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***RESEARCH ARTICLE***

# *Improved gene transfer efficiency to primary and established human pancreatic carcinoma target cells via epidermal growth factor receptor and integrin-targeted adenoviral vectors*

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*In this study we analyzed two ways of retargeting of Ad-vectors to human pancreatic carcinoma with the aim of enhancing the gene transfer efficiency. First, we analyzed the expression of the epidermal growth factor receptor (EGFR) on primary, as well as established pancreatic carcinoma cells by flow cytometry which revealed high expression levels of EGFR on the surface of these cells. We showed that EGFR-retargeted entry pathway using a bispecific fusion protein formed by a recombinant soluble form of truncated Coxsackie and Adenovirus Receptor (sCAR) genetically fused with human EGF (sCAR-EGF) redirects them to the EGFR leading to an enhanced gene transfer efficiency to pancreatic carcinoma cells. Since flow cytometry revealed absence of CAR expression, but the presence of at least one of both αv integrins on the pancreatic carcinoma cells, a second way of targeting was investigated using a genetically modified Ad vector which has an RGD (Arg-Gly-Asp)-containing peptide inserted into the HI-loop of the fiber knob. This RGD targeted Ad (AdlucRGD) revealed efficient CAR-independent infection by allowing binding to cellular integrins resulting in a dramatic enhancement of gene transfer. These findings have direct relevance for Ad-vector based gene therapy strategies for pancreatic carcinoma.* Gene Therapy (2001) **8,** 969–976.

**Keywords:** *pancreatic carcinoma; primary tumor cells; adenovirus vector; targeting; gene therapy*

## *Introduction*

Pancreatic cancer is highly aggressive and ranks fifth among malignancy-associated deaths. Prognosis remains dismal because diagnosis of pancreatic cancer is made late in the clinical course of the disease. Currently, there is no effective treatment for this disease: resection is only available to a small fraction of patients presenting with locally confined tumor.[1] Chemotherapy and radiation also have limited effects on patient survival. Adjuvant combined radiochemotherapy might potentially improve survival and can be considered in unresectable, locally advanced disease. However, the role of chemotherapy in advanced disease is exclusively palliative.[1] Therefore, development of new therapeutic modalities such as gene therapy are necessary to improve patient outcome and serve as a more effective treatment for pancreatic cancer.

Adenoviral vectors have been used for both *in vitro* and *in vivo* gene delivery of pancreatic cancer, mainly because of their ability to infect both dividing pancreatic cancer cells, as well as nondividing tumor cells. Another advantage is that the techniques to produce high-titered preparations of adenovirus vectors are relatively simple. Furthermore, phase I clinical trials employing adenovirus vectors have been started already for pancreatic cancer.[2] As observed for other tumor tissue types, a major concern associated with using adenovirus vectors in pancreatic cancer is the relatively limited infection efficiencies achieved *in vitro*.[3] Furthermore, *in vivo* gene delivery may be limited by other factors, such as vector's access to target cells through local dissemination or through penetration of vessel walls.

Studies on adenoviral entry into host cells have revealed that two cell surface events, attachment and internalization, are required for an adenovirus to enter a cell.[4] The viral fiber protein will first attach to the CAR (Coxsackie and adenovirus receptor) on the surface of a host cell.[5] The virion then enters the cell through the interaction of its penton base with the αvβ3 and αvβ5 integrins on the host cell surface.[4] Expression of these cell surface markers and their correlation with the efficiency of adenovirus-mediated gene transfer have revealed that the presence of integrins αvβ3,[6] αvβ5[7] and CAR[8,9] are important for an efficient gene transfer and efficacy of

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infection. Recently, a relative lack of CAR on both neoplastic[10] and non-neoplastic[11] tissues has been implicated as a limiting factor in succesful adenovirus gene transfer.

To overcome the problems associated with *in vitro* and *in vivo* gene delivery to tumor cells, retargeting of adenovirus has been endeavored as a means to improve its specificity and efficacy. Retargeting allows adenovirus to bind to alternative cellular receptors, resulting in CAR-independent infection. One approach is based on immunological retargeting, which uses an antifiber antibody or antibody fragment that is chemically conjugated to either a cell-specific ligand (receptor) or antireceptor antibody. Using this conjugate approach, successful CAR-independent gene transfer has been achieved by targeting viral infection *in vitro* to several cellular receptors including integrins,[12] the folate receptor,[13] the basic fibroblast growth factor (FGF2) receptor[14] and the epidermal growth factor (EGF) receptor.[10] Retargeting of adenovirus to EGFR was recently shown to enhance gene transfer in primary, low-passage glioma tumor cells as well as in squamous cell carcinoma of the head and neck, which suggests further clinical relevance for retargeting.[10,15]

A potential disadvantage of this conjugate approach may be the introduction of a degree of complexity to the vector system and the concerns regarding the stability of the virus–conjugate complex under *in vivo* conditions such as systemic administration. Therefore, a genetically modified targeted viral particle might be a more attractive vector candidate for clinical application. Insertion of an Arg-Gly-Asp (RGD) motif into the HI-loop of the adenoviral fiber knob results in efficient CAR-independent infection by allowing binding of the virus to cellular integrins.[16,17] The vector containing this fiber, AdlucRGD, achieved dramatically augmented gene delivery to several cell types, both *in vitro*[17] and *in vivo*.[18]

To direct Ad gene delivery specifically to pancreatic carcinoma cells we chose two approaches. First, we have explored the utility of a bispecific fusion protein formed by a recombinant soluble form of truncated CAR (sCAR) genetically fused with human EGF (sCAR-EGF) to target Ad infection to the EGF receptor expressed on established and primary human pancreatic carcinoma cells. An approach based on employment of soluble viral receptor-EGF fusion proteins has ben originally established for targeting retroviral infection to specific cell types.[19,20] We recently showed that sCAR-EGF fusion protein possesses the ability to effectively retarget the vector via the EGF receptor with enhancement of gene transfer efficiency.[21] Second, we have shown that recombinant Ad vector containing fibers with RGD motif in the HI loop is capable of augmenting gene delivery to established and primary pancreatic carcinoma cells via a CAR-independent cell entry mechanism using the integrins as receptor. These findings have direct relevance for Ad vector-based gene therapy strategies for pancreatic carcinoma.

