

SHORT COMMUNICATION

An adenovirus vector with a chimeric fiber incorporating stabilized single chain antibody achieves targeted gene delivery

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Adenovirus (Ad) vectors are of utility for many therapeutic applications. Strategies have been developed to alter adenoviral tropism to achieve a cell-specific gene delivery capacity employing fiber modifications allowing genetic incorporation of targeting motifs. In this regard, single chain antibodies (scFv) represent potentially useful agents to achieve targeted gene transfer. However, the distinct biosynthetic pathways that scFv and Ad capsid proteins are normally routed through have thus far been problematic with respect to scFv incorporation into the Ad capsid. Utilization of stable scFv, which also maintain correct folding and thus

functionality under intracellular reducing conditions, could overcome this restriction. We genetically incorporated a stable scFv into a de-knobbed, fibritin-foldon trimerized Ad fiber and demonstrated selective targeting to the cognate epitope expressed on the membrane surface of cells. We have shown that the scFv employed in this study retains functionality and that stabilizing the targeting molecule, per se, is critical to allow retention of antigen recognition in the adenovirus capsid-incorporated context.

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Adenovirus (Ad) vectors based on the human serotypes 2 and 5 are promising candidates as gene therapy delivery vehicles. In addition to attractive features such as low pathogenicity for humans, lack of integration in host cell genome and the ability to grow to high titers, adenoviruses have unique utility for *in vivo* applications due to their high efficacy compared with other approaches. In this regard, Ad vectors are currently involved in more than one-quarter of all human gene therapy trials and have a proven safe clinical profile. However, their efficacy within the clinic would clearly be improved by the development of Ad vectors with selective tissue targeting capabilities.

In this regard, motifs derived from antibody molecules represent potentially useful agents to achieve this desired goal of cell-specific targeting, as they embody unparalleled affinity and specificity for recognition and binding to target cell surface markers as well as encompassing a broad range of characterized targets. Thus, it is widely recognized that genetically incorporating antibody-derived targeting motifs into the adenoviral capsid represents the optimal means to achieve the

field-wide goal of rendering Ad target cell specific. In particular, single chain antibodies (scFv), generally comprising a variable region of a heavy (V_H) domain fused to a light (V_L) domain of an immunoglobulin via a polypeptide linker, represent the antibody fragment of choice; tropism modification of several viral vectors has been attained via envelop incorporation of scFv.^{1–10} However, attempts to directly incorporate scFv into the Ad capsid have been problematic to this point.¹¹ Consideration of the infectious life cycles and biological pathways of the different viral vectors is required to understand this phenomenon. Specifically viral envelop incorporation of scFv is directly achievable owing to the fact that glycoproteins of such viral vectors are routed in a manner similar to that of scFv, wherein synthesis of these proteins occurs with assembly and folding in the RER. On the other hand, Ad capsid proteins are synthesized in the host cell cytosol followed by cytosol-to-nuclear transport and full virion assembly in the nucleus. Relevant in this context is the fact that the redox state of the cytosol environment is potentially deleterious to scFv due to intra-chain disulfide bridges, found in each variable heavy and light chain. Thus, the nonnative routing imposed on scFv by Ad capsid incorporation methods likely confounds proper scFv folding, thus perturbing the structural configuration required for antigen recognition. This intranuclear assembly of adenovirus thus renders incorporation of scFv into Ad capsids an unprecedented challenge. As a

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provisional alternative to genetic modification, targeting through two-component adaptor systems has demonstrated that the concept of re-targeting is attainable and defined key biologic aspects thereof.^{12–18} Thus, with the advent of genetic development of cytosol stable scFv (known as ‘intrabodies’) (reviewed in Barberis *et al.*¹⁹ and Stocks²⁰), and the translation of technologies to scFvs that target cell surface markers of disease-related cells (e.g. CEA²¹), these potential pitfalls seen thus far with standard scFv may be overcome. It would therefore seem that these stabilized scFv provide a more relevant choice as targeting ligands to be utilized within the Ad capsid configuration.

