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Communication

Modular Adapters Utilizing Binders of Different Molecular Types Expand Cell-Targeting Options for Adenovirus Gene Delivery

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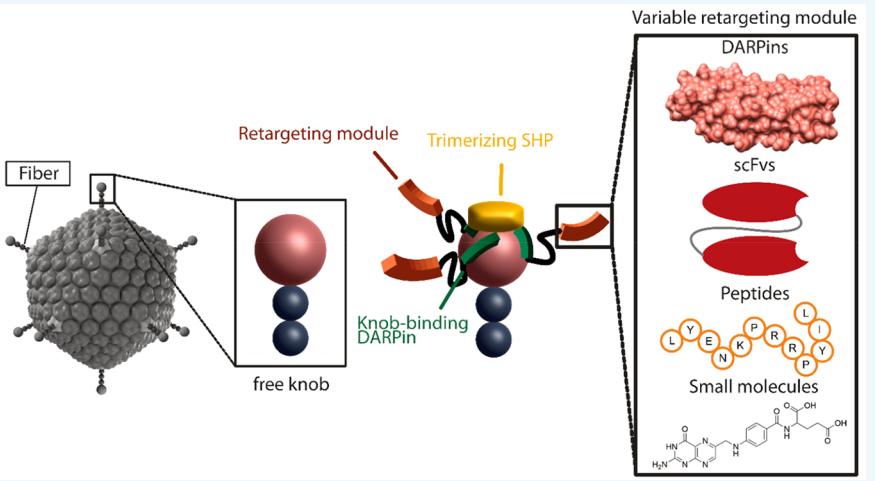

ABSTRACT: Efficient and cell-specific delivery of DNA is essential for the effective and safe use of gene delivery technologies. Consequently, a large variety of technologies have been developed and applied in a wide range of *ex vivo* and *in vivo* applications, including multiple approaches based on viral vectors. However, widespread success of a technology is largely determined by the versatility of the method and the ease of use. The rationally designed adapter technology previously developed redirects widely used human adenovirus serotype 5 (HAdV-C5) to a defined cell population, by binding and blocking the adenoviral knob tropism while simultaneously allowing fusions of an N-terminal retargeting module. Here we expand modularity, and thus applicability of this adapter technology, by extending the nature of the cell-binding portion. We report successful receptor-specific transduction mediated by a retargeting module consisting of either a DARPin, a single-chain variable fragment (scFv) of an antibody, a peptide, or a small molecule ligand. Furthermore, we show that an adapter can be engineered to carry more than one specificity, allowing dual targeting. Specific HAdV-C5 retargeting was thus demonstrated to human epidermal growth factor receptor 2 (HER2), human folate receptor α, and neurotensin receptor 1, effective at vector concentrations as low as a multiplicity of infection of 2.5. Therefore, we report a modular design which allows plug-and-play combinations of different binding modules, leading to efficient and specific mono- or dual-targeting while circumventing tedious optimization procedures. This extends the technology to combinational applications of cell-specific binding, supporting research in gene therapy, synthetic biology, and biotechnology.

Gene therapy is a fast-growing field of biomedical research, recently exceeding more than 4000 ongoing or completed clinical trials.[1] This rapid progress became possible due to successes in numerous DNA delivery methods, including physical gene transfer, synthetic nanoparticles, and viral or cellular vectors.[2,3] Especially for *in vivo* applications, viral vectors have been shown to achieve high transduction rates and sustained expression.[4−6] Adenoviral vectors (AdVs) in particular are among the most frequently applied gene vectors, and they are currently being investigated for multiple clinical applications, including but not limited to the fields of vaccines, oncology, or rare diseases.[1,7−13] Among the more than 100 human adenovirus serotypes, the most prominent and best studied AdV serotype is the human adenovirus serotype C5 (HAdV-C5).[14,15]

