Review Article

Development of gene therapy to target pancreatic cancer

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Pancreatic cancer remains one of the most difficult cancers to treat. Its high propensity to infiltrate and metastasize early from a small primary focus necessitates development of a new therapy which can track down the disseminated cancer cells *in vivo*. Gene therapy may offer new opportunities for a variety of targeting strategies, and we review here some of our work related to the development of targeted gene therapy: 1) Targeting by specific molecular abnormality: Many pancreatic cancer cells show "addiction" to K-*ras* mutation, while normal cells appear resistant to suppression of K-*ras*-mediated signaling by antisense K-*ras* RNA expression adenoviral vector. 2) Targeting by *in vivo* tumor characteristics: In a peritoneal dissemination model, intraperitoneal lipofection/polyfection can deliver and express transgenes highly preferentially in tumor nodules. 3) Targeting by vector: An efficient protocol for construction of an adenovirus expression vector library has been developed, which will enable a direct functional selection of fiber knob-modified targeting vector species for given cells. 4) Targeting by tumor immunity: Several cytokines not only induce direct cytotoxicity, but are also expected to activate specific immunity to achieve targeted suppression of cancer cells *in vivo*. Unlike parenteral administration of short-lived recombinant interferon protein, local interferon gene transfer can provide a target tissue-restricted distribution and sustained expression, which may improve the efficacy/safety balance of cytokine therapy. Cancer gene therapy development is, in general, at the stage of proof of principles and safety. However, it is an art of integrated science. The recent rapid progress of related sciences and technologies will expand the potential and consolidate the clinical reality of gene therapy. (Cancer Sci 2004; 95: 283–289)

Pancreatic cancer ranks fifth as a cause of cancer-related mortality in Japan and the United States. In Japan, the age-adjusted death rate for pancreatic cancer has risen from 2.0 per 100,000 populations in 1955 to 9.4 in 2001.[1)] Pancreatic cancer is one of the most difficult cancers to treat, with an overall prognosis of less than 10% 3-year survival. The reasons for the poor prognosis include: 1) the difficulty of early diagnosis due to its anatomical location and lack of specific early symptoms, 2) the avid infiltrative spread to the surrounding vital organs, such as nerves, great vessels and bile duct, 3) the frequent occurrence of distant metastasis even from a small primary tumor less than 2 cm in diameter, and 4) the poor response to existing chemo-, radio-, endocrine or immune therapy.[2–4)] A high risk group is also difficult to define; there are few established risk factors for pancreatic cancer. Among the most convincing are smoking, which confers a relative risk of about 1.5, and rare cases of pancreatic cancers associated with hereditary pancreatitis or other familial cancer syndromes.[5–7)] At least at the present time, an emphasis on early diagnosis alone may not be sufficient for significant improvement in the current poor prognosis of pancreatic cancer, and a full range of research, starting from very basic proof of concepts and progressing to clinical trials, is necessary to establish a new modality of treatment.

Gene therapy has long been regarded a possible new therapeutic modality, and about 70% of gene therapy clinical trials have been devoted to cancer. Although several protocols were shown to be safe and anecdotally effective in phase I/II studies, no phase III study has proved superiority over existing treatment for cancer.

Gene therapy is defined as a therapy in which gene(s) or gene-transducer cells are introduced to the patient's body for a therapeutic or gene-marking purpose. Therefore, gene therapy by definition is not necessarily a molecular targeting therapy, but the reason for the high expectations lies in the fact that new mechanisms of cancer cell targeting can be integrated into the therapy. Targeting points in cancer gene therapy can be either 1) targeting of genes crucial for cancer cell survival or phenotype to achieve specific killing or suppression of cancer cells, 2) targeting of cells to achieve specific delivery of a cell-killing device, or 3) a combination of both. This mini-review outlines a series of basic, preclinical research efforts in our laboratory to address the issue of the targeting of pancreatic cancer.

Targeting by molecular abnormality

The characteristically high incidence of K-*ras* point mutation may be the most well-known example of specific molecular abnormalities of solid cancer. Some 70–90% of these tumors have been reported to carry mutation, and more than 95% of the mutations are located in codon 12 with the remainder at codons 13 and 61.[8)] No significant difference existed in the incidence of K-*ras* mutation among the different stages of the disease,[9)] and the mutation was also found in mucous cell hyperplasia or chronic pancreatitis.[10–12)] Thus, it seems that K-*ras* point mutation is involved in the initiation or early phase of carcinogenesis, but not in the malignant progression of pancreatic cancer.

The general premise for solid cancer is that the full-blown phenotype of cancer cells depends on the accumulation of multiple genetic changes during multistep carcinogenesis. Genetic alterations of pancreatic cancer other than the K-*ras* mutation include abnormalities of the *p53* gene, loss of expression of the *DCC* gene, somatic mutation of the *APC* gene, loss and suppression of the *DPC4* gene, overexpression of acidic and basic fibroblast growth factors and microsatellite instability.[8)] Considering its potent NIH3T3-transforming activity *in vitro*, K-*ras* gene mutation appeared an obvious and attractive target for gene therapy in pancreatic cancer. However, it was not known if fully developed pancreatic cancer cells still depend on the

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single molecular abnormality, which presumably occurred in the initiating phase of carcinogenesis.

