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A platform for constructing infectivity-enhanced fiber-mosaic adenoviruses genetically modified to express two fiber types

Research Article

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Abbreviations: adenovirus type 3, (Ad3); coxsackie adenovirus receptor, (CAR); fetal bovine serum, (FBS); Green Fluorescent Protein, (GFP); Head and neck squamous cell carcinoma, (HNSCC); plaque forming units, (pfu); relative light units, (RLU); viral particle, (vp)

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Summary

Adenoviruses type 5 have been successfully exploited as gene transfer vectors and numerous vectorological improvements have contributed to increasing efficiency and specificity of adenoviral gene therapy. Despite these improvements, inefficient gene transfer still is an important limitation and is, at least in part, due to the low expression of the primary receptor (CAR) on target cells. Combining two different fiber types (the fiber of Ad5 for CAR-dependent uptake and the fiber of Ad3 for CAR-independent uptake) on an Ad5-based capsid would increase the options for improvement of specificity and efficiency. In this study, we present an approach to engineer fiber-mosaic adenoviruses by cloning the fiber of Ad3 into the Ad5 genome under the control of the Major Late Promoter using native splicing signals. Such fiber-mosaic viruses were efficiently rescued using conventional 293 cells and demonstrated good infection profiles. Pre-incubation with recombinant fiber knob (either derived from Ad5 or Ad3) indicated different mechanisms of entry for the fiber-mosaic viruses. The introduction of an additional entry pathway can be further exploited to overcome low infection efficiency due to low CAR expression. In addition, the technology will be of value in increasing the specificity of adenoviral gene therapy since this approach allows the incorporation of two different retargeting ligands per capsid. Such infectivity enhancement will also prove powerful in the context of replicative agents.

I. Introduction

Adenoviruses are widely used as gene transfer vehicles in gene therapy for several reasons including the easy production to high titers and their efficient infection of both dividing and non-dividing cells. Even though adenoviruses are among the most efficient vectors *in vivo* to date, accounting for 40% of all clinical gene therapy trials (Marshall, 2001), adenoviral cancer gene therapy is limited by the low efficiency of gene transfer. This low gene transfer might at least partially be explained by no/low expression or accessibility of the primary receptor for adenoviruses (coxsackie adenovirus receptor (CAR)) (Douglas et al, 2001).

Redirecting viruses to specific receptors on target cells will improve specificity and possibly also efficiency (Glasgow and Curiel, 2004). Such transductional retargeting has been exploited through complexing the virus to targeting moieties (eg bispecific antibodies) (Rots et al, 2003) or through genetic modification of the knob or penton base (Nicklin and Baker, 2002). Alternatively, several genetic strategies have been developed to stably incorporate retargeting moieties directly into the viral capsid. For fiber modifications, the HI-loop or C-terminal

end have been exploited, and several polypeptides have been successfully incorporated (Belousova et al, 2002). Although successful in improving the characteristics of the vector *in vitro*, the retargeting moiety generally is only expressed by specific tumor types *in vivo*. In this respect, we reasoned that both efficiency and specificity of adenoviral gene transfer would be improved by allowing a virus to infect cells via two ways of entry.

Adenoviruses belonging to subgroup C mainly bind to the CAR receptor and will be internalized after binding of the penton base to integrins. However, subgroup B adenoviruses do not bind to CAR but to other receptor(s), like CD46 (Gaggar et al, 2003), before internalization via integrin-mediated endocytosis takes place (Cuzange et al, 1994). These subgroup B viruses display a different infection profile as has been described in detail for adenovirus type 3 (Ad3) (Stevenson et al, 1997; Kanerva et al, 2002), Ad7 (Gall et al, 1996), Ad17 (Chillon et al, 1997) and Ad35 (Shayakhmetov et al, 2000). Based on the improved infection of primary cancer cells described for Ad3 versus Ad5 (Kanerva et al, 2002; Volk et al, 2003), we choose to exploit the infection mechanism of Ad3. To this end, the Ad3 fiber was cloned into the Ad5 genome using the native fiber splicing signals thus creating a virus expressing both fibers onto the capsid of Ad5 (fiber-mosaic virus). We demonstrate that such fiber-mosaic viruses (AdF3F5) can be rescued and that this virus infects cells through two different mechanisms; one CAR mediated entry which can be blocked by recombinant knob 5 protein and one entry pathway which can be blocked by preincubation with recombinant knob 3 protein.