## Results

### *Detection of CAR-receptors on primary and established human pancreatic carcinoma cells*

It has been shown that human pancreatic carcinoma cell lines are quite refractory to infection of adenovirus vectors due to the low expression level of cell surface molecules involved in adenovirus infection, ie αv-integrins and the recently identified CAR.[3] Therefore, we decided to analyze four established human pancreatic carcinoma cell lines, and two primary human pancreatic carcinoma cells, for cell surface CAR expression by indirect immunofluorescence using an anti-human murine polyclonal serum to CAR. As shown in Figure 1a, both the four pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T or MIA PaCa-2), as well as the two primary pancreatic carcinoma cells (p6.3 and p10.5) displayed very low levels of cell surface CAR expression. This CAR deficiency strongly suggests a low level of adenovirus-directed gene transfer to these primary and established pancreatic carcinoma cells will result if Ad5 vectors with unmodified

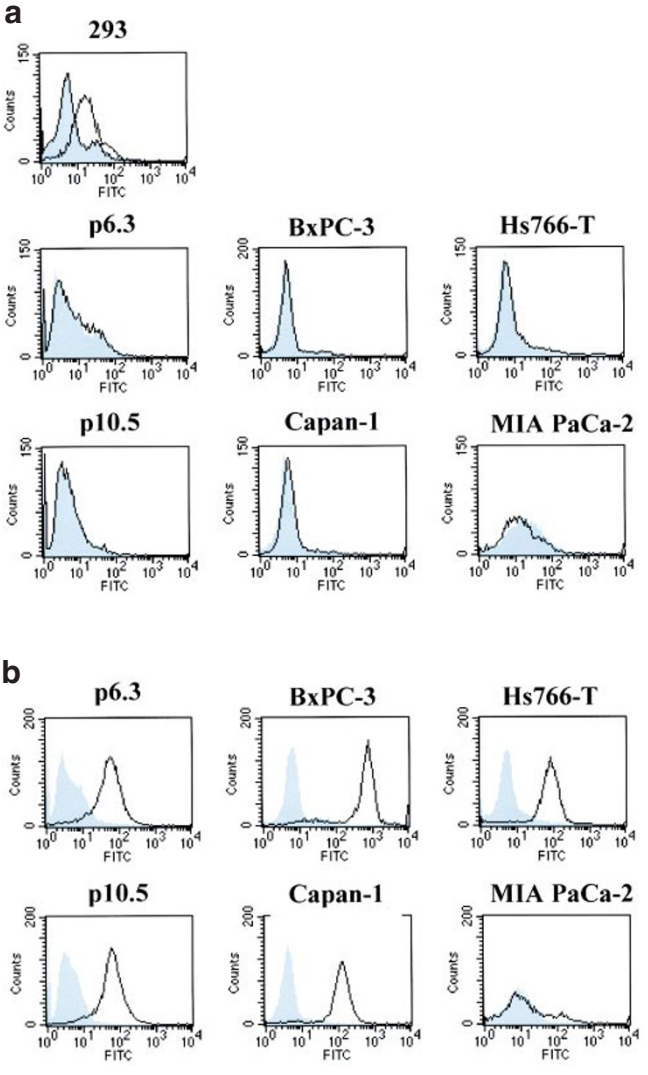


***Figure 1*** *Expression of CAR and EGFR in human pancreatic carcinoma cells. (a) Indirect flow cytofluorometry is shown for the expression of CAR (black line) in the primary human pancreatic carcinoma cells (p6.3 and p10.5), in the established human pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T and MIA PaCa-2) and in the positive control cell line (293). Normal mouse serum is used as a control (blue peak). (b) Indirect flow cytofluorometry is shown for the expression of EGFR (black line) in the primary human pancreatic carcinoma cells (p6.3 and p10.5) and in established human pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T and MIA PaCa-2). Normal mouse IgG is used as a control (blue peak).*

fiber knobs are used (see below), as has been shown for head and neck cancer[22] and for ovarian cancer.[23] On this basis we considered alternative receptors that might be upregulated in pancreatic carcinoma cells and which could be exploited for targeting.

### *Expression of EGF-receptors on primary and established pancreatic carcinoma cells*

The low levels of the native adenovirus receptor CAR in the two primary pancreatic carcinoma cells and the four pancreatic carcinoma cell lines, predicted that they would be refractory to adenovirus infection. Thus, we sought alternative receptors to exploit for targeting. In this regard, the epidermal growth factor (EGF) receptor has shown to be upregulated in a number of human tumors, ie squamous cell carcinoma of the head and neck[15] and in human glioma.[10] Therefore, we evaluated our established pancreatic carcinoma cell lines and the primary pancreatic carcinoma cells for this receptor by flow cytometry using an anti-EGF receptor (EGFR) antibody. Greater than 85% of the cells in three out of four established pancreatic carcinoma cell lines and in both primary pancreatic carcinoma cells analyzed expressed high levels of EGFR (Figure 1b). Based on the observed differences in CAR and EGFR expression levels in pancreatic carcinoma cells (Figure 1), we hypothesized that adenovirus infection would be more efficient if the vector was redirected to EGFR.