When we first addressed the question of whether the K-*ras* mutation could be a molecular target for killing pancreatic cancer cells effectively, ribozyme and siRNA technologies were not yet widely available, and the *in vivo* applicability of antisense oligonucleotides was still controversial. Therefore, we constructed an antisense K-*ras* RNA expression plasmid, AS-K-ras-LNSX, to express a 347-base antisense RNA of the wild-type K-*ras* exons 1, 2 and part of exon 3 (Fig. 1). Unlike mutation-specific oligonucleotides, the wild-type sequence antisense vector should, we assumed, work on a spectrum of K-*ras* mutations and on the wild-type K-*ras* as well. AS-K-ras-LNSX was transduced by lipofection into several human pancreatic cancer cell lines such as AsPC-1, MIAPaCa-2, Panc-1, PSN-1 and BxPC-3. Resequencing of the K-*ras* gene confirmed the wild-type sequence in the BxPC-3 cells and point mutations in the others. Western blot analysis of stable transfectants showed that the antisense vector significantly downregulated the K-*ras* p21 protein in all the pancreatic cancer cell lines except for BxPC-3. In line with the K-*ras* p21 suppression, the growth of pancreatic cancer cell lines with K-*ras* point mutations was inhibited following transduction of AS-K-ras-LNSX (Fig. 1), while the effect of the antisense construct on growth was not significant in BxPC-3.[13, 14] The study suggested that the K-*ras* point mutation is a valid molecular target for at least a certain fraction of pancreatic cancers, and that an antisense RNA expression vector is a possible tool for attacking the target.

An *in vivo* tumor-suppressive effect was demonstrated in a nude mice peritoneal dissemination model with AsPC-1 cells[13] (Table 1). We found that although liposome-mediated *in vivo* gene transfer could exhibit a unique targeting *per se*, as discussed later in this review, the major disadvantage of a synthetic nonviral vector is its low transduction efficiency. For certain *in vivo* gene therapy applications, such as intratumoral injection of an antisense RNA vector for locally advanced pancreatic cancers, vectors with a much higher transduction potency are desired, because targeting can be easily achieved anatomically in such situations. Therefore, we transplanted the antisense K-*ras* RNA unit into an adenovirus vector backbone with a CAG promoter[15] to construct AxCA-AS-K-ras (designated as AxCA-AS in ref. 16). Using this highly active viral vector, we not only confirmed our findings using stable transfectants of the plasmid antisense vector, but also found that the antisense K-*ras* RNA can induce apoptosis in pancreatic cancer cells (unpublished data), thereby categorizing the therapy as potentially cytocidal.

Such a cytocidal effect is also expected for other types of cancers with a high frequency of the K-*ras* mutation. Colorectal cancer is known to have K-*ras* point mutation in about 40–50% of the cases, a frequency second only to pancreatic cancer. Infection of seven human colorectal cancer cell lines with the AxCA-AS-K-ras adenovirus vector resulted in up to 25% reduction of the K-*ras* p21 protein, but the status of K-*ras* point mutation did not appear to be correlated with the growth-suppressive effect of the antisense K-*ras* vector: both K-*ras*-mutation-positive and -negative colorectal cancer cells were growth-suppressed.[17] Obviously, a wider collection of the cell lines should be examined to draw a definitive conclusion, but it appears that pancreatic and colorectal cancers differ in their de-