We have recently reported on a novel intracellular screening system, the yeast genetic system named ‘Quality control’ that allows selection of scFvs, which are stable under reducing conditions.²² These selected scFvs have shown high biochemical stability and solubility not only in the intracellular reducing environment, but also under normal extracellular conditions, and therefore these scFvs serve as acceptor backbones upon which to construct hypervariable loops that specifically recognize antigens. Through this library screening system soluble and stable scFv moieties for various antigens can be rapidly identified.²³ The antigen-specific scFv (referred to as Binder throughout) developed for this study is based on the FW4.4 scaffold.²⁴ It is able to specifically bind to an artificial receptor system composed of a transmembrane spanning part and a short extracellular peptide epitope.

Another key consideration in this context is the necessity to identify (or modify) capsid proteins that are compatible with the incorporation of heterologous ligands of comparable complexity to antibody-derived fragments. Underlying this general concept is the requirement to incorporate the antibody-related species into the adenoviral capsid via genetic capsid modifications. Whereas small peptides have been incorporable at the major capsid proteins penton,²⁵ hexon,^{26,27} and in the so-called HI loop^{28–33} and carboxy terminus of the Ad5 fiber protein,^{34,35} these identified regions within the aforementioned capsid proteins have not been shown to be suitable for incorporation of molecules with the complexity represented by antibody-related molecules.^{33,35} In addition, these resultant adenovirus vectors often have expanded, rather than restricted, cell recognition, as they do not address the question of ablation of CAR tropism. Realization of these limitations recently resulted in new strategies capitalizing on the modular structure of the native fiber protein, the natural determinant of Ad vector tropism. Hence, the development of the so-called ‘knobless’ fiber platforms have been endeavored, whereby modifications are based on the concept of replacing the native fiber with an alternative protein capable of providing the trimerization functions that the knob usually confers. The removal of the structural constraints imposed by the fiber knob would generate a theoretical universal ligand-presenting molecule, by which an expanded repertoire of incorporable and complex ligands can be utilized. Hence, several chimeric fibers based on this design have been constructed by utilizing various trimerizing motifs, including the trimerizing domain of Moloney murine leukemia virus envelope glycoprotein,³⁶ the neck region peptide of human lung surfactant protein D³⁷ and the σ 1 attachment

protein of the mammalian reovirus.³⁸ In this regard, our group has utilized the bacteriophage T4 fibrin protein for fiber replacement,³⁹ which permitted the incorporation of CD40L, a trimeric targeting ligand of comparable complexity, to scFv.⁴⁰ However, the structural integrity of these fibers is lessened^{11,36,37,41} and in our own studies we have noted that targeting with these vectors, while CAR independent and target receptor specific, does not achieve the levels associated with an Ad5 fiber on a CAR expressing cell.^{39,40} To address this point, we have further refined the fiber-fibrin platform, and developed a 566FF chimeric fiber containing the Ad5 tail and Ad5 shaft fused to the 12th coil of the fibrin molecule (as described⁴²). This fiber demonstrating improved incorporation into the viral capsid provided us a new platform for the incorporation of the stabilized scFv (Figure 1).

To conduct preliminary analysis of these 566FF-scFv, the genes were assembled in the mammalian expression plasmid pVSII⁴³ and the resultant plasmids were then used to direct the expression of these proteins in 293T/17 cells. These expression experiments were intended to demonstrate that the designed fiber chimeras, when expressed in mammalian cells, possess structural and functional properties required both for the incorporation of these proteins into Ad virions and for binding to the cognate epitope (RA) present in a recombinant protein, GST-RA. As seen in Figure 2a, a Western blot of the lysates of pVS-transfected 293T/17 cells showed that the 566FF-scFv proteins, as well as the control 566FF-Cd (this control 566FF fiber instead of scFv as the ligand contains the Fc binding domain (termed Cd) from *Staphylococcus aureus* Protein A incorporated into the C terminus and this fiber is known to be structurally stable)⁴² and wild-type Ad5 fiber expressed by the same plasmid system, were of the expected molecular weight without detectable signals indicating degradation. A comparison of the mobilities of the chimeras in denatured and non-denatured samples showed that all the newly designed proteins formed trimers upon self-association. We next examined the RA-binding capability of the Binder scFv in the context of the 566FF chimeras. This was accomplished by an ELISA which used the lysates of 566FF-scFv-expressing 293T/17 cells for a binding assay employing GST-RA as bait. This assay demonstrated that 566FF-Binder chimeras bound to GST-RA, while predictably 566FF-Scaffold did not bind to GST-RA even at the highest concentration used (Figure 2b). In the aggregate, these experiments clearly demonstrated that 566FF-Scaffold and 566FF-Binder proteins despite their size and complexity preserve the key properties essential for incorporation into virions and, with 566FF-Binder, targeting of Ad vectors.