### *EGFR-retargeted gene delivery to primary and established pancreatic carcinoma cells*

The high EGFR levels on both the established, as well as the primary, pancreatic carcinoma cells offer a potential target for a modified adenovirus vector that is capable of utilizing this receptor. To demonstrate the utility of EGFR retargeting we fused a recombinant form of truncated CAR (sCAR) with human EGF as a targeting ligand (sCAR-EGF) and investigated its ability to target Ad infection to the EGF receptor overexpressed on pancreatic carcinoma cells. In a previous study Dmitriev *et al*[21] have shown that sCAR-EGF protein is capable of binding to Ad virions and directing them to EGFR. The sCAR-EGF protein was titered against Ad to ascertain the optimal ratio of targeting protein to virus as measured by improvements in gene transfer (results not shown). To demonstrate EGFR retargeting, both the established as well as the primary pancreatic carcinoma cells were infected with either native AdCMVLuc or sCAR-EGF-complexed AdCMVLuc or sCAR-6His-complexed AdCMVLuc. sCAR-6His serves as a relevant control protein to show that sCAR-EGF promoted gene transfer occurs by an EGFR-specific mechanism and no enhancement is observed in cells exposed to AdCMVLuc complexed with sCAR-6His, as shown earlier.[21] Forty-eight hours after infection, cells were lysed and luciferase activity was measured. As shown in Figure 2, compared with AdCMVLuc alone or with AdCMVLuc complexed with sCAR-6His, AdCMVLuc complexed with sCAR-EGF targeting protein mediated 1.5-, two-, three- and five-fold enhancement of luciferase expression in p10.5, p6.3, Capan-1 and BxPC-3 cells, respectively, both using an MOI of 10 and 100. In Hs-766T cells a 1.5-fold enhancement of luciferase expression is only seen at an MOI of 10. Although the expression level of the EGF receptors on MIA PaCa-2 cells appeared to be very low

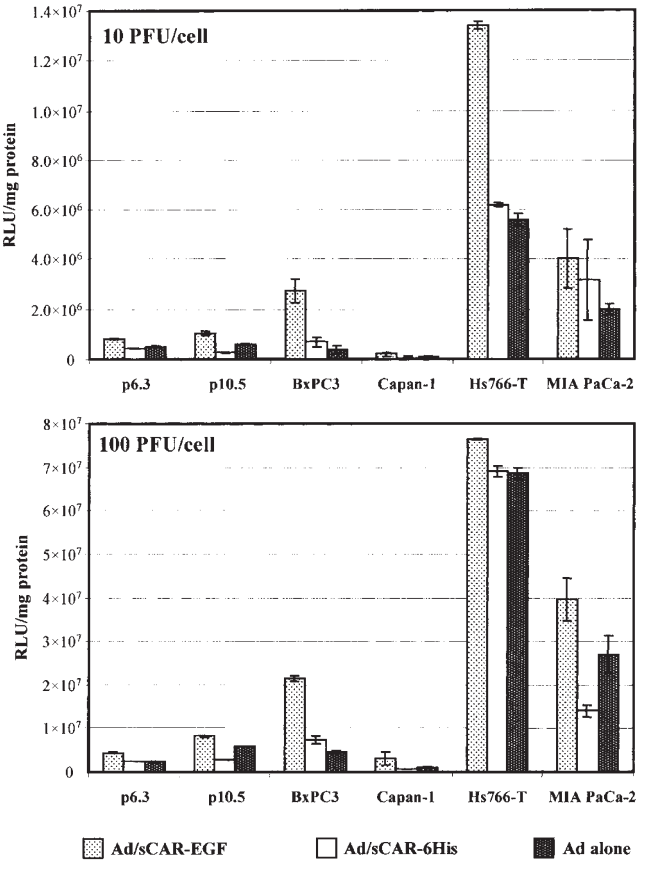


***Figure 2*** *Comparison of EGFR-targeted Ad gene transfer to human pancreatic carcinoma cells. An amount of $3 \times 10^8$ p.f.u. of AdCMVLuc was preincubated with either 15 μg of targeting sCAR-EGF protein (Ad/sCAR-EGF ▨) or with 12 μg of sCAR-6His as a control (Ad/sCAR-6His □) or with PBS (Ad alone ■) before incubation with cells. Then monolayers of primary pancreatic carcinoma cells (p6.3 and p10.5), as well as established pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs 766-T and MIA PaCa-2) were exposed to Ad or Ad/sCAR-ligand complexes at MOI (10 or 100 p.f.u./cell) for 1 h at 37°C. Infection medium was changed for complete medium and the cells were incubated for 48 h at 37°C. The cells were lysed, the protein concentration and luciferase activities of the lysates were determined. The relative light units (RLU) of luciferase/milligram of total cellular protein are shown graphically. The results are shown as the mean of multiple assays. Each point represents the mean ± standard deviation of two determinations. Error bars, s.e.*

(Figure 1b), still a 1.5-fold enhancement of luciferase expression is observed using AdCMVLuc complexed with sCAR-EGF (Figure 2). These results demonstrated that sCAR-EGF targeting protein enables retargeting of Ad vector with several-fold enhancement of gene transfer efficiency specifically to EGFR-positive pancreatic carcinoma cells (both primary and established cell lines). The sCAR-EGF promoted gene transfer occurs by an EGFR-specific mechanism, since no significant enhancement was observed in cells exposed to AdCMVLuc complexed with sCAR-6His (untargeted Ad). Furthermore, the specificity of sCAR-EGF-mediated Ad-targeting was illustrated by failure of the sCAR-EGF to enhance Ad-based gene transfer to EGFR-negative human mammary gland (MDA-MB-453) cells (results not shown; Ref. 21).