This technology of introducing an additional fiber type in adenoviral gene therapy vectors will contribute to optimizing adenoviral gene therapy efficiency (**Figure 1**). Specificity can subsequently be achieved by introducing targeting ligands into the knob of Ad5 (Dmitriev et al, 1998) and/or the knob of Ad3 (Uil et al, 2003). Alternatively, the use of tumor specific promoters will restrict transgene expression or viral replication specifically to target cells (Rots et al, 2003). Especially in the context of replication competent adenoviruses, the fiber-mosaic approach will be beneficial since secondary infection efficiency is thought to be a major problem hampering therapeutic outcome of replicative agents.

II. Materials and methods

A. Cells

Human cervical cancer cells (HeLa) and embryonic kidney cells (293), both expressing high levels of CAR and integrins, were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Head and neck squamous cell carcinoma (HNSCC) cell lines (FaDu and SCC25), glioma lines (U373 and U118) and ovarian cancer cell lines (SKOV) were included for their differential expression of the receptor for Ad3 and Ad5. Cells were cultured at linear phase in recommended media.

B. Construction of recombinant adenoviral plasmid encoding the fiber-mosaic adenovirus AdF3F5

Since incorporation of the fiber monotrimer into the viral capsid is dependent on the tail domain of the fiber, we constructed a chimeric fiber by fusing the tail of Ad5 to the shaft of Ad3. Oligos encoding the first 15 amino acids of the tail of the fiber of Ad5 (based on Ad sequence nts 31042 to 31087 containing the KRAR nuclear localization signal) (Hong and Engler, 1991) were constructed to contain a *NdeI*- 3'end. The shaft and the knob region of Ad3 were obtained by PCR using *Pfu*-polymerase (Stratagene) resulting in a *NdeI*-5'end (underlined) using the following primers: 5'-GTACCCATATGAAGATGAAAGCAGCTC-3' (forward) and 5'-GGGAAGGGGGAGGCAAAATAACTAC-3' (reverse). The tail of Ad5 was then genetically fused to the gene coding for the shaft and the knob of Ad3 and introduced upstream of the wild type Ad5 fiber by cloning into the *PacI* site of pAd70-100dlE3 (kindly provided by Dr. Falck-Pederson, Cornell, New York) (Gall et al, 1996). Digestion with *NdeI* results in a *NdeI-NdeI* fragment containing the shaft and knob of Ad3 followed by

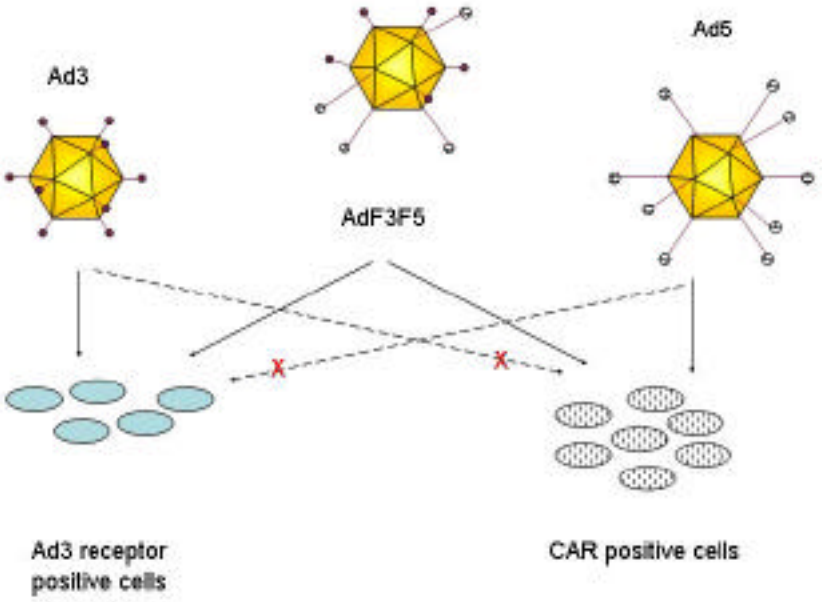


Figure 1. Schematic representation of infectivity-enhancement by fiber mosaic adenoviruses. Adenoviruses expressing two different fiber types on one capsid can make use of two different mechanisms of entry. This approach will circumvent the low expression of the primary receptor, CAR, as described for numerous primary cancer cell types. The technology allows for introduction of two targeting moieties in the same virion.

upstream sequence of wild type Ad5 fiber and the starting genetic sequence encoding the Ad5 tail. This fragment was subcloned into the *NdeI* site of the adenoviral transfer plasmid pNEBpkFSP (Krasnykh et al, 1996) to ensure optimal splicing conditions for the second chimeric fiber. Cloning strategy and resulting construct is shown in **Figure 2**. Enzymes used were obtained from LifeTechnologies and New England Biolabs. Homologous recombination with the adenoviral backbone pVK50 (Krasnykh et al, 1996) containing genes encoding luciferase and Green Fluorescent Protein (GFP) in the E1-region (Seki et al, 2002) resulted in a plasmid encoding AdF3F5.