The rescue and propagation of viruses with Binder and Scaffold scFv chimeric fibers were carried out according to the two-step procedure⁴⁰ with a final round of amplification on 293 cells. Analysis of fiber presence in virions indicated that similar levels of 566FF-Scaffold, 566FF-Binder and Ad5 fiber incorporation could be attained (Figure 3a). The lysate data demonstrate that for both 566FF-scFv fibers there was extensive expression of the fibers, and many of these fibers were degraded indicating the necessity of further optimization. However, it was evident that only full-length 566FF-Scaffold fibers were incorporated into the virions, whereas some

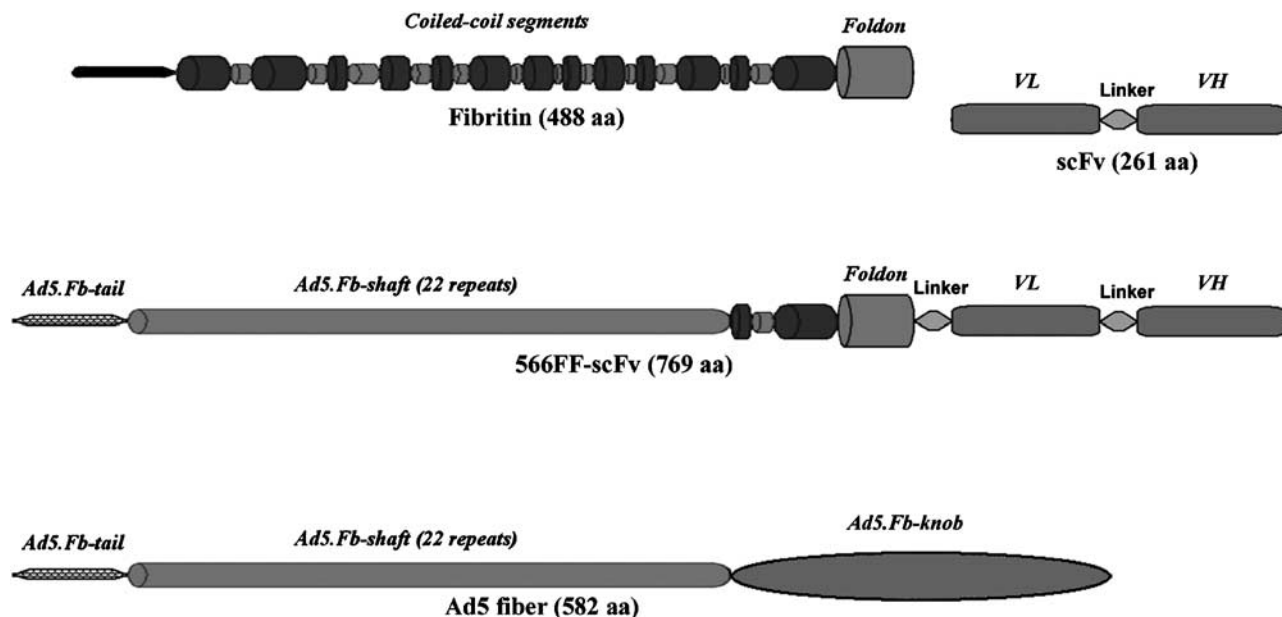


Figure 1 Schema of 566FF-scFv chimeric fiber. Single chain antibodies, either Scaffold scFv (non-Binder) or Binder scFv, were amplified from their respective plasmids using two reaction mixes with the following primer pairs:- (1) 5'-/Phos/GAT CCG ACT ACA AAG ACC AGT CTG-3' (forward) and 5'-/Phos/GGC CCC CGA GGC CGA GGA G-3' (reverse) and (2) 5'-/Phos/GAC TAC AAA GAC CAG TCT G-3' (forward) and 5'-/Phos/TAT TAG GCC CCC GAG GCC GAG GAG-3' (reverse). *BaeI* ends are underlined. For each scFv, following PCR, the fragments were gel purified, then mixed, denatured and re-annealed to create fragments with the *BaeI* sticky ends. This allowed for cloning into pKan-566FF-*BaeI* plasmid so that the scFv (Scaffold or Binder) was in the correct reading frame beside the 566FF chimeric fiber. The schematic composition of 566FF chimeric fiber, with the genetically fused scFv, is shown. The fiber itself is composed of the Ad5 tail and Ad5 shaft fused to the 12th coil of fibrinin.⁴²

fibers incorporated into the 566FF-Binder virions were partially degraded, with loss of either one or both domains of the scFv (products predominantly located around the 62 kDa region). We ruled out the possibility that these degraded products were contaminated with wild-type Ad5 fiber using an antibody that detects only trimeric Ad5 fiber in Western blots. Both nondenatured 566FF-Scaffold and 566FF-Binder virions were negative in this analysis, whereas nondenatured Ad.Luc1 (containing Ad5 fiber) was positive (data not shown). To establish functional properties of the chimeric fibers in the context of virion incorporation, consideration of the RA-binding capability of the 566FF-Binder virion was necessary. This was accomplished by an ELISA, which used purified virions for a binding assay employing GST-RA as bait. This assay demonstrated that Ad.Luc.566FF-Binder bound to GST-RA, despite there being the presence of sub-length