Cell-based Protein Delivery System for the Inhibition of the Growth of Pancreatic Cancer: NK4 Gene-transduced Oral Mucosal Epithelial Cell Sheet

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ABSTRACT

Purpose: Pancreatic resection for pancreatic cancer is the only curative modality, but the high incidence of local recurrence after surgery results in a very poor prognosis. This study aims to develop a new therapeutic tool that could inhibit the growth of remnant cancer cells, which is based on local delivery of NK4 (hepatocyte growth factor antagonist) secreted from an NK4 gene-transduced oral mucosal epithelial cell (OMEC) sheet (NK4-sheet), which is adhered to the resected surface.

Experimental design: OMECs, harvested and cultured according to 3T3 feeder layer technique, were seeded on a collagen mesh-overlayered, biodegradable VICRYL mesh to produce an OMEC sheet. NK4 gene transduction was mediated by recombinant adenovirus (Ad-NK4). Applicability of OMECs for cell-based NK4 delivery was examined. An experimental model using nude mice was established to determine the effect of an NK4-sheet on both tumor growth and angiogenesis.

Results: NK4 secreted from Ad-NK4-transduced OMECs suppressed MRC-5-induced invasion of pancreatic cancer cell lines. Heterotypically implanted gene-transduced OMECs remained for ≥10 days while gradually decreasing.

Conclusion: Autologous OMEC was found to be suited to this purpose because of no secretion of hepatocyte growth factor, ease in harvesting from a patient, reasonably high proliferation potential, and no immune reaction. Although NK4-sheets under development exhibited a low level and short period of NK4 secretion, it is expected that this system may have a great potentiality of protein delivery system to target tissue at clinical situations when it is loaded with multilayered OMECs.

INTRODUCTION

Pancreatic cancer remains difficult to cure, and pancreatic resection is the only curative modality. Pancreatic cancer, even if small, often invades surrounding tissues and induces local recurrence after surgery at a high incidence, resulting in a very poor prognosis (1, 2). Although some adjuvant therapies, such as intraoperative radiotherapy and adjuvant chemotherapy, have been tested, satisfactory results have not been obtained as yet. Therefore, development of a new therapeutic modality to prevent the local recurrence has been awaited.

The local recurrence after pancreatic resection is generally caused by the high migratory and invasive potentials of pancreatic cancer cells. Such malignant behavior is usually affected by many kinds of cytokines. HGF, known to act as a potent scattering factor by binding to c-Met receptor expressed on cells (3, 4). HGF antagonist, NK4, composed of the NH₂-terminal hairpin and four kringle domains of the α-subunit of HGF, binds to the c-Met receptor but does not induce tyrosine phosphorylation of c-Met, resulting in inhibition of mitogenic, motogenic, and morphogenic activities of HGF (5, 6). Recent studies showed that NK4 inhibits growth and migration of vascular endothelial cells stimulated by angiogenic growth factors (7, 8) and that the c-Met receptor is frequently overexpressed in pancreatic cancers (9–11). NK4 injection to a tumor and NK4-encoding adenovirus-mediated gene transduction suppressed the migration, invasion, and growth of pancreatic cancer cells in vitro and in vivo (9, 12–15).

In our previous study, we developed a local delivery system of a protein and an adenoviral vector using in situ photocured gelatin: a bioactive substance-immobilized, tissue-adhesive matrix (16, 17). Protein delivery from photocured gelatin adhered to a living tissue proceeded with time. However, the

Received 12/17/02; revised 3/20/03; accepted 3/26/03.

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1Supported by Promotion Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (contract Grant 97-15) and Grant-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology, Japan (contract Grants A2-12358017 and B2-12470277).

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3The abbreviations used are: HGF, hepatocyte growth factor; OMEC, oral mucosal epithelial cell; SEM, scanning electron microscopy; MOI, multiplicity of infection; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR.
efficiency of gene transduction using this photocured gelatinous matrix was strictly limited because of very low permeation of a giant molecule, i.e., the adenoviral vector, because tissue permeation largely depends on the molecular size of a permeant (18). On the basis of these experimental data, an alternative NK4 delivery system was devised for in situ production of NK4 on the diseased tissue. The working principle of the fabricated device under development is that a hybrid tissue, constructed by ex vivo gene-transduced autologous cells with a biodegradable mesh, is adhered to a resected tissue. NK4 secreted from the gene-transduced cells in the hybrid tissue is transported deep into the target tissue and suppresses the tumor growth. If this works effectively, such a cell-based delivery system, which is a product of tissue engineering and genetic engineering disciplines, will be a promising therapeutic tool.