Subsequent virus production was performed according to the pAdEasy protocol (He et al, 1998). Expression of the two fibers was detected by western blot analysis of 10^{10} boiled viral particles separated on a 10% SDS-PAGE gel using the anti-tail antibody 2D4 (Hong and Engler, 1991) generated at the University of Alabama at Birmingham Hybridoma Core Facility.

C. Viruses

To compare the infection efficiency of AdF3F5 with unretargeted Ad5, AdTL was used containing luciferase and GFP in the E1 region (wild type Ad5 fiber, AdF5) (Alemany and Curiel, 2001). To investigate the infection efficiency relative to knob 3 mediated infection, Ad5/3Luc1 was used expressing a chimeric fiber containing the knob of Ad3 in a Ad5 backbone (AdK3) (Krasnykh et al, 2001). AdK3 encodes luciferase from a different expression cassette and can therefore not be directly compared to AdF5 and AdF3F5. Adenovirus type 3 was obtained from the American Type Culture Collection. All viruses were CsCl purified and quantified for viral particle (vp) number and plaque forming units (pfu) according to standard procedures. The vp/pfu ratios were 3.1, 3.7 and 2.2 for AdF3F5, AdF5 and AdK3, respectively.

D. Inhibition of viral mediated gene transfer by recombinant fiber proteins

Monolayers were grown to 70% confluency in 24 wells plates and incubated with recombinant Ad3 knob (10 µg/ml PBS), Ad5 knob (2 or 10 µg/ml), a combination of both knobs or with plain PBS for 10 minutes at room temperature. Recombinant proteins were obtained as described previously (Krasnykh et al, 1996). Viruses (100 vp/cell) were added in 100 µl cell growth medium containing 2% fetal bovine serum (FBS, hospital pharmacy University Hospital Groningen) and cells were incubated for 1 hour at 37°C. Then, 500 µl growth medium containing 10% FBS was added and cells were incubated for 2 days. Cells were lysed using Cell Culture Lysis Buffer and luciferase activity was measured using a luminometer (Packard, Groningen, the Netherlands), according to manufacturers conditions (Luciferase Assay System, Promega, Leiden, the Netherlands). Data are expressed as relative light units (RLU).

E. Infectivity assays

To compare infection efficiency of the fiber-mosaic adenovirus AdF3F5 with Ad5 infection (AdF5) and with infection of Ad3 (Ad5/3-Luc1), different cell lines were grown to 70% confluent monolayers in 24 wells plates. Viruses were diluted in 100 µl medium containing 2% FBS and cells were infected at 100 vp/cell. After 1 hour of incubation at 37°C, medium containing 10% FBS was added. After 2 days, cells were lysed and luciferase activity was determined. Data are represented as means of triplicates of representative experiments. Students t-Tests were performed to analyze the differences between infection efficiencies of AdF5 and AdF3F5.