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Multiple generations of HAdV-C5 vectors have been developed, including nonreplicative ones such as first-generation and helper-dependent adenoviral vectors, and replicative and oncolytic adenoviral vectors, which all differ in the viral genes retained.[16] However, the outer capsid of all HAdV-C5-derived vectors is identical, dictating the tropism of the vector. Unmodified, the HAdV-C5 enters the cell by binding to the coxsackievirus and adenovirus receptor (CAR), followed by the interaction of the RGD motif of the adenovirus penton base with $\alpha_V\beta_3/\beta_5$ integrins on the cell surface.[14] Although this process has been reported to lead to success for some specific cell types, unmodified vectors are limited by their specificity for only a few cell surface receptors, restricting adenoviral gene delivery to cell and tissue populations that express the natural receptors. To overcome these limitations, various chimeras or capsid-engineering methods have been applied.[17−22] However, the manipulation of the protein structure and the incorporation of additional binding proteins encoded on the viral genome is not only a tedious process, but can drastically reduce viral titers and possibly lead to unpredictable interferences, ultimately resulting in additional validation and purification issues for each target to be investigated.[23−25]

To circumvent this cumbersome process, different bispecific proteins binding to HAdV-C5 capsid proteins and to a cellular receptor of choice were developed. These exogenously produced proteins are altering the HAdV-C5 interactions, while leaving the viral genome unmodified.[26−28] For successful applications, bispecific retargeting proteins require a very strong binding interaction with the vector, preventing dissociation from the viral capsid, resulting otherwise in untargeted virions. Additionally, off-target transduction must be inhibited by blocking the natural tropism, ensuring only targeted vector uptake.[29,30]

Bispecific proteins that bind to the knob domain of the HAdV-C5 fiber protein can fulfill all of these criteria. Fiber-binding bispecific proteins have the additional advantage that they are released after endosomal escape, and thus do not interfere with intracellular viral trafficking.[14] We previously described HAdV-C5-binding bispecific proteins that form a stable trimeric adapter, at the same time inhibiting CAR tropism by binding to the knob as a clamp, without any detectable off-rate.[31] Briefly, each monomer of these adapters consists of three distinct modules.[32] At the N-terminus, the retargeting module is located, which binds to a specific cell-surface marker. The retargeting module is fused by a $(G_4S)_4$ linker to the knob-binding module, consisting of the DARPin 1D3nc. A crystal structure of 1D3nc in complex with the fiber knob demonstrated direct interference of the DARPin with CAR binding to the fiber knob, which was further experimentally validated.[31] Furthermore, the knob-binding module is connected to the trimerization module, formed by the protein SHP from lambdoid phage 21, which is extremely stable against dissociation. This results in adapter binding to the HAdV-C5 knob as a chelate.[31]

Although these described adapters have shown to be effective for various *in vitro* and *in vivo* applications, several limitations still exist.[8,31,33] First, although DARPins are highly stable and rather convenient to handle, not all possible targets have yet an identified DARPin binder, limiting the current adapters to available DARPins—or requiring a new selection. Second, until now the adapters were genetically encoded fusion proteins, limiting possible retargeting modules to proteins or peptides, even though a multitude of medically relevant interaction partners consist of synthetic small organic molecules. Third, only single binding modules have been reported. Expanding the strategy to multiple binding interactions by linking two binding modules would enhance combinatorial possibilities and the potential of more advanced adapter-designs. Fourth, therapeutic proteins are often expressed in eukaryotic expression systems such as Chinese Hamster Ovary (CHO) cells. Including CHO as a possible expression host for our described adapters will expand the adapter platform to proteins requiring mammalian post-translational modifications.

Here we report strategies to overcome these limitations by extending the adapter design to peptides, small molecules, and antibody-derived scFvs and test them on different types of receptors in order to explore the broad applicability of the trimeric adapter system.