In this study, rat OMECs and adenoviral vector-mediated NK5 gene transduction were used. The fabrication of the tissue-engineered, gene-transduced hybrid tissue, the effects on pancreatic cancer cells in vitro, and a xenograft pancreatic cancer model in vivo are reported. Its potential applicability in a clinical setting is discussed.

MATERIALS AND METHODS

OMEC. The harvesting and culture of rat OMECs were according to the “feeder layer technique” developed by Rheinwald and Green et al. (19–21). Briefly, oral mucosal tissues, which were harvested from 3- to 6-week-old Wistar rats, were cut into small pieces and immersed twice in PBS (pH 7.4; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing antibiotics (1000 units/ml Penicillin G potassium, 1 mg/ml kanamycin, and 2.5 μg/ml amphotericin B) for 30 min at 37°C. Then, these tissues were immersed in DMEM (Life Technologies, Inc., Grand Island, NY) containing 0.2% dispase (Sigma-Aldrich Co., St. Louis, MO) at 4°C for 20 h, followed by treatment with 0.25% trypsin and 5 mM EDTA solution for 30 min at room temperature. The enzyme activity was blocked by washing with DMEM containing 10% FBS (CSL Ltd., Victoria, Australia). Then, the specimens were gently stirred in DMEM containing 5% FBS for 30 min and passed through a 50-μm filter to obtain OMECs.

Swiss 3T3 cells, obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and treated with 4 μg/ml Mitomycin C (Wako Pure Chemical Industries, Tokyo, Japan) for 2 h, were seeded on a six-well plate (Corning Inc., Corning, NY) at a density of 1 × 10^5/well. OMECs were seeded over them at a density of 1 × 10^5/well and cultured in 10% CO_2 in epithelium formation medium, which was a 3:1 mixture of DMEM and Ham’s F medium (Nihonseiyaku, Tokyo, Japan) and supplemented with 5% FBS, 5 μg/ml insulin (Wako Pure Chemical Industries), 5 μg/ml transferrin (Wako Pure Chemical Industries), 2 × 10^{-9} M triiodothyronine (Sigma-Aldrich Co.), 10 ng/ml cholera toxin (Sigma-Aldrich Co.), 0.5 μg/ml hydrocortisone (Wako Pure Chemical Industries), 100 units/ml penicillin, 0.1 mg/ml kanamycin, and 0.25 mg/ml amphotericin B. Human recombinant epidermal growth factor (Wako Pure Chemical Industries) was added at 10 ng/ml from 3 days after the inoculation. Seven to 10 days later, the proliferated OMECs became confluent (the yield of cell numbers per rat was 6 × 10^6 to 1 × 10^7 cells) and were subcultured. Cells at the second or third passages were used in the present study.

RNA Preparation and RT-PCR. s.c. tissue, which was used as a positive control, was isolated from the back skin of a Wistar rat with scissors under general anesthesia and cut into small pieces. RNA was extracted from OMECs or the pieces of s.c. tissue with ISOGEN-LS (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer’s protocol. RNA (35 μg) from each sample was reverse transcribed with a Ready-To-Go T-Primed First-Strand kit (Amersham Pharmacia Biotech, Inc., Pisacataway, NJ) according to the manufacturer’s protocol. The rat HGF primers used were 5′-AGTAGGGTGGATGGTAGTT-3′ (sense strand) and 5′-TACAACCTTATGTCGAAAAAT-3′ (antisense strand), which delineated a 672-bp product (22). The rat glyceraldehyde-3-phosphate dehydrogenase primer was purchased from R&D Systems, Inc. (Minneapolis, MN). Subsequently, reverse transcription products were PCR amplified, using the rat HGF primer set or the rat glyceraldehyde-3-phosphate dehydrogenase primer set under the following conditions: at 95°C for 5 min followed by 33 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. The amplified products were subjected to electrophoresis in a 1% agarose gel, stained with ethidium bromide, and photographed using a Molecular Imager FX (Bio-Rad Laboratories, Inc., Richmond, CA).

