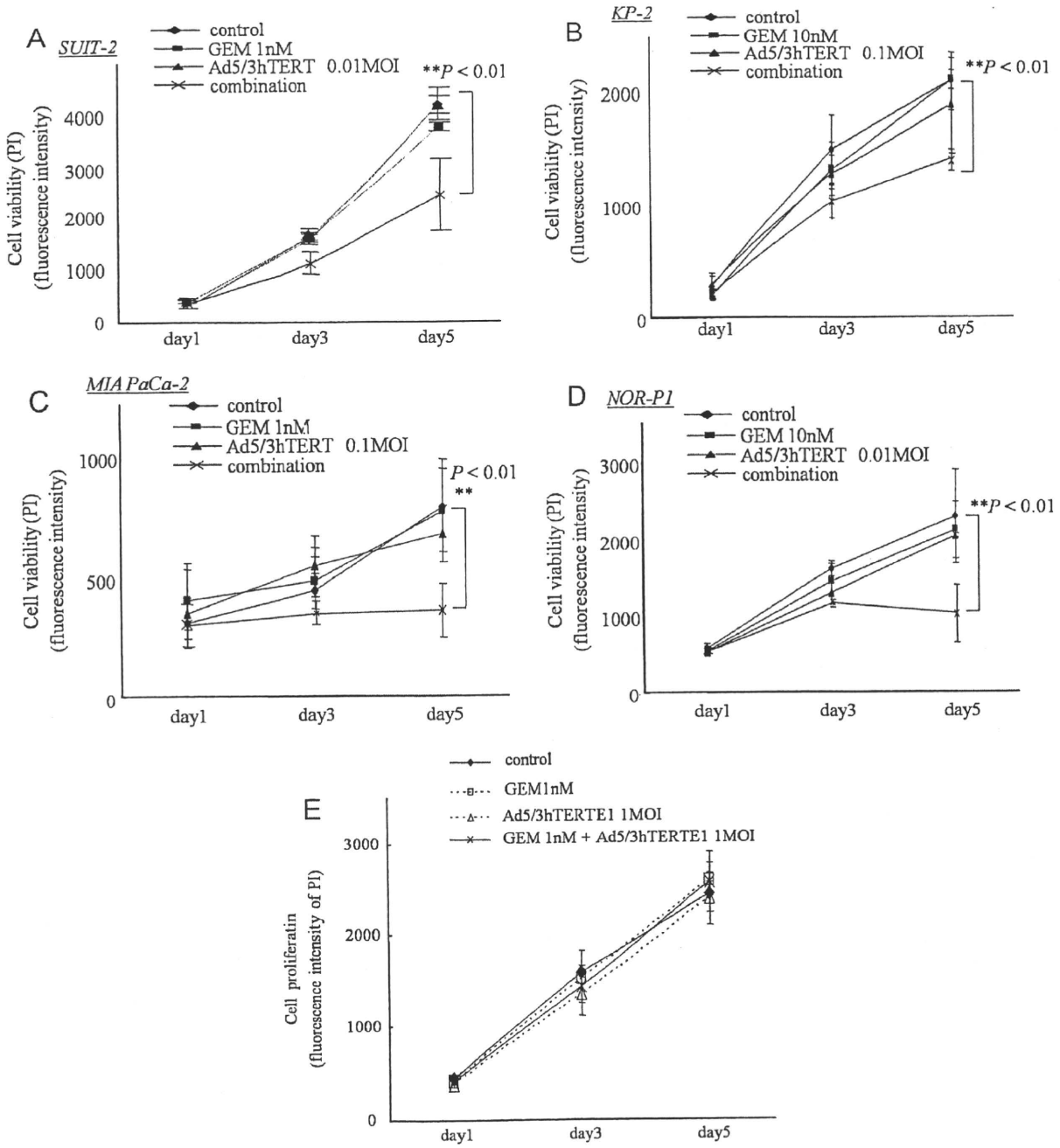

Fig. 1. Combination treatment with GEM and Ad5/3hTERTE1 enhances the cytotoxic effect in pancreatic cancer cells (A, SUIT-2; B, KP-2; C, Mia PaCa-2; D, NORP-1) but not in normal pancreatic fibroblasts (E) *in vitro*. A–E, Control (diamond), no treatment; GEM (square); Ad5/3hTERTE1 (triangle); combination (cross) treated with Ad5/3hTERT and GEM. Cell viability was determined by PI assay on days 1, 3 and 5. Each value represents the mean ± SD of three independent samples.

3.2. GEM-induced E1 expression regulates GEM sensitivity in SUIT-2 cells

The *E1A* gene, which is a component of the *E1* gene, has the ability to induce chemo- and radio-sensitivity [32–37]. Studies have shown that some chemotherapeutic agents affected telomerase activity [25,26] that was correlated with hTERT expression [8]. Ad5/3hTERTE1 encodes the *E1A* and *E1B* genes directly downstream of the hTERT promoter [13]. Therefore, we investigated the effect of GEM on *E1* expression derived from hTERT-CRAd and the activity of the hTERT promoter in hTERT-CRAd as well as the effect of adenovirus *E1* expression on sensitivity to GEM.

First, we investigated the effect of GEM on *E1A* expression in SUIT-2 cells transfected with pAd5/3hTERTE1, which was a plasmid encoding *E1A* and *E1B* under the control of the hTERT promoter, before treatment with or without a low dose of GEM (1 or 10 nM), to exclude any effects of GEM on adenovirus infectivity. *E1A* mRNA levels were significantly increased in cells treated with GEM, compared with those in untreated cells in a dose-dependent manner (Fig. 2A, $P < 0.01$). *E1A* mRNA expression was increased 24 and 48 h after treatment, which was the time taken before newly-replicated virus to be released from relapsed cells re-infected cells again. These data suggest that GEM increases *E1A* expression in an infectivity-independent manner.