As a model system to test our modular targeting, we investigated human epidermal growth factor receptor 2 (HER2), neurotensin receptor 1 (NTR1), and folate receptor α (FTR) as target receptors. HER2 is a receptor tyrosine kinase and has long been explored as an important therapeutic target for gastric and breast cancer.[34] In contrast, NTSR1 is a G protein-coupled receptor, involved in neuromodulatory signaling in the central nervous system and in local hormone signaling in the gastrointestinal tract.[35] It has also been proposed to contribute to cancer progression.[35] Finally, FTR is a GPI-anchored protein of major interest in the field of oncology due to its important role to one-carbon metabolism and its overexpression in various solid tumors.[36]

RESULTS AND DISCUSSION

First, we explored a single-chain variable fragment (scFv) of an antibody for targeting. Since scFvs can readily be engineered from described antibodies, this extends possible adapter applications to all targets, for which an antibody has been developed. We tested here an scFv derived from the HER2-binding humanized mouse antibody hu4D5, which led to the therapeutic antibody trastuzumab.[37] We used the scFv in the VL-linker-VH orientation (termed 4D5 LH) (Supporting Information (SI) ref 3) to also exemplify the modular design by expressing it in eukaryotic cells. 4D5 LH was cloned into the mammalian expression vector pcDNA3.1, and was secreted using the HSA leader peptide. The leader peptide is followed by a His_6-tag, a FLAG-tag, and a 3C protease cleavage site N-terminal to the retargeting module.

We designed the mammalian expression vector to allow for rapid exchange of the retargeting module with single-step cloning by flanking the retargeting module with *Bam*HI and *Hin*dIII sites, allowing facile exchange of the retargeting module without interference with the knob-binding and trimerization modules. Our mammalian expression vector thus allows fast and easy exchange of retargeting modules without the need for long optimization, and it extends possible retargeting modules to proteins that need to be secreted from mammalian cells. Successful expression and purity of all constructs were determined by SDS-PAGE (SI Figure S1).

Next, we extended the retargeting module assembly strategy from genetic fusions to chemical coupling. For subsequent maleimide coupling, we added a single cysteine to the N-terminus of E2_5,[38] a stable nonbinding consensus DARPin functioning as a rigid spacer, and expressed the adapter containing this module in *E. coli*. For facile use, adapters

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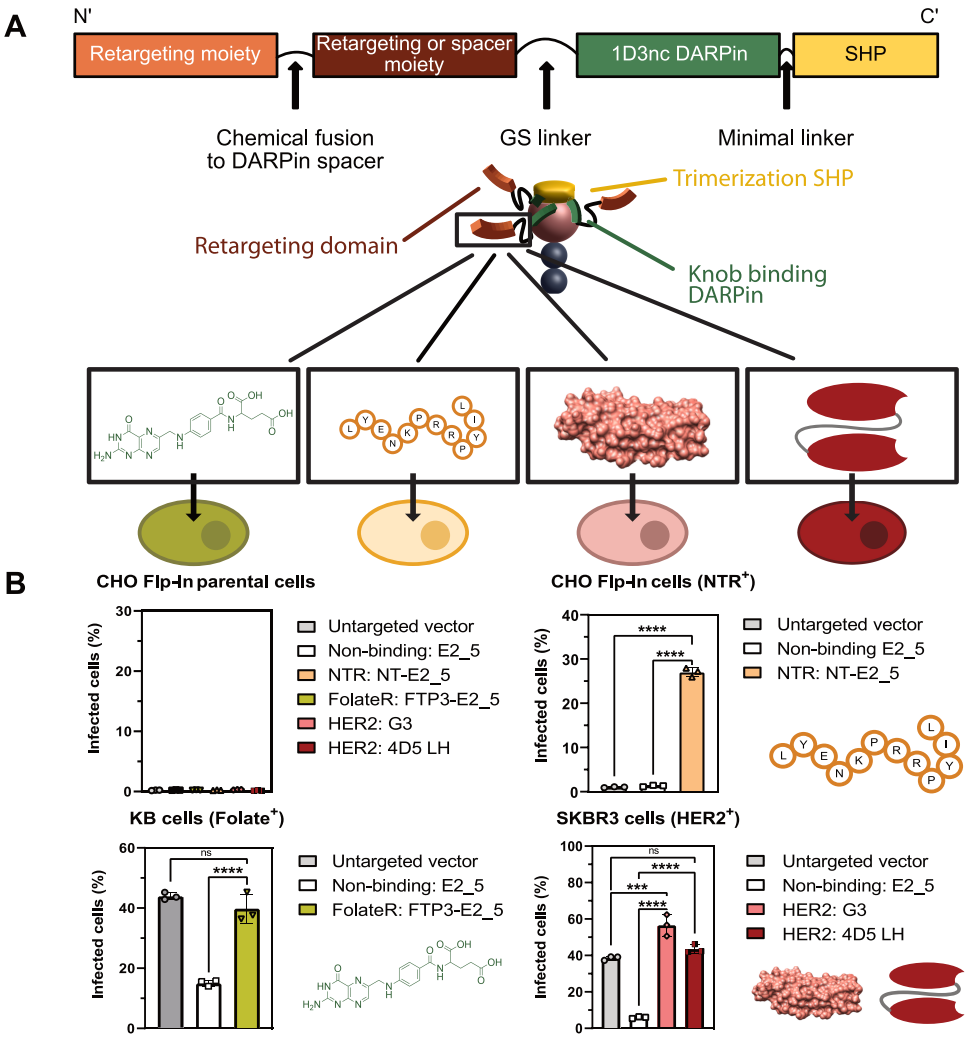