### *Expression of αvβ3 and αvβ5 integrins on primary and established pancreatic carcinoma cells*

Because EGFR retargeting by sCAR-EGF enhanced adenovirus gene transfer with variable efficiency and

with relatively low retargeting indexes (range of enhancement of 1.5- to five-fold, Figure 2), as compared with human squamous carcinoma (SCC-4) cells and human epidermoid carcinoma (A-431) cells,[21] we looked for targeting via other cellular receptors. It has been shown that insertion of an Arg-Gly-Asp (RGD) motif in the HI-loop of the Ad5 knob domain resulted in a viral fiber protein which results in efficient CAR-independent infection by allowing binding of Ad to cellular integrins.[17] The vector containing this fiber (AdlucRGD) indeed achieved augmented gene delivery to several cell types by interaction to cellular αv integrins and thus allowing CAR-independent infection.[17,18] We already determined the low level of expression of the native CAR receptor on the surface of established and primary pancreatic carcinoma cells (Figure 1). Therefore, to assess if a genetically modified Ad containing an RGD motif can efficiently enter the pancreatic carcinoma cells, we ascertained the level of expression of both αvβ3 and αvβ5 integrins on these tumor cells. Flow cytometry was performed to detect expression of both αvβ3 and αvβ5 integrins on established and primary pancreatic carcinoma cells by indirect immunofluorescence using LM609 and P1F6 mAbs, respectively. As shown in Figure 3, expression of αvβ3 integrin is absent in p6.3, BxPC-3 and Capan-1 cells, while a low to moderate αvβ3 expression is seen in p10.5, Hs766-T, MIA PaCa-2 cells. Integrin αvβ5, on the other hand, is present on all pancreatic cancer cells: p6.3, p10.5 and Capan-1 cells express high levels of αvβ5, while the other pancreatic carcinoma cell lines (BxPC-3, Hs766-T, MIA PaCa-2) express moderate levels of this integrin. Thus, the presence of one or both of the αv integrins on the established and primary pancreatic carcinoma cells should allow CAR-independent gene transfer by AdlucRGD.

### *Integrin targeted gene delivery to primary and established pancreatic carcinoma cells*

Our next goal was to examine whether introduction of the RGD motif in the fiber of AdlucRGD resulted in an enhancement of this virus' ability to infect established and primary pancreatic carcinoma cells. Therefore, AdlucRGD was utilized for an assay based on competitive inhibition of Ad-mediated gene delivery by recombinant Ad5 fiber knob protein, known to efficiently block virus binding to CAR.[17] To demonstrate CAR-independent cell entry by AdlucRGD, established and primary pancreatic carcinoma cells were infected with various MOIs with either native AdCMVLuc or integrin-retargeted AdlucRGD in the presence or absence of blocking knob protein. Forty-eight hours after the infection, cells were lysed and luciferase activity was measured. As shown in Figure 4a in both established as well as primary pancreatic carcinoma cells, striking differences between the infection profiles were demonstrated by these two viruses at each MOI. Luciferase expression in the AdlucRGD-infected primary pancreatic carcinoma cells was 100- to 500-fold higher than in the cells infected with AdCMVLuc. Even in the established pancreatic carcinoma cell lines the difference in infection efficiencies demonstrated by these two viral vectors was still between 10- and 100-fold. Of note, comparison of luciferase expression upon infection of 293 cells (high CAR expression) with AdCMVLuc *versus* AdlucRGD did not reveal a substantial enhancement of gene transfer with the AdlucRGD-targeted virus (results not shown).

Furthermore, AdCMVLuc-mediated infection in the presence of Ad5 fiber knob protein revealed a dramatic inhibition. The knob blocked between 55% and 95% of the gene transfer at MOIs of 10 and 100 p.f.u. for primary pancreatic carcinoma cells, while this protein blocked between 40% and 90% of the gene transfer at MOIs of 1, 10 and 100 p.f.u. for established pancreatic carcinoma cell line (Figure 4b). Most importantly, for the primary p10.5 pancreatic carcinoma cells as well as the established Capan-1, Hs766-T and MIA PaCA-2 pancreatic cell lines, recombinant knob protein did not reveal any significant inhibition effect on the levels of luciferase expression directed by AdlucRGD (Figure 4b). Strong inhibition by the fiber knob on AdCMVLuc-mediated luciferase expression suggests that the fiber–CAR interaction is the only pathway this virus can use to infect pancreatic carcinoma cells.

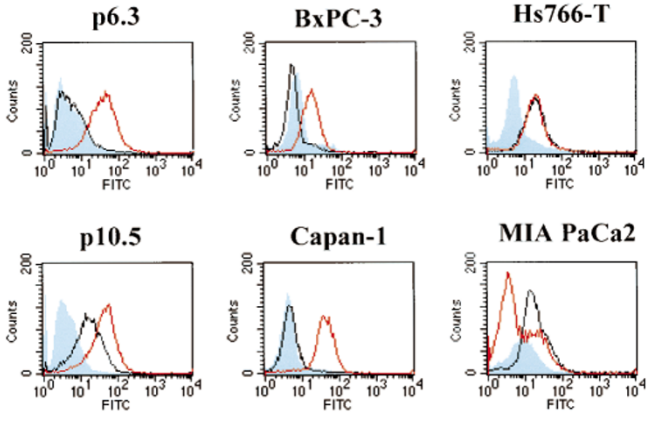


***Figure 3*** *Expression of αv-integrins in human pancreatic carcinoma cells. Indirect flow cytofluorometry is shown for the expression of αvβ3-integrins (black line) and αvβ5-integrins (red line) in primary human pancreatic carcinoma cells (p6.3 and p10.5) and in established human pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T and MIA PaCa-2). Normal mouse IgG is used as a control (blue peak).*