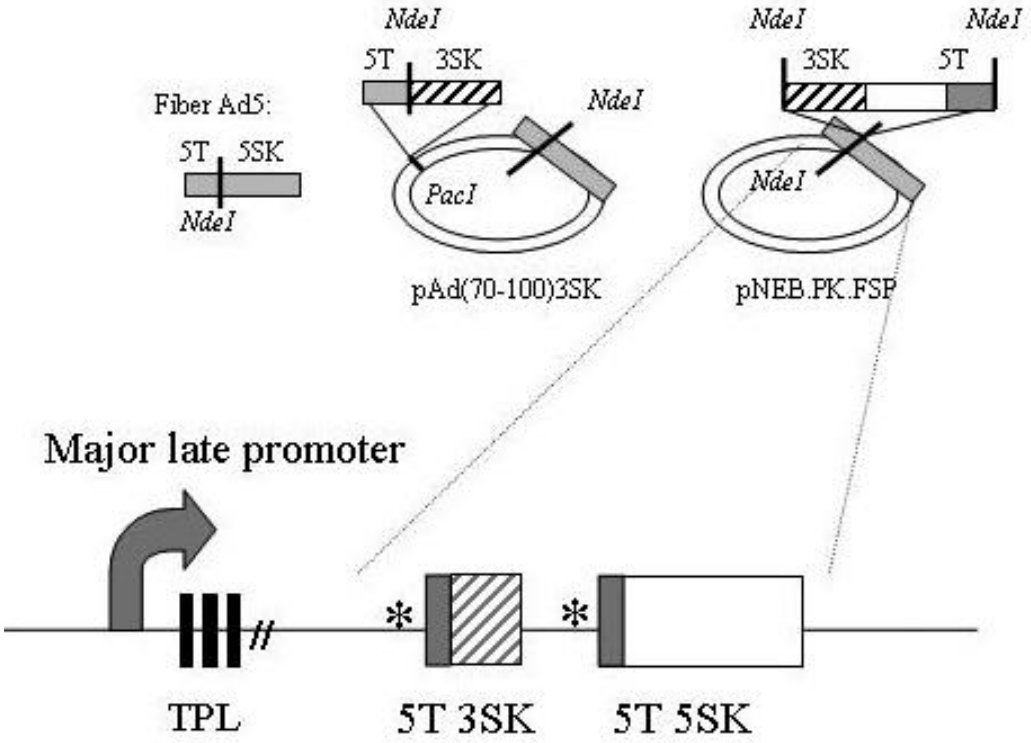


Figure 2. Cloning strategy for the transfer plasmid pNEBpkFSP.F3F5 for construction of fiber-mosaic adenoviruses AdF3F5. The chimeric fiber F3 was made by ligating the 5' part of the tail of Ad5 (T5) to the PCR product of the Shaft and the Knob of Ad3 (SK3). Subsequently, this fragment (5T3SK) was ligated into pAd70-100dlE3 containing wild type fiber (F5). Restriction with *NdeI* of plasmid pAd70-100(5T3SK) results in a fragment containing: 1) 3SK, 2) the wild type splicing sequences (*) upstream of fiber 5 and 3) the initial portion of the tail of wild type fiber 5. Subcloning of this *NdeI*-fragment into pNEBpkFSP resulted in a transfer vector for introduction of an additional fiber-encoding gene into the adenoviral backbone. Both fibers are under the control of the Major Late Promoter, with the tripartite leader marked as black boxes and the wild type splicing sites denoted as *.

III. Results

A. Construction of recombinant adenoviral plasmid encoding the fiber-mosaic adenovirus AdF3F5

To circumvent low infection efficiency which is hampering gene therapy approaches *in vivo*, we constructed an adenovirus with two different fiber types allowing two different mechanisms of cellular entry. Since adenovirus type 5 is the most commonly used vector for adenoviral gene therapy, we developed an approach to incorporate the additional fiber into the capsid of Ad5. To retain the trimerisation properties of adenoviral fiber molecules, we focused on subgroup B viruses which do not use the CAR receptor for entry. Optimal incorporation of the chimeric additional fiber protein into the capsid of Ad5, is ensured by cloning the Shaft and the Knob of Ad3 (3SK fragment) downstream of the initial coding sequence for the Tail of Ad5 (5T) (**Figure 2**).

To achieve equal expression levels of the chimer fiber compared to the wild type fiber, the chimeric fiber (5T3SK) was cloned under the control of the same promoter as the wild type fiber (the native adenoviral Major Late Promoter). To this end, however, the splicing signals of the wild type fiber 5 sequence also needed to be retained. This has been achieved through subcloning of the Ad3ShaftKnob-Ad5Tail *Nde*-fragment into another fiber shuttle plasmid (see Materials and Methods). The fiber-mosaic AdF3F5 viruses could be rescued on 293 cells as efficiently as other first generation adenoviruses (up to 10^{12} viral particles/ml). Western blot analysis subsequently confirmed the presence of both fiber types onto the CsCl-purified virus material. The protein levels, however, were not equal and higher levels of Ad5 fiber were detected compared to Ad3 fiber (**Figure 3**).