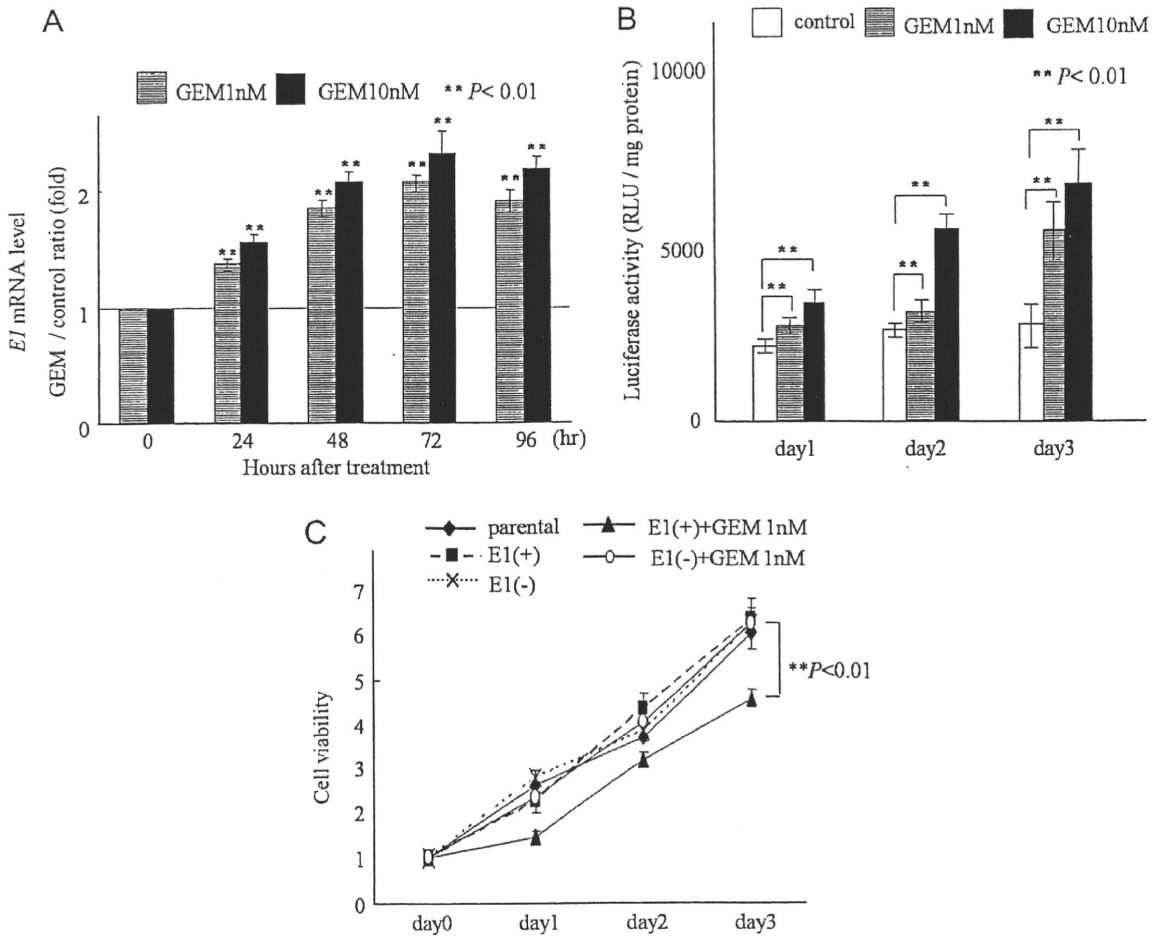

Fig. 2. GEM induces E1 expression via hTERT promoter enhancement and regulates GEM sensitivity in SUIT-2 cells. A, SUIT-2 cells were transfected with pAd5/3hTERTE1(E1(+) plasmid) and treated with or without GEM (1 nM, 10 nM). Total RNA was extracted and the expression of *E1A* mRNA was measured using qRT-PCR normalized to *18S rRNA*. Bars represent relative expression levels as a fold increase, in comparison with non-treated cells. B, SUIT-2 cells were transfected with pAd5/3hTERTLuc (E1(−) plasmid) and treated with GEM. Cell lysates were used for luciferase assay. Luciferase activity was normalized to the protein concentration in the cell lysate. C, cell viability was determined by PI assay. Parental (diamond), non-transfected and no treatment; E1(+) (square) transfected with pAd5/3hTERTE1(E1(+) plasmid) and without GEM; E1(+) with GEM (triangle) transfected with E1(+) plasmid and treated with 1 nM GEM; E1(−) (cross) transfected with pAd5/3hTERTLuc (E1(−) plasmid) and without GEM; E1(−) plus GEM (circle, white), transfected with E1(−) plasmid and 1 nM GEM. Bars represent relative expression levels as a fold increase in comparison with initial expression level. Each value represents the mean ± SD of three independent samples. ** Indicates $P < 0.01$.

To investigate the effects of GEM on hTERT promoter activity, which controls *E1* expression, we investigated changes in hTERT promoter activity in cells treated with GEM. We transfected SUIT-2 cells with a plasmid expressing luciferase, under the control of the hTERT promoter (pAd5/3hTERTLuc), and measured the luciferase activity in cells 1, 2 and 3 days after treatment with GEM. As shown in Fig. 2B, the luciferase activity was significantly higher in cells treated with GEM than in control cells ($P < 0.01$). The enhancement of luciferase activity by GEM was dose dependent, and this result is consistent with the results presented in Fig. 2A. This finding suggests that GEM enhances hTERT promoter activity in a dose-dependent manner, and this enhancement might result in a dose-dependent increase in the expression of adenovirus E1.

Next, we investigated if the increase in E1 expression affected the sensitivity of SUIT-2 cells to GEM. To exclude the effect of adenovirus replication, we used cells transfected with adenovirus plasmid, pAd5/3hTERTE1 (E1(+) plasmid) and pAd5/3hTERTLuc (E1(−) plasmid). We determined the cell viability of parental E1(+) plasmid-transfected and E1(−) plasmid-transfected SUIT-2 cells treated with or without GEM (1 nM). As shown in Fig. 2C there was no difference in cell viability of transfected cells with and without E1 in the absence of GEM. When these cells were treated with GEM, there was a significant cytotoxic effect in E1(+) plasmid-transfected cells, compared to E1(−) plasmid-transfected cells (Fig. 2C, $P < 0.01$), suggesting that adenovirus E1 increased the sensitivity of SUIT-2 cells to GEM as previously reported [23].

3.3. GEM increases infectivity of adenovirus

Adenoviral infectivity, which is important for the infection of neighboring cancer cells by Ad5/3hTERTE1, is one of the major factors in the viral cytotoxic effect. Therefore, we investigated the effect of GEM on adenoviral infectivity. To exclude the effect of adenovirus replication, we used Ad5/3hTERTLuc, which has both the hTERT promoter and chimeric 5/3 fiber as well as Ad5/3hTERTE1, but which had no ability to replicate, and we performed quantitative analyses of viral DNA in cells infected with Ad5/3hTERTLuc. SUIT-2 cells were treated with GEM (1 nM) for 1, 6, 12, 24, 48 and 72 h before infection with Ad5/3hTERTLuc at an MOI of 10. At 48 h after infection, we measured the Ad5/3hTERTLuc DNA content of infected cells, which was quantified by measuring adenovirus hexon DNA. As shown in Fig. 3A, the Ad5/3hTERTLuc DNA content of cells treated with GEM was significantly higher than that of the controls ($P < 0.01$). This increase was particularly apparent more than 48 h after

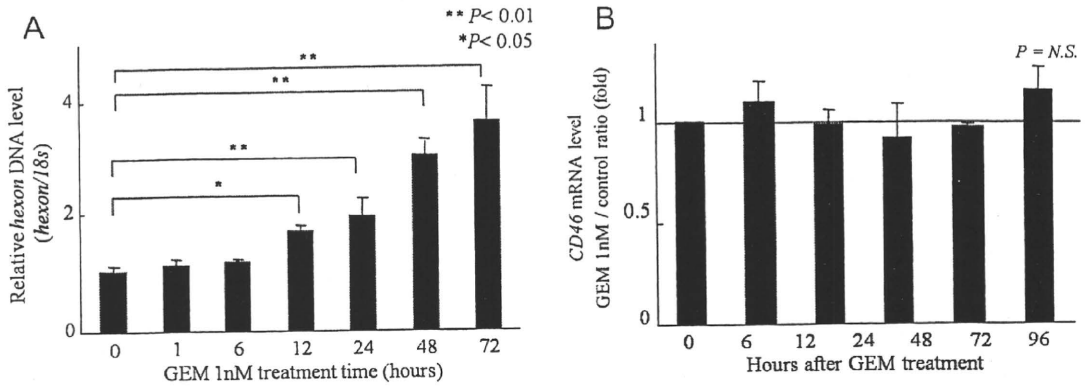

Fig. 3. GEM increased the infectivity of adenovirus in a CD46-independent way. A, SUIT-2 cultures were treated with 1 nM GEM for the indicated number of hours and were infected with Ad5/3hTERTLuc at an MOI of 10. At 48 h post-infection, DNA was extracted. Viral hexon DNA content was quantified using PCR and expressed as a fold increase in comparison with non-treated cells. B, SUIT-2 cells were treated with or without 1 nM GEM and total RNA was extracted. Expression of *CD46* mRNA was measured using qRT-PCR and was normalized to *18S rRNA*. Bars represent relative expression levels as a fold increase in comparison with non-treated cells. Each value represents the mean ± SD of three independent samples. ** Indicates $P < 0.01$, * indicates $P < 0.05$.