Figure 1. Retargeting of HAdV-C5 to cells expressing NTR, Folate receptor, and HER2. A. Schematic depiction of a knob-bound trimeric adapter, consisting of the three domains: From the N-terminus, the exchangeable retargeting domain(s) (orange and dark red), the knob-binding DARPin (green), and the trimeric SHP protein (yellow). The retargeting domain can consist of a small molecule (green), a peptide (yellow), a DARPin (light red), or a scFv (dark red). B. HAdV-C5 encoding iRFP670 was coated with a knob-to-adapter ratio of 1:20 and then added to 22,500 cells with an MOI of 2.5 PFU/cell. Gene delivery was analyzed 48 h post mixture by flow cytometry, and fluorescence was compared to the autofluorescence of the cells. No unspecific uptake was measured on CHO Flp-In parental cells, and all retargeted constructs were able to efficiently transduce the targeted cells. Three independent cell populations were transduced, significance was calculated using one-way ANOVA with Sidak's multiple comparison correction, and error bars represent the SD.

expressed in prokaryotes also carry an N-terminal His_6-tag, and the retargeting module is also flanked by *Bam*HI and *Hin*dIII. Since the adapters do not contain internal cysteines, the unique cysteine at the N-terminus of the retargeting module allows for site-specific coupling and thus expands retargeting possibilities to synthetic constructs or those that would not fold as a fusion protein.

We chose maleimide-coupled folate (FTP3-E2_5) as an example of a small molecule and maleimide-coupled neurotensin (NT-E2_5) as a peptide ligand for exemplifying broad applicability of this coupling strategy. Correct coupling was confirmed by electrospray ionization mass spectrometry (SI Table S2), and purity of the construct was determined by SDS-PAGE (SI Figure S1).

In conclusion, the generated vectors allow fast and easy adaptability of the trimeric construct for various research applications, e.g., screening of HAdV-C5 based gene delivery to a wide variety of different cells, including binders derived from antibodies, peptides or small molecules, which can be attached by genetic fusions or by chemical coupling. Additionally, these vectors circumvent complicated cloning procedures and are compatible with standard expression systems, potentially accelerating testing phases.

Next, the expression of each target receptor or, alternatively, their absence on the cell surface was verified by flow cytometry (SI Figure S2) and specific transduction capabilities of adapters carrying 4D5 LH, NT-E2_5, and FTP3-E2_5 were analyzed (Figure 1A). Additionally, various controls were included in all transduction experiments. Unconjugated E2_5 adapters (non-binding DARPin E2_5) were used as a negative control (as it only blocks the binding site for CAR), HER2-binding DARPin G3 served as a HER2-binding positive control,[39] and untargeted vectors which were not incubated with any adapters were also applied as controls for CAR-mediated uptake. This latter comparison served as a reference to the natural transduction efficiency of HAdV-C5. A reduced transduction when using E2_5-fused adapters that block the CAR-binding site on the knob, compared to untargeted vector, shows