fibers in this viral prep, while predictably the 566FF-Scaffold virions and Ad.Luc1 virions did not bind to GST-RA even at the highest concentration used (Figure 3b). In the aggregate, these experiments clearly demonstrated that 566FF-Scaffold and 566FF-Binder could incorporate into the virions at near wild-type levels. This confirmed our previous study that the 566FF chimeric fiber has utility as a fiber platform for targeting ligand incorporation.⁴² Importantly, the Binder scFv within the context of fiber and virion retained functionality, a key property essential for the targeting of Ad vectors.

It was a requisite to develop an artificial receptor system to express the Binder epitope, termed eRA, and therefore we established CHO cells to express this receptor. Korokhov *et al.*,⁴³ have previously used this

artificial receptor system to mimic the 'natural' situation and have demonstrated its utility in the context of a range of target antigen molecules. Analysis of the isolated clonal populations of cells indicated through Western blot analysis that high protein levels could be attained (data not shown), and via cell surface flow cytometry analysis, with the fusion fibers, we could identify clones with low, medium and high cell surface expression levels (Figure 4, for parental cells (a) and high receptor expressing clone, A16 (b)). In addition, this experiment, utilizing transiently expressed chimeric fibers as eRA-recognizing molecules, demonstrated that the Binder scFv could recognize the cell surface expression of the cognate epitope within the constraints of an Ad capsid protein. We therefore selected Clone A16 (Figure 4b) for further utility in gene delivery experiments. We found that Ad.Luc.566FF-Scaffold vector did not have increased transduction on A16 cells, compared to parental cells, whereas there was a 6–10-fold increase in transduction (dependent on experiment) for the Ad.Luc.566FF-Binder vector, when examined on A16 cells (Figure 4c, 10-fold increase in this representative example). This was specific as the increase was ablated by preincubation of virus with GST-RA and in addition, this ablation was dose dependent (Figure 4d). Further, CHO cells are CAR negative, and thus a direct comparison with an Ad5 virus containing Ad5 fiber could be achieved. We saw a 6.4-fold increase in transduction with Ad.Luc.566FF-Binder in comparison to Ad.Luc.1 on the A16 cells in this experiment (Figure 4c). These experiments clearly demonstrated that a combination of optimized fiber-replaced platform with stabilized scFv allows the feasibility of Ad vector generation with