Cell Culture of Cell Lines. Human pancreatic cell lines, AsPC-1 (23) and SUIT-2 (24), generously donated by Dr. H. Iguchi, National Kyushu Cancer Center (Fukuoka, Japan), were cultured in DMEM supplemented with streptomycin, penicillin, and 10% FBS at 37°C in 5% CO_2. The human fibroblast cell line, MRC-5, which secretes biologically active HGF, was obtained from RIKEN Cell Bank (Ibaragi, Japan). The cells were cultured in DMEM supplemented with streptomycin, penicillin, and 10% FBS at 37°C in 5% CO_2.

Preparation of Collagen Membrane and OMEC Sheet. A biodegradable poly(glycolic acid) mesh (knitted-type VICRYL mesh, polyglactin 910; Ethicon, Inc., Somerville, NJ) was placed on a 24-well plate (Greiner Bio-One Co., Ltd., Frickenhausen, Germany), to which a mixture of 0.5 ml of type I collagen (0.3%, CELLGEN; Koken Corp., Tokyo, Japan) and 0.5 ml of DMEM containing 10% FBS was overlaid on the mesh and incubated at 37°C for 30 min to form a collagenous gel-VICRYL mesh hybrid composite. The thick collagenous gel was compressed by a silicone cap to form a dense collagen mesh entrapped in the VICRYL mesh. Cultured OMECs, dislodged with 0.05% trypsin-EDTA, were seeded on the mesh at a density of 2 × 10^5 cells/cm^2. Two to 3 days later, OMECs formed a subconfluent monolayer. An H&E-stained cross-section of the cell-seeded collagen mesh was observed under a phase-contrast microscope (TE 300; Nikon, Tokyo, Japan). The cell-seeded collagen mesh was fixed in 2% glutaraldehyde (Electron Microscopy Sciences, Washington, PA) for 1 h, then postfixed in 1% osmium tetroxide (Chiyoda Junyaku, Tokyo, Japan) for 1 h, and subsequently dehydrated with a graded series of ethanol, sputter coated with platinum and evaluated by SEM (JEOL, JSM-840A, Tokyo, Japan).

Adenovirus Vectors. The replication-deficient adenovirus vector expressing NK4 (Ad-NK4) used in this study is E1a-, partially E3-deleted vectors based on human adenovirus type 5 (12). Replication-defective E1a, E1b, and E3 recombinant ade-
novirus vector expressing Escherichia coli lacZ (Ad-lacZ) was kindly gifted from Professor H. Ueno (University of Occupational and Environmental Health, Fukuoka, Japan; Refs. 25 and 26). The titers of viral stocks were quantified by a plaque-forming assay using 293 cells and expressed as plaque formation unit.

Secretion of NK4 from OMECs in Vitro. OMECs, which were plated at 2 × 10^5 cells/cm² on a type I collagen-coated 12-well tissue culture plate (Greiner Bio-One Co., Ltd.) for 72 h, were transduced with Ad-NK4 at an MOI of 10, 50, 100, and 200 in a 500-μl medium at 37°C for 2 h. After transduction, the culture supernatant was replaced with a fresh culture medium. Aliquots of the culture medium were collected every 48 h. The concentration of NK4 in the culture medium was determined using an IMMUNIS human HGF enzyme immunoassay kit (Institute of Immunology, Tokyo, Japan). This ELISA kit has no cross-reactivity between human and rodent HGF.

OMEC Proliferation after Ad-NK4 Transduction in Vitro. OMECs, which were seeded at a density of 5 × 10^4/well on a type I collagen-coated 24-well plate and cultured for 72 h, were transduced with Ad-NK4 or Ad-lacZ at an MOI of 100. Transduced OMECs were cultured in epithelium formation medium. Twenty-four and 48 h later, the cells dislodged using 0.05% trypsin-EDTA were counted using a Coulter counter (Beckman Coulter, Inc., Fullerton, CA).