### *Gene delivery of an integrin-targeted Ad vector increases the frequency of infection of pancreatic carcinoma cells*

The previous experiments demonstrated that adenovirus infection of both primary and established pancreatic carcinoma cells can be redirected to the αv-integrins via a CAR-independent pathway, resulting in enhanced reporter gene expression. This observed enhancement of gene transfer with the AdlucRGD virus could have arisen from either a few transduced cells exhibiting more abundant gene expression because an increased number of viruses have infected these cells or from a greater number of pancreatic carcinoma cells that may have been rendered susceptible to infection because of the high amount of expressed αv-integrins on the cell surface. To identify the nature of the enhanced gene expression CAR-independent retargeting was performed using an adenovirus that expressed the green fluorescent protein (GFP) reporter gene. Both primary, as well as established, pancreatic carcinoma cells were infected with either native AdGFP or with the genetically modified AdGFP-RGD at an MOI of 10, 100 and 1000 per cell during 48 h and the number of infected cells were monitored by fluorescent

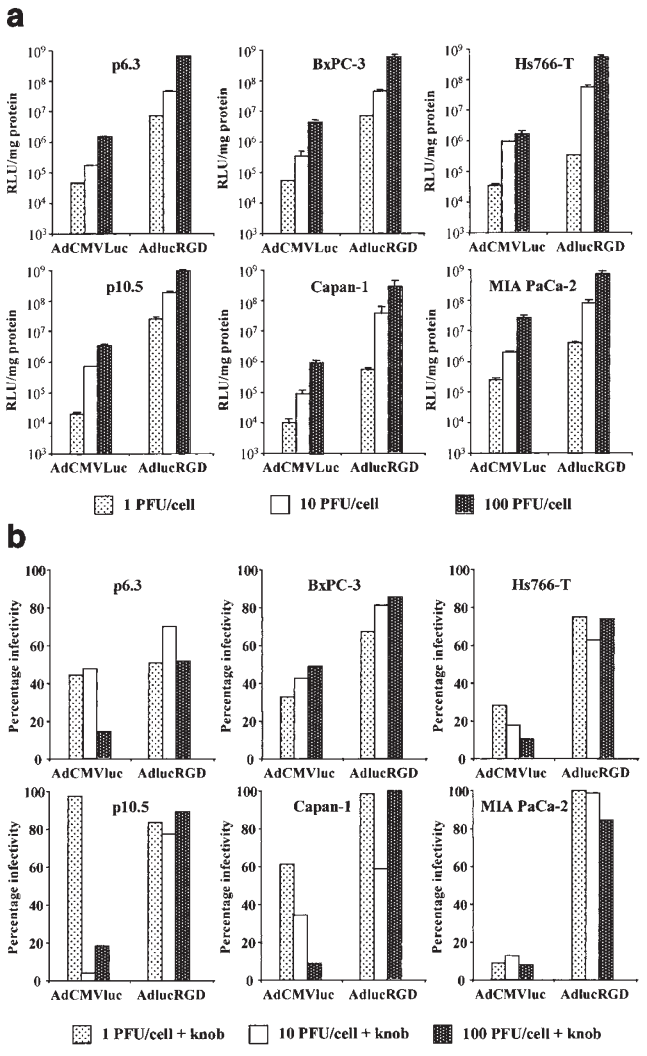


***Figure 4*** *Comparison of Ad gene transfer efficiencies of AdCMVLuc and AdlucRGD to the human pancreatic carcinoma cells. (a) Primary human pancreatic carcinoma cells: p6.3 and p10.5 and established human pancreatic carcinoma cell lines BxPC-3, Capan-1, Hs766-T and MIA PaCa-2 were infected with Ad5CMVLuc or AdlucRGD at an MOI of 1 (▨), 10 (□), and 100 (■) p.f.u./cell. Infection medium was changed for complete medium 1 h after infection and the cells were incubated for 28 h at 37°C. The cells were lysed, the protein concentration and luciferase activity of the lysates were determined. The relative light units (RLU) of luciferase/milligram of total cellular protein are shown graphically. The results are shown as the mean of multiple assays. Each point represents the mean ± standard deviation of two determinations. (b) Primary human pancreatic carcinoma cells: p6.3 and p10.5 and established human pancreatic carcinoma cell lines BxPC-3, Capan-1, Hs766-T and MIA PaCa-2 were preincubated in PBS or PBS containing recombinant Ad5 fiber knob protein for 30 min at 37°C. AdCMVLuc or AdlucRGD were added to the cells at an MOI of 1 (▨), 10 (□), and 100 (■) p.f.u./cell for 1 h, then media was changed and the cells were incubated at 37°C. At 48 h after infection the cells were lysed, the protein concentration and luciferase activity of the lysates were determined. The percentages of luciferase activity detected in the cells infected in the presence of knob calculated with regard to luciferase activity detected in the cells infected in the absence of knob (=100%, not shown on the graph) are depicted graphically.*