GEM treatment, which coincided with the time at which newly formed Ad5/3hTERTE1 was released from cells killed by the first round of Ad5/3hTERTE1 infection, thereby infecting neighboring cells in our present experiment (Fig. 1). These data indicate that GEM increases the efficiency of adenovirus infection, especially in the time Ad5/3hTERTE1 re-infected neighboring cells.

It has been reported that the infectivity of adenovirus may closely correlate with the cell surface density of its receptors, such as CAR or integrins [38,39]. CD46 is also considered to bind to the knob domain of the chimeric Ad 5/3 fiber [15]. Therefore, we measured *CD46* mRNA expression in SUIT-2 cells after GEM (1 nM) treatment, but GEM did not alter the expression levels of *CD46* mRNA, suggesting that the increase of adenoviral infectivity induced by GEM might occur in a CD46-independent way (Fig. 3B).

3.4. Ad5/3hTERTE1, in combination with low doses of GEM, suppresses tumor growth in vivo

To evaluate the combined therapeutic effect of GEM and Ad5/3hTERTE1 on pancreatic cancer *in vivo*, we examined the growth of tumors derived from SUIT-2 cells implanted subcutaneously into nude mice. To evaluate the therapeutic effect of GEM alone, three mice per group were treated with 5, 10, 20 or 40 mg/kg/week GEM by i.p. administration for 4 weeks, from 7 days after SUIT-2 cell implantation. We observed 0 ± 4.08%, 9.4 ± 14.2%, 35.9 ± 6.67% and 41.2 ± 21.4% growth inhibition of SUIT-2 tumors in the four different treatment groups compared to untreated ones. Because there was no inhibitory effect at doses less than 10 mg/kg GEM, 10 mg/kg GEM was used in the following experiment.

Mice were randomly assigned to four treatment groups ($n = 5$ each group). As shown in Fig. 4A and B, combination treatment significantly suppressed tumor growth (94.2 ± 4.9% growth inhibition of tumors 8 weeks after initial treatment, compared with controls). Treatment with either GEM alone or Ad5/3hTERTE1 alone also suppressed tumor growth gradually, on days 21 and later but the inhibitory effect was small (GEM alone, 29.6 ± 6.1%; Ad5/3hTERTE1 alone, 41.2 ± 16.6%). This result demonstrated that Ad5/3hTERTE1, in combination with low doses of GEM, significantly enhanced the anti-tumor effect *in vivo* as well as *in vitro*.

We also measured the expression levels of *E1A* mRNA within implanted SUIT-2 tumors. Seven days after implantation, three mice per group were treated with 0, 10, 20 or 40 mg/kg i.p. GEM and Ad5/3hTERTE1 (5×10^7 pfu). Seven days later, we excised the tumors and measured them. As shown in Fig. 4C, the expression of *E1A* mRNA was increased by GEM in a dose-dependent manner. This result indicated that the *in vivo* anti-tumor effects of combination therapy were enhanced by the same mechanisms as the *in vitro* effects.

4. Discussion

A few earlier reports showed enhancing effects of combination therapy with GEM and various types of CRAds. Raki et al. showed the improved efficacy of GEM in combination with Ad5/3-Δ24 in ovarian cancer [40]. Liu et al. recently reported that hTERT-CRAd enhanced anti-tumor effects of GEM in human lung cancer, and that this enhancement was due to the chemo-sensitizing effects by adenovirus E1 [23]. In the present study, we also showed enhanced anti-tumor effects of GEM and Ad5/3hTERTE1 in pancreatic cancer cells both *in vitro* and *in vivo*. Furthermore, we revealed that this effect was not only due to CRAd-derived E1expression, which induces cells more sensitive to GEM, but was also due to the effect of GEM on hTERT-CRAd, with enhancement of hTERT promoter activity leading to the increase of adenovirus E1 and viral infectivity. These data suggest that the killing effects of this combination therapy are enhanced via multiple bidirectional interactions between hTERT-dependent CRAds and GEM.

E1A gene transfer has been reported to result in increased cell sensitivity to paclitaxel in breast cancer [35], adriamycin in colon and liver cancers [41], cisplatin and etoposide in sarcoma [42], and gemcitabine in hepatocellular carcinoma [33]. Telomerase has been reported to repair broken chromosomes and maintain genomic integrity during cytotoxic stresses due to DNA damage caused by chemotherapeutic agents [26,43,44]. Several studies have reported the effects of chemotherapeutic agents on telomerase activity, but there has been no report to date regarding the effects of GEM on telomerase activity. The present data suggests that GEM increases expression levels of adenovirus E1, possibly through enhancement of hTERT promoter activity, which controls E1 expression of Ad5/3hTERTE1.

Adenovirus E1 consists of *E1A* and *E1B*. However, these viral genes have opposing functions. *E1A* works as an apoptotic gene, but *E1B* works as an anti-apoptotic gene. Leitner et al. reported that oncolytic adenoviral mutants with *E1B19K* gene deletions enhanced GEM-induced apoptosis [24], but we observed that the cells transfected with pAd5/3hTERTE1 (E1(+) plasmid), which was an adenovirus plasmid encoding the full-length of *E1A* and *E1B* under the control of the hTERT promoter, were also sensitized to