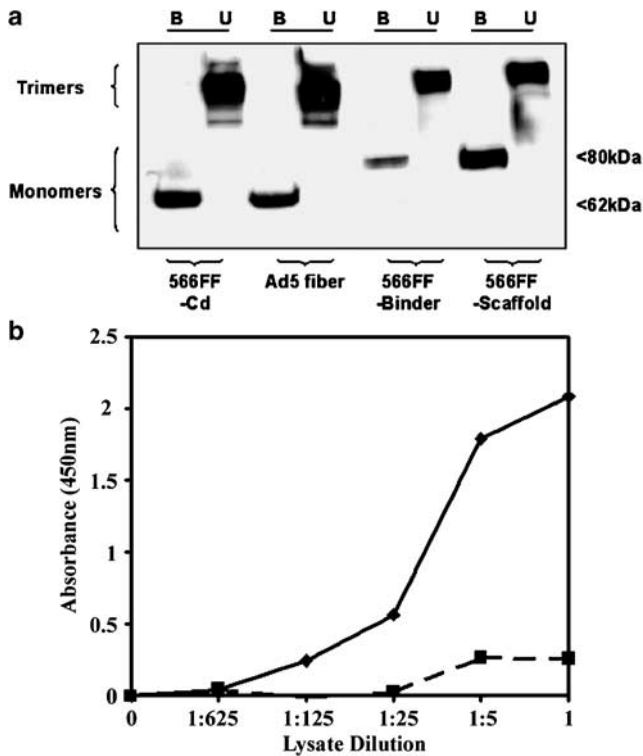


Figure 2 Monomeric and trimerization profiles (a) and binding (b) of transiently expressed 566FF-scFv fiber proteins to GST-RA. 566FF-Binder and 566FF-Scaffold were sub-cloned from the pKan shuttle vectors into the pVS expression plasmid,⁴³ to generate pVS-566FF-Binder and pVS-566FF-Scaffold. 293T/17 cells, grown to confluence in six-well dishes, were transfected with 5 μ g plasmid DNA/well, of either pVSII (Ad5 fiber⁴³), pVS-566FF-Cd (a control 566FF fiber),⁴² pVS-566FF-Binder or pVS-566FF-Scaffold, using DOTAP liposomal transfection reagent (Roche) according to the manufacturer's protocol. Following 72 h of culture, cells were harvested into 200 μ l serum-free media, freeze-thawed three times to release the fibers, and centrifuged supernatant collected. (a) Structural integrity and ability to form homotrimer chimeric fibers in the lysate of 293T/17 cells was assessed by Western blot analysis. Samples, nondenatured (U, unboiled) or denatured (B, boiled), were applied at 10 μ l of lysate. The proteins were separated by 4–20% gradient polyacrylamide gel (Bio-Rad) and transferred to polyvinylidenedifluoride (PVDF) membrane where they were probed with 4D2 antibody (UAB), which detects the tail section of both monomeric and trimeric forms of Ad5 fiber. The blots were developed with the WesternBreeze immunodetection system (Invitrogen) according to the manufacturer's protocol. Samples are indicated in the figure, and as described, monomeric fibers migrate as follows: 566FF-Cd fiber and Ad5 fiber migrates at 62 kDa while 566FF-scFv migrate at 80 kDa. (b) Lysates of 293T cells transiently expressing 566FF-Binder (diamonds) or 566FF-Scaffold (squares) were tested for binding to GST-RA, a recombinant protein containing the scFv-binding epitope, RA, using ELISA methodology as follows. GST-RA was diluted in 50 mM NaHCO₃ [pH 9.6] to obtain 5 μ g/ml and 100 μ l added per well in Nunc-Maxisorp ELISA plates (Nunc). The plates were stored overnight at 4°C and then blocked with PBS containing 0.05% Tween 20 and 0.5% casein for 1 h at room temperature. Wells were washed with PBS containing 0.05% Tween 20. Lysates were diluted (dilutions indicated on x-axis) in binding buffer (TBS with 0.05% Tween 20 and 0.05% casein) and incubated at RT for 1 h in the wells following blocking and detection by the 4D2 antibody. Goat anti-mouse immunoglobulin antibody conjugated with horseradish peroxidase (HRP) (Dako) was used as a secondary Ab and the color was developed with the Sigma FAST *o*-phenylenediamine dihydrochloride tablet kit (Sigma) as recommended by the manufacturer. The color intensity was measured at 450 nm with an EL800 plate reader (Bio-Tek Instruments).