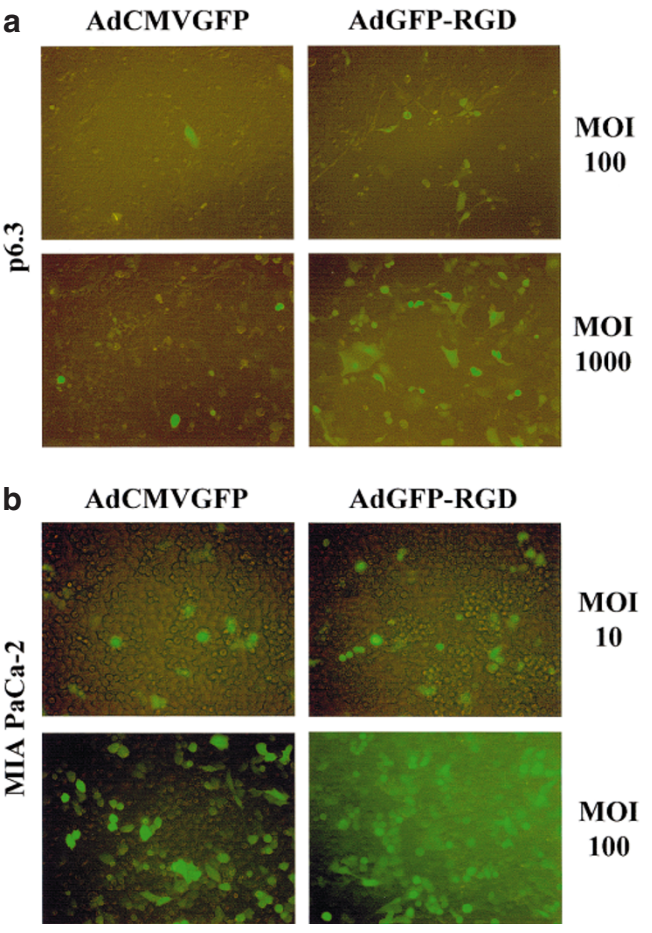


***Figure 5*** *Analysis of the number of infected pancreatic carcinoma cells using AdCMVGFP and AdGFP-RGD. (a) Infection of the primary pancreatic carcinoma cells (p6.3) was peformed at an MOI of 100 or 1000 v.p./cell and (b) the established pancreatic carcinoma cell line (MIA PaCa-2) was infected at an MOI of 10 or 100 p.v./cell. Forty-eight hours after infection the percentage of infected cells was determined using the the fluorescence microscope.*

microscopy. Figure 5 reveals the results of retargeting via the αv-integrins of two representative pancreatic carcinoma cells: the p6.3 primary pancreatic carcinoma cells and the established pancreatic carcinoma cell line MIA PaCa-2. Using the native AdCMVGFP virus only a low amount of pancreatic carcinoma cells were infected, while retargeting via the αv-integrins by the CAR-independent route using AdGFP-RGD resulted in increased numbers of cells that were positive for green fluorescent protein expression both for the p6.3 primary cells (Figure 5a) as well as for the MIA PaCa-2 established pancreatic carcinoma cell line (Figure 5b). As a control, infection of 293 cells (high CAR expression) with AdCMVGFP and AdGFP-RGD viruses revealed a significant lower percentage of gene transfers with AdGFP-RGD compared with untargeted AdCMVGFP (data not shown). These data indicate that retargeting of the adenovirus to the αv-integrins via a CAR-independent cell entry increased the number of infected pancreatic carcinoma cells instead of showing more abundant gene expression in the infected cells.

## Discussion

Human pancreatic carcinoma cell lines have been shown to be relatively resistant to adenovirus mediated gene transfer. These unmodified, first-generation adenovirus vectors have failed to deliver genes at an efficiency that

would be therapeutic in the context of human pancreatic cancer. Pearson *et al*[3] revealed that the low expression levels of the integrins αvβ3, αvβ5, α5, β1 and CAR will limit adenovirus-mediated gene transfer in both lung and pancreatic cancer cell lines and their results were confirmed in this study. Importantly, the two primary (passage number lower than five) human pancreatic carcinoma cells (p6.3 and p10.5) used in this study were revealed to be refractory to adenoviral infection to the same amount as the established, highly passaged pancreatic carcinoma cell lines. For this reason, in this report, we have demonstrated two ways of retargeting of adenovirus vectors as a means to enhance gene transfer efficiency to both primary and established human pancreatic carcinoma cells. First, we showed that EGFR-retargeted entry pathway using the sCAR-EGF protein, which binds to Ad virions and redirects them to EGFR,[21] was able to enhance the gene transfer efficiency of pancreatic carcinoma cells between 1.5- and five-fold, most likely via a non-CAR pathway. Increase of gene transfer by retargeting to EGFR is explained by flow cytometry analyses which revealed that EGFR is overexpressed in both primary, as well as established, pancreatic carcinoma cells. It is very likely that redirecting the virus to the highly expressed EGFR molecule increased the overall number of cells infected, thereby leading to an enhanced gene transfer, as has been shown for head and neck cancer cells.[15] Of importance, immunohistochemical staining of normal and adenocarcinoma pancreatic tissue slides (resection material from patients) with anti-EGFR antibody revealed a moderate to high expression level of EGFR molecules on most (adeno)carcinoma cells of the pancreas compared with the surrounding normal pancreas or normal liver cells (unpublished observations). Therefore, it is not likely that EGFR-targeted Ad vectors will cause any significant toxicity to non-diseased pancreatic or liver tissue. The increase in gene transfer shown here for pancreatic carcinoma is significantly lower than seen by Dmitriev *et al*[21] who revealed that Ad encoding luciferase complexed with sCAR-EGF targeting protein mediated a much higher enhancement of luciferase gene expression in EGFR-positive SKOV3.ip1 (human ovarian carcinoma) cells, EGFR-positive SCC-4 (human squamous carcinoma) cells and EGFR-positive A-431 (human epidermoid carcinoma) cells. This difference is most likely explained by the higher number and/or expression level of the EGF-receptors on these SKVO3.ip1, SCC-4 and A-431 cells compared with the primary and established pancreatic carcinoma cells. Despite the conceptual gains realised by this conjugate approach, this 'two-component' strategy introduces a degree of complexity to the vector system. Moreover, it raises concerns regarding the stability of the virus–conjugate complex under certain *in vivo* conditions.[18] Therefore, we decided to exploit another targeting approach.