Invasion Assay. OMECs, which were seeded at a density of 1 × 10^5 cells/wells on a type I collagen-coated 12-well plate and cultured for 72 h, were transduced with Ad-NK4 or Ad-lacZ at an MOI of 100. After transduction, the culture supernatant was replaced with DMEM containing 2% FBS. Aliquots of the culture medium were collected 3–4 days after transduction (named as NK4-sup and lacZ-sup) and used in invasion assay. The invasion of tumor cells was measured using a 24-well Matrigel invasion double chamber (Becton Dickinson, Bedford, MA). MRC-5 cells were seeded at a density of 1.5 × 10^4 cells/cm² on the outer cup in DMEM containing 10% FBS, and after 24-h cultivation, the medium was replaced by DMEM supplemented with 2% FBS. Pancreatic cancer cells suspended in DMEM containing 2% FBS or NK4-sup were added to the inner cup of Matrigel invasion chamber at a density of 5 × 10^4 cells/cup. After 24-h cultivation, pancreatic cancer cells that degraded the Matrigel and migrated through 8-μm pores of the membrane at the bottom of the inner cup to the opposite side of the membrane were counted after they were stained with H&E. Five microscopic fields (×200) were randomly selected for cell counting.

Establishment of i.m. Cancerous Mass and Gene Expression. Four-week-old female nude mice (BALB/c nu/nu; Kyudo Co., Ltd., Saga, Japan) were i.m. injected with 3 × 10^6 AsPC-1 cells in the left flank on day 0. Three days after s.c. injection, lacZ-sheets (n = 5) or NK4-sheets (n = 5) were implanted s.c. in an upside-down manner. In the control group (n = 5), mice were not treated after injection of cancer cells. Five mice of each experimental group were used for the tumor growth assessment. To quantify the tumor growth, two perpendicular diameters of the resultant s.c. tumors were measured with calipers every 3–5 days. The tumor volume was calculated using the formula: tumor volume (mm³) = 0.52 × [width (mm)]² × [length (mm)]. Another 11 mice were used for examination of angiogenesis (control, n = 4; lacZ-sheet, n = 4; 3-indolyl-β-galactopyranoside (Sigma-Aldrich Co.), according to the method described previously (30). Ten days after implantation, three samples with lacZ-sheet were also subjected to X-gal staining. The resected tumors of each experimental group (n = 3) were washed once in PBS and homogenized with radioimmunoprecipitation assay buffer consisting of 10% 10 × radioimmunoprecipitation assay buffer (Upstate Biotechnology, Inc., Lake Placid, NY), 1 mm sodium orthovanadate (Wako Pure Chemical Industries), and 1 mm phenylmethylsulfonyl fluoride (Wako Pure Chemical Industries), and tumor lysates were obtained. Serum samples were obtained from mice implanted with NK4-sheets (n = 3). After protein determinations were performed, the amounts of NK4 in the tumor lysates and sera were analyzed by ELISA, as described above. Seven and 10 days after implantation of NK4 sheets, the amounts of NK4 in the tumor lysates were also examined by ELISA (n = 3).
NK4-sheet, \( n = 3 \). Fourteen days after the injection of cancer cells, the tumor was removed and embedded in paraffin for immunohistochemical staining. Tissue sections were incubated with a rabbit polyclonal antibody against von Willebrand factor (DAKO Corp., Carpinteria, CA), and immunoreaction was visualized by staining with a 3,3'-diaminobenzidine-peroxidase complex (DAKO EnVision System; DAKO). The number of blood vessels was counted under a light microscope at \( \times 200 \) magnification. At least 15 randomly selected fields were examined per section.

Statistical Analysis. Statistical analysis was performed with ANOVA. Post hoc comparisons were made by the Scheffe analysis. Differences were considered significant at \( P < 0.05 \).