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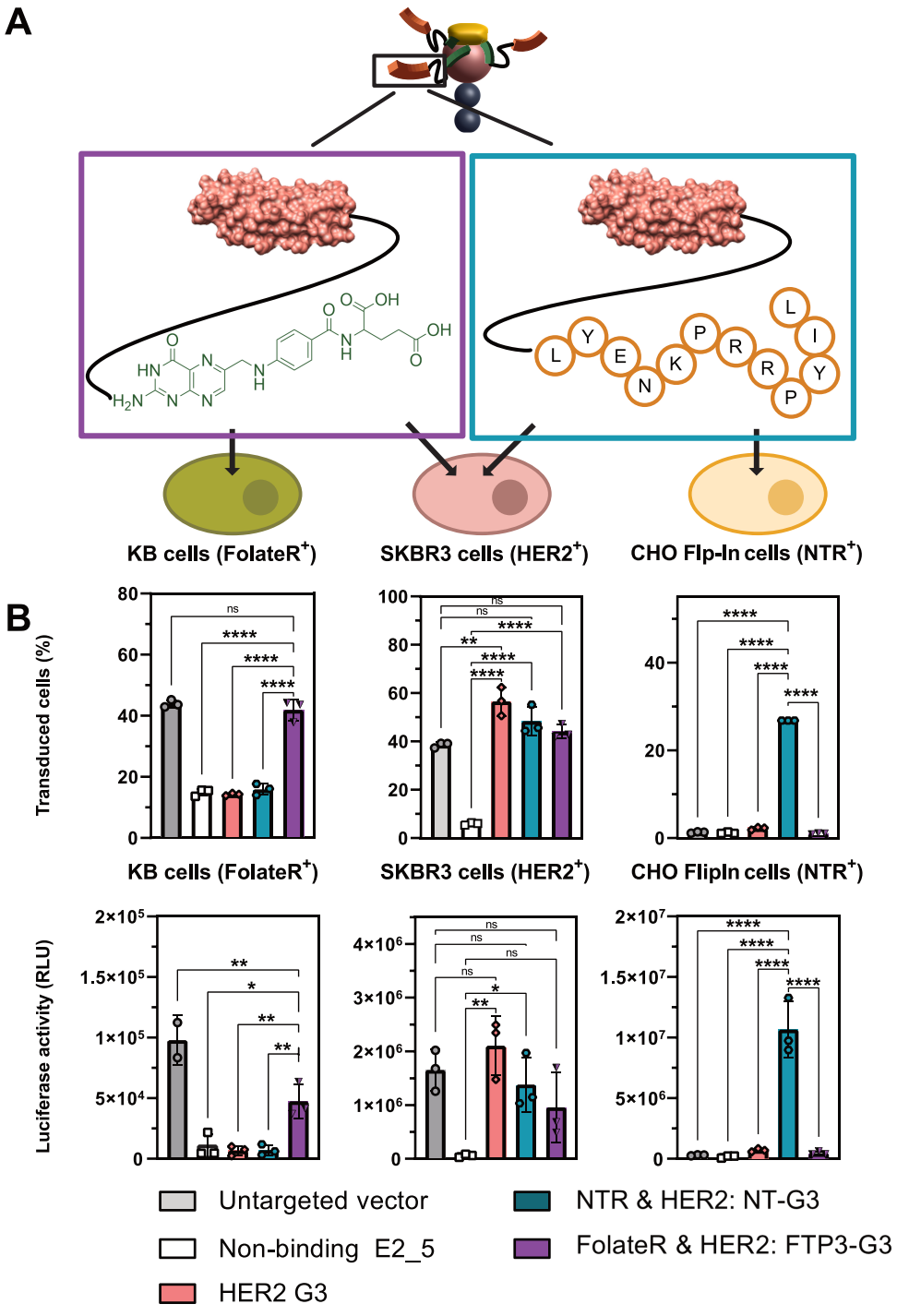