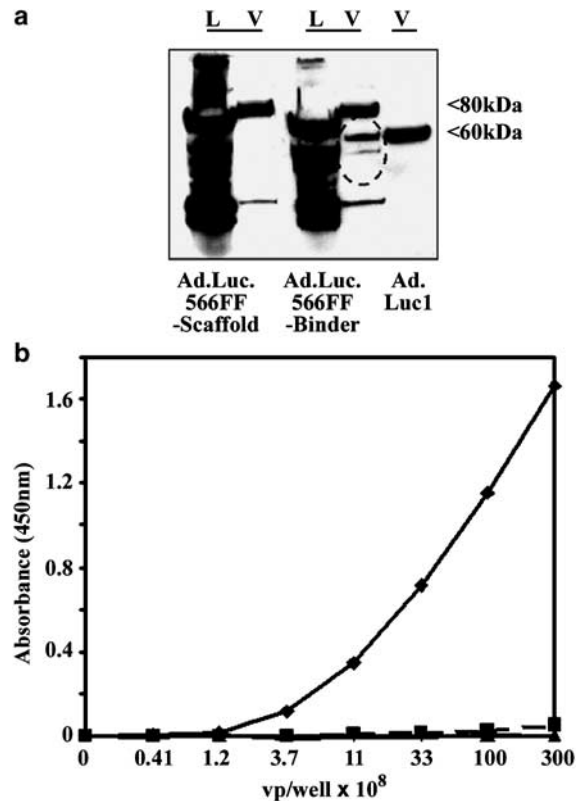


Figure 3 Structural (a) and functional (b) analysis of rescued viruses encoding variants of 566FF-scFv fiber proteins. The generated plasmids, pKan.566FF-Binder and pKan.566FF-Scaffold, were linearized with an *Eco*RI digest and recombined with *Sma*I digested pVK700 Ad5 modified genome⁴⁰ using standard bacterial methods. Resultant recombinant Ad vectors were digested with *Pac*I to release the viral genome and used to transduce 293-F28 cells,⁴⁴ a fiber complementing cell as the fibers are CAR ablated, to rescue viruses, Ad.Luc.566FF-Binder and Ad.Luc.566FF-Scaffold. These rescued viruses were in a mosaic format (i.e. the virion capsids contained modified fibers and Ad5 fibers in varying amounts). A large-scale amplification on 293 cells to retrieve non-mosaic viruses (i.e. those containing only the fiber encoded for in the genome) was performed. Standard methods for CsCl purification of mosaic and non-mosaic virions were undertaken. (a) Viruses were propagated on 293 cells and lysates (10 μ l) and purified virions (5×10^9 vp) from these cells were analysed by Western blot using the 4D2 primary antibody to detect the presence of fibers as described previously. Lysate and virion samples are indicated as L and V, respectively, with viruses further indicated in the figure. Full-length 566FF-scFv chimeric fibers migrate at 80 kDa, while Ad5 fiber migrates at 62 kDa and degradation products of 566FF-scFv migrate at 62 kDa and lower (see circled region). (b) Abilities of Ad.566FF-Binder (diamond) and Ad.566FF-Scaffold (square) virions to bind to GST-RA were compared with Ad.Luc.1 virions (triangle) at the vp/well as indicated on the graphs. ELISA was performed as described in Figure 2 with these following modifications. Purified virions were detected by anti-Ad2 polyclonal antibody (NIAID), with the secondary HRP antibody being goat anti-rabbit immunoglobulin antibody (Dako).

novel tropism. Importantly, accrued gene transfer was precisely specific for the target cell receptor.