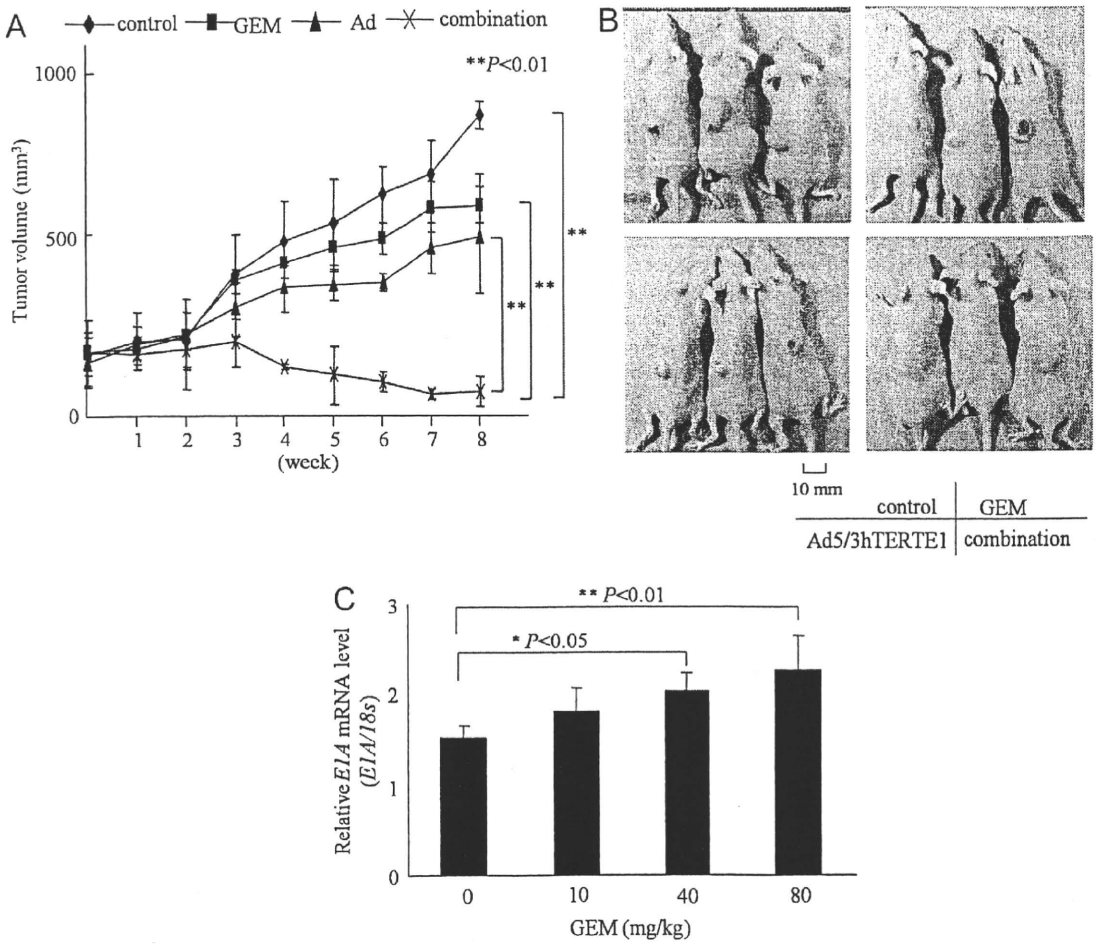

Fig. 4. Ad5/3hTERTE1 in combination with a low dose of GEM suppressed tumor growth, and GEM-enhanced expression levels of *E1A* mRNA *in vivo*. A, Five mice from each group were subcutaneously injected on their backs with 5×10^6 SUIT-2 cells. Seven days later (day 0), they were administered either nothing (control, diamond), GEM 10 mg/kg i.p. (GEM only, square), 5×10^7 pfu Ad5/3hTERTE1 p.t. (Ad5/3hTERTE1 only, triangle), or both Ad5/3hTERTE1 and GEM (combination, cross), followed by weekly injections over 8 weeks. The size of tumors was measured weekly, and the volume of tumors was calculated according to the formula: tumor volume = $ab^2/2$, (a: longest diameter, b: the shortest diameter). B, The combination treatment group showed significant suppression of tumor growth compared with other groups 8 weeks after initial administration. C, Six mice were subcutaneously injected on their backs with 5×10^6 SUIT-2 cells. Seven days later (day 0), they were administered 5×10^7 pfu of Ad5/3hTERTE1 p.t. and GEM (0, 10, 20, 40 mg/kg) i.p. Seven days after administration, mice were sacrificed and the tumors were excised. Total RNA was extracted and quantitative analysis of *E1A* mRNA was performed. Each value represents the mean ± SD of three independent samples. ** Indicates $P < 0.01$, * indicates $P < 0.05$.

GEM, similar to the case of the *E1B19K*-deleted adenovirus reported by Leitner et al. [24]. This finding is consistent with the report of Liu et al. [23]. These data suggest that GEM-enhanced hTERT promoter activity may lead to an increase in adenovirus E1, and renders pancreatic cancer cells more sensitive to GEM.

We also found that GEM enhanced Ad5/3hTERTE1 infectivity. We used Ad5/3hTERTE1 that binds and enters through receptors such as CD46 to increase tropism. We found that the Ad5/3hTERTLuc content in cells pre-treated with GEM was increased. Furthermore, this increase in infectivity was most notable 48–72 h after GEM treatment. This timing is coincident with the time at which the adenovirus begins to re-infect neighboring cancer cells. These data suggest that GEM-enhanced infectivity in neighboring target cells increases the number of re-infected cells, leading to the main cytotoxic effects of combination therapy. With respect to the receptor of the Ad5 vector, CAR, Hemminiki et al. reported that the effects of chemotherapeutic agents on *CAR* mRNA was variable depending on the specific combination of agents and cells, and that increased CAR expression allowed increased virus entry into cells [45]. In this study, we used the chimeric Ad5 vector, Ad5/3, which is considered to bind to CD46, but not to CAR. Therefore, we investigated whether GEM enhanced infectivity of Ad5/3hTERTE1 via an increase in *CD46* expression. However, the present data revealed that *CD46* mRNA expression in pancreatic cancer cells was not altered after GEM treatment and suggested that the increase in efficiency of infection might be independent of the expression level of CD46, unlike the case of CAR. We need to conduct further research to determine the effect of GEM on the infectivity of adenovirus with chimeric fiber 5/3.

In conclusion, our present data demonstrates that a combination of a low dose of GEM and a low dose of hTERT-promoter-dependent CRAd produces enhanced anti-tumor effects *in vivo* as well as *in vitro*. Although previous studies have demonstrated that combination treatment with chemotherapy and oncolytic virotherapy demonstrated promising efficacies due to the ability of adenovirus E1 to sensitize cells to chemotherapeutics [23,33,35], this is the first report describing that even at a low dose, GEM contributes to the enhanced cytotoxic effects of this combination therapy via enhancement of E1 expression derived from hTERT-CRAds and an increase in infectivity of CRAds. In clinics, a maximal therapeutic effect with minimal side effects is the best approach to attacking cancers, and this combined therapy may fit these requirements. Furthermore, a phase I/II trial of intratumoral endoscopic ultrasound (EUS) injection of therapeutic adenovirus, with intravenous injection of GEM in unresectable pancreatic carcinoma showed a feasible result and suggested that transgastric EUS-guided injection was a practical method for delivering biological agents to pancreatic cancer [22]. Our present study indicates that combining hTERT-promoter-dependent CRAds and GEM has the potential to be an ideal therapeutic approach for pancreatic cancer.

Conflict of interest

There is no financial interest or other relationship of a commercial nature in the content of this work.