Figure 2. Bispecific targeting of adapter-coated HAdV-C5. A. Graphical depiction of the bispecific targeting, using the G3 DARPin (light red) fused to either folate (FTP3-G3 in violet) or NT (NT-G3 in cyan). B. Transduction efficiency and specificity measured on folate receptor-expressing KB cells, HER2-expressing SKBR3 cells, and NTR-expressing CHO Flp-In cells. HAdV-C5 was coated with a knob-to-adapter ratio of 1:20 and then added to 22,500 cells with a MOI of 2.5 PFU/cell. Gene delivery was analyzed 48 h post mixture by flow cytometry with HAdV-C5 encoding iRFP670 (upper row) or luciferase (lower row). Three independent cell populations were transduced, significance was calculated using one way ANOVA with Sidak's multiple comparison correction, and error bars represent the SD.

effective knob binding and therefore successful manipulation of virion−host interactions.[31,40]

The purified constructs were incubated with a first-generation ΔE1/E3 HAdV-C5 vector, encoding either infrared fluorescent protein 670 (iRFP) or luciferase, forming a stable adapter−vector complex. After incubating a 20-fold excess of adapter per HAdV-C5 fiber knob for 1 h at 4 °C, the adapter-vector complexes were added to different cell populations using a multiplicity of infection (MOI) of 2.5 plaque-forming units (PFU) per cell. After 16 h incubation, the medium was exchanged and vectors remaining in the medium were washed away. Cells were then kept for another 24 h to allow for sufficient expression of reporter until cells were taken for transduction analysis. We added our adapter−vector complex either to CHO Flp-In parental cells, CHO Flp-In expressing neurotensin receptor 1 (NTR+), folate receptor α expressing

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KB cells, or the HER2-expressing breast cancer cell line SKBR3. All constructs were also investigated for unspecific vector uptake. Since CHO Flp-In parental cells do not express our targeted receptors, any transduction of CHO Flp-In parental cells would therefore have to happen due to unspecific interactions.

As predicted, none of the tested variants showed transduction of CHO Flp-In parental cells, demonstrating that targeted HAdV-C5 transduction depends on specific binding (Figure 1B). Next, we analyzed inhibition of transduction using the control adapter E2_5 to confirm that knob domains of HAdV-C5 are covered, which is a prerequisite for demonstrating specific binding interactions for transduction. Indeed, the use of nonbinding E2_5 adapters did block fiber knob-mediated transduction in KB and SKBR3 cells drastically, although residual transduction through independent uptake mechanisms remains possible (Figure 1B). CHO cells do not express CAR, and therefore, there is no interaction which could be blocked. Thus, no further reduction was observed using the nonbinding E2_5 control, compared to the untargeted vector transduction.

In contrast, transduction was indeed observed where specific binding was expected. Not only did the scFv-containing adapter 4D5 LH permit transduction of SKBR3 cells, but also coupling of the small molecule folate to the DARPin E2_5 spacer (FTP3-E2_5) increased the transduction capability of HAdV-C5 KB cells, which express folate receptor. This demonstrates efficient and specific transduction using antibody fragments or chemically synthesized small molecules as targeting modules.

Chemically coupled peptides were also shown to be effective as targeting agents. When maleimide-activated NT was coupled to DARPin E2_5 (NT-E2_5), the vector−adapter complex transduced NTR1-expressing CHO Flp-In cells, resulting in 27% positive transduced cells. Notably, the same HAdV-C5 could not detectably transduce CHO Flp-In without a targeting adapter.

Taken together, we propose the trimeric adapter system as a facile tool for rational engineering of targeted HAdV-C5 transduction, which is effective for adapters produced by different expression hosts, for genetic fusions or for chemical coupling, and it works with widely different molecular sizes of the retargeting moiety.

To explore the truly modular design, we combined HER2-specific transduction, mediated through the DARPin G3, and transduction via chemical coupling of folate or neurotensin. Generating bivalent targeted transduction allows for more complex designs. We replaced our spacer E2_5 with the HER2-binding DARPin G3 while maintaining the unique cysteine. Using the same thiol-maleimide coupling, we thus generated a polyvalent adapter, binding the HAdV-C5 knob, HER2, and additionally either neurotensin receptor 1 by coupling to NT (NT-G3), or folate receptor α by coupling to folate (FTP3-G3) (Figure 2A).