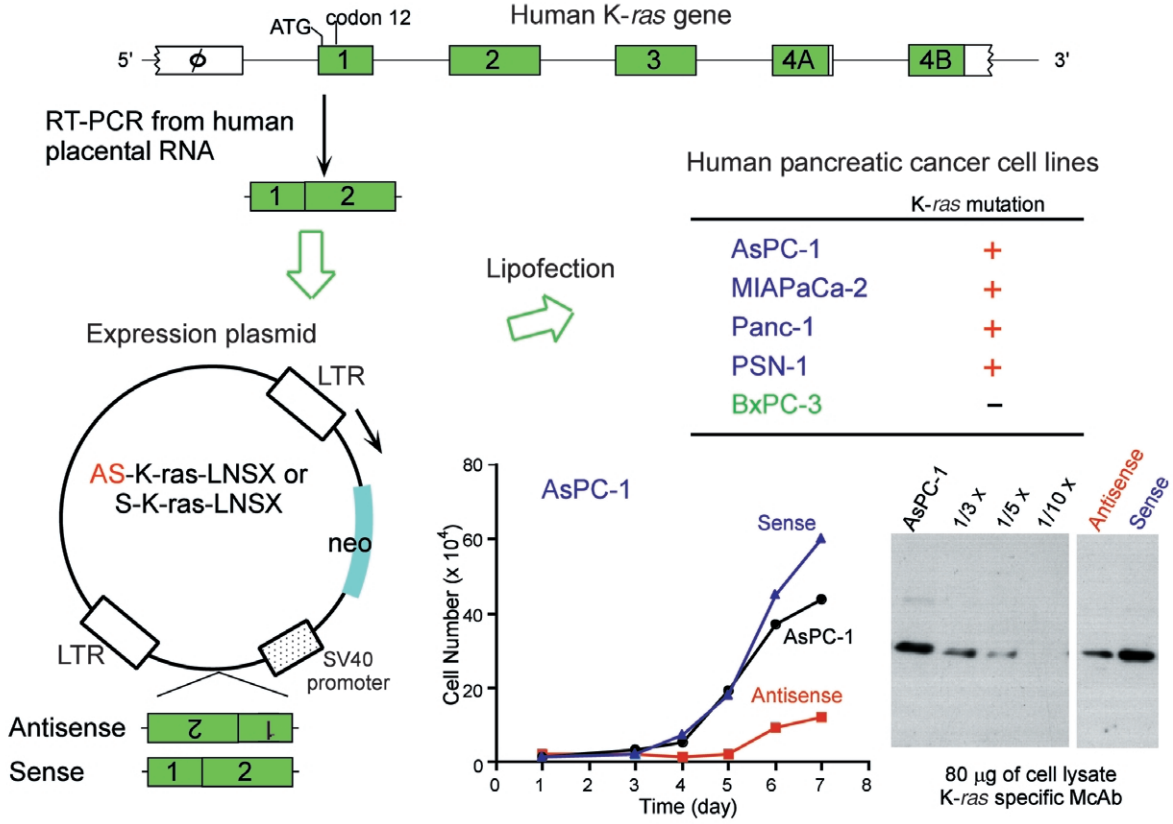

Fig. 1. Suppression of cell growth and K-*ras* p21 protein in pancreatic cancer cells transfected with antisense K-*ras* expression plasmid. A K-*ras* cDNA fragment spanning exons 1, 2 and part of exon 3 was cloned from normal human placental mRNA and placed downstream of the SV40 early promoter in an antisense or a sense orientation (AS-K-ras-LNSX and S-K-ras-LNSX, respectively). The plasmids were transfected into pancreatic cancer cell lines by lipofection, and G418-resistant colonies were pooled and used for cell growth and western blot analyses. Titration of the western blot signals showed that the antisense vector downregulated the level of the K-*ras* p21 protein to ca. 1/3 of the parental cells.

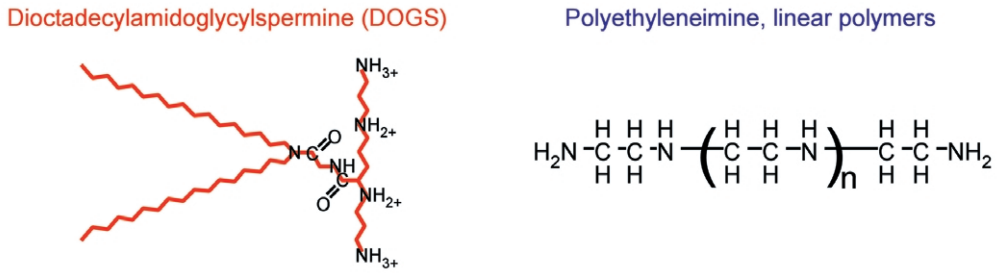

Fig. 2. Structures of dioctadecylamidoglycylspermine (DOGS) and linear form of polyethyleneimine (PEI).

pendency on K-*ras* signaling in the absence of K-*ras* mutation. The reason for the difference is not known, but it is noteworthy that K-*ras* mutation seems to occur in the mid or later stage of colorectal carcinogenesis, and in only about half of all cases, suggesting that the aberration of the K-*ras* signaling has a different meaning in the two types of cancers.

Because our antisense RNA sequence is directed against the wild-type K-*ras* sequence, the effect of the AxCA-AS antisense K-*ras* adenoviral vector was evaluated on five primary cultures of normal human cells: human umbilical vein endothelial cells, lung microvascular endothelial cells, hepatocytes, smooth muscle cells and mesangial cells. The high-efficiency infection with the adenoviral vector did not lead to a significant growth inhibition of these normal cells.[17)]

Our data suggested a dependence of the majority of pancreatic and colorectal cancer cells on growth mechanisms governed by the K-*ras* protein, while normal cells appear more resistant and adaptable to the stagnation of the K-*ras* signaling. Evidence has been accumulating that cancer cells are often "addicted to" the continued activity of specific activated or overexpressed oncogenes for maintenance of their malignant phenotype.[18)] Moreover, it remains an enigma why the occurrence of K-*ras* mutation is so selective to certain types of cancers, such as pancreas, colorectal and thyroid cancers, but is rare in many other cancers, such as those of the breast, stomach, esophagus, prostate and liver.

Taken together, these observations suggested that K-*ras* signaling is distinct, at least partly, in different types of cells. Several *ras*-mediated signaling pathways have been discovered, such as those leading to the activation of the RAF/MEK/ERK kinase cascade, the G proteins Rac and Rho, PI3K and Akt activation and to the regulation of Ca^{2+} metabolism.[19)] However, in addition to the tissue specificity, it is also possible that the signaling cascade elicited by the mutated K-*ras* gene is different from that activated by the wild-type K-*ras* gene. Therefore, cataloging of the genes mobilized specifically by K-*ras* mutation in the context of pancreatic carcinogenesis is necessary to understand the targeting nature of the antisense wild-type K-*ras* RNA expression. We first applied differential display analysis to pancreatic cancer cells stably transfected with AS-K-ras-LNSX,[20)] but the adenovirus transient transduction system offers a unique opportunity to address this issue, because its high gene transfer efficiency (more than 80–90% transduction can be achieved in many cell lines) enables analysis of populations of native cells of many different kinds, but not G418-selected clones.

Four pancreatic cancer cell lines with K-*ras* point mutations were infected with the AxCA-AS-K-ras adenoviral vectors, and the changes of gene expression were analyzed by using oligonucleotide-based microarrays containing 12,626 genes. Among the genes showing more than 2-fold differences in the expression levels between the control- and antisense-K-*ras*-transduced cells, 7 genes were commonly up-regulated and 4 genes, *syntaxin 1A*, *p120ctn*, *G-protein coupled receptor RE2* (*GPR-RE2*) and *phenylethanolamine N-methyltransferase* (*PNMT*), were commonly down-regulated in three or all of the four pancreatic cancer cell lines transduced with AxCA-AS-K-ras.[16)]

Although further optimization of the analysis may be necessary to increase the sensitivity of the screening, this knockdown system is expected to capture the authentic genes regulated by K-*ras* mutation in the context of genuine pancreatic cancer cells, unlike the "knock-in" system, i.e., overexpression of the exogenous K-*ras* gene in cells without K-*ras* mutation. This line of research may also lead to the identification of a molecular target better than K-*ras* mutation itself.