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References

[1] C.J. Yeo, J.L. Cameron, T.A. Sohn, et al., Six hundred fifty consecutive pancreaticoduodenectomies in the 1990s: pathology, complications, and outcomes, Ann. Surg. 226 (1997) 248–257. discussion 57–60.
[2] M. Tempero, J.P. Arnoletti, E. Ben-Josef, et al., Pancreatic adenocarcinoma. Clinical practice guidelines in oncology, J. Natl. Compr. Cancer. Netw. 5 (2007) 998–1033.
[3] M.W. Saif, Pancreatic cancer: highlights from the 42nd annual meeting of the American Society of Clinical Oncology, 2006, JOP 7 (2006) 337–348.
[4] S. Boeck, D.P. Ankerst, V. Heinemann, The role of adjuvant chemotherapy for patients with resected pancreatic cancer: systematic review of randomized controlled trials and meta-analysis, Oncology 72 (2007) 314–321.
[5] R. Alemany, C. Balague, D.T. Curiel, Replicative adenoviruses for cancer therapy, Nat. Biotechnol. 18 (2000) 723–727.
[6] J. Feng, W.D. Funk, S.S. Wang, et al., The RNA component of human telomerase, Science 269 (1995) 1236–1241.
[7] L. Harrington, T. McPhail, V. Mar, et al., A mammalian telomerase-associated protein, Science 275 (1997) 973–977.
[8] T.M. Nakamura, G.B. Morin, K.B. Chapman, et al., Telomerase catalytic subunit homologs from fission yeast and human, Science 277 (1997) 955–959.
[9] N.A. Lanson Jr., P.L. Friedlander, P. Schwarzenberger, J.K. Kolls, G. Wang, Replication of an adenoviral vector controlled by the human telomerase reverse transcriptase promoter causes tumor-selective tumor lysis, Cancer Res. 63 (2003) 7936–7941.
[10] J.M. Bergelson, J.A. Cunningham, G. Droguett, et al., Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5, Science 275 (1997) 1320–1323.
[11] A. Kanerva, G.V. Mikheeva, V. Krasnykh, et al., Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells, Clin. Cancer Res. 8 (2002) 275–280.
[12] J.G. Wesseling, P.J. Bosma, V. Krasnykh, et al., Improved gene transfer efficiency to primary and established human pancreatic carcinoma target cells via epidermal growth factor receptor and integrin-targeted adenoviral vectors, Gene Ther. 8 (2001) 969–976.
[13] J. Uchino, K. Takayama, A. Harada, et al., Infectivity enhanced, hTERT promoter-based conditionally replicative adenoviruses are useful for SCLC treatment, Cancer Gene Ther. 12 (2005) 737–748.
[14] V.N. Krasnykh, G.V. Mikheeva, J.T. Douglas, D.T. Curiel, Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism, J. Virol. 70 (1996) 6839–6846.
[15] D. Sirena, B. Lilienfeld, M. Eisenhut, et al., The human membrane cofactor CD46 is a receptor for species B adenovirus serotype 3, J. Virol. 78 (2004) 4454–4462.
[16] I.V. Ulasov, M.A. Tyler, S. Zheng, Y. Han, M.S. Lesniak, CD46 represents a target for adenoviral gene therapy of malignant glioma, Hum. Gene Ther. 17 (2006) 556–564.
[17] H. Juhl, F. Helmig, K. Baltzer, H. Kalthoff, D. Henne-Bruns, B. Kremer, Frequent expression of complement resistance factors CD46, CD55, and CD59 on gastrointestinal cancer cells limits the therapeutic potential of monoclonal antibody 17–1A, J. Surg. Oncol. 64 (1997) 222–230.
[18] A. Kanerva, A. Hemminki, Adenoviruses for treatment of cancer, Ann. Med. 37 (2005) 33–43.
[19] D. Kirn, Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned?, Gene Ther 8 (2001) 89–98.
[20] F.R. Khuri, J. Nemunaitis, I. Ganly, et al., A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer, Nat. Med. 6 (2000) 879–885.
[21] J.P. Lamont, J. Nemunaitis, J.A. Kuhn, S.A. Landers, T.M. McCarty, A prospective phase II trial of ONYX-015 adenovirus and chemotherapy in recurrent squamous cell carcinoma of the head and neck (the Baylor experience), Ann. Surg. Oncol. 7 (2000) 588–592.
[22] J.R. Hecht, R. Bedford, J.L. Abbruzzese, et al., A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma, Clin. Cancer Res. 9 (2003) 555–561.
[23] D. Liu, T. Kojima, M. Ouchi, et al., Preclinical evaluation of synergistic effect of telomerase-specific oncolytic virotherapy and gemcitabine for human lung cancer, Mol. Cancer Ther. 8 (2009) 980–987.
[24] S. Leitner, K. Sweeney, D. Oberg, et al., Oncolytic adenoviral mutants with E1B19K gene deletions enhance gemcitabine-induced apoptosis in pancreatic carcinoma cells and anti-tumor efficacy in vivo, Clin. Cancer Res. 15 (2009) 1730–1740.
[25] G.S. Shieh, A.L. Shiau, Y.T. Yo, et al., Low-dose etoposide enhances telomerase-dependent adenovirus-mediated cytosine deaminase gene therapy through augmentation of adenoviral infection and transgene expression in a syngeneic bladder tumor model, Cancer Res. 66 (2006) 9957–9966.
[26] N. Sato, K. Mizumoto, M. Kusumoto, et al., Up-regulation of telomerase activity in human pancreatic cancer cells after exposure to etoposide, Brit. J. Cancer 82 (2000) 1819–1826.
[27] N. Sato, K. Mizumoto, K. Beppu, et al., Establishment of a new human pancreatic cancer cell line, NOR-P1, with high angiogenic activity and metastatic potential, Cancer Lett. 155 (2000) 153–161.
[28] M.G. Bachem, E. Schneider, H. Gross, et al., Identification, culture, and characterization of pancreatic stellate cells in rats and humans, Gastroenterology 115 (1998) 421–432.
[29] Y. Adachi, P.N. Reynolds, M. Yamamoto, et al., A midkine promoter-based conditionally replicative adenovirus for treatment of pediatric solid tumors and bone marrow tumor purging, Cancer Res. 61 (2001) 7882–7888.
[30] L. Zhang, K. Mizumoto, N. Sato, et al., Quantitative determination of apoptotic death in cultured human pancreatic cancer cells by propidium iodide and digitonin, Cancer Lett. 142 (1999) 129–137.
[31] N. Sato, N. Maehara, K. Mizumoto, E. Nagai, T. Yasoshima, K. Hirata, M. Tanaka, Telomerase activity of cultured human pancreatic carcinoma cell lines correlates with their potential for migration and invasion, Cancer 91 (2001) 496–504.