Genetically modified vectors containing short peptide targeting sequences at the C-terminus of the adenoviral knob domain (which binds to CAR) have been produced, which revealed expanded viral tropism *in vitro* by targeting to, for example, integrins.[24] However, the C-terminus of the knob domain is located at the base which is not an ideal position to interact efficiently with cellular receptors. Recently it has been reported that the use of an alternate region of the knob domain, the HI-loop, is a more rational site for inserting targeting motifs.[16] This region is not directly involved in trimerization, it contains mostly hydrophilic amino acids and is of different length in different Ad serotypes. Furthermore, the HI-loop is flexible and is exposed on the exterior of the knob. Insertion of an RGD motif in this region resulted in a viral fiber protein which results in efficient CAR-independent infection by allowing binding of Ad to cellular integrins.[17] The use of RGD-modified viral vectors (AdlucRGD and AdGFP-RGD) in this study is based on flow cytometry data using anti-CAR and anti-αvβ3/αvβ5 antibodies which revealed no expression of CAR, but shows the presence of at least one of the αv-integrins on the surface of the primary and established pancreatic carcinoma cells. The resulting modified virus (AdlucRGD) revealed a dramatic increase in gene transfer efficiency of both primary and established pancreatic carcinoma cells.[16,17] The absence of CAR expression and the presence of the αv-integrins on the surface of these cells explains the enhanced luciferase expression of AdlucRGD compared with parental AdCMVLuc. Furthermore, the ability of the knob protein to block infection using parental AdCMVLuc as well as the lack of inhibition in the presence of this knob protein on the levels of luciferase expression directed by AdlucRGD, clearly demonstrated that RGD-modified Ad has enhanced binding to pancreatic carcinoma cells lacking CAR, leading to enhanced gene expression. The observation that knob inhibited luciferase expression of AdlucRGD in p6.3 cells and to a lesser extent in BxPC-3 cells, suggests that in these particular pancreatic carcinoma cells either knob may interfere with binding of AdlucRGD to the integrins or that a minor fraction of AdlucRGD enters these cells via the CAR.

Of note, infection of pancreatic carcinoma cells with AdGFP-RGD revealed that the number of infected pancreatic carcinoma cells has been increased rather than a few cells exhibiting more abundant gene expression as compared with parental AdGFP. The importance of both latter findings lay in the fact that fewer AdRGD virus particles need to be administered *in vivo* to obtain the same therapeutic effect, thereby decreasing the vector-related toxicity. Importantly, although Ad5lucRGD has expanded tropism,[18] this genetically modified virus is not tumor-specific. However, in a study where both primary ovarian tumor explants, as well as nontumor mesothelial tissue samples, from patients were infected with Adluc and AdlucRGD, the mesothelial tissue samples expressed low luciferase activity both with the Ad5lucRGD vector as with AdCMVLuc.[23] Therefore, studies will be initated to assess the efficacy of infection of normal pancreatic epithelial cells obtained from human resection material with Ad targeted to EGFR, as well as Ad targeted to integrins (AdlucRGD).

To our knowledge this is the first study where enhanced gene transfer by EGFR, as well as integrin-targeted adenovirus vectors has been demonstrated in primary pancreatic carcinoma cells. Of note, the infection conditions were selected for representing a high level of stringency that a gene transfer vector would have to overcome in the clinical context. The observed level of enhancement as seen with the EGFR-retargeted vector to a minor extent and with the RGD-modified Ad vector to a major extent, would thus support the use of these vectors in human gene therapy clinical trials for pancreatic carcinoma. Thus, this study seeks to validate a strategy

that will address a critical shortcoming in cancer gene therapy. The key finding in this study is that gene transfer to both primary pancreatic cancer cells, as well as established pancreatic carcinoma cell lines, is significantly enhanced by utilizing an RGD-modified retargeted vector. As integrins have been frequently shown to be overexpressed by various epithelial tumors, as described for head and neck cancer[22] and ovarian cancer,[23] our novel vector strategy could potentially be exploited in the context of pancreatic cancer. Most importantly, the levels of gene transfer of the RGD-modified adenovirus in surrounding nontumor pancreatic epithelial cells provides a rationale for further studies with this targeted vector in preclinical efficacy studies that would lead to human clinical trials.

## Materials and methods

### Tumor cells

The established human pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T and MIA PaCa-2; >20 passages) were purchased from Boehringer Ingelheim, Belgium. These cells were cultured in Dulbecco's minimal essential medium (DMEM) (Mediatech, Herndon, VA, USA) with 10% fetal bovine serum (FBS) (Summit Biotechnology, Ft Collins, CO,USA), 1% L-glutamine and 1% penicillin/streptomycin (Gibco BRL, Life Technologies, Rockville, MD, USA). The primary human pancreatic carcinoma cells (p6.3 and p10.5; <5 passages) were obtained from Dr E Jaffee, Johns Hopkins University School of Medicine, Baltimore, MD, USA. All cell lines were cultured at 37°C in 5% carbon dioxide atmosphere.