Targeting based on *in vivo* characteristics of intraperitoneal tumor nodules

Peritoneal dissemination is one of the major metastasis modes at advanced stages of pancreatic, gastric or ovarian cancers, but no effective therapy has been established. The *in vivo* efficacy of the antisense K-*ras* RNA expression unit was examined in a nude mouse peritoneal dissemination model induced by an intraperitoneal inoculation of AsPC-1 pancreatic cancer cells. The antisense RNA expression plasmid AS-K-ras-LNSX was mixed with a lipofection reagent, DOGS lipopolyamine[21)] (Fig. 2), and the DNA-DOGS complex was then injected intraperitoneally 3 times. Twenty-eight days after tumor cell inoculation, the nude mice were sacrificed and disseminated tumor nodules were found to be significantly suppressed in the antisense vector-injected group[13)] (Table 1).

There are two main classes of synthetic non-viral vectors: 1) cationic lipids such as *N*[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride (DOTMA), dioctadecylamidoglycylspermine (DOGS) or 1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide (DMRIE), and 2) polymeric DNA-binding cations such as poly-L-lysine, protamine, cationized albumin and polyethyleneimine (PEI)[21, 22)] (Fig. 2). PEI is the organic macromolecule with the highest cationic-charge-density potential; every third atom is an amino nitrogen that can be protonated, which makes the polymeric network an effective "proton sponge" at virtually any pH. PEI was examined in detail *in vivo*, because in our experiments, PEI was more efficient than the cationic liposomes examined, and it is possible to synthesize the simple polymer in large quantities in-house. Surprisingly, the transgene was preferentially expressed in the disseminated cancer nodules in the peritoneal cavity.[23)] The plasmid DNA of the luciferase marker gene driven by the potent and tissue-non-specific hybrid promoter CAG was complexed with PEI and injected into the peritoneal dissemination model mouse. High luciferase activities were observed only in tumors on the mesentery and pancreas, and low activities were detected in some organs such as the spleen, stomach and skeletal muscle (Fig. 3). Other organs such as the brain, lung, heart, liver, kidney, testis and small intestine did not show any luciferase activity. PCR analysis showed that the injected DNA was delivered to various organs, but the distributed DNA became undetectable by 6 months after the gene transfer. Blood

Table 1. Tumors in the peritoneal cavity of mice treated with AS- or S-K-ras-LNSX complexed with liposomes[1)]

AS-K-ras-LNSX					S-K-ras-LNSX			
Mouse No.	Tumors on				Mouse No.	Tumors on		
	Mesentery	Pancreas	Hepatic hilus			Mesentery	Pancreas	Hepatic hilus
1	–	–	–		1	++	++	+
2	–	–	–		2	++	+++	+
3	–	–	–		3	+	+	–
4	–	–	–		4	+	–	+
5	–	–	–	$P<0.01$	5	–	–	–
6	–	–	–		6	++	–	–
7	++	–	–		7	–	++	+
8	–	–	–		8	+	–	–
9	–	–	–		9	+	–	–
10	–	–	–		10	++	+	–
11	–	+	–					
12	–	–	–					
	2/12					9/10		

+: 0–3 mm in size and <3 in number
++: 3–10 mm in size or 3–10 in number
+++: >10 mm in size or >10 in number

1) Twenty-two BALB/c nude mice were injected intraperitoneally with 6×10^5 AsPC-1 cells at day 0, and 12 of the mice were given AS-K-ras-LNSX:liposome (DOGS) complex 3 times at 12 h intervals during days 3–4. As a control, S-K-ras-LNSX was used in the other 10 mice. The mice were sacrificed at day 28 and examined for evidence of the tumor in the peritoneal cavity.

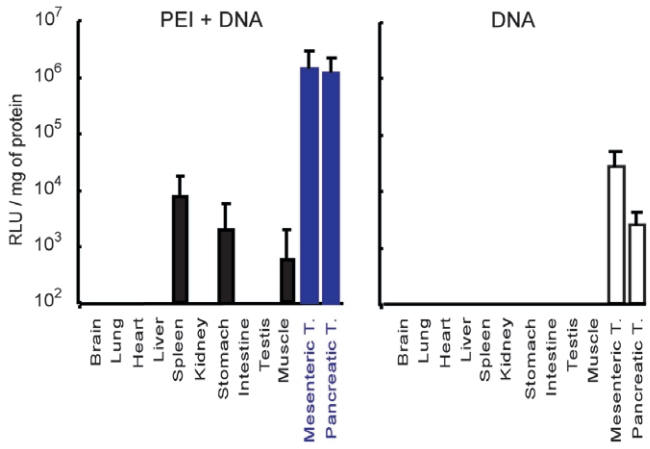

Fig. 3. Tissue distribution of luciferase expression after the intraperitoneal injection of DNA: PEI complex. After intraperitoneal transplantation of AsPC-1 cells, BALB/c nude mice were intraperitoneally injected with luciferase expression plasmid (pCAG-luci) complexed with PEI. Results were expressed as light unit per mg of tissue protein. (Left) pCAG-luci:PEI complexes were injected 3 times into the peritoneal cavity of 5 mice. (Right) pCAG-luci plasmids were injected 3 times into the peritoneal cavity of 5 mice. Mesenteric T., tumors on the mesentery; pancreatic T., tumors on the pancreas.

chemistry and histological analysis showed no significant toxicity in the injected mice.