[32] R. Sanchez-Prieto, M. Quintanilla, A. Cano, et al., Carcinoma cell lines become sensitive to DNA-damaging agents by the expression of the adenovirus E1A gene, Oncogene 13 (1996) 1083–1092.
[33] W.P. Lee, D.I. Tai, S.L. Tsai, et al., Adenovirus type 5 E1A sensitizes hepatocellular carcinoma cells to gemcitabine, Cancer Res. 63 (2003) 6229–6236.
[34] E. White, Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus, Oncogene 20 (2001) 7836–7846.
[35] N.T. Ueno, C. Bartholomeusz, J.L. Herrmann, et al., E1A-mediated paclitaxel sensitization in HER-2/neu-overexpressing ovarian cancer SKOV3.ip1 through apoptosis involving the caspase-3 pathway, Clin. Cancer Res. 6 (2000) 250–259.
[36] D. Chattopadhyay, M.K. Ghosh, A. Mal, M.L. Harter, Inactivation of p21 by E1A leads to the induction of apoptosis in DNA-damaged cells, J. Virol. 75 (2001) 9844–9856.
[37] X.M. Rao, M.T. Tseng, X. Zheng, et al., E1A-induced apoptosis does not prevent replication of adenoviruses with deletion of E1b in majority of infected cancer cells, Cancer Gene Ther. 11 (2004) 585–593.
[38] S. Hemmi, R. Geertsen, A. Mezzacasa, I. Peter, R. Dummer, The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures, Hum. Gene Ther. 9 (1998) 2363–2373.
[39] Y. Li, R.C. Pong, J.M. Bergelson, et al., Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy, Cancer Res. 59 (1999) 325–330.
[40] M. Raki, A. Kanerva, A. Ristimaki, et al., Combination of gemcitabine and Ad5/3-Delta24, a tropism modified conditionally replicating adenovirus, for the treatment of ovarian cancer, Gene Ther. 12 (2005) 1198–1205.
[41] Y. Li, D.C. Yu, Y. Chen, et al., A hepatocellular carcinoma-specific adenovirus variant, CV890, eliminates distant human liver tumors in combination with doxorubicin, Cancer Res. 61 (2001) 6428–6436.
[42] Z. Zhou, S.F. Jia, M.C. Hung, E.S. Kleinerman, E1A sensitizes HER2/neu-overexpressing Ewing's sarcoma cells to topoisomerase II-targeting anticancer drugs, Cancer Res. 61 (2001) 3394–3398.
[43] J. Flint, C.F. Craddock, A. Villegas, et al., Healing of broken human chromosomes by the addition of telomeric repeats, Am. J. Hum. Genet. 55 (1994) 505–512.
[44] G.G. Sharma, A. Gupta, H. Wang, et al., HTERT associates with human telomeres and enhances genomic stability and DNA repair, Oncogene 22 (2003) 131–146.
[45] A. Hemminki, A. Kanerva, B. Liu, et al., Modulation of coxsackie-adenovirus receptor expression for increased adenoviral transgene expression, Cancer Res. 63 (2003) 847–853.

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ORIGINAL ARTICLE

Gemcitabine synergistically enhances the effect of adenovirus gene therapy through activation of the CMV promoter in pancreatic cancer cells

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Adenovirus-mediated gene therapy shows remarkable promise as a new strategy for advanced pancreatic cancer, but satisfactory clinical results have not yet been obtained. To improve this gene therapy, we investigated the effects of gemcitabine (GEM) on transgene expression by adenoviral vectors and their biological effects. We used Ad-lacZ and adenoviral vector-expressing NK4 (Ad-NK4) as representative adenoviral vectors. These vectors express β-galactosidase (β-gal) and NK4 (which inhibits the invasion of cancer cells), respectively, under the control of the CMV promoter. Cells were infected with the individual adenoviruses and then treated with GEM. GEM increased β-gal mRNA expression and β-gal activity, and increased NK4 expression in both culture media and within infected cells, in dose-dependent manners. The increased expression of NK4 delivered by Ad-NK4 had biological effects by inhibiting the invasion of cancer cells. GEM also enhanced NK4 expression in SUIT-2 cells transfected with an NK4-expressing plasmid, suggesting that GEM enhanced CMV promoter activity. In *in vivo* experiments, NK4 expression within subcutaneously implanted tumors was increased in GEM-treated mice compared with control mice. These results suggest that adenovirus-mediated gene therapy with GEM may be a promising approach for treating pancreatic cancer, and that this combination therapy may decrease the risks of side effects.

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Introduction

Pancreatic cancer is highly lethal, and associated with an annual incidence almost equal to its annual death rate owing to late diagnosis, aggressive tumor growth, invasion and metastasis, and a high rate of relapse after adjuvant therapy.[1–3] Even among patients with resectable pancreatic cancer, nearly all die from the disease within 7 years after surgery, and conventional chemotherapy and radiotherapy show limited effectiveness.[4,5] Therefore, new strategies for pancreatic cancer, such as molecular target therapies and gene therapies, are needed and beginning to show remarkable promise.[6,7]

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Although adenovirus-mediated gene therapy is one of the promising approaches for cancer treatment because of the high transduction efficiency,[8] the great results demonstrated at the laboratory level are not always obtained in clinical settings.[9] Therefore, it is still necessary to develop devices for improving the efficiency of the gene introduction.

We earlier reported that gene therapy with an adenoviral vector-expressing NK4 (Ad-NK4), which acts as a hepatocyte growth factor (HGF) antagonist and an angiogenesis inhibitor, inhibited the *in vitro* invasion and *in vivo* growth of human pancreatic cancer cells.[10] We also reported that Ad-NK4 combined with gemcitabine (GEM), which is now the first-line chemotherapeutic agent for pancreatic cancer, remarkably suppressed the growth and metastasis of human pancreatic cancer cells.[11] In the latter study, however, we did not examine the detailed mechanism of the enhanced suppression of tumor growth, such as the interaction between GEM and adenovirus-mediated gene therapy. On the other hand, we recently reported that radiation could enhance

adenovirus-mediated gene therapy by increasing adenovirus infectivity and CMV promoter activity, which regulated the expression of target genes.[12] In support of these findings, genotoxic stresses, such as chemotherapeutic agents and irradiation, have been reported to enhance the expression of transgenes under the control of the CMV promoter.[13–15] To date, however, the effects of GEM on the adenovirus-mediated transduction of target genes have remained unknown.

In this study, we investigated the effects of GEM on the expression levels of transgenes under the control of the CMV promoter using Ad-lacZ and Ad-NK4 as representative adenoviral vectors. Furthermore, we investigated the effects of GEM on CMV promoter activity. The data obtained suggest that adenovirus-mediated gene therapy combined with GEM may be a promising approach for the treatment of pancreatic cancer, and that this combination therapy may decrease the risks of side effects.

Materials and methods

Cultured cells and reagents

The following three human pancreatic cancer cell lines were used: SUIT-2 and KP-2 (generous gifts from Dr H Iguchi, National Shikoku Cancer Center, Matsuyama, Japan) and MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with streptomycin (100 μg ml^{-1}), penicillin (100 U ml^{-1}), and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 90% air and 10% CO_2. GEM (2′,2′-difluorodeoxycytidine) was kindly provided by Eli Lilly and Company (Indianapolis, IN).

Construction of recombinant adenoviruses

A recombinant adenoviral vector-expressing human NK4 was constructed as described earlier.[5,16–18] A control vector expressing the bacterial β-galactosidase (β-gal) gene (lacZ) was constructed by the same procedure. The recombinant adenoviruses (denoted as Ad-NK4 and Ad-lacZ, respectively) were propagated in HEK293 cells. The adenovirus titers in plaque-forming units (pfu) were determined by plaque-formation assays with HEK293 cells. The multiplicity of infection (MOI) was defined as the ratio of the total number of pfu used in a particular infection to the total number of cells to be infected.

Treatment with adenoviruses in combination with GEM

Cells were seeded in plates and cultured in DMEM supplemented with 10% fetal bovine serum for 24 h. The cells were then infected with Ad-lacZ or Ad-NK4 at various MOIs for 1 h, followed by replacement of the culture media with fresh DMEM supplemented with 10% fetal bovine serum. GEM was dissolved in phosphate-buffered saline and added to the fresh media at various concentrations. After 24 h, the GEM-containing media were replaced with fresh media without GEM.