B. Infectivity assays

1. Functional validation of AdF3F5.

To identify the pathway of entry of AdF3F5, different cell lines (HeLa cells (expressing high levels of both CAR and the receptor for Ad3), FaDu and SCC25 cells (both expressing low levels of CAR and high levels of Ad3 receptor)) were incubated with AdF3F5, AdF5 and AdK3 after preincubation with recombinant knob 3 and/or knob 5 protein as described in Material and Methods. Presence of knob 3 did slightly inhibit infection of AdF3F5 (expressing both Ad3 and Ad5 fibers) and of AdK3 (expressing the knob of Ad3, displaying an Ad3 infection spectrum) on HeLa cells (14 and 10% inhibition, respectively) (**Figure 4**). However, more pronounced inhibition of knob 3 mediated infection was observed on FaDu (AdF3F5: 31% and AdK3: 58% inhibition) and SSC25 (27 and 77%, respectively). Preincubation with recombinant knob 5 protein efficiently inhibited infection of AdF3F5 and AdF5 (wild type Ad5 fiber) on all cell lines, especially on HeLa cells (over 90%). Combination of both recombinant knob proteins inhibited the infection efficiency of AdF3F5 even further on FaDu and SCC25 cells. Preincubation with knob 3 occasionally increased infection efficiency of AdF5, whereas knob 5 could marginally inhibit infection of AdK3 (shown for FaDu in **Figure 4b**).

2. Determination of infection efficiency of AdF3F5 on cancer cells.

Some cancer types are known to be less susceptible to infection with Ad5 compared to others due to low CAR levels. To test improved infectivity of the fiber-mosaic AdF3F5 on different cancer cell types, cell lines were infected with AdF3F5, AdF5 and AdK3 (**Figure 5**). Infection with AdF3F5 was as efficient as AdF5 on HeLa and FaDu, while an 2- to 3-fold increase in efficiency was observed for AdF3F5 compared to AdF5 on U373, U118

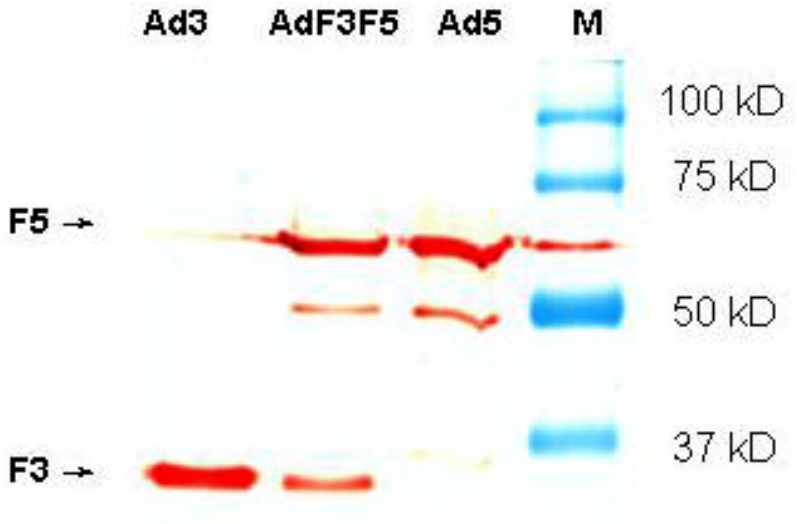


Figure 3. Western blot detecting adenoviral fiber molecules. Boiled CsCl-purified viruses (10^{10} viral particles) were separated on SDS-PAGE gel, transferred to PVDF membrane and stained with 2D4 anti-tail antibody. Ad3 virions showed a band for the fiber molecule at 35 kDa, whereas the fiber of Ad5 was detected around 65 kDa. For the fiber-mosaic AdF3F5, two bands were detectable: one strong band at the size of the fiber of Ad5, whereas a weaker band could be detected at the size of Ad3 fibers.