The unexpected targeting capability was not restricted to PEI. PSN-1 is a pancreatic cancer cell line established in our laboratory and has 3- to 6-fold amplification of activated K-*ras* gene. The cells express the highest level of the p21 protein among the pancreatic cancer cell lines examined and were relatively resistant to the antisense K-*ras* RNA expression in the nude mouse peritoneal dissemination model. Accordingly, we introduced a *herpes simplex virus thymidine kinase* (HSV-tk) gene expression plasmid under the control of the CAG promoter as a DNA-DOGS lipopolyamine[21)] complex. Ganciclovir (GCV) was then administered for 8 days, and the mice were examined for tumor development at the 24th day after the tumor inoculation. While all 24 control mice showed macroscopic peritoneal dissemination and solid tumors on the pancreas, 8 of the 14 mice treated with the HSV-tk and GCV were free of tumors, and only a few small tumors were observed in the remaining 6 mice.[24)] No treatment-related toxicity was observed. A semi-quantitative RT-PCR analysis suggested that the HSV-tk transgene was expressed in about 10% of the tumor cells, but not in the normal pancreas or in the small intestine.

We speculated that at least a part of the mechanisms for tumor-preferential expression of a transgene following intraperitoneal lipofection/polyfection pertains to an anatomical barrier: the peritoneum and underlying connective tissue. The interaction of cancer cells with the peritoneum induces exfoliation of the mesothelial lining during the early process of peritoneal metastasis, and such disruption of the intact peritoneal barrier may predispose the tumor nodules to efficient gene transfer. To test this hypothesis, the peritoneum and underlying fibrous layer (renal capsule) covering the right kidney were surgically peeled off, and pCAG-luci:PEI complexes were then instilled directly onto the surface of the renal parenchyma. As a control, the same *in vivo* gene transfer was attempted on the left kidney with an intact surface. No luciferase expression was detected in the left kidney, whereas the right kidney showed a significant luciferase activity.[23)] It appears that the peritoneal lining captures the plasmid DNA:PEI complexes and prevents the spread of the gene transfer into the underlying organ parenchyma. An additional important factor which may contribute to the apparent tumor-preference is that the transduced gene is more readily expressed in rapidly proliferating cells such as cancer cells than in normal cells with low mitogenic activity. Therefore, the observed highly efficient targeting by intraperitoneal lipofection/polyfection may need further examination in an animal model with *de novo* tumor development and peritoneal dissemination.

Targeting by vector

Although the simple vector scheme of intraperitoneal lipofection/polyfection is attractive, the current low transduction efficiency of the synthetic non-viral vector poses a major disadvantage in the cost of clinical application. An alternative approach is the installation of more specific and active targeting mechanisms in the viral vector. Such vectors may also be administered systemically via the blood circulation to reach distant hematological metastasis foci. In particular, modification of the CAR (Coxsackie-adenovirus receptor)-specificity of the fiber knob protein of the adenovirus vector has attracted a num-

ber of investigators as a promising way to combine high transduction efficiency of the viral vector and targetability *in vivo* (Fig. 4A). Several cell surface-binding ligands, such as polylysine, proteoglycan-binding peptides, integrin-binding peptides, hormones and phage library-derived peptides,[25–27] have been engineered at the fiber knob to redirect the cell tropism of the vector.

However, the limited list of the existing ligand-receptor combinations is not always applicable to target many different types of cancer. For systematic development of a targeting adenoviral vector tailored to a given cancer, a rapid expression cloning protocol of a fiber knob-modified adenovirus vector library is required. As a first step to this goal, we developed a simple and efficient method for constructing adenovirus cDNA expression libraries[28] (Fig. 4B). This protocol is based on a Cre/lox-mediated *in vitro* recombination between adenoviral shuttle plasmid cDNA libraries and adenoviral genomic DNA tagged with terminal protein. Highly optimized packaging cell clones were also selected. In a model experiment, EGFP clones mixed at the frequency of 0.003% in the shuttle plasmid library were able to be efficiently identified and converted to an adenoviral vector, indicating that high-complexity libraries harboring low abundance cDNAs can be produced. The usefulness of this system was also demonstrated by the isolation of cDNA for CD2 (frequency, less than 1 in 0.3×10^4 transcripts in T cells) from human T cells. This effective and versatile method enables functional cloning for a variety of purposes. Construction of an adenovirus vector library with a randomly modified fiber knob is in progress to isolate vector clones with a high targeting potential.