In summary, the modification of viral tropism is one of the major paradigms in generating clinically relevant gene delivery vectors. Highly attractive targeting moieties that provide utility for this concept are antibody-related molecules, in particular scFv. However, the biosynthetic incompatibility of scFv and nuclear viral

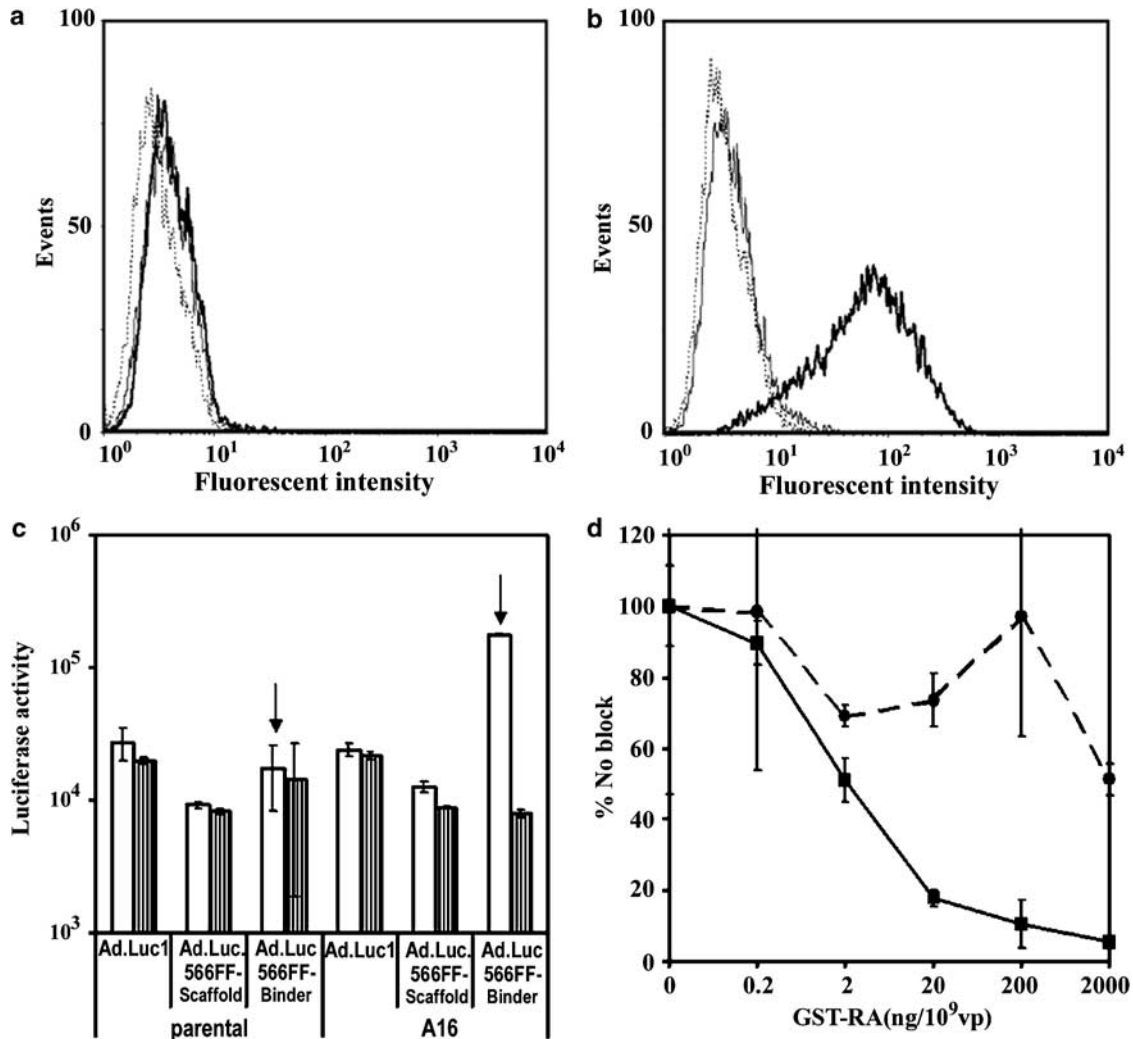


Figure 4 Evaluation of the efficiency and specificity of gene transfer by the rescued viruses. (a–b) Generation of a stable cell line to express the artificial receptor containing the scFv cognate epitope (eRA) was as follows. Complimentary ss oligos for the epitope (IDT) were annealed and inserted into *SfiI-SacII* digested pDisplay (Invitrogen), a plasmid that expresses artificial receptors, resulting in generation of pDisplay-eRA plasmid. Cells, which express eRA, were generated by transfection of CHO cells with pDisplay-eRA plasmid and subsequent selection with 1 mg/ml G418 (Mediatech Inc.). Clonal populations expressing eRA were identified by Western blotting of cell lysates with an antimyc antibody (Invitrogen), which recognizes one of the tags within the pDisplay receptor. The blots were developed with the WesternBreeze immunodetection system as before. Parental (a) and clonal CHO cells (b, clone A16) were analyzed for cell surface expression of eRA. Cells, 2×10^5 cells/sample, were treated either as controls (dotted line), or with the fusion proteins, 566FF-Scaffold (thin line) or 566FF-Binder (thick line) derived from pVS-transfected 293T/17 cells. All samples were probed with 4D2 antibody (final concentration of $0.5 \mu\text{g}/\mu\text{l}$) for 1 h at 4°C and followed for a final 1 h at 4°C with a secondary Alexa-fluor conjugated goat anti-mouse antibody (Molecular Probes). Cells were fixed in 1% paraformaldehyde before analysis on a FACSCalibur machine (Becton Dickinson). (c and d) Evaluation of transduction ability of scFv-targeted Ad vector. Parental CHO and clonal CHO-eRA (A16) cells were grown to confluence in 24-well plates. Prior to transduction, viruses were either untreated or pretreated with GST-RA ($2 \mu\text{g}$ per 1×10^9 vp (c)) or a range of GST-RA ($0\text{--}2 \mu\text{g}$ (d)) for 15 min at RT. Cells were then incubated on ice for 1 h with unblocked or blocked Ad.Luc1, Ad.Luc.566FF-Scaffold or Ad.Luc.566FF-Binder at 1000 vp/cell, then washed twice with