RESULTS

Fabrication of OMEC Sheet. OMECs, which were harvested and cultured according to the 3T3 feeder layer technique (19–21), were seeded on the collagen mesh-overlayered VICRYL mesh to produce OMEC sheets. The gross appearance of the OMEC sheet after 3 days culture was thin and flexible (Fig. 2A). A phase-contrast microscopy image of a cross-section of an H&E-stained OMEC sheet showed that a monolayer of OMECs is formed on the collagen mesh (Fig. 2B). An SEM of the cross-section showed that the OMEC sheet was composed of three layers: (a) OMECs; (b) collagen mesh; and (c) the VICRYL mesh (Fig. 2C). OMECs cultured on collagen mesh were polygonal and had regular microplicae similar to microridges with a cell membrane protrusion at the cell border as reported by Günter Lauer et al. (Fig. 2D; Ref. 31). To examine whether rat OMECs expressed rat HGF mRNA, RT-PCR was performed. The rat s.c. tissue showed a detectable level of rat HGF mRNA but not OMECs. OMECs cultured on collagen mesh were polygonal and had regular microplica similar to microridges with a cell membrane protrusion at the cell border as reported by Günter Lauer et al. (Fig. 2D; Ref. 31).

Ad-NK4-transduced OMECs: In Vitro Release and Proliferation. OMECs were transduced with Ad-NK4 at an MOI of 10, 50, 100, and 200, and aliquots of supernatants were collected every 2 days and analyzed by ELISA (Fig. 4). The amount of NK4 secreted from Ad-NK4-transduced OMECs reached a maximal peak on day 4 and then gradually decreased, irrespective of MOI. The amount at the maximal peak was the highest at an MOI of 100–200. NK4 was not detected in supernatants of nontransduced OMECs and Ad-lacZ-transduced OMECs (data not shown). Ad-NK4 transduction was carried out at an MOI of 100 for additional experiments.

To examine the effect of Ad-NK4 on proliferation of OMECs, the proliferation profile of Ad-NK4-transduced OMECs was compared with those of nontransduced OMECs and Ad-lacZ-transduced OMECs (Fig. 5). Little statistical difference among them was observed within 48-h observation period. This indicates that neither viral transduction nor autocrine NK4 causes any significant effect on proliferation of OMECs.

Invasion Assay. To determine whether NK4 secreted from Ad-NK4-transduced OMECs inhibits the invasion of hu-
human pancreatic cancer cells, an invasion assay was conducted using a Matrigel invasion chamber in the presence or absence of the cell-lined fibroblast that secretes HGF, MRC-5, in the outer cup of the chamber. The supernatant of Ad-NK4-transduced OMECs (NK4-sup), Ad-lacZ-transduced OMECs (lacZ-sup), or the medium (DMEM containing 2% FBS) was added into the inner cup that was preseeded with pancreatic cancer cells (SUIT-2 and AsPC-1). After 24-h cultivation, the number of the invaded cells, which were migrating to the opposite side of the filter membrane (average pore size, 8 μm) through the Matrigel, was counted. In the absence of MRC-5 in the outer cup, regardless of the type of cancer cell, there was little difference between the numbers of invaded cancer cells cultured in the medium, lacZ-sup, and NK4-sup. In the presence of MRC-5, the numbers of invaded SUIT-2 cells cultured in the medium and lacZ-sup tended to be quite higher than those in the absence of MRC-5, whereas the number of invaded cells in NK4-sup was almost the same as that in the absence of MRC-5. For AsPC-1 cells, the same trend was observed. Values are expressed as means ± SD (n = 3).

**DISCUSSION**

A series of our ongoing studies aimed at reducing retroperitoneal recurrence of pancreatic cancer is utilization of NK4, which has dual biological functions: an HGF antagonist and angiogenetic inhibitor. Our previous study has shown that the controlled release of these substances from photocured gelatinous matrix, which was adhered to a resected surface, was assessed using in situ photocured gelatinous matrix (16). The
sustainably released model protein could diffuse and be trans-
ported deep into the target tissue with time (Fig. 10 A). The
release rate and period were controlled by formulation param-
eters of a photocured gelatin solution and the porosity of pho-
tocured gelatinous matrix, which were reported in our previous
study in detail. Because NK4 is available only for the experi-
mental purpose at this time, the NK4 gene-transduction method
was devised in our previous study (17). However, when Ad-
NK4 was immobilized into a photocured gelatinous matrix,
Ad-NK4 diffused out from the matrix but was not transported
into the target tissue (Fig. 10 B). Because only cells present at the
outermost surface of the target tissue were partially transduced,
"passive"-diffusion-driven mechanism does not operate for a
giant molecule, such as an adenovirus. As an alternative strategy
for the therapy for local recurrence, we devised a cell-based
protein delivery system, which was intraoperatively implanted
with a gene-transduced cell-monolayer sheet as a vehicle for
gene therapy.