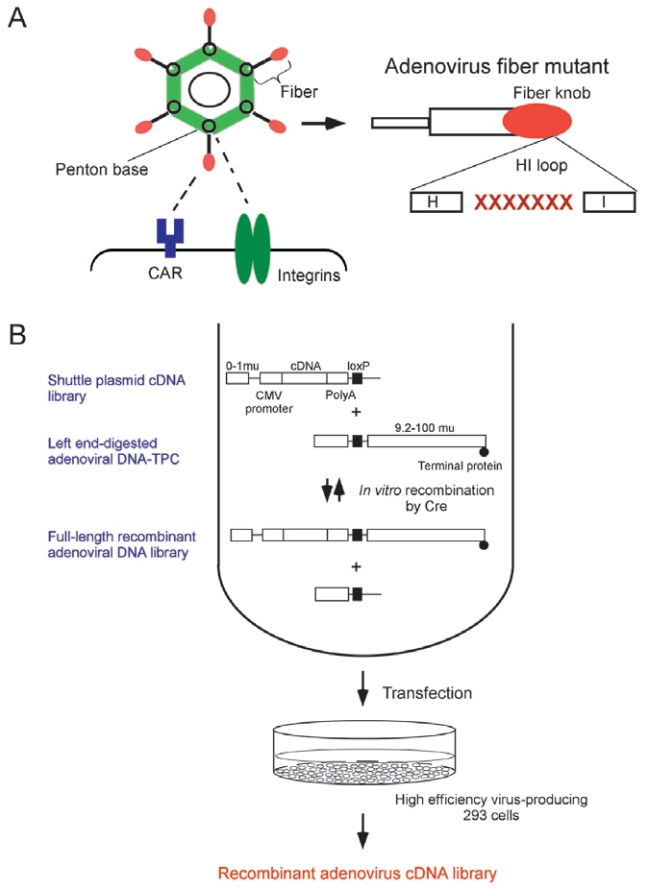

Fig. 4. A) Natural pathway of adenoviral entry and fiber knob modification to redirect cell tropism. The adenoviruses enter susceptible cells through two distinct sequential steps: the initial high affinity binding of adenovirus serotype 5 to the primary cellular receptors (CAR) occurs via the C-terminal knob domain of the fiber protein. The subsequent internalization of the virion by receptor-mediated endocytosis is potentiated by the interaction of Arg-Gly-Asp (RGD) peptide in the penton base with secondary host cell receptors, integrins. A fiber knob-modified adenovirus vector displaying an extra 7-amino acid sequence in the HI loop is shown as an example. The development of targeting vectors may require ablation of endogenous tropism as well as the introduction of a ligand for novel tropism. B) Construction of an adenovirus cDNA expression library. The linearized shuttle plasmid cDNA library is mixed with left end-digested adenoviral DNA tagged with terminal protein. Cre recombinase produces a full-length recombinant adenoviral DNA library *in vitro*, which is subsequently transfected into 293 cells to generate an adenovirus vector library.

A variation of targeting by the vector mechanism is the use of tissue/cell-specific promoters. We previously designed and tested two examples of such promoters, von Willebrand factor promoter to target endothelial cells[29] and modified rat probasin promoter to target human prostate cancer cells, including those that acquired androgen independence or resistance to endocrine therapy.[30] Not surprisingly, there was a trade-off between the promoter specificity and potency. Our promoters may be sufficient to drive a sensitive cell-killing device such as the HSV-tk gene, or presumably to turn on Cre recombinase to activate or deactivate a loxP-regulated expression unit in the target cells. However, in general, a more potent promoter would be desirable, and such a promoter has not been developed for gene therapy targeted to the pancreatic ductal carcinoma. Although our experience with oligonucleotide microarray analysis suggested a significant heterogeneity in gene expression among the different pancreatic cancer cell lines,[16] it is still necessary to accumulate expression profiling data of surgical specimens of pancreatic cancer. Laser capture microdissection may be required for this cancer, which tends to show an infiltrative growth. Together with the fine body mapping of the systematic expression profiling project, we can expect identification of many novel tissue-specific promoters.

Targeting by use of the immune system

Last but not least, an *in vivo* targeting of cancer can be achieved by way of tumor immunity. Although pancreatic cancer is not a classical example of a highly immunogenic tumor, a comprehensive survey using transcriptome or proteome technologies may open up new possibilities for identifying tumor antigens. Moreover, the recent advent of an allogenic hematopoietic stem cell transfer protocol is expected to introduce fresh immune effector and regulator cells of donor origin to boost an immunological assault targeted to the cancer.[31]

Among the various strategies of immune gene therapy, we have been interested in the direct injection of cytokine gene expression vectors into the tumor *in vivo*. In addition to the direct cytotoxicity at the injection site, several cytokines may induce or augment tumor specific immunity. For instance, interferon-α and -β activate an adaptive immune response by stimulating increased expression of MHC antigens on cancer cells and by activation of CTL and dendritic cells. The cytokines also enhance an innate immune response by stimulation of macrophages and NK cells.[32] In the expectation of activation of tumor immunity, parenteral therapy with interferon-α protein, mostly by subcutaneous or intramuscular injection as a systemic administration, has been used for the treatment of a number of cancers including hematological malignancies, melanoma, renal carcinoma and Kaposi's sarcoma.[33, 34] For pancreatic cancer, interferon-α protein was shown to inhibit the growth of the cells,[35, 36] and recent clinical trials showed some antitumor activity of this protein, but the effect was not significant enough to enlist the cytokine as a standard therapy for this cancer. In general, an improved therapeutic effect and safety can be expected for cytokine gene therapy, because a local injection of the cytokine

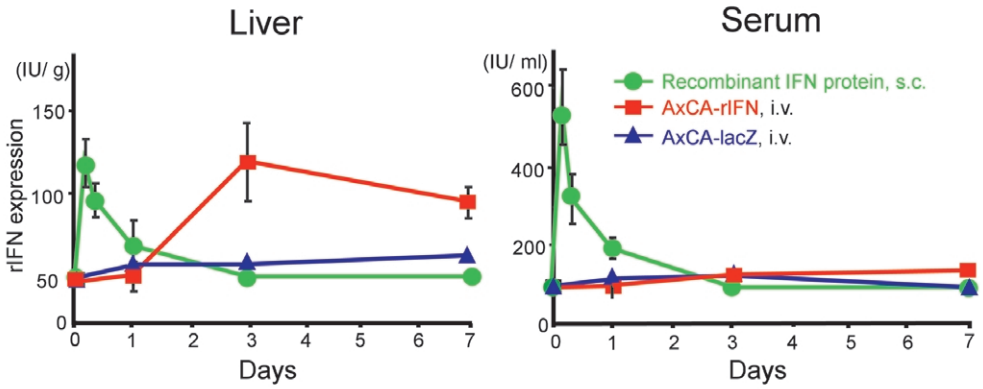

Fig. 5. Time course of rat IFN-α in the liver and serum. After treatment with DMN for 3 weeks, rats were injected once with recombinant rat IFN-α (0.1 MIU, subcutaneous injection, n=3), rat IFN-α-expressing adenovirus vector (AxCA-rIFN; 1×10^7 p.f.u., injection from tail vein, n=3), or lacZ-expressing adenovirus vector (AxCA-lacZ; 1×10^7 p.f.u., injection from tail vein, n=3).

cDNA-expressing vector can achieve sustained and increased local concentrations of the cytokine in the target sites, while keeping unwanted systemic distribution at a low level.[37] It is expected that local antigen release via tumor cell killing, coupled with the enhanced antigen presentation, will help specific tumor immunity.