Quantitative analysis of β-gal mRNA levels by one-step real-time reverse transcription–polymerase chain reaction

Total RNA was extracted from cultured cells using a High Pure RNA Isolation kit (Roche Diagnostics, Mannheim, Germany) with DNaseI (Roche Diagnostics) treatment, according to the manufacturer's instructions. We designed specific primers as follows: *β-gal*, forward primer, *5′-cacggcacatacacttgctg-3′* and reverse primer, *5′-atcgccatttgaccactacc-3′*; *18S rRNA*, forward primer, *5′-gtaacccgttgaaccccatt-3′* and reverse primer, *5′-ccatcca atcggtagtagccg-3′*. We performed BLAST searches to ensure the specificities of these primers. One-step quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was performed using a QuantiTect SYBR Green Reverse Transcription–PCR kit (Qiagen K.K., Tokyo, Japan) with a Light Cycler Quick System 350S (Roche Applied Science, Mannheim, Germany) as described earlier.[19] Each sample was run in triplicate and the expression of each gene was presented as the ratio between the expression of each target gene mRNA and that of *18S* rRNA.

Assessment of transgene distributions by evaluation of β-gal expression

After treatment of SUIT-2 cells with Ad-lacZ and GEM as described above, the treated cells were rinsed twice with phosphate-buffered saline and fixed with 0.25% glutaraldehyde in phosphate-buffered saline for 15 min at 4 °C. β-gal activity was detected by immersing the cells in 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal)-staining solution (5 mM K_4FeCN, 5 mM K_3FeCN, 2 mM $MgCl_2$, 1 mg ml^{-1} X-gal) for 12 h at 37 °C.

Extraction of proteins from Ad-NK4-infected cells

SUIT-2 cells were infected with Ad-NK4 and treated with GEM as described above. After these treatments, the cells were lysed in 500 l of ice-cold lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 5 μg ml^{-1} leupeptin, 1 mM phenylmethyl sulfonyl fluoride, and 0.5% (v/v) Triton X-100). Cell debris was removed by centrifugation at 14 000 *g* for 20 min at 4 °C and the supernatants were collected. The protein concentrations of the supernatants were measured by the absorbances at 280 nm using an ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE) and adjusted to 2.0 mg ml^{-1} with lysis buffer.

Electroporation

pcDNA-3-NK4 (2.5 μg; an NK4-expressing plasmid) was mixed with 5×10^6 SUIT-2 cells and electroporated with Nucleofector (Amaxa Biosystems GmbH, Koln, Germany) according to the manufacturer's instructions.

Measurement of NK4 expression levels

At 24 h after infection of pancreatic cancer cells with Ad-NK4 or transfection with an NK4-expressing plasmid with or without GEM, the media were exchanged for fresh media. The conditioned media and proteins extracted from cells infected with Ad-NK4 were measured using

a human HGF ELISA kit (IMMUNIS HGF EIA; Institute of Immunology, Tokyo, Japan), according to the manufacturer's recommendations.

Cell viability assay

Cell viability was evaluated by the fluorescence intensity of propidium iodide (PI) as described earlier.[20] Cells were plated at 2×10^4 cells per well in 24-well tissue culture plates (Becton Dickinson Labware, Bedford, MA) and cultured overnight. After determination of the initial cell numbers, the cells were infected with Ad-NK4 at various MOIs for 1 h and then treated with GEM at various concentrations for 24 h as described above. PI (30 μM) and digitonin (600 μM) were added to each well to label all nuclei with PI. The fluorescence intensity, corresponding to the total cells, was measured using a CytoFluor II multi-well plate reader (PerSeptive Viosystems Inc., Framingham, MA) with 530-nm excitation and 645-nm emission filters. Cell viability was defined as the ratio of fluorescence intensity at each time point to that measured at the beginning of the experiment. All experiments were performed in triplicate wells and repeated at least three times.

Invasion assay

SUIT-2 cells were infected with Ad-NK4 at an MOI of 10 and then treated with 0, 1, 10, or 100 nM GEM for 24 h as described above. The conditioned culture media were collected on days 1 and 3. Invasion of tumor cells was measured as the number of cells invading through Matrigel-coated transwell inserts (Becton Dickinson, Franklin Lakes, NJ) as described earlier.[21] Briefly, fresh untreated SUIT-2 cells were seeded in 24-well plates at a density of 5×10^4 cells per cm^2 in 100 μl of DMEM mixed with 150 μl of conditioned culture media in the inner chamber, and cultured with 150 μl of conditioned culture media in the outer chamber. After 48 h of incubation in the presence of 3 ng ml^{-1} HGF, cells that had invaded to the lower surface of the Matrigel-coated membrane were fixed with 70% ethanol, stained with hematoxylin and eosin, and counted in five randomly selected fields under a light microscope (Nikon, ECLIPSE TE2000, Tokyo, Japan).

In vivo analysis of NK4 expression levels in tumors

Six-week-old female nude mice were subcutaneously injected with 5×10^6 SUIT-2 cells (200 μl) in the back. Six mice were used in each experimental group. After 7 days (day 0), the mice were administered 2×10^9 pfu Ad-NK4 pertumorally, with intraperitoneal administration of GEM (0, 10, 20, 40, and 80 mg kg^{-1}). At 3 days after the administration, three mice in each group were killed and their tumors were excised for protein extraction. On day 7, the other three mice in each group were administered the same treatment. These mice were killed on day 10, and their tumors were also excised for protein extraction. The tumors were homogenized in ice-cold lysis buffer, and NK4 was measured by ELISA as described above.

Statistical analysis

Statistical significance was evaluated by the nonparametric Mann–Whitney *U*-test. Statistical significance was defined as values of $P<0.05$ based on a two-tailed test.

Results

GEM enhances the transgene expression of β-gal delivered by Ad-lacZ

First, to investigate the effects of GEM on the transgene expression of a target gene delivered by an adenoviral vector, we measured the expression levels of β-gal delivered by Ad-lacZ with or without GEM. SUIT-2 cells were infected with Ad-lacZ at an MOI of 10 for 1 h. After the infection, the cells were cultured with or without GEM for 24 h at various concentrations. The culture media were then replaced with fresh DMEM supplemented with 10% fetal bovine serum without GEM. We evaluated the β-gal mRNA levels by qRT–PCR and the β-gal activities by X-gal staining. The β-gal mRNA expression level increased in the GEM-treated cells in a dose-dependent manner (Figure 1a, $P=0.002$ and 0.008 for 10 and 100 nM GEM, respectively, on day 3), and the number of cells with positive X-gal staining also increased in a dose-dependent manner (Figure 1b, $P=0.03$, 0.03, and 0.001 for 1, 10, and 100 nM GEM, respectively, on day 3). These results indicate that GEM enhances the expression of β-gal delivered by Ad-lacZ in a dose-dependent manner.