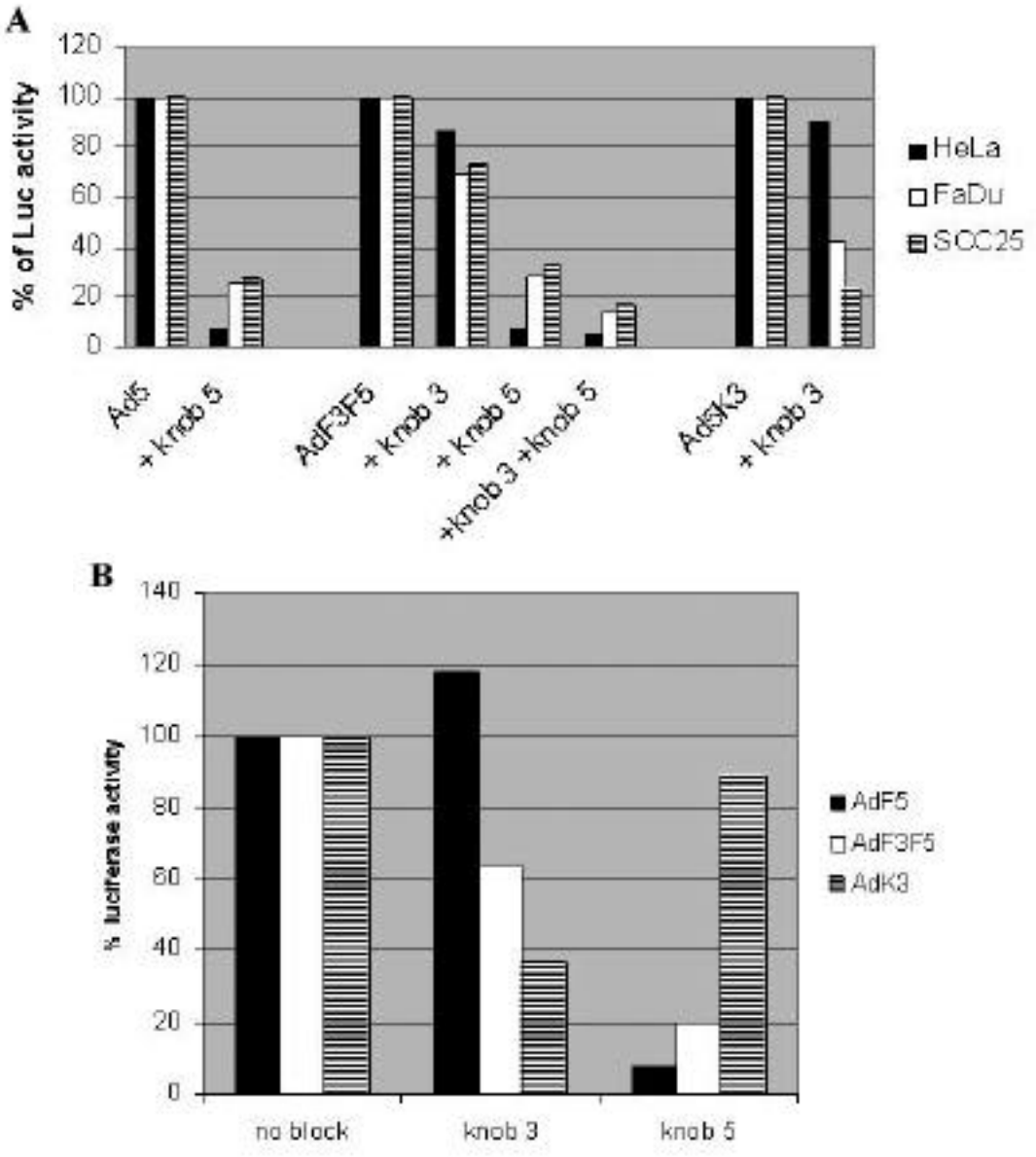
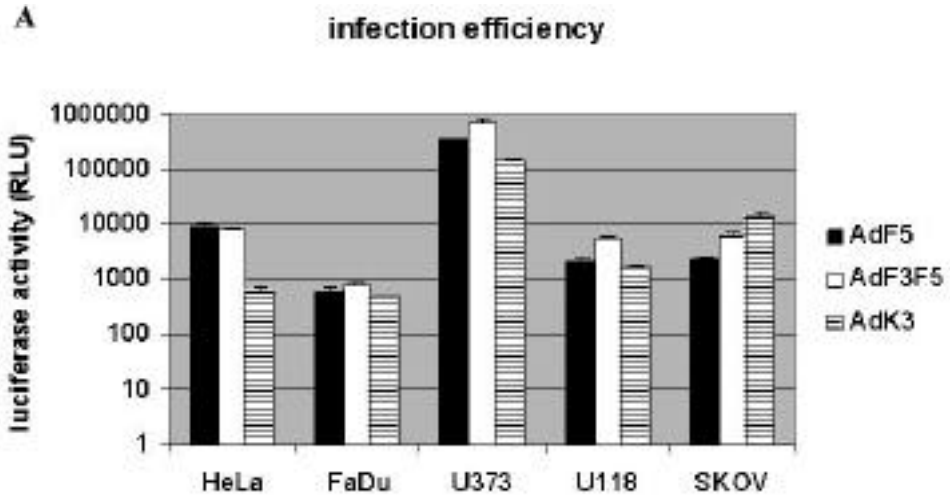


Figure 4. Functional validation of AdF3F5. a) HeLa, FaDu and SCC25 cells were infected with AdF5, AdF3F5 and AdK3 (100 vp/cell) after pre-incubation with recombinant knob 3 (10 µg/ml) and/or knob 5 (2µg/ml). After 2 days, luciferase readings were performed. Data are expressed as percentage of relative light units, 100% being no knob block present. **b**) to investigate cross-inhibition of the knobs, FaDu cells were infected with AdF5, AdF3F5 and AdK3 (100 vp/cell) after pre-incubation with recombinant knob 3 or knob 5 (10µg/ml).