We observed this favorable DDS (drug delivery system) effect of cytokine gene therapy in a rat liver fibrosis model induced by dimethylnitrosamine[37] (Fig. 5). Subcutaneous interferon-α protein injection led to only a transient elevation of the cytokine in both the liver and serum, after which the cytokine was rapidly degraded without any substantial therapeutic effect. By contrast, when an adenovirus vector expressing the rat interferon-α gene (AxCA-rIFN) was injected intravenously into the rats, the gene transfer produced a significant amount of interferon-α in the liver, but not in the serum. The injection of AxCA-rIFN prevented the progression of the cirrhosis, and improved the survival rate of the treated rats. Since the liver is a frequent metastatic organ of pancreatic cancer, an increased concentration of interferon-α in the liver may be a useful strategy for preventing and treating hepatic metastasis of pancreatic cancer.

Perspectives

As with many other types of difficult-to-cure cancers, a multi-disciplinary approach holds out hope for a significant improvement in the therapeutic outcome. Expanding the list of available weapons based on different modes of actions will also promote development of rational clinical protocols for effective and safe combinations. The first cancer gene therapy clinical study in 1991 for melanoma was followed by a few years of frenzy for early clinical trials, mostly in the United States. In 1995, the Orkin-Motulsky Report to the director of the NIH emphasized the necessity of further promotion and investment in basic research, vector development in particular.[38] We then encountered two major incidents of therapy-related adverse effects, one with an adenoviral vector in 1999[39] and the other with a retroviral vector in 2002.[40] These lessons showed that carefully designed and regulated clinical trials are definitely necessary to learn what we cannot learn from preclinical research alone. Gene therapy development is thus a typical example of full-line translational research, from very basic molecular biology to human clinical trials. It is also evident that gene therapy is an art of integrated medical sciences, and today's rapid progress in various scientific frontiers such as genetics/genomics, vectorology, stem cell biology and immunology will together accelerate the departure of cancer gene therapy from its infancy of proof of principles and safety, towards the reality of standard clinical practice.

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1. Cancer Statistics in Japan, FFCR, 2003.
2. Haller DG. Future directions in the treatment of pancreatic cancer. *Semin Oncol* 2002; **29**: 31–9.
3. Ryan DP, Willett CG. Management of locally advanced adenocarcinoma of the pancreas. *Hematol Oncol Clin North Am* 2002; **16**: 95–103.
4. Kaufman HL, DiVito J, Horig H. Immunotherapy for pancreatic cancer: current concepts. *Hematol Oncol Clin North Am* 2002; **16**: 159–97.
5. Ghadirian P, Lynch HT, Krewski D. Epidemiology of pancreatic cancer: an overview. *Cancer Detect Prev* 2003; **27**: 87–93.
6. Isaksson B, Jonsson F, Pedersen NL, Larsson J, Feychting M, Permert J. Lifestyle factors and pancreatic cancer risk: a cohort study from the Swedish twin registry. *Int J Cancer* 2002; **98**: 480–2.
7. Michaud DS. The epidemiology of pancreatic, gallbladder, and other biliary tract cancers. *Gastrointest Endosc*, 2002; **56**: S195–200.
8. Sakorafas GH, Tsiotou AG, Tsiotos GG. Molecular biology of pancreatic cancer; oncogenes, tumor suppressor genes, growth factors, and their receptors from a clinical perspective. *Cancer Treat Rev* 2000; **26**: 29–52.
9. Motojima K, Urano T, Nagata Y, Shiku H, Tsunoda T, Kanematsu T. Mutations in the Kirsten-*ras* oncogene are common but lack correlation with prognosis and tumor stage in human pancreatic carcinoma. *Am J Gastroenterol* 1991; **86**: 1784–8.
10. DiGiuseppe JA, Hruban RH, Offerhaus GJ, Clement MJ, van den Berg FM, Cameron JL, van Mansfeld AD. Detection of K-*ras* mutations in mucinous pancreatic duct hyperplasia from a patient with a family history of pancreatic carcinoma. *Am J Pathol* 1994; **144**: 889–95.
11. Cerny WL, Mangold KA, Scarpelli DG. K-*ras* mutation is an early event in pancreatic duct carcinogenesis in the Syrian golden hamster. *Cancer Res* 1992; **52**: 4507–13.
12. Yanagisawa A, Ohtake K, Ohashi K, Hori M, Kitagawa T, Sugano H, Kato Y. Frequent c-Ki-*ras* oncogene activation in mucous cell hyperplasias of pancreas suffering from chronic inflammation. *Cancer Res* 1993; **53**: 953–6.
13. Aoki K, Yoshida T, Sugimura T, Terada M. Liposome-mediated *in vivo* gene transfer of antisense K-*ras* construct inhibits pancreatic tumor dissemination in the murine peritoneal cavity. *Cancer Res* 1995; **55**: 3810–6.
14. Aoki K, Yoshida T, Matsumoto N, Ide H, Sugimura T, Terada M. Suppression of Ki-ras p21 levels leading to growth inhibition of pancreatic cancer cell lines with Ki-*ras* mutation but not those without Ki-*ras* mutation. *Mol Carcinog* 1997; **20**: 251–5.
15. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991; **108**: 193–9.
16. Ohnami S, Aoki K, Suzuki K, Ohnami S, Hatanaka K, Yoshida K, Sasaki H, Yoshida T. Expression profiles of pancreatic cancer cell lines infected with antisense K-*ras* expressing adenoviral vector. *Biochem Biophys Res Commun* 2003; **309**: 798–803.
17. Nakano M, Aoki K, Matsumoto N, Ohnami S, Terada M, Hibi T, Yoshida T. Suppression of colorectal cancer growth using an adenovirus vector expressing an antisense K-*ras* RNA. *Mol Ther* 2001; **3**: 491–9.
18. Weinstein IB. Addiction to oncogenes-the Achilles heal of cancer. *Science* 2002; **297**: 63–4.
19. Villalonga P, Lopez-Alcala C, Bosch M, Chiloeches A, Rocamora N, Gil J, Marai R, Marshall CJ, Bachs O, Agell N. Calmodulin binds to K-Ras, but not to H- or N-Ras, and modulates its downstream signaling. *Mol Cell Biol*