GEM enhances the transgene expression of NK4 delivered by Ad-NK4 in both culture media and within adenovirus-infected cells

Next, we investigated the effects of GEM on the expression levels of NK4 delivered by Ad-NK4. SUIT-2 cells were infected with Ad-NK4 at an MOI of 10 and cultured with or without GEM as described above for Ad-lacZ. After replacement of the GEM-containing media with fresh media without GEM, we measured the NK4 expression levels in culture media and among intracellular proteins extracted from the infected cells on days 1, 2, and 3. GEM significantly increased the NK4 expression level in culture media in a dose-dependent manner (Figure 2a, $P=0.045$, 0.0014, and <0.0001 for 1, 10, and 100 nM GEM, respectively, on day 3), similar to the case for Ad-lacZ. In particular, the increase in expression was remarkable for 100 nM GEM. GEM also significantly increased the intracellular NK4 expression level in infected cells in a dose-dependent manner (Figure 2b, $P<0.001$ and 0.001 for 10 and 100 nM GEM, respectively, on day 1). In the presence of 100 nM GEM, the intracellular NK4 expression level peaked on day 1 and then decreased on days 2 and 3. It was possible that NK4 proteins accumulated within Ad-NK4-infected cells were released into the culture media when the cells were killed by the high dose of GEM, thereby leading to the increased NK4 expression level in the culture media. We investigated the viability of GEM-treated SUIT-2 cells, and found that cells treated with 100 nM GEM showed a slight decrease in viability on day 2 and a

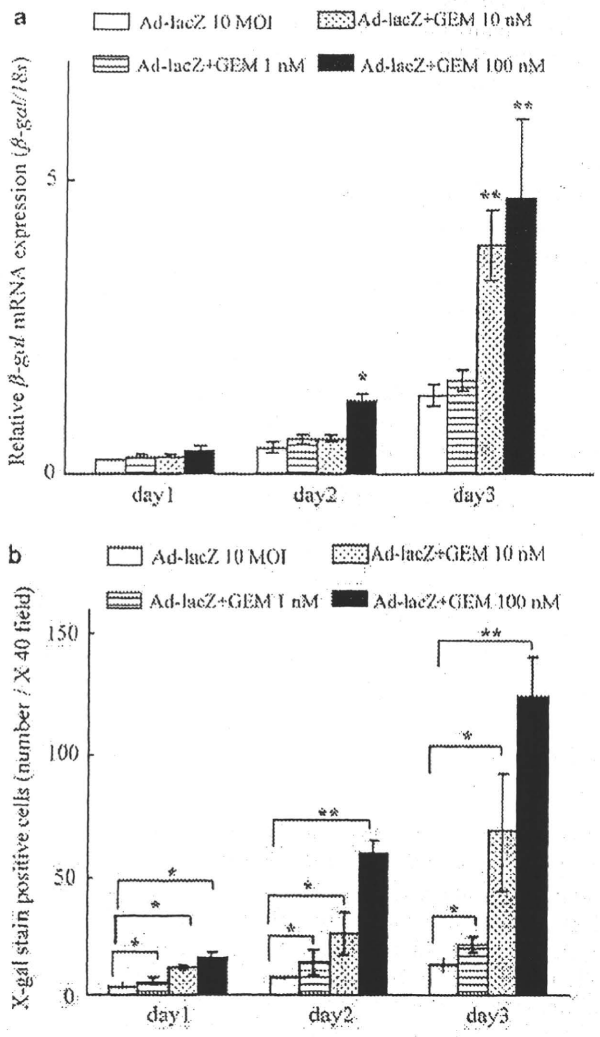

Figure 1 GEM enhances the transgene expression of β-gal delivered by Ad-lacZ. SUIT-2 cells were infected with Ad-lacZ (MOI of 10) for 1 h and treated with GEM (0, 1, 10, and 100 nM) for 24 h. The culture media were then replaced with fresh media without GEM. (**a**) Total RNA samples were extracted on the indicated days. The expression levels of *β-gal* mRNA were measured by qRT–PCR and normalized by the corresponding expression level of *18S rRNA*. Bars represent relative expression levels as the fold changes in comparison with untreated cells. Each value represents the mean ± s.d. of three independent samples. (**b**) β-gal activity was assessed by X-gal staining and counted numbers of β-gal-positive cells (magnification, × 100). Each value represents the mean ± s.d. of five independent fields. $^{*}P<0.05$, $^{**}P<0.01$.

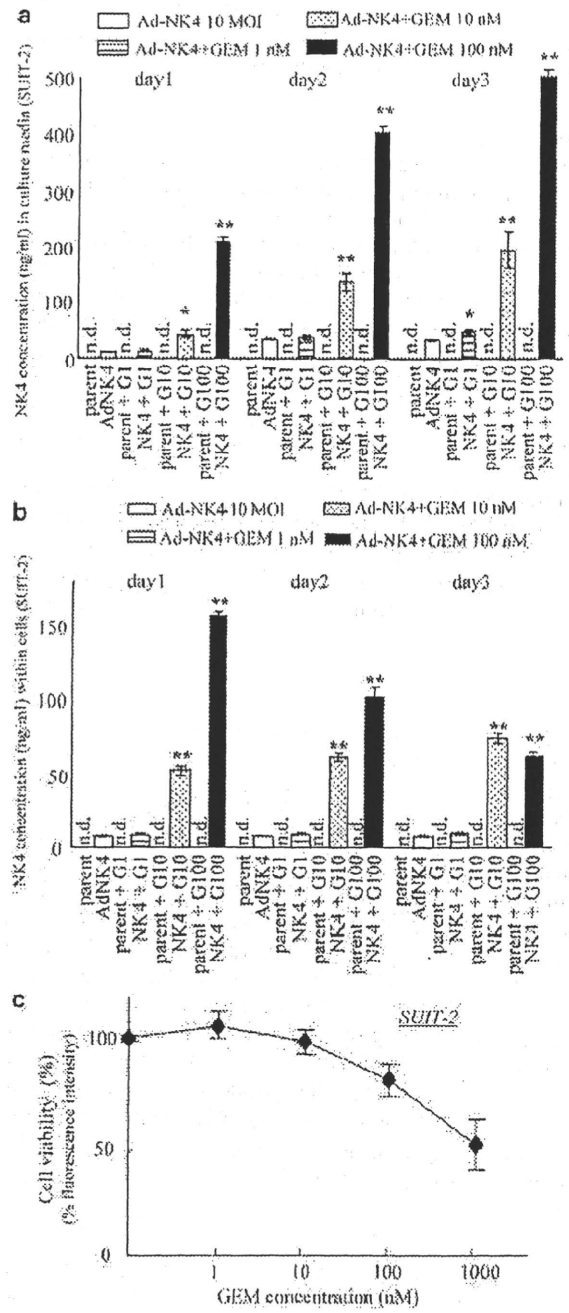

Figure 2 GEM enhances the transgene expression of NK4 delivered by Ad-NK4 in both the culture media and within adenovirus-infected SUIT-2 cells *in vitro*. (**a**, **b**) SUIT-2 cells were infected with Ad-NK4 (MOI of 10) for 1 h and treated with GEM (0, 1, 10, and 100 nM) for 24 h. The culture media were then replaced with fresh media without GEM. The NK4 expression levels were measured in the culture media (**a**) and within cells (**b**) on days 1, 2, and 3. (**c**) SUIT-2 cells were treated with GEM for 24 h, followed by replacement of the culture media with fresh media without GEM. After 72 h, the cell viabilities were determined by PI assays as the ratio of the fluorescence intensity. 'n.d.' in the graphs means 'not detectable'. Bars represent relative cell viabilities as the fold changes in comparison with control cells. Each value represents the mean ± s.d. of three independent samples. $^{*}P<0.05$, $^{**}P<0.01$.

18.8 ± 6.9% reduction in viability on day 3 (Figure 2c). Interestingly, the increase in intracellular NK4 expression in SUIT-2 cells began to be notable on day 1 (Figure 2b), although GEM did not kill the cells at any of the concentrations examined on day 1 (Figure 2c). Furthermore, low doses of GEM, such as 10 nM, did not kill SUIT-2 cells even on day 3 (Figure 2c), but still