For cell-based NK4 delivery, the selection of cell type used
is essential. The requirement for a cell type is that such a cell
does not inherently secrete HGF. Our survey experiment of
mesenchymal cells as a possible candidate for a cell source
showed that fibroblasts produced HGF at a relatively high level,
and concomitantly, no suppressive effect of Ad-NK4-transduced
fibroblasts on invasion of cancer cells was observed. On the
other hand, autologous OMECs were found to be best suited to
this purpose because of the following: (a) no secretion of HGF
nor expression of HGF mRNA (Fig. 3); (b) reasonably high
proliferation potential that is not affected by adenoviral trans-
duction and NK4 (Fig. 5); (c) ease in harvesting from a patient;

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Table 1  Time course of the amount of NK4 in the tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Amount of NK4 (ng/gram protein)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>4 day</td>
</tr>
<tr>
<td>Control</td>
<td>Undetectable</td>
</tr>
<tr>
<td>lacZ</td>
<td>Undetectable</td>
</tr>
<tr>
<td>NK4</td>
<td>155.86 ± 60.83</td>
</tr>
</tbody>
</table>

* Not examined.

Fig. 7  Gene-transduced OMEC sheet in experimental tumor models. Cross-sectional image of Ad-lacZ-transduced OMEC sheet (lacZ-sheet) stained by X-gal and H&E. A, 4 days after implantation; B, 10 days after implantation. Arrowheads, X-gal-positive OMECs; bar, 100 µm. O, OMECs; C, collagenous gel; M, VICRYL mesh; T, tumor.

Fig. 8  Tumor growth of s.c. injected AsPC-1 cells. Three days after cancer cell injection, tumors were covered with lacZ-sheet or NK4-sheet. In the control group, mice were not treated. A, development of tumor arising from AsPC-1 cells. Values are expressed as means ± SD (n = 5, *P < 0.05). B, gross appearance 14 days after AsPC-1 cell injection of each group.
and (d) no immune reaction (19–21, 32, 33). Indeed, NK4 secreted from Ad-NK4-transduced OMECs suppressed invasion of pancreatic cancer cells, which was induced by MRC-5 in vitro (Fig. 6). Moreover, the proliferation of OMECs was not affected by viral transduction and autocrine NK4 (Fig. 5), although OMECs express c-Met receptor (33).

Our preliminary study using a prototype gene-transduced cell sheet indicated that heterotopically implanted gene-trans-
duced OMECs remained for ≥10-day observation period, and NK4 was delivered into the tumor by Ad-NK4-transduced OMEC sheets, although gene expression significantly decreased with time. One possible reason for the decrease in gene expression was transient expression of adenoviral transduction. Despite a low level of NK4 delivery from Ad-NK4-transduced OMEC sheets into the tumor, these sheets inhibited angiogenesis and tumor growth (Figs. 7 and 8). However, the tumor growth gradually increased with time, which must be attributable to a time-dependent decrease in the amount of NK4. The antiangiogenic effect observed in this study was also observed in our previous studies, which were verified by i.p. injection or intratumoral injection of NK4 or Ad-NK4 (9, 12–15).

In conclusion, Ad-NK4-transduced OMECs, from which secreted NK4 diffuses into a tumor tissue (Fig. 10A), appear to offer a new therapeutic modality for local recurrence of pancreatic cancer. The shortcomings of a prototype technology, such as the low level and short period of NK4 production, may be overcome by high density seeding of gene-transduced cells, e.g., multilayering on a microporous matrix as schematically shown in Fig. 10D. It is highly envisaged that the combination of a high cell seeding and more powerful vector enabling longer term NK4 secretion, such as retrovirus vectors, overcome the shortcomings of the prototype cell-sheet device in this study. On the basis of such a hypothesis-driven strategy, we may offer a new therapeutic choice to improve the survival of pancreatic cancer patients.

ACKNOWLEDGMENTS

We thank K. Yasutake and E. Koga for excellent technical assistance, H. Okino for technical advice, and T. Kanemura for scanning electron examination.

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