### Viruses, antibodies and recombinant proteins

The E1-, E3-deleted adenovirus vector expressing the firefly luciferase from the cytomegalovirus (CMV) immediate–early promoter, AdCMVLuc[25] was obtained from Robert Gerard (University of Leuven, Leuven, Belgium). Ad vector AdlucRGD, containing recombinant fiber-RGD protein and expressing firefly luciferase was generated by transfection of 293 cells with *Pac*I-digested pVK703.[18] Ad vector AdCMVGFP, encoding green fluorescent protein, and AdGFP-RGD, containing recombinant fiber-RGD protein and expressing GFP were obtained from Dr M Parameshwar (University of Alabama at Birmingham, AL, USA). Viruses were propagated and plaque-titered on the permissive cell line 293 and purified by double cesium chloride gradients.[26] Virus preparations were dialyzed against phosphate-buffered saline (PBS), aliquoted, and stored at −80°C. Titers were determined using standard plaque assays and the number of viral particles was determined by measuring the optical density at 260 nm. For AdCMVLuc: $4.2 \times 10^{11}$ p.f.u./ml and $8.1 \times 10^{12}$ v.p./ml (v.p. to p.f.u. ratio: 19.3) and for AdlucRGD: $5.6 \times 10^{10}$ p.f.u./ml and $1.7 \times 10^{11}$ v.p./ml (v.p. to p.f.u. ratio: 32.9).

Murine polyclonal serum to baculovirus-produced human soluble CAR protein were generated at the University of Alabama at Birmingham, Hybridoma Core Facility. Murine mAb 425 to human EGFR was a generous gift from Zenon Steplewski (Thomas Jefferson University, Philadelphia, PA, USA) and was described earlier.[27] Murine mAb LM609 to αvβ3 integrin and P1F6 to αvβ5 integrin were purchased from Chemicon (Temecula, CA, USA). Recombinant fiber knob from Ad5 was obtained from Dr V Krasnykh (University of Alabama at Birmingham). Recombinant sCAR-6His and sCAR-EGF proteins were constructed as described.[21]

### Flow cytometry

Confluent cells were released with versene or cell dissociation buffer (Gibco BRL, Life Technologies) or by trypsinizing of the cells using 0.05% trypsin/0.53 mM EDTA for 3 min or less (2 ml per T75 flask). The trypsinized cells were quenched with 10-fold volume of cold DMEM-medium containing 10% fetal bovine serum and pelleted at 1200 r.p.m. for 5 min. Cells were resuspended in cold PBS with 1% bovine serum albumin (BSA) and counted. Cells were spinned (1200 r.p.m. for 5 min) and aliquoted in PBS + BSA at $2 \times 10^6$ cells/ml. Cells ($2 \times 10^5$) were incubated with either mAb 425 (5 μg/ml) (anti-EGFR) or with murine anti-CAR serum (1:250) or with murine mAb LM609 (anti-αvβ3) or with mAb P1F6 (anti-αvβ5) for 1 h at 4°C. A normal murine serum and control IgG were used as a negative control. Cells were then washed with buffer and incubated with secondary FITC-labeled goat anti-mouse immunoglobulin G (Jackson, West Grove, PA, USA) at a concentration of 5 μg/ml for 1 h at 4°C. After washing, $10^4$ cells per sample were analyzed using flow cytometry performed at the University of Alabama at Birmingham FACS Core Facility. Data were expressed as the geometric mean fluorescence intensity of the entire gated population. The positive population cells was determined by gating the right-hand tail of the distribution of the negative control sample for each individual cell line at 1%. This gate setting was then used to determine the percentage of CAR-, αvβ3-, αvβ5- or EGFR-positive cells in each individual cell line.

### Adenovirus vector-mediated gene transfer

To assess native or EGFR-retargeted adenovirus infection efficiency, $5 \times 10^4$ cells were plated in 24-well plates and allowed to adhere overnight at 37°C. An amount of $3 \times 10^8$ p.f.u. of AdCMVLuc was preincubated with either 15 μg of sCAR-EGF protein or 12 μg of sCAR-6His as a control or with PBS, before incubation with cells for 30 min at room temperature. Then monolayers of pancreatic carcinoma cells were exposed to Ad/sCAR–ligand complexes and Ad without ligand complexes at various MOIs (10 and 100 p.f.u. cell) cell for 1 h at 37°C. The virus was removed and the cells were incubated for 48 h in complete media.

To assess native or RGD-modified adenovirus efficiency, $5 \times 10^4$ cells were plated in 24-well plates and allowed to adhere overnight at 37°C. To demonstrate the specificity of infection, half of the cells were blocked with recombinant Ad5 knob protein (10–15 μg/well, diluted in PBS) for 30 min at 37°C and the other half were incubated with PBS as a control. Subsequently, both the blocked cells, as well as the unblocked cells, were infected with native AdCMVLuc or AdlucRGD at various MOIs (1, 10 and 100) per cell for 1 h at 37°C. The virus was removed and the cells were incubated for 48 h in complete media.

Cell lysates were assayed for luciferase expression 48 h after infection in a Berthold Luminometer using the Luciferase Assay System (Promega, Madison, WI, USA), and the protein concentration was determined using the

Pierce Protein Assay according to the manufacturer's protocols.

To evaluate the number of transfected cells, $5 \times 10^4$ cells were plated in 24-well plates and allowed to adhere overnight at 37°C. The cells were infected with AdCMVGFP and AdGFP-RGD at various MOIs (10, 100 and 1000) per cell for 1 h at 37°C. The virus was removed and the cells were incubated for 48 h in complete media. Subsequently, the media was removed and PBS was added to the wells and the percentage of infected cells were visualized under a fluorescence microscope.

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