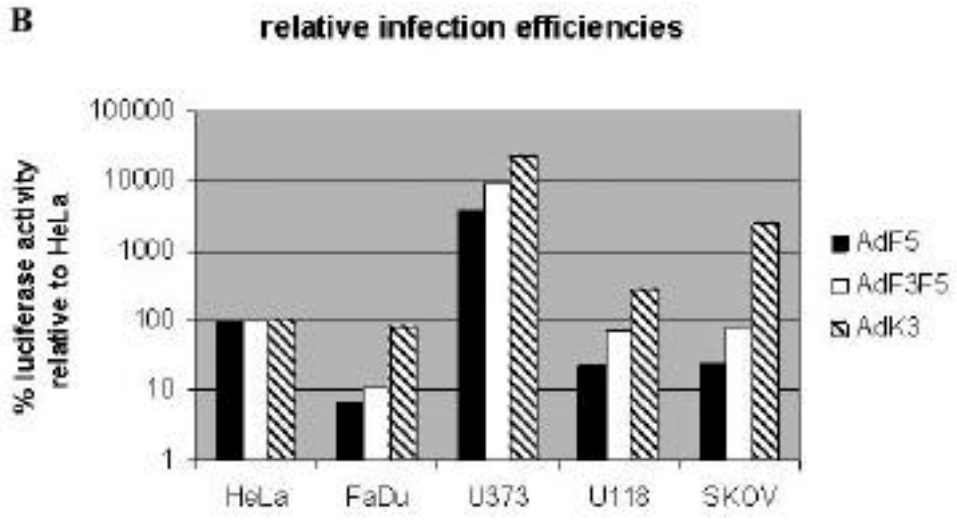


Figure 5. Absolute and relative infection efficiencies of fiber-mosaic Ad on Ad3 receptor positive cells. Different cell lines were incubated with AdF5, AdF3F5 and AdK3 viruses (100 vp/cell) and after two days infectivity efficiency was measured by determining luciferase activity. Data are represented as mean values of triplicates + SD. To directly compare the different viruses, infectivity on HeLa cells has been set at 100% in Figure 5b.

and on SKOV cells ($p \leq 0.01$). Since the receptor levels of Ad3 and Ad5 receptors on HeLa cells is similar, the infection of the three viruses on HeLa cells was set at 100% to determine the relative infection efficiency of AdF3F5 compared to AdK3. Although AdF3F5 showed improved infection efficiency over AdF5, infection efficiency of AdF3F5 was not improved compared to AdK3 for any of the cell lines tested.

IV. Discussion

Low infection efficiency is hampering cancer gene therapy from showing its full potential and vectorological improvements are warranted. Moreover, virotherapy (the conditional viral replication resulting in oncolysis) would greatly benefit from infectivity enhanced agents as shown by incorporation of different targeting moieties in fibers of replication competent viruses (Hemminki et al, 2001; Bauerschmitz et al, 2002; Kawakami et al, 2003). These studies, however, again are limited to the expression of one receptor type on tumor cells. In this study, we describe the feasibility to grow fiber-mosaic adenoviral agents targeting two different receptors simultaneously. We showed that infection with this fiber-mosaic virus shows advantage over AdF5 infection. Although no increase in efficiency was observed compared to infection with AdK3 for low CAR cell lines, the technology provides a flexible platform allowing increase of specificity by introduction of two different targeting ligands.

Tropism of adenoviruses is determined by the knob of the fiber protein and the penton base. We hypothesized that the introduction of an additional different fiber type provides a way of introducing an additional mechanism of cellular entry of virus, thereby increasing efficiency of infection and/or broadening the infection spectrum. Since Ad3 has been demonstrated to efficiently infect several CAR deficient (primary) tumor types (Stevenson et al, 1995; Kanerva et al, 2002; Volk et al, 2003), we choose the Ad3 fiber molecule to be incorporated into the capsid of type 5 adenoviruses in addition to the wild type Ad5 fiber.