By successful transduction with the HER2-targeting module G3 and the chemically coupled module (NT or folate), we investigated the plug-and-play design to bivalent targeting. After complexation of NT-G3 or FTP3-G3 with HAdV-C5 encoding iRFP or luciferase, we compared transduction efficiencies on KB, SKBR3, and CHO Flp-In NTR+ cells (Figure 2B). To compare the transduction level, we also added adapter−vector complexes with unconjugated E2_5 (nonbinding), or vector only (untargeted vector).

In line with our previous results, only on-target transduction was observed, and off-target transduction was inhibited by the blocked CAR interaction, independent of the cell type. Using iRFP as a reporter, we quantified the percentage of transduced cells, while luciferase activity allowed for determination of reporter gene expression level with high sensitivity. Measuring both quantities thus allowed us to observe differences in the number of cells taking up the vector and the amount of total functional vector uptake. We therefore conclude that specific and efficient transduction of all binding modules was maintained in this bispecific format.

Although various research and clinical studies showed promising results using HAdV-C5, an easy-to-use and widely applicable adapter technology would expand currently explored solutions. Applications of DNA delivery via natural HAdV-C5 is restricted by the tropism of the virion, limiting its use to target cells expressing CAR and other HAdV-C5 binding proteins. However, many applications may need a more defined population than cells that express CAR. Consequently, the primary goal for enhancing the success and applications of targeted HAdV-C5 gene delivery is to create a technology applicable to existing HAdV-C5 constructs while mediating receptor-specific targeting. Ideally, the technology would be able to incorporate various types of binding molecules, is flexible in their expression host, remains robust and modular in design, allows to generate multispecific targeting, and still remains easy to use.

Besides the adapter, to overcome HAdV-C5 liver tropism and immunogenicity for *in vivo* applications, a shield based on a trimerized scFv binding to the hexon can be applied.[40] This shield was shown to diminish the undesired intrinsic tropism caused by hexon interactions, while the tropism remains adapter-specific.

Here, we have focused our work on *in vitro* applications and shown their usefulness for cell culture assays. As the next step, future studies should be conducted focusing on an in-depth analysis of *in vivo* approaches[8,40,41] of the new modular adapters.

Excess of applied adapters could potentially lead to binding competition or unknown stimuli *in vivo*. To circumvent this, excess adapters could be removed through additional purification steps of the vector−adapter mixture via gel filtration techniques or beads coated with fiber knob. Previous *in vivo* studies have given no indications of adapter toxicity,[8,40,41] even though this cannot be ruled out for every target. *In vivo* studies must also investigate the potential immunogenicity of the adapters themselves. While DARPins, scFvs, and many peptides and small molecules can show negligible immunogenicity and are used in numerous clinical trials, their behavior in the context of the adapter needs further study.

CONCLUSIONS

We propose our trimeric modular adapter system now to be used as a mono- or polyvalent adenoviral retargeting strategy, utilizing a variety of different targeting modules, thereby extending the scope of currently targetable cells and receptors. Importantly, since the adapters are brought in to form a complex with the vector *after* expression, the technology is easy to use and can be combined with all existing and investigated HAdV-C5 vectors allowing for fast implementation in already existing strategies. This suggests potential applications for targeted transduction in various fields of

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science, utilizing peptides, antibody fragments, or small molecules.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.2c00346.

Detailed description of experimental procedures, including expression, bioconjugation techniques, methodologies, and the design and execution of experiments (PDF)

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Author Contributions

#P.C.F. and F.B. contributed equally to this work. P.C.F. conducted experimental design, planning, execution of experiments and writing of the manuscript. F.B. conducted experimental design, planning, execution of experiments and helped writing the manuscript. F.W. and D.B. provided valuable experimental data and helped writing the manuscript. B.D. planned the experimental design and execution and helped writing the manuscript. A.P. supervised the study, acquired funding and edited the final manuscript.

Notes

The authors declare the following competing financial interest(s): A.P. is a cofounder and shareholder, and D.B. is an employee and shareholder of Vector BioPharma, which is commercializing the technology described.

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