2001; **21**: 7345–54.
20. Ohnami S, Matsumoto N, Nakano M, Aoki K, Nagasaki K, Sugimura T, Terada M, Yoshida T. Identification of genes showing differential expression in antisense K-*ras*-transduced pancreatic cancer cells with suppressed tumorgenicity. *Cancer Res* 1999; **59**: 5565–71.
21. Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc Natl Acad Sci USA* 1989; **86**: 6982–6.
22. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc Natl Acad Sci USA* 1995; **92**: 7297–301.
23. Aoki K, Furuhata S, Hatanaka K, Maeda M, Remy JS, Behr J-P, Terada M, Yoshida T. Polyethylenimine-mediated gene transfer into pancreatic cancer dissemination in the murine peritoneal cavity. *Gene Ther* 2001; **8**: 508–14.
24. Aoki K, Yoshida T, Matsumot N, Ide H, Sugimura T, Terada M. Gene therapy for peritoneal dissemination of pancreatic cancer by liposome-mediated transfer of herpes simplex virus thymidine kinase gene. *Hum Gene Ther* 1997; **8**: 1105–13.
25. Krasnykh V, Douglas JT. Targeted adenoviral vectors I: transductional targeting. In: Curiel DT, Douglas JT, editors. Adenoviral vectors for gene therapy. San Diego: Academic Press; 2002. p. 205–45.
26. Nicklin SA, White SJ, Watkins SJ, Hawkins RE, Baker AH. Selective targeting of gene transfer to vascular endothelial cells by use of peptides isolated by phage display. *Circulation* 2000; **102**: 231–7.
27. Belousova N, Krendelchtchikova V, Curiel DT, Krasnykh V. Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein. *J Virol* 2002; **76**: 8621–31.
28. Hatanaka K, Ohnami S, Miura Y, Yoshida K, Aoyagi K, Sasaki H, Asaka M, Terada M, Yoshida T, Aoki K. A simple and efficient method for constructing an adenoviral cDNA expression library. *Mol Ther* 2003; **8**: 158–66.
29. Ozaki K, Yoshida T, Ide H, Saito I, Ikeda Y, Sugimura T, Terada M. Use of von Willebrand factor promoter to transducer suicidal gene to human endothelial cells, HuVEC. *Hum Gene Ther* 1996; **7**: 1483–90
30. Furuhata S, Ide H, Miura Y, Yoshida T, Aoki K. Development of a prostate-specific promoter for gene therapy against androgen-independent prostate cancer. *Mol Ther* 2003; **3**: 366–74.
31. Hogan WJ, Storb R. Therapeutic applications of non-myeloablative hematopoietic stem cell transplantation in malignant disease. *Immunol Res* 2003; **28**: 1–11.
32. Chawla-Sarkar M, Lindner DJ, Liu Y-F, Williams BR, Sen GC, Silverman RH, Borden EC. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* 2003; **8**: 237–49.
33. Pfeffer LM, Dinarello CA, Herberman RB, Williams BR, Borden EC, Bordens R, Walter MR, Nagabhushan TL, Trotta PP, Pestka S. Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons. *Cancer Res* 1998; **58**: 2489–99.
34. Gutterman JU. Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci USA* 1994; **91**: 1198–205.
35. Matsubara N, Fuchimoto S, Orita K. Antiproliferative effects of natural human tumor necrosis factor-alpha, interferon-alpha, and interferon-gamma on human pancreatic carcinoma cell lines. *Int J Pancreatol* 1991; **8**: 235–43.
36. Watanabe T, Fuchimoto S, Matsubara N, Iwagaki H, Orita K. Anti-proliferative effect on human pancreatic cancer cells of natural human tumour necrosis factor-beta combined with natural human interferon-alpha or interferon-gamma. *J Int Med Res* 1992; **20**: 112–20.
37. Suzuki K, Aoki K, Ohnami S, Yoshida K, Kazui T, Kato N, Inoue K, Kohara M, Yoshida T. Adenovirus-mediated gene transfer of interferon α improves dimethynitrosamine-induced liver cirrhosis in rat model. *Gene Ther* 2003; **10**: 765–73.
38. http://www.nih.gov/news/panelrep.html
39. http://www.fda.gov/fdac/features/2000/500_gene.html
40. Marshall E. Gene therapy, a suspect in leukemia-like disease. *Science* 2002; **298**: 34–5.