We demonstrated that two different fiber proteins can be incorporated into one viral capsid and that such fiber-mosaic viruses can be efficiently rescued using conventional methods. Although the additional fiber was cloned under the control of the same Major Late Promoter, using the same upstream splicing sequences as the wild type fiber, incorporation of the chimeric fibers onto the capsid was less efficient then for the wild type fiber, as detected by western blot. This might be explained by a packaging bias of one fiber type over the other as previously described for naturally occurring fiber-mosaic adenoviruses (Schoggins et al, 2003). However, in our approach the tail of both fiber types starts with the first amino acids of the tail of wild type Ad5 to avoid inefficient incorporation. The imbalanced incorporation most likely is explained by the low protein expression of fiber observed after infection by Ad3 (Albiges-Rizo et al, 1991). However, we continued with this fiber chimera since the shorter size of the fiber of Ad3 allowed easy biochemical discrimination with the wild type Ad5 fiber.

Blocking experiments demonstrated that these fiber-mosaic viruses exploit two ways of entry. Importantly, infection efficiency was not impaired by incorporation of the additional fiber into the capsid. As both the knob of Ad5 (Belousova et al, 2002) as well as the knob of Ad3 (Uil et al, 2003) can be exploited to introduce targeting moieties, fiber-mosaic viruses represents a powerful platform for constructing efficient, but specific gene therapy agents. Improved gene transfer efficiency by introducing two retargeting moieties onto the viral capsid has previously been obtained by incorporation of both the RGD and a polylysine motif into the fiber (Wu et al, 2002), supporting our hypothesis.

Previously, we obtained fiber-mosaic adenoviruses expressing both the fiber of Ad5 and a chimeric fiber consisting of the tail and the shaft of Ad5 fiber and the

knob of Ad3 by co-culture of Ad5 and AdK3. The resulting viruses incorporated both fibers on the same virion as has also been described for co-culture of other serotypes in the early 70s (Norrby and Gollmar, 1971). These AdF5:AdK3 fiber-mosaic viruses demonstrated an expanded infection spectrum (Takayama et al, 2003). Interestingly, the viruses also showed an improved infection efficiency on various cell types tested compared to Ad5, suggesting synergism between knob 5 and knob 3. In this study, we did not observe such profound synergism, probably since the knob of Ad3 is expressed on the short shaft of Ad3; Binding to the receptor of Ad3 through the short shaft might prevent simultaneous binding to the CAR via the long shaft. Any receptor-cross talk resulting in synergism will therefore be prevented.

Although co-infection results in fiber-mosaic viruses, the production is laborious and most likely not very reproducible. The approach to genetically construct a fiber-mosaic virus expressing two different fibers is therefore preferred. Naturally occurring human fiber-mosaic adenoviruses have been identified and belong to subgroup F enteric viruses. These viruses (serotype 40 and 41) contain two separate genes encoding a short fiber of 200A (41 kDa) and a long fiber of 340A (61 kDa) (Kidd et al, 1993; Favier et al, 2002). These viruses therefore are very similar to the one described here as the fiber of Ad3 is 160A and the fiber of Ad5 is 370A. The long fiber of Ad40 and Ad41 binds to the CAR receptor whereas binding of the short fiber is CAR-independent (Roelvink et al, 1998). The concept of tandem fiber genes to construct fiber-mosaic viruses had previously been shown feasible (Schoggins et al, 2003; Pereboeva et al, 2004). In an elegant approach, Perebouva et al, introduced the option of binding targeting ligands to the second fiber type through a biotin-acceptor peptide (Pereboeva et al, 2004). Schoggins et al, reported on the construction of a fiber-mosaic adenovirus type 5 co-expressing the fiber of Ad7 either with the fiber of Ad5 or with the short fiber of Ad41. Like AdF3F5, the fiber-mosaic F7F5 virus showed similar infection efficiencies compared to Ad5 (Schoggins et al, 2003). The infectivity of the Ad5 based fiber-mosaic adenovirus expressing the fiber of Ad7 and the short fiber of Ad41 virus was dramatically impaired *in vitro*. Also *in vivo*, a 2-log lower transduction of the liver was observed. Similarly, a 10-fold reduction in liver transduction has been reported for an Ad5 based adenovirus expressing the shaft and the knob of Ad3 on its capsid (Vigne et al, 2003). In this respect, fiber-mosaic viruses based on Ad5 show promise as a platform for engineering efficient gene therapy agents with a liver-off profile.

In conclusion, we demonstrated that viruses expressing two different fiber types can be constructed and efficiently rescued. Both fiber types are functional in infecting cells, which opens the way for infecting a broader spectrum of tumors. The next step is to increase the specificity of this potent vector by introducing targeting moieties and/or tumor specific promoters to selectively express a trangene or to restrict viral replication.

Acknowledgments

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Group picture of the Department of Therapeutic Gene Modulation. Dr. Marianne G. Rots is the third person shown in the first row from right to left.