PBS, and returned to normal culture media and conditions. After 24 h, cells were lysed with Luciferase Reporter Lysis Buffer (Promega) and assayed for luciferase activity, measured as relative light units according to the manufacturer's protocol (Promega Luciferase Kit). All the measurements were done in triplicate and are shown as means with error bars representing standard deviations. (c) This is a representative example of three individual experiments performed. Viruses are indicated as control (white bars) or pretreated with GST-RA (vertical lined bars). Further details of samples are indicated in the figure. Arrows indicate the 10-fold increase in transduction efficiency when Ad.Luc.566FF-Binder is incubated on A16 cells compared to parental cells. (d) This experiment was performed once. Viruses transduced on A16 cell lines are represented as follows; Ad.Luc.566FF-Scaffold (dashed line) or Ad.Luc.566FF-Binder (solid line).

capsid proteins as well as absence of identified locale in Ad capsid tolerant to incorporation of complex molecules have provided a challenge to generate an Ad vector that can target through capsid incorporated scFv. Thus, as a means to circumvent this hindrance in targeting Ad vectors with capsid-incorporated scFv we chose to utilize a stable scFv, which has been selected to be resistant to

any cytosol-induced alterations, in combination with radical reconstructions of Ad fiber allowing restrictions on the size and complexity of incorporable targeting ligands to be reduced. We have thus shown that the scFv employed in this study retains functionality and, on this basis, it is clear that stabilizing the targeting molecule, *per se*, allows retention of antigen recognition in the

adenovirus capsid-incorporated context. Importantly, the concept of vector engineering exemplified in this research illustrates the necessity to understand the key cell biology aspects when considering targeting motifs for genetic incorporation into the Ad capsid. Our studies clearly validate the feasibility of achieving Ad-mediated targeted gene delivery via capsid incorporation of scFv. This study thus represents achievement of a universally recognized field milestone and provides the basis of advanced generation Ad vectors compatible with the goal of achieving targeted, cell-specific gene delivery.

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